HEMOPOIETIC STEM CELLS IN RHESUS MONKEYS

SURFACE ANTIGENS, RADIOSENSITIVITY, AND RESPONSES TO GM-CSF

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HEMOPOIETIC STEM CELLS IN RHESUS MONKEYS

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(Hemopoietische stamcellen in Rhesus apen: oppervlakte antigenen, stralingsgevoeligheid en reacties op GM-CSF)

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ABBREVIATIONS

Ara-C	cytosine arabinoside
BFU-E	burst forming unit-erythrocyte
BM	bone marrow
C'	complement
cDNA	complementary DNA
CFU	colony forming unit
CFU-C	colony forming unit-culture
CFU-E	colony forming unit-erythrocyte
CFU-EO	colony forming unit-eosinophilic granulocyte
CFU-G	colony forming unit-granulocyte (neutrophil)
CFU-GM	colony forming unit-granulocyte, monocyte/macrophage
CFU-M	colony forming unit-monocyte/macrophage
CFU-MEG	colony forming unit-megakaryocyte
CFU-mix	colony forming unit-mixed
CFU-S	colony forming unit-spleen
CSF	colony stimulating factor
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte, monocyte/macrophage-colony stimulating factor
M-CSF	monocyte/macrophage-colony stimulating factor
multi-CSF	multi lineage-colony stimulating factor
Ep	erythropoietin
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLS	forward light scatter
GARA	goat anti rat
Gy	Gray

GvHD	graft versus host disease
HBSS	Hanks balanced salt solution
HGF	hemopoietic growth factor
HLA	human leukocyte antigen
HSC	hemopoietic stem cell
IFN	interferon
IL	interleukin
LD	lethal dose
LSM	leukocyte separation medium
MCA	monoclonal antibody
MHC	major histocompatibility complex
MRA	marrow repopulating ability
NK	natural killer
PE	phycoerythrin
PHSC	pluripotential hemopoietic stem cell
PLS	perpendicular light scatter
RBE	relative biological effectiveness
RELACS II	Rijswijk experimental light activated cell sorter II
RhLA	Rhesus monkey leukocyte antigen
TBI	total body irradiation
TNF	tumor necrosis factor

CHAPTER I
GENERAL INTRODUCTION

1.1. Hemopoietic stem cells and progenitor cells.

Peripheral blood cells have a limited life span. Therefore, to sustain life it is necessary to have a continuous production of new blood cells. Hemopoiesis is maintained from a small population of pluripotential hemopoietic stem cells. Those cells, defined as PHSCs, have the capacity of both self-renewal and differentiation into specific lineages of committed hemopoietic progenitor cells, which eventually differentiate into mature blood cells. This concept, depicted in Figure 1.1, has experimentally been substantiated by a number of observations: Bone marrow (BM) cells, and in mice also spleen cells, protect lethally irradiated animals against death from BM failure[1,2,3]. Protection is due to the production of donor derived peripheral blood cells[4,5,6]. Only a small proportion of the injected cells contributes to production of blood cells. Transplantation of a small number of syngeneic BM cells following lethal irradiation results in the appearance of a small number of macroscopically visible colonies of hemopoietic cells in the spleen[7]. Such a colony, which is the progeny of **one single cell**[8] termed spleen colony forming unit (CFU-S), may grow out to a **complete hemopoietic system**[9], including lymphocytes[10,11]. Thus such a CFU-S has some characteristics of the putative PHSC. However, CFU-S have also been shown to be a heterogeneous group of cells[12], which are not all capable to repopulate a lethally irradiated recipient. Roughly, two major populations of CFU-S can be distinguished, one producing colonies around day 8, and the other

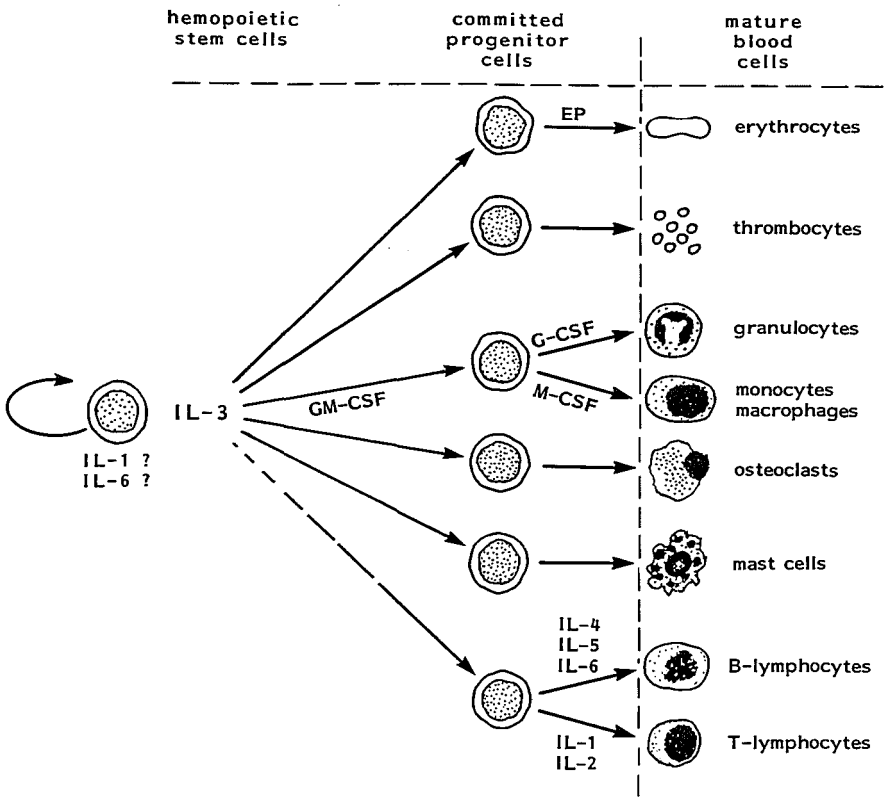

Figure 1.1 **Schematic representation of blood cell formation.**

around day 12. Although these populations partially overlap, a number of differences between the two populations are generally accepted: Injection of day 12 spleen colonies into irradiated secondary recipients results in larger numbers of daughter CFU-S than injection of day 8 CFU-S, indicating that the day 12 CFU-S have a higher self-renewal capacity[13]. Furthermore, day 8 CFU-S can be distinguished from day 12 CFU-S by differences in cell surface antigens[14,15,16], in physical parameters, and in cell cycle status and metabolism[17,18,19,20,21]. Both populations have been demonstrated to be heterogeneous for those parameters. Analysis of BM regeneration following BM transplantation with grafts from 5-

fluorouracil treated donor mice have indicated the existence of a more primitive HSC (i.e. a pre-CFU-S)[17] which may give rise to CFU-S in irradiated recipients (marrow repopulating ability, MRA).

Hemopoietic progenitor cells have been studied extensively *in vitro* using semi-solid colony culture systems. In such cultures, individual progenitor cells of a particular lineage proliferate and differentiate into a clone of mature cells, which can be detected as a colony. Such a progenitor cell is defined as a colony-forming unit in culture (CFU-C). Initially, only colonies, composed of granulocytes and macrophages have been described[22,23], but more recently, other culture systems have been developed for progenitor cells of erythrocytes[24], eosinophils[25], megakaryocytes[26,27], and for colonies composed of lymphocytes[28,29]. The nomenclature of all these colony forming cells has been described in an UICC-ICREW Report[30]: An affix added to the term CFU- indicates the cells present in such a colony, for instance GM for granulocyte/macrophage and Meg for megakaryocytes. A CFU-mix forms multilineage colonies ***in vitro***[31].

Recent data suggest, that the capacity for self-renewal is not strictly limited to pluripotent cells, but is shared by committed progenitor cells and even more differentiated cells[32].

The current concept is that of a PHSC which is a resting, metabolically inactive, primitive cell. This cell can be triggered into cell cycle, thereby having a probability (p) of self-renewal and a probability (1-p) of maturation. Following one or more maturation divisions, lineage commitment occurs, which is irreversible. In this concept, a p-value of 0.5 in steady state implies, that the number of PHSCs is constant throughout life, whereas a p-value < 0.5 results in exhaustion of the PHSC pool. The extreme possibility, that p = 0 in steady state would implicate, that either the number of PHSCs at birth, or the number of cell divisions between PHSCs and mature cells is extremely high[33,34,35]. The evidence for the capacity of PHSC self replication, summarized above, argues against a p-value being 0.

It is poorly understood, how PHSCs become committed to restricted pathways of differentiation. Restriction in differentiation potentials could be **intrinsically**

determined either in a stochastic[36] or in an hierarchically branched manner[37]. Alternatively, restriction in differentiation potentials could be determined by **external influences**, such as stromal cells and cytokines[38,39]. Generally, the probability of self renewal is considered to be lower in more mature progenitor cells as compared to more primitive cells[32]. The precise relationships between the various committed lineages remain unresolved, and different hierarchical orders can be argued[40].

The process of hemopoiesis is tightly regulated, so that functional end cells are produced in response to peripheral demands. In this process, hemopoietic growth factors play a role, which will be discussed in the next section.

Characterization of HSCs is essential for the development of methods to eliminate unwanted cells, such as T lymphocytes or malignant cells from BM grafts without loss of the critical HSCs. Furthermore, characterization and purification of HSCs is of importance for investigations concerning the influence of hemopoietic growth factors (see section 1.3) on self replication and/or differentiation of HSCs. In addition. characterization and purification of HSC is necessary for studies on the genetic control of stem cell differentiation, which needs to be elucidated to make gene therapy in hemopoietic stem cells feasible.

Studies in mice have shown, that PHSCs are archetypal round cells, ultrastructurally distinct from lymphocytes[41]. The existence of a similar BM cell has been confirmed in rats, Rhesus monkeys, and humans[42,43,44]. The subsequent use of cell surface markers and light activated cell sorting has enabled the preparation of highly purified murine HSC suspensions. By using this technology, grafts of only 10^2 cells appeared to be sufficient to protect a lethally irradiated mouse from BM failure[45,46]. Among the cell surface markers available to isolate immature human hemopoietic cells, the CD34 antigen, identified by the MCAs My-10[47], 12-8[48], BI-3C5[49], ICH3[50], and other MCAs, is of outstanding interest, since it is mainly expressed on immature hemopoietic cells of various differentiation lineages, including hemopoietic progenitor cells such as CFU-mix, CFU-GM, and BFU-E. Moreover, CD34 is present only on 1-4% of human BM cells and is not co-expressed with lymphatic markers (CD2, CD3, CD4, CD5, CD7, and CD8) and myeloid markers (CD11, CD14, CD15, CD16,

and CD18)[51].

The monoclonal antibody ICH3 is distinct from the other three in that it is a high avidity antibody of IgG 2a isotype that recognizes a peptide epitope and does not modulate or have any effector function such as complement (C') lysis. These properties make ICH3 preferentially suited for positive selection of HSCs for BM transplantation. My-10 and BI-3C5 do not cross react with Rhesus monkey BM cells at all[52]. In Chapter III, the cross reactivity of the MCA ICH3 will be described. This MCA has been used for positive selection of HSCs. In most of the studies in rodents, the enrichment of HSCs was monitored by a quantitative functional assay, that is, the spleen colony assay (SCA). In outbred species, such as Rhesus monkeys and humans, the most primitive detectable cell is measured as CFU-mix. However, the development of a complete hemopoietic system out of the progeny of a CFU-mix has never been demonstrated. Thus, the relationship between the CFU-mix and PHSCs is less well established than the relationship between the CFU-S and PHSCs. Studies on the nature of human PHSCs have been limited by the lack of a suitable assay, and by a restricted possibility for *in vivo* studies. Recently, an *in vivo* HSC assay in Rhesus monkeys has been developed in our laboratory[53]. This assay enables a quantitative comparison between enrichment of repopulating HSC as calculated from the *in vivo* regeneration rate and the enrichment of progenitor cells as calculated from *in vitro* assays. In Chapter III, this assay will be used to monitor the positive selection of HSCs, based upon CD34 expression.

1.2. Radiosensitivity of hemopoietic stem cells.

Irradiation of the total body (TBI) with ionising radiation causes a dose dependent BM suppression. The severity and duration of the resulting pancytopenia depends on the number of repopulating HSCs that survive the irradiation. Following a BM ablative dose of TBI, hemopoiesis can only be restored by BM transplantation. Following lower doses of TBI, BM transplantation might reduce mortality and morbidity by shortening the duration of pancytopenic period[54,55].

Therefore, a number of victims of accidental irradiation at nuclear power stations at Vinca (Yugoslavia) and Chernobyl (Soviet Union) have been treated with BM transplantation[56,57,58]. However, the conditions for a beneficial effect of BM transplantation in victims of radiation accidents are not accurately known[59]. The number of HSCs, that survive the irradiation and the damage to other organ systems are the major factors determining the benefit of BM transplantation. Observations in untransplanted victims of radiation accidents and nuclear warfare resulted in estimates of the LD_{50} for TBI in humans varying from 2 - 6 Gy[60], but these values have no general significance, since survival of such victims is not only dependent on the number of HSCs that survived the irradiation, but also on the degree of damage to other body systems, and on the efficacy of supportive care, including prevention and treatment of infections and transfusion of blood products. Furthermore, accidental irradiation is, in general, not distributed and absorbed homogeneously, resulting in a surviving HSC fraction which may be higher than after a comparable dose of homogeneous TBI. Unfortunately, there is no appropriate assay for human, and information about the radiosensitivity and frequency of human HSCs is scanty. Reliable information on the radiosensitivity and frequency of the HSCs is essential for the development of treatment regimens for victims of radiation accidents, and for improvement of treatment modalities for cancer patients requiring TBI.

In rodents, survival of HSCs after irradiation has been determined using the spleen colony assay. In larger animals only data on the radiosensitivity of CFU-Cs are available. The radiation sensitivity of HSCs and progenitor cells in different species has been extensively reviewed by Hendry and Lord[61]. Representative data from this review are summarized in Table 1.1. Most of these studies concern measurements in the range between 0 and 5 Gy. The differences between the reported D_0 values of the CFU-S could be explained by differences in dosimetry, differences in genetics, age and sex of the mice. In the case of *in vitro* irradiations differences in oxygenation[62] or pH[63] may also have influenced the reported radiosensitivity. Recently, the D_0 value of day 7 CFU-S, day 12 CFU-S and MRA of mice have been compared, yielding a higher D_0 value for cells that are considered to be more primitive (for 300 kV X-rays 0.75, 0.94 and

1.18 Gy [64,65] respectively). This increase in radiosensitivity during maturation is unexpected, since more primitive cells are generally regarded to be more radiosensitive.

A dose of 10 Gy TBI, given at a dose rate of 0.02 Gy/min has been reported to result in a similar cell kill as a dose of 7.2 Gy TBI, given as a flash irradiation[66]. However, other authors could not demonstrate such an effect of the dose rate on the survival of CFU-S[67,68]. A small shoulder in the dose-survival curve was demonstrated by almost all authors. This shoulder is characterized by an extrapolation number of about 2 for Á (range 1.0-4.0) as well as X-rays (range 0.8-5.2). Apparently, cells capable of protecting lethally irradiated mice have approximately the same radiosensitivity as CFU-S.

In most studies, CFU-C appeared to be less radiosensitive than CFU-S. The canine CFU-C have a lower D_0 value than the human and the murine CFU-C. This might be due to a higher radiosensitivity of the canine CFU-C, but the influence of culture conditions on the D_0 value of CFU-C cannot be ruled out[69]. No data are available on the radiosensitivity of Rhesus monkey CFU-C.

The only estimate of radiosensitivity of HSCs in larger animals was provided by Vriesendorp and van Bekkum[70]. Based on a comparison of $LD_{50/30days}$ for TBI to the autologous BM rescue dose in lethally irradiated animals, it was postulated, that the radiosensitivity of HSCs was similar for mice, rats, dogs, and Rhesus monkeys, but that the frequency of HSCs was negatively correlated with body weight.

Intensive supportive care, including prevention of infections by prophylactic gastrointestinal decontamination, balanced fluid supply, and thrombocyte transfusions, permit the study of endogenous regeneration patterns following doses of TBI, that were formerly assumed to be midlethal or lethal. By comparison of such endogenous regeneration patterns to the regeneration following well defined autologous BM grafts, the radiation sensitivity of HSCs can be directly assessed. In Chapter IV, such an assessment is described for Rhesus monkeys, and the consequences for the clinical management of radiation casualties and for clinical BM transplantation will be pointed out.

TABLE 1.1 RADIOSENSITIVITY OF HEMOPOIETIC STEM CELLS AND PROGENITOR CELLS IN DIFFERENT SPECIES

Species	cell type	*In vitro* irradiation Radiation type	D_0 (Gy) mean ± s.e.	Extrapolation number	*In vivo* irradiation D_0 (Gy) mean ± s.e. (R = Röntgen)	Extrapolation number	Reference
Mouse	CFU-S	γ*	1.15 ± 0.08	2			7
			1.05 ± 0.13	2.5	0.95 ± 0.09	1.5	71
			0.93 ± 0.07	1.9 ± 0.8	0.98 ± 0.09	1.7 ± 0.6	72
				1.55	1.2		73
					0.86 ± 0.06	2.5 ± 0.7	74
					1.16 ± 0.10	1.2 ± 0.3	68
		X**	0.62	4.0	0.73	2.4	75
			1.04 ± 0.04	1.0	1.05	1.2	76
			0.67 ± 0.07	2.2 ± 1.2	0.70 ± 0.04	2.4 ± 0.5	72
					0.81 ± 0.05	0.9	77
	Repopulating stem cell+	X	0.84 (R/100)	2.5	1.05 ± 0.24	1	78 79
	MRA	X			1.18		65
	CFU-C	γ	1.60	1			80
					1.60 ± 0.15	1.0 ± 0.2	81
		X			0.7 (R/100)	1	82
					1.57 ± 0.11	1.09	77
	BFU-E	X			0.69 ± 0.09	0.89	77
	CFU-MIX	X			1.44 ± 0.30	1.03	77
Rat	CFU-S	γ	0.94	4.1	0.97	5.2	83
Dog	CFU-C	γ			0.61 ± 0.02	1	84
					0.70 (R/100)	0.8	85
	BFU-E	X	0.153 ± 0.018	1.05 ± 0.28			86
	Repopulating stem cell	X			0.6	1	70

* ^{60}Co or ^{137}Cs γ-rays

** 200 - 300 kV X-rays

+ measured by 30 day survival of lethally irradiated animals.

Cont. Table 1.1

Species	cell type	Radiation type	D_0 (Gy) mean ± s.e.	Extra-polation number	D_0 (Gy) mean ± s.e. (R = Röntgen)	Extra-polation number	Reference
Rhesus monkey	Repopu-lating stem cell	X			0.6	1	70
Man	CFU-C	γ	1.37	1.0			80
				1.60	1.0		69
		X	1.46 ± 0.12	1.0			87
	BFU-E	X	1.27 ± 0.11	1.0			87

1.3. Cytokines involved in the lympho-hemopoietic system.

1.3.1. Regulation of the production of blood cells.

In humans, the process of hemopoiesis results in an average daily production of about 10^{11} new blood cells[88]. In spite of this large daily production of at least 8 different blood cell types with markedly different life spans and therefore different production rates, the peripheral blood cell counts undergo only moderate fluctuations, even during states of increased demand, such as blood loss or infection. This is only possible, because hemopoiesis is tightly regulated. Furthermore, certain perturbations, such as resulting from infections, require, that specific blood cell types, such as lymphocytes, exert specific functional activities.

Hormone-like peptide compounds, termed cytokines, influence cell proliferation and functional activity of the lymphohemopoietic system. Three different nomenclatures have been developed to designate these regulators: Interleukins (ILs), Interferons (IFNs) and Hemopoietic Growth Factors

TABLE 1.2 HUMAN CYTOKINES AFFECTING THE FUNCTION AND PRODUCTION OF BLOOD CELLS

Factor	Synonyms	Biological effects	Cellular Source	Reference
INTERFERONS				
IFN-α and IFN-ß		Induces Class I expression. Stimulates NK activity. Antiproliferative effects. Induction of fever.	activated T Lymphocytes	89 - 95
IFN-γ		Induces Class I and II expression. Stimulates NK activity. Augments or inhibits effects of other cytokines	activated Lymphocytes	96 - 99
INTERLEUKINS				
IL1	Hemopoietin 1, Leukocytic endogenous mediator. Osteoclast activating factor, Lymphocyte activating factor.	Synergism with other HGFs. Induces synthesis of G-CSF GM-CSF, M-CSF. Osteoclast activation. Natriuresis. Acute phase response.	Macrophages Keratinocytes Glial cells. Endothelial cells Lymphocytes. Neutrophils	100 - 134
IL2	T cell growth factor	Proliferation of activated T cells. Induces synthesis of other cytokines.	T Lymphocytes	135 - 143
IL3	Multi-CSF	See below		
IL4	B cell stimulating factor 1 B cell growth factor	Proliferation of activated B cells. Induces Class II expression on B cells. Proliferation of T cells and mast cells. Induces synthesis of G- and M-CSF in fibroblasts.	activated T Lymphocytes	144 - 146
IL5	B cell growth factor 2	Stimulates growth and differentiation of B lymphocytes and eosinophils	T lymphocytes	147 - 148
IL6	B cell stimulating factor 2 B cell differentiating factor IFN-ß-2 Hepatocyte stimulating factor	Synergism with GM-CSF and IL3 in colony induction.	activated T Lymphocytes Fibroblasts	89 - 91 149 - 162

Cont. Table 1.2

Factor	Synonyms	Biological effects	Cellular Source	Reference
HEMOPOIETIC GROWTH FACTORS				
Erythro-poietin (Ep)	Hemopoietine	Stimulates CFU-E and a subpopulation of BFU-E.	Kidney cells	163 - 177
Multi-CSF	IL3, Stemcell Activating Factor (SAF)	Stimulates stem cells and progenitor cells of all lineages.	T-lymphocytes	178 - 187
GM-CSF	CSF-α, Pluripoietin, CSF-2	Stimulates progenitor cells of several lineages. Induces M-CSF synthesis. Stimulates mature neutrophil functions.	T-lymphocytes Monocytes Fibroblasts	188 - 225
G-CSF	CSF-ß,	Stimulates neutrophil progenitors and neutrophil function.	Macrophages Fibroblasts Endothelial cells	226 - 239
M-CSF	CSF-1 urinary CSF	Stimulates monocyte/ macrophage progenitors. Induces synthesis of IFN, TNF and G-CSF.	Macrophages Fibroblasts Endothelial cell	240 - 248

(HGFs), the latter including the colony stimulating factors (CSFs) and some of the ILs and IFNs. Originally, these peptides have been named according to their biological effect. For instance, B-cell differentiating factor induces differentiation of activated B-cells, and T-cell growth factor stimulates T-cells to proliferate. This descriptive nomenclature appeared to be unsatisfactory, since most of these cytokines exert multiple biological activities. Therefore, a nomenclature has been developed that employs the term IL followed by a number.

IFNs were originally identified on the basis of the capacity to interfere with viral replication in infected cells. Three types of IFN are known: α–IFN, β-IFN, which is structurally related to α–IFN and γ–IFN.

CSFs stimulate HSCs and/or progenitor cells to proliferate and differentiate

in vitro, which under appropriate culture conditions results in colony formation. The nomenclature of the CSF resembles the nomenclature of the CFUs: a prefix indicates the major type of colony produced. G-CSF stimulates the formation of granulocyte colonies, M–CSF stimulates the formation of monocyte colonies, multi-CSF stimulates the formation of a variety of colonies. The present nomenclature of CSFs remains somewhat confusing. For example, GM-CSF was initially described as a factor stimulating the formation of granulocyte and/or macrophage colonies[188], whereas it is now clear, that it induces also colonies of other cell lineages[189]. For some of the HGFs , such as M-CSF, the amino acid sequence homology of the protein is high between the human and the murine factor is high with functional cross reactivity, i.e. stimulation of murine cells by the human factor and/or inversely, has been demonstrated. Other factors, such as IL-3 have much less homology and interspecies cross reactivity does not occur.

The three different nomenclatures are imperfect and partially overlapping: IL-3 is a synonym of multi-CSF, and IL–6 (B cell growth factor and hepatocyte stimulator factor) which is identical to IFN-β2 also acts synergistically with GM–CSF and IL–3 in the stimulation of colony formation[154].

Nowadays, a number of those cytokines have been isolated and the genes, coding for them have been cloned. This allows for the production and purification of these hormones on a large scale, enabling *in vivo* manipulation of the hemopoietic and immune system by the administration of pharmacological amounts of these substances. The most important factors and their major characteristics are listed in Table 1.2, and will be discussed below.

α and ß IFN induce Class I histocompatibility antigen expression, augment natural killer (NK) activity[92], and have fever inducing and anti-proliferative properties. **γ IFN** induces the expression of Class I and Class II histocompatibility antigens on a variety of cells. Furthermore, γ-IFN augments NK activity, augments or inhibits the activity of other cytokines[96].

The direct antiproliferative effects of interferons and the ability of these cytokines to activate NK cells have provided the rationale for clinical trials of IFNs in cancer patients[92-95].

IL2, a 17 to 22 kD glycoprotein, which has a central role in the immune response[137], can be considered as the prototype of an interleukin. The gene encoding for human IL2 has been cloned[143]. IL2 initiates proliferation of activated T-cells[138,139]. Activation of T cells by the specific antigen results in the appearance of high-affinity receptors for IL2. The binding of IL2 to these receptors result in T-cell proliferation. Elimination of the antigen results in the involution of these receptors and the cessation of proliferation[135]. IL2 further induces the secretion of other cytokines, such as IFNÁ, tumor necrosis factor (TNF) and IL4[140]. IL2 enhances the cytolytic activity of natural killer cells[141]. This property has led to clinical trials with IL2 in cancer patients. IL2 has been used alone and in combination with lymphokine-activated killer cells. Although this immunotherapeutic approach has severe side effects, it may cause marked tumor regression in some patients[142]. The value of this treatment remains to be established.

IL4 was originally identified on the basis of its ability to induce proliferation of T lymphocytes and mast cells, to increase the production of immunoglobulins (Ig) as well as the expression of histocompatibility Class II antigens. Binding of antigens to the immunoglobulins on the cell surface of B cells results in proliferation of these cells[144,145]. More recently, IL4 was found to induce the synthesis of G- and M-CSF in fibroblasts[146].

IL5, which stimulates both proliferation and differentiation of B lymphocytes, also stimulates the growth of eosinophilic colonies[147,148].

Recently, the gene encoding human **IL6**, previously described as B cell differentiating factor or B cell stimulating factor 2, and nowadays termed has been cloned[153]. This factor was originally termed T cell replacing factor since it induced immunoglobulin synthesis in stimulated B cells in the absence of T lymphocytes[149]. It appeared to be identical to interferon-β_2[89,90], hepatocyte stimulating factor[91], and 26 kDa protein[155]. IL6 has a role in the

terminal differentiation of plasma cells from B lymphocytes[149]. Furthermore, IL6 induces the IL2 receptor[157] as well as IL2 production[158] in stimulated T-cells. In addition, IL6 can directly induce the growth of T-cells[159]. Other biological functions of IL6 include a stimulation of the production of acute phase proteins in the liver[91], induction of differentiation of neural cells[160], and a stimulation of ACTH secretion by the pituary[161]. Finally, IL6 influences normal and malignant HPC: it supports colony formation by CFU-GM, acting synergistically with IL3 and GM-CSF[150-154]. Recently IL6 appeared identical to MG1-2, a factor that induces differentiation in leukemic cells[249].

A human **IL1** cDNA has been cloned from messenger RNA of peripheral blood monocytes[102]. Later two different human IL1 proteins appeared to exist: IL1 α and IL1 ß. The cDNA coding for IL1 and the cDNA coding for IL1 ß were both isolated from a human macrophage cDNA library[103]. The two proteins are for 26% of their amino-acids homologous[104]. Stimulation of monocytes with lipopolysacharide results in a 40-fold increase in IL1 ß production, whereas the increase in IL1 α is only 2- to 3 fold, suggesting, that IL1 ß is the predominant form. In addition to the secreted IL1, a membrane- bound form of IL1 has been described, which is predominantly IL1 α[105-107]. Both IL1 α and IL1 ß have a molecular weight of 17 kD. IL1 was first identified as a lymphocyte-activating factor (LAF), enhancing lymphocyte proliferation in response to mitogens[108], but more recently many different effects on a variety of target cells have been described: IL1 acts as a cofactor for Con A activation of T cells[109], as an inducer of IL2 and interferon γ production[110], as a growth and differentiation factor for B cells[111], and as a chemotactic factor for neutrophils, monocytes/macrophages and lymphocytes[112]. Furthermore, it stimulates the proliferation of fibroblasts[113], and induces the production of collagen and interferon ß by those cells[114]. IL1 plays a crucial role in the acute phase response: it initiates fever by inducing an increase in hypothalamic prostaglandin E_2 synthesis[115], it

triggers the synthesis of acute phase proteins in the liver[116], and amino acid release from muscles[117-118]. Other biological effects of IL1 include natriuresis[119], osteoclast activation[120], increase in procoagulant activity[121], an increase in release of platelet activating factor[122] and plasminogen activator inhibitor[123]. Finally IL1 plays an important role in the regulation of hemopoiesis. It induces the production of GM-CSF, G-CSF, and M-CSF by a variety of cell types, such as endothelial cells, fibroblasts[124-126] monocytes/macrophages[126,127] and BM stromal cells[128,129]. In addition, IL1 acts synergistically with G-CSF[130], M-CSF[131], GM-CSF[132].

IL1 administration in mice stimulates granulocyte production[133,134,196] and accelerates hemopoietic regeneration following TBI or chemotherapy[130,133,196,250]. As IL1 induces the production of a number of cytokines, it is difficult or impossible to discriminate between the biological effects of IL1 itself and effects which are mediated by other cytokines.

1.3.2. Hemopoietic Growth Factors (HGFs).

Humoral factors have been recognized to play an important role in hemopoiesis. Evidence for a regulation by soluble factors, i.e. HGFs, dates back to 1906. The injection of serum from bled rabbits increased the number of blood cells in normal rabbits, indicating, that a soluble factor stimulates blood cell production[163].Later, this factor appeared to be involved exclusively in erythrocyte production and was termed **erythropoietin**[164] (**Ep**). The isolation and identification of Ep as a glycoprotein was described in 1960[165]. In contrast to other HGFs, detailed information on *in vivo* and *in vitro* characteristics of Ep has been obtained using purified material from urine, before its production with recombinant DNA technology was developed: Ep has been recognized as a physiological regulator of erythropoiesis. This is based on the observation, that artificially induced polycytemia shuts off erythropoiesis, which can be restored by Ep injections[166]. Further-

more, hypoxia stimulates Ep production, resulting in an increased erythropoiesis[167-169]. Purification of Ep to apparent homogeneity[170]was performed in 1977. Recently the gene, coding for Ep has been cloned[171], enabling the production of Ep on a scale, sufficient for clinical applications (see 1.3.3). Similar to the native peptide, recombinant Ep primarily stimulates the proliferation and differentiation of CFU-E and a subset of BFU-E[172]. The numbers of CFU-E in BM rise in response to bleeding, hypoxia and injection of Ep, whereas the numbers of BFU-E are hardly influenced by such treatments[173-176]. Thus, Ep mediates the regulation of the developmentally late events in erythropoiesis. Ep can be regarded as the prototype HGF: It increases the production of a well defined type of blood cells by stimulation of proliferation and differentiation of the progenitors of these cells. Furthermore, the production of Ep is dependent on the demand for the blood cells in question.

Recently, a human **Multi-CSF** cDNA clone has been isolated from a cDNA library prepared from stimulated human lymphocytes. The cDNA was identified on the basis of the sequence homology within the non-coding region of the murine multi-CSF[178]. Independently, others have isolated such a clone from a lymphoid cell line (MLA-144), using the gibbon IL3 cDNA as a hybridization probe[180]. This protein has a molecular weight of 14 to 30 kilodalton (kD) (133 amino acids). Sequence homology between murine and human coding regions appeared to be only 45%, which is considerable less than the homology between other human and murine growth factors or lymphokines. Mouse Interleukin-3 (IL3) has been described as a proliferation stimulus for a variety of precursor cells: CFU-S, CFU-MIX, CFU-GM, CFU-EO, mast cells and, in the presence of Ep, BFU-E[181-185]. In unfractionated human BM cell suspensions, human IL3 induces more colonies than in purified, CD34 positive cells. These results indicate that accessory, CD34 negative cells, which do not form colonies by themselves, enhance colony formation. This enhancement can be explained by the production of other growth factors in response to IL3, or by direct cell-cell contact[187]. Finally, human IL3 stimulates the *in vitro* growth of leukemic cells from a majority

of the patients with acute myeloid leukemia (AML)[179].

Human **GM-CSF** will be described in more detail in section 1.4, and a number of *in vivo* experiments with recombinant human GM-CSF will be described in Chapter V, VI and VII.

Human **G-CSF** has been purified from medium conditioned by a human bladder carcinoma cell line (5637)[226] and from a human squamous carcinoma cell line (CHU-2)[227]. A synthetic oligonucleotide was used to isolate a G-CSF cDNA clone from a library of CHU-2 mRNA. A similar approach resulted in the isolation of a G-CSF clone from a cDNA library of the bladder carcinoma cell line (5637)[228]. The mature protein has a molecular weight of 18-22 kD and consists of 174 amino acids. G-CSF predominantly stimulates granulocyte progenitors, although the induction of mixed colonies have been described[228]. However, the induction of mixed colonies could not be demonstrated in cultures of highly enriched progenitor cells, suggesting, that mixed colony-formation in G-CSF stimulated cultures is mediated by accessory cells. In addition to the influence of G-CSF on granulocyte progenitors, G-CSF also enhances functional activities of mature granulocytes, such as phagocytosis, superoxide release and chemotaxis[230]. In contrast to Multi-CSF, human and murine G-CSF are almost completely homologous and hence show biological and receptor binding cross reactivity to normal and leukemic murine and human cells[231].

Human **M-CSF** has been purified from human urine[240] Oligonucleotide probes were used to isolate several clones from a genomic library[241]. One of these clones was used to screen a 4-kb cDNA library made from mRNA extracted from a stimulated human pancreatic tumor cell line (MIA Paca). This resulted in the isolation of a cDNA clone, which could be expressed in a monkey kidney cell line (COS) and in a Chinese Hamster Ovary cell line (CHO), resulting in production of a dimeric polypeptide, indistinguishable from urinary M-CSF[242]. The mature protein is a dimer consisting of two identical subunits with a molecular weight of 35-45 kD each. Human M-CSF induces colonies derived from both human and murine macrophage

progenitors[245]. M-CSF stimulates mature monocytes and macrophages to produce interferon (IFN), tumor necrosis factor (TNF), and various other CSFs. Factors regulating macrophage production can be isolated from the uteri of pregnant mice[246]. Since the receptor for M-CSF has been demonstrated on a choriocarcinoma cell line, it has been suggested that M-CSF plays also a role in placenta growth[247].

The mechanisms which control the production of hemopoietic growth factors are largely unresolved. Possibly, IL1, which is produced in response to endotoxins[251] and other bacterial products[252,253], plays a crucial role in this regulation[101]. However, a feed-back mechanism, a most essential element for a regulatory function, has so far only been elucidated for Ep and not for IL1 or any of the other growth factors. To resolve the physiological regulatory function, a feed-back mechanism between production and demand should be demonstrated. HSCs in the BM may occupy niches in which the **local** production and action of compartmentalized growth factors modulate proliferation and differentiation. Such niches may provide local control. The stromal aminoglycans may be involved in such a local control by selective retention of HGFs[254]. Furthermore, stromal cell lines produce soluble factors, which support the growth of multi-lineage colony formation, but can be distinguished from multi-CSF and GM-CSF. These factors still support the growth of multi-lineage colony formation, indicating the existence of other stimulating mechanisms than provided by the hitherto identified HGFs[255,256]. A direct contact between stromal cells and HSCs is postulated to influence self-replication and proliferation[257,258]. The mechanisms by which overall coordination is exerted in all those processes, remain to be elucidated.

1.3.3. Clinical applications of hemopoietic growth factors.

Several hemopoietic growth factors have become available by recombinant DNA technology on a scale that allows for their clinical use. Potential applications are summarized in Table 1.3. Some of these applications are currently the subject of clinical trials.

TABLE 1.3 POTENTIAL APPLICATIONS OF HEMOPOIETIC GROWTH FACTORS

- Substitution of deficient factors.
- Mitigation of pancytopenia induced by radio- and chemotherapy.
- Mitigation of pancytopenia in relation to bone marrow disease.
- Treatment and classification of leukemia.
- *In vitro* and *in vivo* stem cell assays.
- *In vitro* and *in vivo* stem cell multiplication.
- Genetic modification of stem cells.

Therapeutic use of hemopoietic growth factors includes substitution of deficient factors as well as pharmacological manipulation of hemopoiesis. The most commonly known growth factor deficiency is anemia in end-stage renal disease. In recent clinical trials, **Ep** substitution has been successfully used to correct anemia resulting from renal failure[259,260]. In patients with congenital agranulocytosis (Kostmann's Disease), which is considered as a possible **G-CSF** deficiency, treatment with recombinant **G-CSF** has been reported to result in an increase in the numbers of neutrophils[261].

Pharmacological manipulation of hemopoiesis could be beneficial in conditions characterized by a lack of peripheral blood cells. Since most of these conditions are not caused by a deficiency of one or more of those factors,

supraphysiological serum- and tissue concentrations are required to achieve a therapeutic effect. Furthermore, systemic administration will probably result in an abnormal tissue distribution compared to local production under physiological conditions. This might result in unpredictable side effects.

BM suppression is one of the most important dose limiting factors in chemotherapy and in treatment modalities involving TBI. Irradiation- or chemotherapy induced pancytopenia is characterized by a partial eradication of normal BM. As a consequence, the residual HSCs will be stimulated to renew themselves and to differentiate in order to restore homeostasis. The time required for such a regeneration is dependent on the number of surviving HSCs and progenitor cells, the proliferation rate, and the fraction of cells in cycle. Pharmacological manipulation of hemopoiesis could reduce this pancytopenia by stimulation of HSCs and progenitor cells. The same principle, visualised in Figure 1.2, could mitigate the pancytopenia occurring in the first weeks following BM transplantation (mechanism 1). **G-CSF** has been used successfully to reduce chemotherapy induced neutropenia in clinical trials with patients suffering from transitional-cell carcinoma of the urothelium[232] and in patients suffering from a variety of other malignant diseases[233-235].

Preclinical studies with G-CSF in healthy, hamsters have shown a tenfold rise in peripheral blood granulocyte counts, and a dose dependent thrombopenia. This thrombopenia appeared to be reversible in spite of continuation of the treatment. After a single dose of G-CSF the granulocytosis was noticed as early as two hours after administration, indicating mobilisation of granulocytes from the BM into the peripheral blood[236]. Similar results have been obtained in cynomolgus monkeys. Furthermore a shortening of cyclophosphamide induced granulocytopenia has been reported[226]. G-CSF also stimulates granulocyte production after BM transplantation[238].

GM-CSF has also been used successfully to reduce shortage of peripheral blood cells under several circumstances. This will be discussed in section 1.4. The influence of other hemopoietic growth factors on chemo- and radiotherapy induced pancytopenia is subject of ongoing studies. Since synergism

between different growth factors has been demonstrated in vitro[262,263,264], as well as in mice[265,266], maximal reduction of pancytopenia can be expected from a combination of synergistically acting hemopoietic growth factors. However, in patients with hematological malignancies, such treatments should be considered with great caution, as hemopoietic growth factors have been shown to induce proliferation of leukemic cells in vitro[179,221-224].

Treatment with hemopoietic growth factors is expected to be beneficial in BM insufficiency caused by a wide variety of diseases such as aplastic anemia, myelodysplasia, BM infiltration by malignant cells, acquired immunodeficiency syndrome (AIDS) and other infections. In such conditions, the regulation of hemopoiesis is disturbed and growth factor responses might be different from normal regenerating BM.

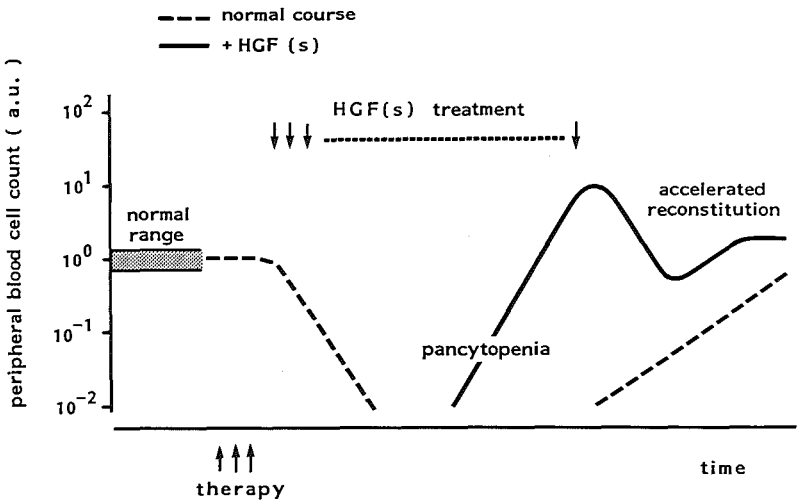

Figure 1.2 **Putative effects of hemopoietic growth factors (HGFs) following high dose radio-/chemotherapy, electively followed by BM transplantation**: stimulation of lymphohemopoietic reconstitution (mechanism 1). For explanation, see text.

The induction of leukemic cell proliferation by hemopoietic growth factors may provide a means to make those cells more sensitive for S-phase specific cytostatic agents, such as cytosine arabinoside (Ara-C). Recently, enhancement of *in vitro* Ara-C cytotoxicity on AML clonogenic cells by **multi-CSF** pretreatment has been demonstrated[267]. This application could also result in an increased toxicity for normal BM cells, recruited into cycle as well. Consequently a longer duration of the pancytopenia can be expected, unless BM toxicity is not the dose limiting factor, such as in conditioning regimens for BM transplantation. The patterns of responsiveness of clonogenic leukemic cells to growth factors show a striking variability[179]. Correlation of those patterns with morphological, clinical, and immunological datamight result in a better understanding of malignant growth, and to new classification criteria for leukemia.

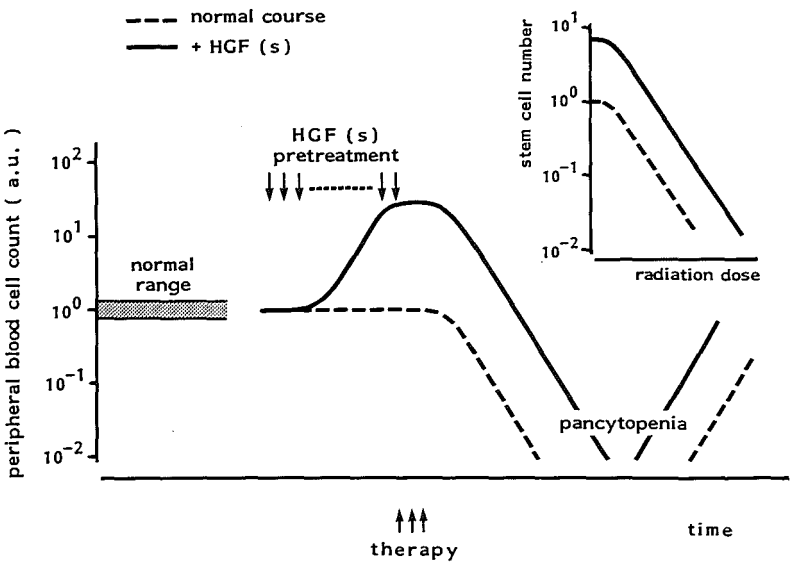

Figure 1.3 **Putative effects of hemopoietic growth factors (HGFs) before high dose radio-/chemotherapy**: radioprotection by increases in peripheral blood cells (mechanism 2) and in stem cells (mechanism 3). For explanation, see text.

Hemopoietic growth factors which stimulate the proliferation of HSCs are, in principle, useful for the development of HSC assays *in vitro*. In patients with pancytopenia, induced by irradiation and or chemotherapy, regeneration of peripheral blood cells without BM transplantation will be dependent on the number of residual repopulating HSCs. A response of peripheral blood cell counts upon *in vivo* administration of appropriately selected HGFs could possibly be used in such patients to estimate the number of repopulating HSCs *in vivo*, which enables the prediction of endogenous regeneration. This will be discussed for GM-CSF in Chapter VII.

Graft failure is one of the principle problems of BM transplantation. In human patients receiving autologous BM transplantation, the number of transplanted HSCs is probably lower than in allogeneic BM transplantation due to previous exposure to anticancer agents and due to storage. In allogeneic BM transplantation, GvHD can be prevented by T-cell depletion, but this procedure results in an increased risk of graft rejection (reciprocal interference[268]), especially in MHC mismatched donor recipient combinations. The probability of graft failure following BM transplantation can be reduced by increasing the graft size[269], but the number of HSC that can be harvested is limited. This problem could be circumvented by selective multiplication of HSCs *in vitro*. In serum free, liquid cultures of mouse BM, containing murine IL3 and IL1, an actual increase of CFU-S has been demonstrated. However, the increase of day 8 CFU-S was higher than that of day 12 CFU-S, indicating the induction of differentiation rather than that of self-replication. Under those culture conditions repopulating HSCs were maintained, whereas the number of T-lymphocytes was largely reduced. This has been demonstrated by semiallogeneic BM transplantation with a limited number of cultured BM and spleen cells resulting in sustained chimerism in the absence of GvHD[270]. Control mice, which received the same cell suspensions before culturing invariably died from GvHD. Pilot experiments in Rhesus monkeys have demonstrated the maintenance of repopulating HSCs as well[270]. However, the actual application of such a method for BM transplantation in humans still requires considerable developmental research

in preclinical models such as the Rhesus monkey.

In addition to stimulation of hemopoiesis following chemotherapy or irradiation (mechanism 1, Figure 1.2), treatment with hemopoietic growth factors before chemotherapy or TBI could expand the number of peripheral blood cells, resulting in a later onset of the pancytopenia (mechanism 2, Figure 1.3). However, since most blood cells have a very short lifespan, this mechanism may only be relevant for some cell types such as lymphocytes and thrombocytes. Pretreatment with HGFs could also expand the HSC and progenitor cell compartment, resulting in a reduction of pancytopenia (mechanism 3, Figure 1.3). However, such a pretreatment with HGFs stimulates HSC into cycle, making them more sensitive for cycle specific cytotoxic agents[271,272]. Again, considerable research will be required to select the appropriate (combination of) growth factor(s) and administration schedules, and to avoid possible undesired effects.

The development of retrovirus mediated gene transfer into the hemopoietic system requires DNA replication and, hence, *in vitro* self-replication of HSCs. Therefore, definition of the culture conditions for selective multiplication of HSCs will be required for this type of genetic modification of hemopoietic cells.

1.4. Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF).

1.4.1. Biochemistry and molecular biology of human GM-CSF.

The complementary DNA (cDNA) of human GM-CSF has been cloned from a cDNA library which was constructed from messenger RNA (mRNA) of the Mo cell line (a HTLV transformed T-cell line) in p91203(B) expression vectors. These constructs were transfected into COS-1 monkey cells and screened for GM-CSF expression by testing the ability of the COS-1 supernatant to stimulate colony formation by the KG-1 human myeloid leukemia cell line and by normal human BM. The GM-CSF cDNA contains a

single long open reading frame of 432 nucleotides, encoding 144 amino acids. Comparison of this sequence with that of natural GM-CSF, purified from Mo cell-conditioned medium suggested, that GM-CSF is synthesized as a precursor from which 17 amino acids are cleaved to yield the 127 amino acids mature protein. After purification, the mature protein is heterogenous when analyzed by sodium dodecyl sulfate-plyacrylamide gel electrophoresis (SDS-PAGE), migrating with an apparent molecular weight of 18 to 24 kD. This is probably due to variable glycosylation; the protein contains two potential NH_2-glycosylation sites. The nucleotide sequence of the isolated cDNA consists of 754 nucleotides including 8 nucleotides of 5' and 314 nucleotides of 3' non coding regions. Sequence homology between murine and human GM-CSF is 70 % for nucleotides and 60 % for amino acids[191] Independently, almost identical cDNA have been isolated by other groups[192-194]. The minor differences between those cDNAs may represent allelic variation.

By in situ hybridisation, the gene has been localized to chromosome 5q21-32[195]. The gene encoding for IL3[273] has been localized in the same region. The genes encoding for GM-CSF and IL3 have been demonstrated to be separated by only 9 kilobases of DNA[186]. Also the genes encoding for M-CSF[274], endothelial cell growth factor[275], IL5[276], the receptor of platelet derived growth factor[277], and for the c-fms proto-oncogene, (the receptor for M-CSF[278,279,280]), are localized to this region of chromosome 5. Thus all of these genes are deleted in the 5q- syndrome. It is temptating to speculate on a possible relationship between deletions of genes encoding HGFs and/or receptors in the 5q- syndrome and the pathogenesis of myelodysplastic syndromes.

Sequences coding for the human GM-CSF protein were expressed in Escherichia Coli, resulting in a high yield synthesis of nonglycosylated human GM-CSF. This preparation contains an extra methionine residue at position 1 and has the same biologic activity as purified natural GM-CSF[133,196].

1.4.2. Influence of human GM-CSF on bone marrow cells in culture.

GM-CSF was initially described as a factor stimulating the formation of granulocyte and/or macrophage colonies[188]. It is now clear, that GM-CSF also induces colonies of other cell lineages[189]. In contrast to semipurified natural G-CSF, recombinant GM-CSF induces colony formation at day 14 rather than at day 7, just like semipurified natural GM-CSF[197-198]. The day 14 colonies are derived from CFU-GM, CFU-M, CFU-G, and CFU-EO. GM-CSF alone does not stimulate erythroid or mixed colonies. However, addition of GM-CSF to cultures containing Ep results in the formation of two- to threefold higher numbers of erythroid colonies (BFU-E) than in cultures containing only Ep, and in the formation of small numbers of CFU-MIX[189]. After elimination of adherent cells, GM-CSF does not enhance erythroid colony formation[199]. Furthermore, if clones, initiated in GM-CSF containing cultures, are subsequently transferred to cultures stimulated with GM-CSF and Ep erythroid colony formation does not occur[197]. Those data indicate, that the influence of GM-CSF on erythroid colony formation requires the presence of accessory cells, which may produce (an) additional HGF(s). GM-CSF has also been found to stimulate megakaryocytopoiesis *in vitro*[200]. In cultures of CD34 positive, monocyte depleted human BM cells, GM-CSF predominantly stimulates CFU-Eo, but no CFU-GM, CFU-M or CFU-G[281].

GM-CSF stimulates *in vitro* colony formation and a limited differentiation of AML cells from most patients[179,221-224]. GM-CSF also stimulates the growth of several human leukemic cell lines such as HL-60 and KG-1. In the former, but not in the latter, GM-CSF induces differentiation[199].

In addition to its effects on progenitor cells, GM-CSF also induces functional changes in mature cells. These effects on neutrophils include enhancement of phagocytosis[201], chemotaxis[202,203], lysozyme secretion, and superoxide production in response to bacterially derived f-Met-Leu-Phe[204,205]. In monocytes, GM-CSF enhances cytotoxicity *in vitro*[206], superoxide production, phagocytosis, and class II MHC expression[207]. Some of

these functional changes are probably related to GM-CSF induced expression of CD11b[208] a surface glycoprotein that is essential for adhesion dependent granulocyte functions such as phagocytosis, aggregation and chemotaxis[209].

1.4.3. Clinical applications of GM-CSF.

GM-CSF has been used in clinical trials to examine its benefits following chemotherapy, electively supported by autologous BM transplantation[211-214]. In patients treated with 8-32 μg/kg GM-CSF, the average duration of leukopenia was two days shorter than in historical controls. Conflicting results were obtained on the effect of GM-CSF on thrombocyte counts. Doses up to 16 μg/kg were tolerated well, but those 4 patients who received a dose of 32 μg/kg/day developed edema. Also other phase I trials, which are not effectivity studies, point to a reduction of chemotherapy related pancytopenia.

In patients with pancytopenia due to myelodysplasia, lower doses (30-250 $\mu g/m^2$/day, approximately 0.8-7 μg/kg) resulted in a rise in leukocytes, whereas higher doses (120-500 $\mu g/m^2$, approximately 3-15 μg/kg) induced a rise in hematocrit values thrombocyte counts and a reduction in transfusion dependency[215].

In leukopenic patients with AIDS, administration of GM-CSF resulted in an increase of granulocytes, eosinophils and monocytes. Following high doses also a rise of lymphocyte numbers was described, but significant changes in reticulocyte- and thrombocyte counts were not noted[216].

Victims of a recent irradiation accident have been treated with GM-CSF[217], but the efficacy of this treatment is difficult to interpret, since adequate control groups are lacking, and the irradiation dose on the BM is difficult to assess following accidental irradiation which is in general inhomogeneous.

Preclinical data in primate models included an increase in peripheral blood cell counts in healthy, not pretreated animals[218,219] and a reduction of the pancytopenic period following TBI and autologous BM transplantation[220,225].

1.5. The Rhesus monkey as a preclinical model for studies on hemopoiesis.

For nearly 30 years the Rhesus monkey (Macaca mulatta) has been used for investigations on BM transplantation. Since the early 1960s it was felt, that the gap between mouse models and clinical BM transplantation had to be bridged by a species that was more similar to man.

Human and Rhesus monkey BM have many similar properties: human and Rhesus monkey BM contain approximately the same numbers of CFU-C[282]. After albumin density centrifugation of human and Rhesus monkey BM, HSCs and progenitor cells can be found in the same fractions[283]. In addition, in contrast to mouse BM, a high content of T cells is present in human as well as subhuman primate BM[284], capable to induce severe Graft versus Host Disease (GvHD), even in MHC identical donor recipient combinations[285]. Furthermore, the Major Histocompatibility Complex (MHC) of the Rhesus monkey, termed RhLA, closely resembles the human HLA system[286]. A number of monoclonal antibodies (MCA) directed against human cell surface antigens cross react with antigens on the same cells of Rhesus monkeys[287,288,289].

The similar properties of monkey and human BM have been used to test many techniques involved in BM transplantation before their introduction into clinical practice: methods for cryopreservation of HSCs[290], conditioning regimens[291], and mitigation of GvHD, by methotrexate[292], by antilymphocyte globulin[293,294], by MHC matching[295], by decontamination of the digestive tract and by T cell depletion[296,297].

The frequency of HSCs in mammalian BM is supposed to be inversely correlated with body weight, implying a lower HSC frequency in man than in Rhesus monkeys[298]. This idea is based on extrapolation of differences in the LD_{50} of TBI in different species and on the minimal number of transplanted BM cells per kg required for survival after high dose TBI. It is not clear

whether these differences can be directly related to stem cell numbers. Until now, other differences in Rhesus monkey and human hemopoiesis have not been demonstrated.

Clinical treatment of patients, including full supportive care, can be imitated under standard conditions in Rhesus monkeys.

The disadvantages of the monkey model are mostly technical, and the high costs of experiments with Rhesus monkeys prevent the use of large numbers of animals.

For the reasons mentioned above, the majority of data obtained in Rhesus monkeys, can be extrapolated to the clinical situation with more confidence than data obtained in more generally used laboratory animals, making the Rhesus monkey the most reliable animal for preclinical studies on hemopoiesis and BM transplantation.

1.6. Rationale of the study.

The hemopoietic system is highly sensitive to cytotoxic damage, such as chemotherapy and ionising irradiation. Therefore survival following high dose chemotherapy and/or TBI, electively in combination with BM transplantation is only possible because the system has a great regenerative capacity. Hemopoietic regeneration following therapeutic or accidental cytotoxic damage, cannot always be predicted since quantitative information on the process of regeneration is insufficient. Self replication and differentiation of HSCs and progenitor cells are essential in this process. Therefore a sufficient number of HSCs is required for regeneration. Unfortunately, a suitable assay for human HSCs is not available. Cell surface markers expressed on HSCs could possibly be used for the development of such an assay. Furthermore characterization of HSC surface markers can be used for the preparation of BM grafts as discussed in section 1.1. In the experimental work described in chapter III, a recently developed *in vivo* HSC assay is used to investigate the expression of the CD34 antigen on

repopulating HSCs.

Another approach to estimate the number of HSCs and to predict endogenous regeneration following cytotoxic damage, is a calculation based on the sensitivity of HSCs for chemotherapy or irradiation. Radiosensitivity can be characterized quantitatively by the relationship between the irradiation dose and the fraction of surviving HSCs. In Chapter IV this relationship will be described for 6 MV and for 300 kV X-rays. Such quantitative information on HSC numbers is essential for the assessment of the role of BM transplantation in the treatment of victims of radiation accidents.

Even if the radiosensitivity and the original number of HSCs is precisely known, inadequate dosimetric information and inhomogeneous irradiation might hamper a precise estimate of the number of surviving HSCs and a reliable prediction of endogenous regeneration in victims of radiation accidents. A response of peripheral blood cell counts upon *in vivo* administration of appropriately selected HGFs could possibly be used in such patients to estimate the number of residual HSCs *in vivo*, which enables the prediction of endogenous regeneration. This application of HGFs, and especially GM-CSF, will be discussed in Chapter VII.

If the number of surviving and/or transplanted HSCs is sufficient for regeneration, this process is a matter of self replication and differentiation. HGFs might accelerate this process by stimulating the proliferation of HSCs and progenitor cells. Furthermore HGFs can stimulate the functional activity of peripheral blood cells. In Chapter V, the effect of GM-CSF on peripheral blood cells in normal monkeys will be described. In Chapter VI and VII, the possible beneficial influence of GM-CSF on hemopoietic regeneration following TBI with or without BM transplantation will be described in relation to the number of surviving HSCs.

REFERENCES

1. Jacobson, L.O., Marks, E.K., Robson, M.J., Gaston, O.E. and Zirkle, R.E. (1949) The role of the spleen in radiation injury. Proc. Soc. Exp. Biol. Med. 70:740-742.

2. Lorenz, E., Uphof, D.E., Reid, T.R., and Shelton, E. (1951) Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J. Nat. Cancer Inst. 12:197-201.

3. Jacobson, L.O., Simmons, E.L., Marks, E.K., and Eldredge, J.H. (1951) Recovery from radiation injury. Science 133:510.

4. Ford, C.E., Hamerton, J.L., Barnes, D.W.H., and Loutit, J.F. (1956) Cytological identification of radiation chimeras. Nature 177:452-454.

5. Vos, O., Davids, J.A.G., Weyzen, W.W.H., and Bekkum, D.W. van (1956) Evidence for the cellular hypothesis in radiation protection by bone marrow cells. Acta Physiol. Pharmacol. Neerl. 4:482-486.

6. Nowell, P.C., Cole, L.J., Habermeyer, J.G., and Roan, P.L. (1956) Growth and continued function of rat marrow cells in X-radiated mice. Cancer Res. 16:258-261.

7. Till, J.E., and McCulloch, E.A. (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Rad. Res. 14:213-222.

8. Becker, A.J., McCulloch, E.A., and Till, J.E. (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplantated mouse marrow cells. Nature 197:452-454.

9. Trentin, J.J., and Fahlberg, W.J. (1963) An experimental model for studies of immunological competence in irradiated mice repopulated with "clones" of spleen cells. In: Conceptual Advances in Immunology and Oncology, 16th Annual Symposium (1962). Texas, Harper & Row, New York, p66-74.

10. Nowell, P.C, Hirsch, B.E., Fox, D.H., and Wilson, D.B. (1970) Evidence for the existence of multipotential lympho-hematological stem cells in the adult rat. J. Cell. Physiol. 75:151.

11. Lemischka, I.R., Raulet, D.H., and Mulligan, R.C. (1986). Developmental potential and dynamic behaviour of hemopoietic stem cells. Cell 45:917-927.

12. Magli, M.C., Iscove, N.N., and Odartchenko, N. (1982) Transient nature of early hemopoietic spleen colonies. Nature 295:527-529.

13. Wolf, N.S., and Priestly, G.V. (1986) Kinetics of early and late spleen colony development. Exp. Hematol. 14:676-682.

14. Visser, J.W.M., Bol, S.J.L., and Engh, G. van den. (1981) Characterization and enrichment of murine hemopoietic stem cells by fluorescence activated cell sorting. Exp. Hematol. 9:644-655.

15. Harris, R.A., Hogarth, P.M., Wadeson, L.J., Collins, P., McKenzie, I.F.C., and Penington, D.G. (1984) An antigenic difference between cells forming early and late haematopoietic spleen colonies (CFU-S). Nature 307:638-641.

16. Monette, F.C., and Stockel, J.B. (1981) Immunological evidence for murine hematopoietic stem cell subpopulations differing in self renewal capacity. Stem cells 1:38-52.

17. Hodgson, G.S., and Bradley, T.R. (1979) Properties of hemopoietic stem cells surviving 5-Fluoro-uracil treatment: Evidence for a pre-CFU-S cell. Nature 281:381-382.

18. Hodgson, G.S., and Bradley, T.R. (1984) In vivo kinetic status of hemopoietic stem cells and progenitor cells as inferred from labelling with bromodeoxyuridin. Exp. Hematol. 12:683-687.

19. Hodgson, G.S., Bradley, T.R., and Radley, J.M. (1982) The organization of hemopoietic tissue as inferred from the effects of 5-fluorouracil. Exp. Hematol. 10:26-35.

20. Zant, G. van. (1984) Studies on hemopoietic stem cells spared by 5-fluorouracil. J. Exp. Med. 159:679-690.

21. Baines, P., and Visser, J.W.M. (1983) Analysis and separation of murine bone marrow stem cells by H33342 Fluorescence-Activated Cell Sorting. Exp.Hematol. 11:701-708.

22. Pluznik, D.H., and Sachs, L. (1965) The cloning of normal "mast" cells in tissue culture. J. Cell. Comp. Physiol. 66:319-324.

23. Bradley, T.R., and Metcalf, D. (1966) The growth of mouse bone marrow cells in vitro. Aust. J. Exp. Biol. Med. Sci. 44:287-300.

24. Stephenson, J.R., Axelrad, A., McLeod, D.L., and Schreeve, M.M. (1971) Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro. Proc. Nat. Acad. Sci. U.S.A. 68:1542-1546.

25. Metcalf, D., Parker, J., Chester, H.M., and Kincade, P.W. (1974). Formation of eosinophilic granulocytic colonies by mouse bone marrow cells in vitro. J. Cell. Physiol. 84:275-290.

26. Nakeff, A., Dicke, K.A., and Noord, M.J. van (1975) Megakaryocytes in agar cultures of mouse bone marrow. Ser. Haematol. 8:4-21.

27. Metcalf, D., MacDonald, H.R., Odartchenko, N., and Sordat, B. (1975) Growth of mouse megakaryocyte colonies in vitro. Proc. Nat. Acad. Sci. U.S.A. 72:1744-1748.

28. Metcalf, D., Warner, N.L., Nossal, S.J.V., Miller, J.F.A.P., Shortman, K., and Rabellino, E. (1975) Growth of B lymphocyte colonies in vitro from mouse lymphoid organs. Nature 255:630-632.

29. Rosenszajn, L.A., Shoham, D., and Kalechman, I. (1975) Clonal proliferation of PHA-stimulated human lymphocytes in soft agar culture. Immunology 29:1041-1055.

30. Lajtha, L.G. (1979) Haemopoietic stem cells: Concepts and definitions. Blood Cells 5:447-459.

31. Fausser, A.A., and Messner, H.H. (1979) Identification of megakaryocytes, macrophages, and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. Blood 53:1023-1027.

32. Grossman, Z. (1986) The stem cell concept revisited: self-renewal capacity is a dynamic property of hemopoietic cells. Leukemia Res. 10:937-950.

33. Kay, H.E.M. (1965) How many generations. Lancet II:418-419.

34. Brecher, G., Beal, S.L., and Schneiderman. (1986) Renewal and release of hemopoietic stem cells: Does clonal selection exist? Blood Cells 12:103-112.

35. Kay, H.E.M. (1986) Commentary: Clonal succession revisited. Blood cells 12:113-117.

36. Till, J.E., McCulloch, E.A., and Siminovitch, L.A. (1964) A stochastic model of stem cell proliferation, based on the growth of spleen colony forming cells. Proc. Natl. Acad. Sci. USA 51:29-36.

37. Johnson, G.R. (1981) Is erythropoiesis an obligatory step in the commitment of multipotential hematopoietic stem cells? In: Experimental hematology today. Eds.: Baum, S.J., Ledley, G.D., and Kahn, A. New York: Springer-Verlag p. 13-20.

38. Dexter, T.M. (1982) Stromal cell associated hemopoiesis. J. Cell. Physiol. 1(suppl):87-94.

39. Metcalf, D., and Burgess, A.W. (1982) Clonal analysis of progenitor cell commitment to granulocyte or macrophage production. J. Cell. Physiol. 3:275-283.

40. Brown, G., Bunce, C.M., Howie, A.J., and Lord, J.M. (1987) Editorial: Stochastic or ordered lineage commitment during hemopoiesis? Leukemia1:150-153.

41. Bekkum, D.W. van, Noord, M.J. van, Maat, b., and Dicke, K.A. (1971) Attempts at identification of hemopoietic stem cells in mouse. Blood 38:547-558.

42. Bekkum, D.W. van (1977) The appearance of the multipotential hemopoietic stem cell. In: Experimental hematology today. Eds.: Baum,S.J. and Ledney,G.D. Springer-Verlag, New York. p. 3-19.

43. Dicke, K.A., Noord, M.J. van, Maat, B., Schaefer, U.W, and Bekkum, D.W. van, (1973) Identification of cells in primate bone marrow resembling the hemapoietic stem cell in the mouse. Blood 42:195-208.

44. Dicke, K.A., Noord, M.J. van, Maat, B., Schaefer, U.W., and Bekkum, D.W. van (1973) Attempts at morphological identification of the hemopoietic stem cell in primates and rodents. In: Hemopoietic stem cells, Ciba Foundation Symposium, Vol 13. Elsevier, Amsterdam. p. 47-69.

45. Visser, J.W.M, Bauman, J.G.J, Mulder, A.H., Eliason, J.F., and Leeuw, A.M. de (1984) Isolation of murine pluripotent hemopoietic stem cells. J. Exp. Med. 59:1576-1590.

46. Spragrude, G.J. (1989). Enrichment of murine stem cells: diverging roads. Immunology today. 10:344-350.

47. Civin, C.I., Strauss, L.C., Broval, C., Fackler, M.J., Schwartz, J.F., and Shaper, J.H. (1984) Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against Kg-1a cells. J. Immunol. 133:157-165.

48. Andrews, R.G., Singer, J.W., and Bernstein, I.D. (1986) Monoclonal antibody 12-8 recognizes a 115-kd molecule present on both unipotent and multipotent hematopoietic colony-forming cells and their precursors. Blood 67:842-845.

49. Tindle, R.W., Nichols, R.A.B., Chan, L., Campana, Catowski, D., and Birnie, G.D. (1985) A novel monoclonal antibody BI-3C5 recognizes myeloblasts and non-B

non-T lymphoblasts in acute leukemias and CGL blast crisis, and reacts with immature cells in normal bone marrow. Leukemia Res. 9:1-9.

50. Watt, S.M., Karhi, K., Gatter, K., Furley, A.J.W., Katz, F.E., Healey, L.E., Atass, L.J., Bradley, N.J., Sutherland, D.R., Levinski, R., and Greaves, M.F. (1987) Distribution and epitope analysis of the cell membrane glycoprotein (HPCA-1) associated with human hemopoietic progenitor cells. Leukemia 1:417-426.

51. Civin, C.I., Banquerigo, M.L., Strauss, L.C., and Loken, M.R. (1987). Antigenic analysis of hematopoiesis. VI. Flow cytometric characterization of My-10-positive progenitor cells in normal human bone marrow. Exp. Hematol. 15:10-17.

52. Gerritsen, W.R. Personal communication.

53. Gerritsen, W.R., Wagemaker, G., Jonker, M., Kenter, M., Wielenga, J.J., Hale, G., Waldmann, H., and Bekkum, D.W. van. (1988) The repopulating capacity of bone marrow grafts following pretreatment with monoclonal antibodies against T lymphocytes in rhesus monkeys. Transplantation 45:301-307.

54. Lapidot, T., Singer, T.S., and Reissner, Y. (1988) Transient engraftment of T cell-depleted allogeneic bone marrow in mice improves survival rate following lethal irradiation. Bone Marrow Transplantation 3, 157-164.

55. Monroy, R.L., Vriesendorp, H.M., and MacVittie, T.J. (1987) Improved survival of dogs exposed to fission neutron irradiation and transplanted with DLA identical bone marrow. Bone Marrow Transplantation 2:375-384.

56. Jammet, H., Mathé, G., and Pendic, B. (1959) Etude de six cas d'irradiation total d'aigue accidentale. Rev. Franc. Etudes Clin. Biol. 4:210-228.

57. Gale, R.P. and Reissner, Y. (1988) The role of bone-marrow transplantation after nuclear accidents. Lancet I:923-926.

58. Baranov, A., Gale, R.P., Guskova, A., Piatkin, E., Selidovkin, G., Muravyova, L., Champlin, R.E., Danilova, N., Yevseeva, L., Petrosyan, L., Pushkareva, S., Konchalovsky. Gordeeva, A., Protasova, T., Reissner, Y., Mickey, M.R., and Terasaki, P.I. 1989. Bone marrow transplantation after the Chernobyl nuclear accident. New Engl. J. Med. 321:205-212.

59. Gale, R.P. (1987) The role of bone marrow transplantation following nuclear accidents. Bone marrow transplantation 2:1-6.

60. Barret, A. (1984) Total Body Irradiation and LD_{50} in man. In: Response of different species to total body irradiation. Eds.: Broerse, J.J., and MacVittie, T.J. Martinus Nijhoff publishers. Boston, Dordrecht, Lancaster. p. 205-208.

61. Hendry, J.H., and Lord, B.I. (1983) The analysis of the early and late response to cytotoxic insults in the hemopoietic cell hierarchy. In: Cytotoxic Insult to Tissue. Effects on cell lineages. Eds.: Potten, C.S., and Hendry, J.H. Churchill Livingstone. Edinburgh, London, Melbourne and New York. p. 1-67.

62. Broerse, J.J., Barendsen, G.W., and Kersen, G.R. (1968) Survival of cultured human cells after irradiation with fast neutrons of different energies in hypoxic and oxygenated conditions. Int. J. Radiat. Biol. 6:559-572.

63. Freeman, M.L., Boone, M.L.M., Ensley, B.A., and Gilette, E.L. (1981) The influence of environmental pH on the interaction and repair of heat and radiation damage. Int. J. Radiat. Oncol. Biol. Phys. 7:761-764.

64. Meyne, E.I.M., Davids, J.A.G., Ploemacher, R.E., Vos, O., and Huiskamp, R. (1989). The effects of 1 MeV fission neutrons and X-rays on murine haematopoietic stem cells. Int. J. Radiat. Biol. 55:1040. (abstr.)

65. Meijne, E.I.M., Davids, J.A.G., Ploemacher, R.E., Vos, O., and Huiskamp, R. (1989). The effects of 1 MeV fission neutrons and X-rays on hematopoietic stem cell compartments in the mouse. 22nd annual meeting of the European Society for Radiation Biology. Book of Abstracts p 131.

66. Dutreix, J., Gluckman, E., and Brule, J.M. (1982). Biological problems of total body irradiation. J. Eur. Radiother. 3:165-173.

67. Fu, K.K., Philips, T.L., Kane, L.J., Smith, V. (1975) Tumor and normal tissue response to irradiation in vivo: variation with decreasing dose rates. Radiology 114:709-716.

68. Belletti, S., Gallini, R.E., Magno, L. (1979) The effects of dose-rate on hematopoietic stem cells: preliminary results. Int. J. Rad. Oncol. Biol. Phys. 5:403-405.

69. Broxmeyer, H.E., Galbraith, P.R., and Baker, F.L. (1976) Relationship of colony-stimulating activity to apparent kill of human colony-forming cells by irradiation and hydroxyurea. Blood 47:403-411.

70. Vriesendorp, H.M. and van Bekkum, D.W. (1980) Role of total body irradiation in conditioning for bone marrow transplantation In: Immunobiology of Bone Marrow Transplantation. Eds.: Thierfelder, S., Rodt, H., and Kolb, H.J. Springer Verlag, Berlin, Heidelberg, New York. p.349-364.

71. McCulloch, E.A, and Till, J.E. (1962) The sensitivity of normal bone marrow cells to Á-rays in vivo and in vitro. Rad. Res. 16:822-832.

72. Hendry, J.H. (1972) The response of hemopoietic colony-forming units and lymphoma cells irradiated in soft tissue (spleen) or bone cavity (femur) with single doses of X-rays, Á-rays or D-T neutrons. Br. J. Rad. 45:923-932.

73. Carlson, D.E., and Thornton, J. (1977) Depth-related differences in bone marrow cell inactivation with ^{60}Co Á-rays. Radiation Research 70, 564-574.

74. Wu, C., and Lajtha, L.G. (1975). Haemopoietic stem-cell kinetics during continuous irradiation. Int. J. Rad. Biol. 27:41-50.

75. Putten, L.M. van, Lelieveld, P., and Broerse, J.J. (1969) Effects of 300 kV X-rays and 15 MeV neutrons in single and fractionated doses on hemopoietic stem cells in mouse bone marrow. In: Time and dose relationships in radiation biology as applied to radiotherapy. NCI-AEC conference Carmel, California. Brookhaven Laboratory, Upton, New York. p. 161-166.

76. Sillini, G. (1967) Control of hemopoiesis and the action of radiation on the blood forming organs. In: Effects of ionising irradiation on haemopoietic tissue. IAEA, Vienna, p 52.

77. Imai, Y., Nakao, I. (1987). In vivo radiosensitivity and recovery pattern of the hematopoietic precursor cells and stem cells in mouse bone marrow. Exp. Hematol 15:1987:890-895.

78. McCulloch, E.A, and Till, J.E. (1960) The radiation sensitivity of normal mouse

bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Rad. Res. 13:115.

79. Smith, L.H., Vos, O. (1962) Sensitivity and protection of bone marrow cells X-irradiated in vitro. Int. J. Rad. Biol. 5:461-470.

80. Senn, J.S., and McCulloch, E.A. (1970) Radiation sensitivity of human bone marrow cells measured by a cell culture method. Blood 35:56-60.

81. Testa, N.G., Hendry, J.H., Lajtha, L.G. (1973) The response of mouse haemopoietic colony-formers to acute or continuous gamma irradiation. Biomedicine express 19:183-186.

82. Wilson, F.D., O'Grady, L., McNeil, C.J., and Munn,S.L. (1974) The formation of bone marrow derived fibroblastic plaques in vitro: preliminary results contrasting these populations to CFU-C. Exp. Hematol. 2:343-354.

83. Comas, F.V. (1970) The radiosensitivity of rat bone marrow cells. Int. J. Rad. Biol. 17:549-557.

84. Nothdurft, W., Fliedner, T.M. (1980) Acute and delayed effects of total body irradiation on the granulocytic progenitor cell compartment in dogs. Rad. Envir. Biophys. 17:284 (abstract).

85. Wilson, F.D., Stitzel, K.A., Klein, A.K., Shifrine, M., Graham, R., Jones, M., Bradley, E., Rosenblatt, L.S. (1978) Quantitative response of bone marrow colony-forming units (CFU-C and PFU-C) in weanling beagles exposed to acute whole body Á-irradiation. Rad. Res. 74:289-297.

86. Kreja, L., Baltschukat, K., and Nothdurft. (1989). In vitro studies of the sensitivity of canine bone marrow erythroid burst-forming units (BFU-E) and fibroblast colony-forming units (CFU-F) to X-irradiation. Int. J. Radiat. Biol. 55:435-444.

87. Grilli, G., Nothdurft, W., and Fliedner, T. M. (1982). Radiation sensitivity of human erythropoietic and granulopoietic progenitor cells in the blood and in the bone marrow. Int. J. Radiat Biol. 41:685-687.

88. Wagemaker, G. (1985) Hemopoietic cell differentiation. In: Bone marrow transplantation. Biological mechanisms and clinical practice. Eds.: Bekkum, D.W. van, and Löwenberg, B. p. 1-72.

89. Zilberstein, A., Ruggieri, R., Korn, J.H., and Revel, M. (1986) Structure and expression of cDNA and genes for human interferon-beta-2, a distinct species inducible by growth-stimulatory cytokines. EMBO J. 5:2529-2537.

90. Damme, J.van, Opdenakker, G., Simpson, R.J., Rubira, M.R., Cayphas, S., Vink, A., Billiau, A., and Snick, J. van. (1987) Identification of the human 26-kD protein, interferon beta 2 (IFN-ß-2), as a B-cell hybridoma/plasmocytoma growth factor induced by interleukin 1 and tumor necrosis factor. J. Exp. Med. 165:914-919.

91. Gauldie, J., Richards, C., Harnish, D., Lansdorp, P., and Bauman, H. (1987) Interferon beta-2 is identical to monocyte derived hepatocyte stimulating factor and regulates the full acute phase response in liver cells. Proc. Natl. Acad. Sci. USA 84:7251-7255.

92. Herberman, R.B. (1986) Effects of biological response modifiers on effector cells with cytotoxic activity against tumors. Semin. Oncol. 13:195-199.

93. Foon, K.A., Roth, M.S., and Bunn, Jr. P.A. (1986) Alpha interferon treatment of low-grade B-cell non-Hodgkin's lymphoma's, cutaneous T-cell lymphomas, and chronic lymphocytic leukemia. Semin. Oncol. 13:Suppl 2:35-42.

94. Talpaz, M.,McCredie, K., Kantarjian, H., Trujillo, J., Keating, M., and Gutterman, J. (1986) Chronic myelogenous leukemia: haematological remissions with alpha interferon. Br.J.Haematol 64:87-95.

95. Quesada, J.R., Reuben, J., Manning, J.T., Hersh, E.M., and Gutterman, J.U. (1984) Alpha interferon for induction of remission in hairy-cell leukemia. N.Engl J.Med. 310:15-18.

96. Dinarello, C.A., and Mier, J.W. (1987) Current concepts: Lymphokines. N.Engl. J. Med. 317:940-945.

97. Pace, J.L., Russel, S.W., Schreiber, R.D., Altman, A., and Katz, D.H. (1983) Macrophage activation: Priming activity from a T-cell hybridoma is attributable to interferon-Á. Proc. Natl. Acad. Sci. USA 80:3782-3786.

98. Sidman, C.L., Marshall, J.D., Shultz, L.D., Gray, P.W., and Johnson, H.M. (1984) Á-interferon is one of several direct B cell-maturing lymphokines. Nature 309:801-804.

99. Leibson, H.J., Gefter, M., Zlotnik, A., Marrack, P., and Kappler, J.W. (1984) Role of Á-interferon in antibody producing responses. Nature 309:799-801.

100. Dinarello, C.A. (1984) Interleukin-1 and the pathogenesis of the acute- phase response. N. Engl. J. Med. 311:1413-1418.

101. Fibbe, W.E. (1988) Interleukin-1, a regulator of hemopoiesis. Thesis, University of Leiden, Leiden, The Netherlands.

102. Auron, P.E., Webb, A.C., Rozenwasser, L.J., Mucci, S.F., Rich, A., Wolff, S.M., and Dinarello, C.A. (1984) Nucleotide sequence of human monocyte interleukin-1 precursor cDNA. Proc. Natl. Acad. Sci. USA 81:7907-7911.

103. March, C.J., Mosley, B., Larsen, A., Cerreti, D.P., Braedt, G., Price, V., Gillis, C.S., Henney, S.R., Kronheim, K., Grabstein, P.J., Conlon, P.J., Hopp, T.P., and Cosman, D. (1985) Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature 315:641-647.

104. Oppenheim, J.J., Kovacs, E.J., Matsushima, K., and Durum, S.K. (1986) There is more than one interleukin 1. Immunol. Today 7:45-56.

105. Lepe-Zuniga, B., and Gery, I. (1984) Production of intracellular and extracellular interleukin-1 by human monocytes. Clin. Immunol. Immunopathol. 31:222-230.

106. Watsuskina, K., Taguski, M., Kovacs, E.J., Young, H.A., and Oppenheim, J.J. (1987) Intracellular localization of human monocyte associated interleukin-1 activity and release of biologically active IL-1 from monocytes by trypsin and plasmin. J. Immunol. 136:2883-2891.

107. Dinarello, C.A. (1988) Biology of interleukin-1. FASEB J. 2:108-115.

108. Gery, I., Gershon, R.K., and Waksman, B.H. (1972) Potentiation of the T-lymphocyte response to mitogens. I. The responding cell. J. Exp. Med. 136:128-142.

109. Larsson, E.L., Iscove, N.N., and Coutinho, A. (1980) Two distinct factors are

required for induction of T-cell growth. Nature 283:664-666.

110. Smith, K.A., Lachmann, L.B., Oppenheim, J.J., and Favata, M.F. (1980). The functional relationship of the interleukins. J. Exp. Med. 151:1551.

111. Pike, B.L., and Nossal, G.J.V. (1985) Interleukin 1 can act as a B-cell growth and differentiation factor. Proc. Natl. Acad. Sci. USA. 82:8153-8157.

112. Luger, T.A., Charon, J.A., Colot, M., and Oppenheim, J.J. (1983) Chemotactic properties of partially purified human epidermal cell-derived thymocyte-activating factor (TAF) for polymorphonuclear and mononuclear cells. J. Immunol. 131:816-820.

113. Schmidt, J.A., Misel, S.B., Cohen, D., and Green, I. (1982) Interleukin 1 a potential regulator of fibroblast proliferation. J. Immunol. 128:2177-2182.

114. Krane, S.M., Dayer, J.M., Simon, L.S., and Byrne, S. (1985) Mononuclear cell-conditioned medium containing mononuclear cell factor (MCF), homologous with interleukin-1, stimulates collagen and fibronectin synthesis by adherent rheumatoid synovial cells: effects of prostaglandin E_2 and indomethacin. Coll. Relat. Res. 5:99-117.

115. Dinarello, C.A., and Wolff, S.M. (1982) Molecular basis of fever. Am. J. Med. 72:799-819.

116. Pepys, M.B., and Baltz, M.L. (1983) Acute phase proteins with special reference to C-reactive proteins (pentaxins) and serum amyloid A protein. Adv. Immunol. 34:141-212.

117. Yang, R.D., Moldawer, L.L., Sakamoto. (1983) Leukocyte endogenous mediator alters protein dynamics in rats. Metabolism 32:654-660.

118. Baracos, V., Rodeman, H.P., Dinarello, C.A., and Goldberg, A.L. (1983) Stimulation of muscle protein degradation and prostaglandin E_2 release by leukocytic pyrogen (interleukin-1). A mechanism for the increased degradation of muscle proteins during fever. N. Engl. J. Med. 308:553-558.

119. Caverzasio, J., Rizzolli, R., Dayer, J.M., and Bonjour, J.P. (1987) Interleukin-1 decreases renal sodium readsorption: possible mechanisms of endotoxin-induced natriuresis. Am. J. Physiol. 252:943-946.

120. Dewhirst, F.E., Stashenko, P.P., Mole, J.E., and Tsuramachi, T. (1985) Purification and partial sequence of human osteoclast activating factor. Identity with interleukin 1 beta. J. Immunol 135:2562-2568.

121. Bevilacqua, M.P., Pober, J.S., Majeau, G.R., Cotran, R.S., and Gimbrone, M.J. Jr. (1984) Interleukin-1 induces biosynthesis and cell surface expression of procoagulant activity on human vascular endothelial cells. J. Exp. Med. 160:618-623.

122. Dejana, E., Brevario, F., Erroi, A., Bussolino, F., Mussoni, L., Gramse, M., Pintucci, G., Dinarello, C.A., Damme, J. van, and Mantovani, A. (1987) Modulation of endothelial cell function by different molecular species of interleukin-1 Blood 69:695-699.

123. Nachman, R.L., Hajjar, K.A., Silverstein, R.L., and Dinarello, C.A. (1986) Interleukin-1 induces endothelial cell synthesis of plasminogen activator inhibitor. J. Exp. Med. 163:1595-1600.

124. Zucali, J.R., Broxmeyer, H.E., Dinarello, C.A., Gross, M.A., and Weiner. (1987) Regulation of early human hemopoietic (BFU-E and CFU-GEMM) progenitor cells in vitro by Interleukin-1 induced fibroblast conditioned medium. Blood 69:33-37.

125. Zucali, J.R., Dinarello, C.A., Oblon, D.J., Gross, M.A., Anderson, L., and Weiner, R.S. (1986) Interleukin-1 stimulates fibroblasts to produce granulocyte-macrophage colony-stimulating activity and prostaglandin E_2. J. Clin. Invest. 77:1857-1863.

126. Lee, M., Segal, G.S., and Bagby, G.C. (1987) Interleukin-1 induces bone marrow derived fibroblasts to produce multilineage hematopoietic growth factors. Exp. Hemat. 15:983-988.

127. Fibbe, W.E., Damme, J. van, Billiau, A., Voogt, P.J., Duinkerken, N., Kluck, P.M.C., and Falkenburg, J.H.F. (1986) Interleukin-1 (22-K factor induces release of granulocyte-macrophage colony-stimulating activity from human mononuclear phagocytes. Blood 68:1316-1321.

128. Renninck, D., Yang, J., Gemmel, L., and Lee, F. (1987) Control of hemopoiesis by a bone marrow stromal cell clone: lipopolysacharide and interleukin-1 inducible production of colony stimulating factors. Blood 69:682-691.

129. Fibbe, W.E., Damme, J. van, Billiau, A., Goselink, H.M., Voogt, P.J., Eden, G. van, Ralph, P., Altrock, B.W., and Falkenburg, J.H.F. (1988) Interleukin-1 induces human marrow stromal cells in long term culture to produce granulocyte colony-stimulating factor and macrophage colony-stimulating factor. Blood 71:430-435.

130. Moore, M.A.S., and Warren, D.J. (1987) Synergy of interleukin 1 and granulocyte colony-stimulating factor: in vivo stimulation of stem-cell recovery and hematopoietic regeneration following 5-fluorouracil treatment of mice. Proc. Natl. Acad. Sci. USA. 84:7134-7138.

131. Kriegler, A.B., Bradley, T.R., Januszewicz, E., Hodgson, G.S., and Elms, E.F. (1982) Partial purification and characterization of a growth factor for macrophage progenitor cells with high proliferative potential in mouse bone marrow. Blood 60:503-508.

132. Mochizuki, D.Y., Eisenman, J.R., Conlon, P.J., Larsen, A.D., and Tushinski, F.J. (1987) Interleukin 1 regulates hematopoietic activity, a role previously ascribed to hemopoietin 1 Proc. Natl. Acad. Sci. USA. 84:5267-5271.

133. Stork, L., Barczuk, L., Kissinger, M., and Robinson, W. (1989) Interleukin-1 accelerates murine granulocyte recovery following treatment with cyclophosphamide. Blood 73:938-944.

134. Stork, L.C., Peterson, V.M., Rundus, C.H. and Robinson, W.A. (1988) Interleukin-1 enhances murine granulopoiesis in vivo. Exp. Hematol. 16:163-167.

135. Cantrell, D.A. and Smith, K.A. (1983) Transient expression of interleukin 2 receptors: consequences for T cell growth. J. Exp. Med. 158:1895-1911.

136. Touw, I.(1986) In vitro growth characteristics of human lymphoid malignancies in primary culture. Thesis. University of Rotterdam, Rotterdam, The Netherlands.

137. Robb, R.J. 1984. Interleukin 2: the molecule and its function. Immunol. Today 5:203-210.

138. Morgan, D.A., Ruscetti, F.W.,and Gallo, R.C. (1976) Selective in vitro growth of T lymphocytes from normal human bone marrows. Science 193:1007-1008.

139. Mier, J.W., and Gallo, R.C. (1980) Purification and some characteristics of human T-cell growth factor from phytohemagglutinin-stimulated lymphocyte conditioned media. Proc. Natl. Acad. Sci. USA 77:6134-6138.

140. Howard, M., Matis, L., Malek, T.R., Sherack, E., Kell, W., Cohen, D., Nakanishi, K., and Paul, W.E. (1983) Interleukin-2 induces antigen-reactive T-cell lines to secrete BCGF-I. J. Exp. Med. 158:2024-2039.

141. Grimm, E.A., Mazumder, A., Zhang, H.Z., and Rosenberg, S.A. (1982) J.Exp. Med. 155:1823-1841.

142. Rosenberg, S.A., Lotze, M.T., Muul, L.M., Leitman, S., Chang, A.E., Ettinghauzen, S.E., Matory, Y.L., Skibber, J.M., Skiloni, E., and Vetto, M.T. (1985) Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N.Engl. J. Med. 313:1485-1492.

143. Taniguchi, T, Matsui, H., Fujitta, T., Takaoka, C., Kashima, N.,Yoshimoto, R., and Hamuro, J. 1983. Structure and expression of a cloned cDNA for human interleukin-2. Nature 302:305-310.

144. Lee, F., Yokota, T., Otsuka, A.Meyerson, P., Villaret, D., Coffman, R., Mosmann, T., Renner, D., Roehm, N., Smith, C., Zlotnik, A., and Arai, K-I.(1986) Isolation and characterization of a mouse interleukin cDNA clone that expresses B-cell stimulatory activities and T-cell and mast-cell-stimulating activities. Proc. Natl. Acad. Sci. USA 83:2061-2065.

145. Yokota, T., Otsuka, T., Mosman T., Banchereau, J., DeFrance, T., Blanchard, D., de Vries, J.E., Lee, F., and Arai, K-I. (1986) Isolation and characterization of a human interleukin cDNA clone, homologous to mouse B-cell stimulatory factor 1, that expresses B-cell- and T-cell-stimulating activities. Proc. Natl. Acad. Sci. USA. 83:5895-5898.

146. Paul, W.E. (1987) Interleukin 4/B cell stimulatory factor 1: one lymphokine, many functions. FASEB J. 1:456-461.

147. Cosman, D. (1988) Colony-stimulating factors in vivo and in vitro. Immunology Today 9:97-98.

148. Kinashi, T., Harada, N., Severinson, E., Tanabe, T., Sideras, P., Konishi, M.,Azuma, C., Tominaga, A., Bergstedt-Lindqvist, S., Matsuda, F., Yaoita, Y., Takatsu, K., and Honjo, T. (1986) Cloning of complementary DNA encoding T-cell replacing factor and identity with B cell growth factor II. Nature 324:70-73.

149. Muraguchi, A., Kishimoto, T., Miki, Y, Kuritani, T., Kaieda, T., Yoshizaki, K., and Yamamura, Y. 1981. T cell-replacing factor (TRF)-induced IgG secretion in human B blastoid cell line and demonstration of acceptors for TRF. J. Immunol. 127:412-416.

150. Ikebuchi, K., Wong, G.G., Clark, S.C., Ihle, J.N., Hirai, Y., and Ogawa, M. (1987) Interleukin 6 enhancement of interleukin-3 dependent proliferation of multipotential hemopoietic progenitors. Proc. Natl. Acad. Sci. USA. 84:9035-9039.

151. Wong, G.G., Witek, J.A.S., Temple, P.A., Kriz, R., Ferenz, C., Hewick, R.M., Clark, S.C., Ikebuchi, K., and Ogawa, M. (1988). Stimulation of hematopoietic colony formation by human interleukin-6. J. Immunol. 140:3040-3044.

152. Wong, G.G., and Clark, S.C. (1988) Multiple actions of interleukin 6 within a cytokine network. Immunology Today 9:137-139.

153. Hirano, T, Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., Koyama, K., Iwamatsu, A., Tsunasawa, S. Sakiyama, F., Matsui, H., Takahara, Y., Taniguchi, T., and Kishimoto, T. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature 324:73-75.

154. Hoang, T., Haman, A., Goncalves, O., Wong, G.G., and Clark, S.C. (1988) Interleukin-6 enhances growth factor-dependent proliferation of acute myeloblastic leukemia. Blood 72:823-826.

155. Haegeman, G., Content, J., Volckaert, G., Derynck, R., Taveneir, J., and Fiers, W. 1986. Structural analysis of the sequence encoding for an inducible 26-kDa protein in human fibroblasts. Eur. J. Biochem. 159:625-632.

156. Zilberstein, A., Ruggieri, R, Korn, J.H., and Revel, M. (1986). Structure and expression of cDNA and genes for human interferon-beta-2, a distinct species inducible by growth stimulatory cytokines. Cell 45:659-666.

157. Noma, T., Mizuta, T., Rosen, A, Hirano, T., Kishimoto, T., and Honjo, T. (1987). Enhancement of the interleukin 2 receptor expression on T cells by multiple B-lymphotropic lymphokines. Immunol. Lett. 15:249-254.

158. German, R.D., Jacobs, K.A., Clark, S.C., and Raulet, D.H. (1987). B-cell stimulatory factor ($_2$ interferon) functions as a second signal for interleukin 2 production by mature murine T cells. Proc. Natl. Acad. Sci. USA 84:7629-7635.

159. Ceuppens, J.L., Baroja, M.L., Lorre, K., Damme, J.V., and Billiau, A. 1988. Human T cell activation with phytohemagglutinin: The function of IL-6 as an accessory signal. J. Immunol. 141:3868-3874.

160. Satoh, T., Nakamura, S., Taga, T., Matsuda, T., Hirano, T., Kishimoto, T., and Kaziro, Y. 1988. Induction of neural differentiation in PC12 cells by B cell stimulatory factor 2/interleukin 6. Mol. Cell. Biol. 8:3546-3549.

161. Naitoh, Y., Fukata, J., Tominaga, T., Nakai, Y., Tamai, S., Mori, K., and Imura, H. 1988. Interleukin-6 stimulates the secretion of adrenocorticotrope hormone in conscious, freely moving rats. Biochem. Biophys. Res. Com. 155:1459-1463.

162. Kishimoto, T. (1989). The biology of interleukin-6. Blood 74:1-10.

163. Carnot, P., and Deflandre, C. (1906) Sur l'activité hémopoiétique du serum au cours de la régénération du sang. C. R. Acad. Sci. 143:384-389.

164. Bonsdorf, E., and Jalavisto, E., (1948) A humoral mechanism in chronic erythrocytosis. Act. Physiol. Scand. 16:150-154.

165. White, W.F., Gurney, C.W., Goldwasser, E., and Jacobson, L.O. (1960) Studies on erythropoietin. Rec. Prog. Hormone Res. 16:219-262.

166. Jacobson, L.O., Goldwasser, E., Plzak, L.F., and Fried, W. (1957). Studies on erythropoiesis. IV. Reticulocyte response of hypophysectomized and polycytemic rodents to erythropoietin. Proc. Soc. Exp. Biol. Med. 94:243.

167. Reissmann, K.R. (1950). Studies on the mechanisms of erythropoietic stimulation in

parabiotic rats during hypoxia. Blood 5:372-378.

168. Gordon, A.S., and Kleinberg, W. (1937). Red cell and reticulocyte counts in guinea pigs following exposure to low pressures. Proc. Soc. Exp. Biol. Med. 37:507.

169. Erslev, A. (1953). Humoral regulation of red cell production. Blood 8:349-357.

170. Miyake, T., Kung, C.K.H., and Goldwasser, E. (1977). Purification of human erythropoietin. J. Biol. Chem. 252:5558-5562.

171. Jacobs, K., Shoemaker, C., Rudersdorf, R., Neill, S.D. Kaufman, R.J., Mufson, A., Sheera, J. Jones, S.J., Hewick, R., Fritsch, E.F., Kawakita, M. Shimizu, T., and Miyake, T. (1985) Isolation and characterization of genomic and cDNA clones of human erythropoietin. Nature 313:806-810.

172. Eaves, A.C., and Eaves. (1984). Erythropoiesis in culture. Clin. Haematol. 13:371-391.

173. Gregory, C.J. (1976) Erythropoietin sensitivity as a differentiation marker in the hemopoietic system: studies on three erythropoietic colony responses in culture. J. Cell. Physiol. 89:289-302.

174. Gregory, C.J., Tepperman, A.D., McCulloch, E.A., and Till, J.E. (1974) Erythropoietic progenitors capable of colony formation in culture: response of normal and genetically anemic W/W^{V} mice to manipulation of the erythron. J. Cell Physiol. 84:1-12.

175. Iscove, N.N. (1977) The role of erythropoietin in regulation of population size and cell cycling of early and late erythroid precursors in mouse bone marrow. Cell Tissue Kinet. 10:323-334.

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

177. Sief, C.A., Emerson, S.G., Mufson, A., Gesner, T.G., and Nathan, D.G. (1986) Dependence of highly enriched human bone marrow progenitors on hemopoietic growth factors and their response to recombinant erythropoietin. J. Clin Invest. 77:74-78.

178. Dorssers, L., Burger, H., Bot, F., Delwel, R. , Geurts van Kessel, A.H.M., Löwenberg, B., and Wagemaker, G. (1987) Characterization of a human multilineage colony-stimulating factor cDNA clone identified by a conserved noncoding sequence in mouse interleukin-3. Gene 55:115-124.

179. Delwel, R., Dorsers, L., Touw, I., Wagemaker, G., and Löwenberg, B. (1987. Human recombinant multilineage colony stimulating factor (interleukin-3): stimulator of acute myelocytic leukemia progenitor cells in vitro. Blood 70:333-336.

180. Yang, Y-C., Ciarletta, A.B., Temnle, P.A., Chung, M.P., Kovacic, S. Witek-Gianotti, J.S., Leary, A.., Kriz, R., Donahue, R.E., Wong, G.G., and Clark, S.C. (1986) Human IL-3 (multi-CSF): identification by expression cloning of a novel hemopoietic growth factor related to murine IL-3. Cell 47:3-10.

181. Ihle, J.N., Keller, J., Oroszlan, S., Henderson, L.E., Copeland, T., Fith, F., Prystowski, M.B., Goldwasser, E., Schrader, J.W. Palaszynsky, E., Dy, M., and Lebel, B. (1983) Biologic properties of homogenous IL3. J. Immunol. 13:282-287.

182. Dorssers, L., Burger, H., and Wagemaker, G. (1984). Identity of murine stem cell activating factor (SAF) and interleukin-3 (IL-3) and common specificity for pluripotent stem cells. Exp. Hematol. 12:357.

183. Hapel, A.J., Fung, M.C., Johnson, R.M., Young, I.G., Johnson, G., Metcalf, D. (1985) Biologic properties of molecularly cloned and expressed murine interleukin-3. Blood 65:1453-1459.

184. Broxmeyer, H.E., Williams, D.E., Cooper, S., Shadduck, R.K., Gillis, S., Waheed, A., Urdal, D.L., and Bickel, D.C. (1987) Comparative effects in vivo of recombinant murine interleukin 3, natural murine colony-stimulating factor-1, and recombinant murine granulocyte-macrophage colony stimulating factor on myelopoiesis in mice. J. Clin. Invest. 79:721-730.

185. Metcalf, D., Begley, C.G., Nicola, N.A., Johnson, G.R. (1987) Quantitative responsiveness of murine hemopoietic populations in vitro and in vivo to recombinant multi-CSF (IL3). Exp. Hematol. 15:288-295.

186. Yang, Y-C, Kovacic, S., Kriz, R., Wolf, S., Clark, S., Wellems, T.E., Nienhuis, A., and Epstein, N. (1988) The human genes for GM-CSF and IL3 are closely linked in tandem on chromosome 5. Blood 71:958-961.

187. Bot, F.J., Dorssers, L., Wagemaker, G., and Löwenberg, B. (1988) Stimulating spectrum of human recombinant Multi-CSF (IL3) on human marrow precursors: importance of accessory cells. Blood 71:1609-1614.

188. Nicola, N.A., Metcalf, D., Johnson, G.R., Burgess, A.W. (1979) Separation of functionally distinct human granulocyte-macrophage colony-stimulating factors. Blood 54:614-627.

189. Sieff, C.A., Emerson, S.G., Donahue, R.E., Nathan, D.G., Wang, E.A., Wong, G.G., and Clark, S.C. (1985) Human recombinant granulocyte-macrophage colony-stimulating factor: a multilineage hemopoietin. Science 230:1171-1173.

190. Metcalf, D. (1986) The molecular biology and function of the granulocyte-macrophage stimulating factors. Blood 67:257-267.

191. Wong, G.C., Witek, J.S., Temple, P.A., Wilkens, K.M., Leary, A.C., Luxenberg, D.P., Jones, S.S., Brown, E.L., Kay, R.M., Orr, E.C., Shoemaker, C., Golde, D.W., Kaufman, R.J., Hewick, R.M., Wang, E.A., and Clark, S.C. (1985) Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins Science 228:810-815.

192. Kaushanski, K., O'Hara, P.J., Berkner, K., Segal, G.S., Hagen, F.S., and Adamson, J.W. (1986) Genomic cloning, characterization and multilineage expression of granulocyte-macrophage colony-stimulating factor. Proc. Natl. Acad. Sci. USA. 83:3101-3105.

193. Lee, F., Yokota, Otsuka, T., Gemmel, L., Larson, N., Luh, J., Arai, K.I., and Renninck, D. (1985) Isolation of cDNA for a human granulocyte-macrophage colony stimulating factor by functional expression in mammalian cells. Proc. Natl. Acad. Sci. USA. 82:4360-4362.

194. Cantrell, M.A., Anderson, D., Ceretti, D.P., Price, V., McKereghan, K., Tushinski, R.J., Mochizuki, D.Y., Larsen, A., Grabstein, K., Gillis, S., and Cosman, D., (1985). Cloning, sequence, and expression of a human granulocyte/macrophage colony-stimulating factor. Proc. Natl. Acad. Sci. USA. 82:6250-6254.

195. Huebner, K., Isobe, M., Croce, C.M., Golde, D.W., Kaufman, S.E., and Gasson, J.C. (1985) The human gene encoding GM-CSF is at 5q21-32, the chromosome region deleted at the 5q- anomaly. Science 230:1282-1285.

196. Burgess, A.W., Begley, C.G., Johnson, G.R., Lopez, A.F., Williamson, D.J., Mermod, J.J., Simpson, R.J., Schmitz, A., and DeLamarter, J.F. (1987) Purification and properties of bacterially synthesized human granulocyte-macrophage colony stimulating factor. Blood 69:43-51.

197. Metcalf, D., Begley, C.G., Johnson, G.R., Nicola, N.A., Vadas, M.A., Lopez, A.F., Williamson, D.J., Wong, G.G., Clark, S.C., and Wang, E.A. (1986) Biologic properties in vitro of a human recombinant granulocyte-macrophage colony-stimulating factor. Blood 67:37-45.

198. Metcalf, D., Johnson, G.R., and Mandel, T. (1979). Colony formation in agar by multipotential hemopoietic cells. J. Cell. Physiol. 98:401-420.

199. Tomonoga, M., Golde, D.W., and Gasson, J.C. (1986) Biosynthetic (recombinant) granulocyte-macrophage colony-stimulating factor:Effect on normal bone marrow and leukemic cell lines. Blood 67:31-36.

200. Bruno, E., Briddel, R., and Hoffman, R. (1988) Effects of recombinant and purified hemopoietic growth factors on human megakaryocyte colony formation. Exp. Hemat. 16:371-377.

201. Fleischmann, J., Golde, D.W., Weisbart, R.H., and Gasson, J.C. (1986) Granulocyte-macrophage colony stimulatory factor enhances phagocytosis of bacteria by human neutrophils. Blood 68:708-711.

202. Gasson, J.C., Weisbart, R.H., Kaufman, S.E., Clark, S.C., Hewick, R.M., Wong, G.G., and Golde, D.W. (1984) Purified human granulocyte-macrophage colony stimulating factor: direct action on neutrophils. Science 226:1339-1342.

203. Weisbart, R.H., Golde, D.W., and Gasson, J.C. (1986). Biosynthetic human GM-CSF modulates the number and affinity of neutrophil f-met-leu-phe receptors. J. Immunol. 137:3584-3592.

204. Weisbart, R.H., Golde, D.W., Clark, S.C., Wong, G.G., and Gasson, J.C. (1985) Human granulocyte macrophage stimulating factor is a neutrophil activator. Nature 314:361-363.

205. Weisbart, R.H., Kwan, L., Golde, D.W., and Gasson, J.C. (1987) Human GM-CSF primes neutrophils for enhanced metabolism in response to the major physiological chemoattractants. Blood 69:18-21.

206. Grabstein, K.H., Urdal, D.L., Tushinski, R.J., Mochizuki, D.Y., Price, V.L., Cantrell, M.A., Gillis, S., and Conlon, P.J. (1986) Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. Science 232:506-508.

207. Coleman, D.L., Chodakewitz, J.A., Bartiss, A.H., and Mellors, J.W. (1988) Granulocyte-macrophage colony-stimulating factor enhances selective effector functions of tissue derived macrophages. Blood 72:573-578.

208. Socinski, M.A., Cannistra, S.A., Sullivan, R., Elias, A., Antman, K., Schnipper, L., and Griffin, J.D. (1988) Granulocyte-macrophage colony-stimulating factor induces the expression of the CD11b surface adhesion molecule on human

granulocytes in vivo. Blood 72:691-697.

209. Sanchez-Madrid, F., Nagg, J.A., Robbins, E., Simon, P, Springer, T.A. (1983). A human leukocyte differentiation antigen family with distinct ‡-subunit and a common ß-subunit: The lymphocyte-function-associated antigen (LFA-1), the C3bi complement receptor (OKM1/Mac-1), and the p150,95 molecule. J. Exp. Med. 158:1785-1794.

210. Socinski, M.A., Cannistra, S.A., Elias, A., Antman, L., Schnipper, L., Griffin, J.D. (1988). Granulocyte-macrophage colony stimulating factor expands the circulating haemopoietic progenitor cell compartment in man. The Lancet I:1194-1198.

211. Brandt, S.J., Peters, W.P, Atwater, S.K., Kurtzberg, J., Borowitz, M.J., Jones, R.B., Shpall, E.J., Bast, R.C., Gilbert, C.J., and Oette, D.H. (1988) Effects of recombinant human granulocyte-macrophage colony-stimulating factor on hemopoietic reconstitution after high dose chemotherapy and autologous bone marrow transplantation. N. Engl. J. Med 318:869-876.

212. Nemutis, J., Singer, J.W., Buckner,C.D., Hil, R., Storb, R., Thomas, E.D., and Appelbaum, F.R. (1988) Use of recombinant Granulocyte-Macrophage Colony-Stimulating Factor in autologous marrow transplantation for lymphoid malignancies. Blood72:834-836.

213. Antin, J.H., Smith, B.R., and Rosenthal, D.S. (1987) Phase I/II study of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) in bone marrow failure. Blood 70:Suppl 1:129a.

214. Antman, K.S., Griffin, J.D., Elias, A., Socinski, M.A., Ryan, L., Cannistra, S.A., Oette, D., Whitley, M., Frei, E. III, and Schnipper, L.E. (1988) Effect of recombinant human granulocyte-macrophage colony-stimulating factor on chemotherapy-induced myelosuppression. N. Engl. J. Med. 319:593-598.

215. Vadhan-Ray, S., Keating, M., LeMaistre, A., Hittelman, W.N., McCredie, K., Trujillo, J.M., Broxmeyer, H.E., Henney, C., and Gutterman, J.U. (1987) Effects of recombinant human granulocyte-macrophage colony-stimulating factor in patients with myelodysplastic syndromes. N. Engl. J. Med. 317:1545-1552.

216. Groopman, J.E., Mitsuyasu, R.T., DeLeo, M.J., Oette, D. H., and Golde, D.W. (1987) Effect of recombinant human granulocyte-macrophage colony stimulating factor on myelopoiesis in the acquired immunodeficiency syndrome. N. Engl. J. Med. 317:593-598.

217. Butturini, A., Gale, R.P., Lopes, D.M., Cunha, C.B., Ho, W.G., Sanpai, J.M., Souza, P.C. de, Cordiero, J.M., Neto, C., Souza, C.E.P. de, Tabak, D.G., Burla, A., and the Navy Hospital Radiation Team. (1988) Use of recombinant granulocyte-macrophage colony stimulating factor in the brazil radiation accident. Lancet II:471-475.

218. Donahue, R.E., Wang, E.A., Stone, D.K., Kamen, R., Wong, G.G., Seghal, P.K., Nathan, D.G., and Clark, S.C. (1986) Stimulation of hematopoiesis in primates by continuous infusion of recombinant GM-CSF. Nature 321:872-875.

219. Mayer, P., Lam, C., Obenhaus, H., Liehl, E., and Besemer, J. (1987) Recombinant human GM-CSF induces leukocytosis and activates peripheral blood polymorphonuclear neutrophils in nonhuman primates. Blood 70:206-213.

220. Nienhuis, A.W., Donahue, R.E., Karlsson, S., Clark, S.C., Agricola, B., Antinoff,

N., Pierce, J.E., Turner, P., Anderson, W.F., and Nathan, D.G. (1987) Recombinant human granulocyte-macrophage colony-stimulating factor shortens the period of neutropenia after autologous bone marrow transplantation. J. Clin. Invest. 80:573-577.

221. Griffin,J.D., Young, D., Herrmann, F., Wiper, D., Wagner, K., and Sabbath, K.D. (1986) Effects of recombinant human GM-CSF on proliferation of clonogenic cells in acute myeloblastic leukemia. Blood 67:1448-1453.

222. Vellenga, E., Delwel, H.R., Touw, I.P., and Löwenberg, B. (1987) Patterns of myeloid leukemia growth in response to recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF). Exp hematol 15:652-656.

223. Vellenga, E., Young, D.C., Wagner, K., Wiper, D., Ostapo-vicz, D., and Griffin, J.D. (1987) The effects of GM-CSF and G-CSF in promoting growth of clonogenic cells in acute myeloblastic leukemia. Blood 69:1771-1776.

224. Pébusque, M.J., Lopez, M., Torres, H., Carotti, A., and Guilbert L. (1988) Growth response of human myeloid leukemia to colony stimulating factors. Exp. Hematol. 16:360-366.

225. Monroy, R.L., Skelly, R.R., MacVittie, Davis, T.A., Sauber, J.J., Clark, S.C., and Donahue, R.E. (1987) The effect of recombinant GM-CSF on the recovery of monkeys transplanted with autologous bone marrow. Blood 70:1696-1699.

226. Welte, K., Bonilla, M., Gillio, A., Boone, T.C., Potter, G.K., Gabrilove, J.L., Moore, M.A., O'Reilly, R.J., and Souza, L.M. (1987) Recombinant human granulocyte colony-stimulating factor. J. Exp. Med. 165:941-948.

227. Nagata, S., Tsuchiya, M., Asano, S., Kaziro, Y., Yamasaki, T., Yamamoto, O., Hirata, Y., Kubota, N., Oheda, M., Nomura, H., and Ono, M. (1986) Molecular cloning and expression of c DNA for human granulocyte colony-stimulating factor. Nature 319:415-418.

228. Souza, L.M., Boone, T.C., Gabrilove, J., Lai, P.H., Zsabo, K.M., Murdock, D.C., Chazin, V.R., Bruszewski, J., Lu, H., Chen, K.K., Barendt, J., Platzer, E., Moore, M.A.S., Mertelsman, R., and Welte, K. (1986) Recombinant human granulocyte colony-stimulating factor: Effects on normal and leukemic myeloid cells. Science 232:61-65.

229. Suda, T., Suda, J., Kajigaya, S., Nagata, S., Asano, S., Saito,M., Miura, Y. (1987) Effects of recombinant murine Granulocyte Colony Simulating Factor on granulocyte-Macrophage and Blast colony formation. Exp. Hematol. 15:958-965.

230. Wang, J.M., Chen, Z.G., Colella, S., Bonilla, M.A., Welte, K., Bordignon, C. and Montovani, A. (1988) Chemotactic activity of recombinant human Granulocyte Colony-Stimulating Factor. Blood 72:1456-1460.

231. Nicola, N.A., Begley, C.G., and Metcalf, D. (1985) Identification of the human analogue of a regulator that induces differentiation in murine leukemic cells. Nature 314:625-628.

232. Gabrilove, J.L., Jakubowski, A.J., Scher, H., Sternberg, C., Wong, G., Grous, J., Yagoda, A., Fain, K., Moore, M.A.S., Clarkson, B., Oettgen, H.F., Alton, K., Welte, K., and Souza, L. (1988) Effect of granulocyte colony-stimulating factor on neutropenia and associated morbidity due to chemotherapy for carcinoma of the urothelium. N. Engl. J. Med 318:1414-1422.

233. Bronchud, M.H., Scarffe, J.H., Thatcher, N., Crowther, D., Souza, L.M.,Alton, N.K., Testa, N.G., and Dexter, T.M. (1987) Phase I/II study of recombinant human granulocyte colony-stimulating factor in patients receiving intensive chemotherapy for small cell lung cancer. Br. J. Cancer 56:809-813.

234. Morstyn, G., Campbell, L., Souza, L.M., Alton, N.K., Keech, J., Green, M., Sheridan, W., and Metcalf, D. (1988) Effect of granulocyte colony stimulating factor on neutropenia induced by chemotherapy. Lancet I:667-672.

235. Cohen, A.M., Zsebo, K.M., Inoue, H., Hines, D., Boone, T.C., Chazin, V.R., Tsai, L., Ritch, T., and Souza, L.M. (1987) In vivo stimulation of granulopoiesis by recombinant human granulocyte colony-stimulating factor. Proc. Natl. Acad. Sci. USA. 84:2484-2488.

236. Shimamura, M., Kobayashi, Y., Yuo, A., Urabe, A., Okabe, T., Komatsu, Y., Itoh, S., and Takaku, F. (1987) Effect of human recombinant granulocyte-colony-stimulating factor on hematopoietic injury in mice induced by 5-fluorouracil. Blood 69:353-355.

237. Yuo, A., Kitagawa, S., Okabe, T., Urabe, A., Komatsu, Y., Itoh, S., and Takaku, F. (1987) Recombinant human granulocyte colony-stimulating factor repairs the abnormalities of neutrophils in patients with myelodysplastic syndromes and chronic myelogenous leukemia. Blood 70:404-411.

238. Gillio, A.P., Bonilla, M.A., Potter, G.K., Gabrilove, J.L., O'Reilly, R.J., Souza, L.M., and Welte, K. (1987) Effects of recombinant human Granulocyte-Colony Stimulating Factor on hematopoietic reconstitution after autologous bone marrow transplantation in primates. Transpl. Proc. 19:153-156.

239. Strife, A., Lambek, C., Wisniewski, D., Gulati, S., Gasson, J.C., Golde, D.W., Welte, K., Gabrilove, J.L., and Clarkson, B. (1987) Activities of four purified growth factors on highly enriched human hemopoietic progenitor cells. Blood 69:1508-1523.

240. Motoyoshi, K., Takaku, F., Mizoguchi, H., and Miura, Y. (1978) Purification and some properties of colony stimulating factor from normal human urine. Blood 52:1012-1020.

241. Kawasaki, E.S., Ladner, M.B., Wang, A.M., Arsdell, J. van, Warren, M.K., Coyne, M.Y., Schweickart, V.L., Lee, M.T., Wilson, K.H., Boosman,A., Stanley, E.R., Ralph, P., and Mark, D.F. (1985) Molecular cloning of a complementary DNA encoding human macrophage specific colony stimulating factor (CSF-1). Science 230:291-296.

242. Wong, G.G., Temple, P.A., Leary, A.C., Witek-Gianotti, J.S., Yang, Y.-C., Ciarletta, A.B., Chung, M., Murtha, P., Kriz, R., Kaufman, R.J., Ferenz, C.R., Sibley, B.S., Turner, K.J., Hewick, R.M., Clark, S.C., Yanai, N., Yokota, H., Yamada, M., Saito, M., Motoyoshi, K., and Takaku, S. (1987) Human CSF-1: Molecular cloning and expression of 4-kb cDNA encoding the human urinary protein. Science 235:1504-1509.

243. Ralph, P., Warren, M.K., Lee, M.T., Csejtey, J., Weaver, J.F., Broxmeyer, H.E., Williams, D.E., Stanley, E.R., and Kawasaki, E.S. (1986) Inducible production of human macrophage growth factor CSF-1. Blood 68:633-639.

244. Warren, K.M., and Ralph, P. (1986) Macrophage growth factor CSF-1 stimulates human monocyte production of interferon, tumor necrosis factor, and colony stimulating activity. J. Immunol. 137:2281-2285.

245. Motoyoshi, K., Suda, T., Kusumoto, K., Takaku, F., and Miura, Y., (1982) Granulocyte-macrophage colony-stimulating factor and binding activities of purified urinary colony-stimulating factor to murine and human bone marrow cells. Blood 60:1378-1386.

246. Stanley, E.R., Cifone, M., Heard, P.M., and Defendi, V. 1976. Factors regulating macrophage production and growth. Identity of colony-stimulating factor and macrophage growth factor. J.Exp.Med. 143:631-647.

247. Rettenmier, C.W., Sacca, R., Furman, W.L., Roussel, M.F., Holt, J.T., Nienhuis, A.W., Stanley, E.R., and Sherr, C.J. (1986) Expression of the human c-fms proto-oncogene product (colony-stimulating factor-1 receptor) on peripheral blood mononuclear cells and choriocarcinoma cell lines. J. Clin. Invest. 77:1740-1746.

248. Masaoka, T., Motoyoshi, K., Takaku, F., Kato, Harada, M., Kodera, Y., Kanamaru, A., Moriyama, Y., Ohno, R., Ohira, M., Shibata, H., and Inoue, T. (1988) Administration of human urinary colony stimulating factor after bone marrow transplantation. Bone Marrow Transpl. 3:121-127.

249. Lipton, J.H., Sachs, L. 1981. Characterization of macrophage- and granulocyte-inducing proteins for normal and leukemic myeloid cells produced by the krebs ascites tumor. Biochem. Biophys. Acta 673:552-569.

250. Neta, R., Douches, S., and Oppenheim, J.J. (1986). Interleukin 1 is a radioprotector. J. Immunol. 136:2483.

251. Duff, G.W., and Atkins, E. (1982) The detection of endotoxin by in vitro production of endogenous pyrogen: comparison with limulus amoebocyte lysate gelation. J. Immunol. Meth. 52:323-331.

252. Oppenheim, J.J., Togawa, A., Chedid, L, and Mizell, S. (1980) Components of mycobacteria and muramyl dipeptide with adjuvant activity induce lymphocyte activating factor. Cell. Immunol. 50:71-81.

253. Ikejima, T., Dinarello, C.A., Gill, D.M.,and Wollf, S.M. (1984) Induction of human interleukin-1 by a product of staphylococcus aureus associated with toxic shock syndrome. J. Clin. Invest. 73:1312-1320.

254. Gordon, M.Y., Riley, G.P., Watt, S.M., and Greaves, M.F. (1987) Compartmentalization of a haemopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature 326:403-405.

255. Li, C.L., and Johnson, G.R. (1985). Simulation of multipotential, erythroid and other haemopoietic progenitor cells by adherent cell lines in the absence of detectable multi-CSF (IL3). Nature 316:633-636.

256. Song, Z.X., Shadduck, R.K., Innes, D.J.Jr., Waheed, A., and Quesenberry, P.J. (1985) Hemopoietic factor production by a cell line (TC-1) derived from adherent murine marrow cells. Blood 66:273-281.

257. Spooncer, E., Heyworth, C.M., Dunn, A., Dexter, T.M. (1986). Self-renewal and differentiation of interleukin-3-dependent multipotent stem cells are modulated by stromal cells and serum factors. Differentiation 31:111-118.

258. Pulsoni, A., Delwel, R., Salem, M., Touw, I., and Löwenberg, B. (1989). Cell to cell contact enhances the proliferation of acute myeloid leukemia (AML) cells in vitro without an apparent role of adhesion glycoproteins LFA1, MAC1, and P150-95.

259. Eschbach, J.H., Egrie, J.C., Downing, M.R., Browne, J.K., and Adamson, J.W. (1987) Correction of the anemia of end stage renal disease with recombinant human erythropoietin: results of a combined Phase I and II clinical trial. N. Engl. J. Med. 316:73-78.

260. Urabe, A., Takaku, F., Mizoguchi, H., Kubo, K., Ota, K., Shimizu, N., Tanaka, K., Mimura, N., Nihei, H., Koshikawa, S., Akizawa, T., Akiyama, N., Otsubo, O., Kawaguchi, Y., and Maeda, T. (1988) Effect of recombinant human erythropoietin on the anemia of chronic renal failure. Int. J. Cell Cloning 6:179-191.

261. Bonilla, M.A., Gillio, A.P., Ruggeiro, M., Kernan, N.A., Brochstein, J.A., Abboud, M., Fumagalli, L., Vincent, M., Gabrilove, J.L., Welte, K., Souza, L.M., and O'Reilly, R.J. (1989). Effects of recombinant human granulocyte colony-stimulating factor on neutropenia in patients with congenital agranulocytosis. N. Engl. J. Med. 320:1574-1580.

262. Quesberry, P.J. (1986) Synergistic hematopoietic growth factors. Int. J. Cell Clon. 4:3-15.

263. Kelleher, C., Miyauchi, J., Wong, G., Clark,S.C., Minden, M.D., and McCulloch E.A. (1987) Synergism between recombinant growth factors, GM-CSF and G-CSF, acting on the blast cells of acute myeloblastic leukemia. Blood 69:1498-1503.

264. Paquette, R.L., Zhou, J-Y, Yang, Y-C, Clark, S.C., and Koeffler, H.P. (1988) Recombinant gibbon interleukin-3 acts synergistically with recombinant human GM-CSF and G-CSF in vitro. Blood 71:1596-1600.

265. Williams, D.E., Hangoc, G., Boswell, H.S., Shadduck, R.K., Gillis, S., Waheed, A., and Broxmeyer, H.E. (1987) The effects of purified recombinant murine interleukin-3 and/or purified natural murine csf-1 in vivo on the proliferation of murine high- and low-proliferative potential colony forming cells: demonstration of in vivo synergism. Blood 70:401-403.

266. Malcolm, A.S., and Warren, D.J. (1987) Synergy of interleukin 1 and granulocyte colony-stimulating factor: In vivo stimulation of stem cell recovery and hematopoietic regeneration following 5-fluorouracil treatment of mice. Proc. Natl. Acad. Sci. USA 84:7134-7138.

267. Lista, P., Porcu, P., Avanzi, G.C., and Pegoraro, L. (1988) Interleukin 3 enhances the cytotoxic activity of 1-ß-D-arabinofuranosylcytosine (Ara-C) on acute myeloblastic leukemia AML) cells. Br. J. Hematol. 69:121-123.

268. Vriesendorp, H.M., Wagemaker, G., and van Bekkum, D.W. (1981). Engraftment of allogeneic bone marrow. Transplant. Proc. 13:643.

269. Vriesendorp, H.M. (1985) Engraftment of hemopoietic cells. In: Bone marrow transplantation. Biological mechanisms and clinical practice. Eds.: Bekkum, D.W. van, and Löwenberg, B., Marcel Dekker Inc., New York and Basel p.73-145.

270. Wagemaker, G. Selective multiplication of hemopoietic stem cells for bone marrow transplantation in mice and Rhesus monkeys. Transpl. Proc. 19:2721-2725.

271. Sinclair, W.K. and Morton, R.A. (1966). X-ray sensitivity during the cell generation cycle of cultured Chinese hamster cells. Radiat. Res. 29:450-474.

272. Teraisma, T, and Tolmach, L.J. (1963). Variations in several responses of HeLa cells

to x-irradiation during the division cycle. Biophys. J. 3:11-33.

273. Le Beau, M.M., Epstein, N.D., O'Brien, S.J., Nienhuis, A.W., Yang, Y.C., Clark, S.C., and Rowly, J.D. (1987) The interleukin 3 gene is located on human chromosome 5 and is deleted in myeloid leukemias with a deletion of 5q. Proc. Natl. Acad. Sci. USA 84:5913-5917.

274. Le Beau, M.M., Pettenati, M.J., Lemons, R.S., Diaz, M.O., Westbrook, C.A., Larson, R.A., Sherr, C.J., and Rowly, J.D. (1986) Assignment of the GM-CSF, CSF-1, and FMS genes to human chromosome 5 provides evidence for linkage of a family of genes regulating hematopoiesis and for their involvement in the deletion (5q) in myeloid disorders. Cold Spring Harbour Symp. Quant. Biol. L.I. 899.

275. Jaye, M., Howk, R., Burgess, W., Ricca, G.A., Chiu, I.M., Ravera, M.W., O'Brien, S.J., Modi, W.S., Maciag, T., and Drohan, W.N. 1986) Human endothelial cell growth factor: Cloning, nucleotide sequence,, and chromosome localization. Science 233:541-545.

276. Sutherland, G.R., Baker, E., Callen, D.F.,Campbell, H.D., Young, I.G., Sanderson, C.J., Garson, O.M., Lopez, A.F., and Vadas, M.A. (1988). Interleukin-5 is at 5q31 and is deleted in the 5q- syndrome. Blood 71:1150-1152.

277. Yarden, Y., Escobedo, J.A., Kuang, W.J., Yang-Feng, T.L., Daniel, T.O., Tremble, P.M., Chen, E.Y., Ando, M.E., Harkins, R.N., Francke, U., Fried, V.A., Ullrich, A., and Williams, L.T. (1986). Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature 323:226-232.

278. Nienhuis, A.W, Bunn, H.F., Turner, P.H., Gopal, T.V., Nash, W.G., O'Brien, S.J., and Sherr, C.J. 1985. Expression of the human c-fms proto-oncogene in hematopoietic cells and its deletion in the 5q-syndrome. Cell 42:421-428.

279. Sariban, E., Mitchell, T., and Kufe, D. 1985. Expression of the c-fms proto-oncogene during human monocytic differentiation. Nature 316:64-66.

280. Sherr, C.J., Rettenmier, C.W., Sacca, R., Roussel, M.F., Look, A.T., and Stanley, E.R. (1985) The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor CSF-1. Cell 41, 665-676.

281. Bot, F., van Eyk, L., Schipper,P., and Lowenberg, B. (1989). Human Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) stimulates immature marrow precursors but no CFU-GM, CFU-G, or CFU-M. Exp. Hematol. 17:292-295.

282. Bekkum, D.W. van. (1978) The rhesus monkey as a preclinical model for bone marrow transplantation. Transpl. Proc. 10:105-111.

283. Moore, M.A.S., Williams, N., and Metcalf, D. (1972) Purification and characterization of the in vitro colony forming cell in monkey hemopoietic tissue. J. Cell. Physiol. 79:283-293.

284. Dicke, K.A., Tridente, G., and Bekkum, D.W. van. (1969). Transplantation 8:678.

285. Putten, L.M. van, Bekkum, D.W. van, Vries, M.J. de, and Balner, H.. (1967). The effect of preceding blood transfusions on the fate of homologous bone marrow grafts in lethally irradiated monkeys. Blood 30:749-757.

286. Balner, H., and Vreeswijk, W. van. (1975). The major histocompatibility complex of

rhesus monkeys: a short review. J. Med. Primatol. 4:382.

287. Haynes, B.F., Dowell, B.L., Hensley, L.L., Gore, I., and Metzgar, R.S. (1982) Human T cell antigen expression by primate T cells. Science 215:298-300.

288. Clark, E.A., Martin, P.J., Hansen, J.A., and Ledbetter, J.A. (1983) Evolution of epitopes on human and non-human primate lymphocyte cell surface antigens. Immunogen. 18:599-615.

289. Jonker, M., and Meurs, G.J.E. van. (1984) Monoclonal antibodies specific for B cells, cytotoxic/suppressor T cells and a subset of cytotoxic/suppressor T cells in the rhesus monkey. In Leukocyte typing Eds. Bernard, A., Boumsel, L., Dauset, J., Milstein, C., and Schlossman S.F. Springer Verlag, Berlin, p.328.

290. Schaeffer, U.W., Dicke, K.A., And Bekkum, D.W. van. (1972) Recovery of hemopoiesis in lethally irradiated monkeys by frozen allogeneic bone marrow grafts. Rev. Europ. Etudes Clin. et Biol. 17:483-488.

291. Bekkum, D.W., (1984) Conditioning regimens for marrow grafting. Seminars in hematology 21:81-90.

292. Muller-Bérat, C.N., Putten, L.M., and Bekkum, D.W. van (1966) Ann. N.Y. Acad. Sci. 129:340.

293. Bekkum, D.W. van, Balner, H., Dicke, K.A., Berg, F.C van den, Prinsen, G.H., and Hollander, C.F. (1972) The effect of pretreatment of allogeneic bone marrow graft recipients with antilymphocytic serum on the acute graft-versus-host reaction in monkeys. Transplantation 13:400-407.

294. Merrit, C.B., Darrow, C.C., Vaal, L., and Rogentine Jr., G.N. (1972) Bone marrow transplantation in Rhesus monkeys following irradiation. Transplantation 14:9-13.

295. Bekkum, D.W. van, Dicke, K.A., and Balner, H. (1970) Exp. Hematol. 20:126.

296. Wagemaker, G. (1986) Problems and prospects of histoincompatible bone marrow transplantation studied in rhesus monkeys. In: Minimal residual disease in acute leukemia. Proc. Second Int. Symp. on detection and treatment of minimal residual disease in acute leukemia. Eds.: Hagenbeek, A., and Löwenberg, B. Rotterdam, Dordrecht, Martinus Nijhoff, p. 363-373.

297. Gerritsen, W.R., Jonker, M., Wagemaker, G., and Bekkum, D.W. van. (1986). The influence of T-cell depletion by monoclonal antibodies on repopulating hemopoietic stem cells and their efficacy in preventing GvHD in rhesus monkeys. In: Experimental hematology today - 1985. Eds.: Baum, S.J., Pluznik, D.H., and Rozenszajn, New York, Springer, p.128-132.

298. Vriesendorp, H.M., and Bekkum, D.W. van. (1984). Susceptibility to total body irradiation. In: Response of different species to total body irradiation. Eds.: Broerse, J.J., and MacVittie, T.J. Martinus Nijhoff, Boston, Dordrecht p. 43-57.

CHAPTER II
MATERIALS AND METHODS

2.1. Experimental animals.

Rhesus monkeys (*Macaca mulatta*) were bred at the Primate Center TNO, Rijswijk, The Netherlands[1]. Both male and female animals were used for the experiments. The monkeys weighed 2.5-4 kg and were 2-4 years old at the time of the experiment. They were all typed for RhLA-A, -B and -DR antigens, as described[2]. Furthermore, all animals used in this study were seronegative for Simian Immunodeficiency Virus (SIV) , as tested at the Virus Reference Laboratory, Southwest Foundation for Biomedical Research, San Antonio, TX, U.S.A. Leukocyte counts, differential counts, red blood cell counts, hemoglobin levels, hematocrit values, thrombocyte counts and reticulocyte counts were determined as described in section 2.10.
Only monkeys with normal values for those parameters were used for the experiments described in this thesis.

2.2. Bone marrow procurement and preparation of cell suspensions.

For autologous bone marrow (BM) graft harvesting, the monkeys were anaesthetized with 10 mg ketamine (Ketamin R, Tesink B.V., Oudewater, The Netherlands) and 1 mg acepromazine (Vetranquil R, Sanofi santé animale, Paris, France). BM aspirates of 1-3 ml were collected by puncturing the femoral shafts. For allogeneic BM graft harvesting, the donor monkey was sacrificed and the

bones were pressed as described[3]. The BM was suspended in PBS or HBSS, containing 500 U Heparin (Thromboliquin^R, Organon Technika, Boxtel, The Netherlands) and 0.1 mg/ml Deoxyribonuclease I (Calbiogem, San Diego, CA, U.S.A.).

T-cell depleted stem cell concentrates were produced as illustrated in Figure 2.1. Mononuclear cell suspensions were prepared by neutral density centrifugation, using lymphocyte separation medium (LSM, Organon Technika Corporation, Durham, NC, U.S.A., density: 1.077 g/cm^3; 18°C, 500 g, 20 minutes). The erythrocytes were resuspended into 25-50 ml pyrogen free NaCl 0.9% and reinfused into the animal as soon as possible. Further enrichment of stem cells and progenitor cells was achieved by a discontinuous albumin density gradient. T-cell depleted stem cell concentrates were produced by albumin density gradient centrifugation. This procedure generally resulted in a 10-30 fold concentration of precursor cells and in an elimination of about 80-90% of the T-lymphocytes and 90-95 % of all nucleated cells[4]. This procedure was followed by T cell depletion, either by sedimentation of cells which bind sheep red blood cells, hereafter referred to as E-rosette centrifugation[5], or by complement (C') mediated lysis of CAMPATH I positive cells[6]. Both procedures resulted in an additional 1-2 log T-cell depletion. All cell suspensions were cultured for CFU-C, as described below.

For most experiments, these T-cell depleted stem cell concentrates were cryopreserved, since the conditioning regimen that was used for autologous BM transplantation required two days. The BM cells were thawed immediately before use by stepwise dilution. By using this method, significant losses of CFU-C did not occur[7]. Therefore, it was assumed, that significant losses of PHSC due to the cryopreservation procedure could also be avoided.

These stem cell concentrates were used as autologous BM grafts or for further purification of stem cells on the basis of the expression of cell surface markers.

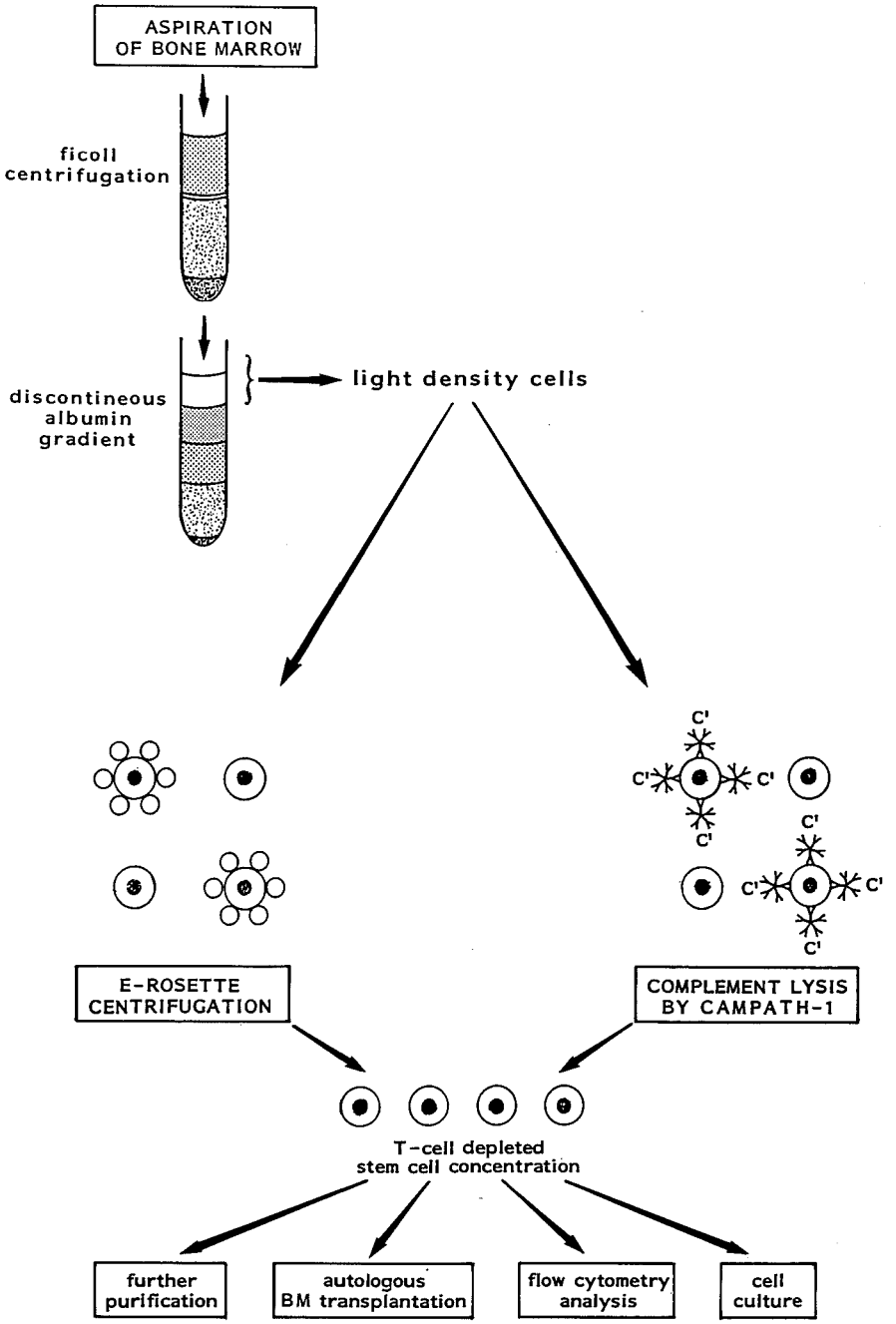

Figure 2.1 **Preparation of T-lymphocyte depleted stem cell concentrates from Rhesus monkey BM cells.**

2.3. Monoclonal antibodies.

ICH3, a murine monoclonal antibody (MCA), which recognizes an epitope of the CD34 antigen, also termed Human progenitor cell Antigen 1 (HPCA 1) was a gift of Dr R. Levinski (Institute of Child Health, London, U.K.). This antigen is expressed on 1-4% of human bone marrow cells, including hemopoietic progenitor cells such as CFU-blast, CFU-mix, CFU-GM, and BFU-E. ICH3 was produced as described earlier[8]. Cells were labelled by adding 2 µl ICH3 (1 mg/ml IgG_{2a}) to 5 x 10^6 pelleted cells and the mixture was incubated for 30 minutes at 4°C. After washing with Hanks Balanced Salt Solution (HBSS), whith 1% fetal calf serum (FCS), 5 X 10^6 cells were stained with 50 µl anti-IgG_{2a}, conjugated to fluorescein isothiocyanate (FITC) (Nordic, Tilburg, The Netherlands), and diluted 1:30 in HBSS/FCS, for 30 minutes at 4°C.

CAMPATH I, a rat MCA directed against human and Rhesus monkey[9] B- and T lymphocytes, was a gift of Dr H. Waldman (Department of Pathology, University of Cambridge, U.K.). Cells were labelled by adding 0.1 ml CAMPATH I (0.1 mg/ml, Ig M) to 10^6 pelleted cells and incubated for 30 minutes at 4°C, followed by C' mediated lysis, using autologous serum as a source of complement[6], or by staining of the positive cells with goat anti rat (GARA), conjugated to FITC.

DR positive cells were labelled with anti-HLA-DR conjugated to phycoerythrin (PE) (Becton & Dickinson, Immunocytometry Systems, Mountain View, CA, U.S.A.) (10 µl, 25 µg/ml, IgG_{2a} to 5 x 10^6 cells.)

CD4 and CD8 positive cells were labelled with PE conjugated Leu 3A (2 µl, 10 µg/ml, IgG_1 to 10^6 cells) and Leu 2A (Becton & Dickinson) (2 12.5 µg/ml IgG_{2a} to 10^6 cells), respectively.

CD11b positive cells were labelled with Mol/FITC (Coulter Immunology, Hialeah, FL, U.S.A.) (10 µl, 1 µg/ml to 5 x 10^6 cells) for fluorescence analysis or with Mol/biotin (Coulter). Cells labelled with Mol/biotin were washed and stained with Streptavidin/PE (Becton & Dickinson) (1 µl, 250 µg/ml to 10^6 cells). For immunomagnetic cell separation Mol (Coulter) (2µl/10^6 cells) was

used.

2.4. Immunofluorescence analysis and cell sorting.

A modified fluorescence activated cell sorter (FACS II, Becton & Dickinson) with the laser at 488 nm (0.5 W) was used for analysis and sorting[10]. In some experiments, four parameters were analyzed. For these experiments, an eight parameter cell sorter, termed Rijswijk Experimental Light Activated Cell Sorter II (RELACS II) was used. The sheet fluid consisted of PBS. Forward light scatter (FLS) intensity, which is related to cell size, was measured with the laser beam blocking bar in its narrowest orientation, and linearly amplified. Perpendicular light scatter (PLS) intensity, which is related to cell structure, was measured by an S-11 type photomultiplier (Thorn EMI, Middlesex, U.K.) and linearly amplified. Based on FLS and PLS, several subpopulations of BM cells can be distinguished[11], as indicated in Figure 2.2. FLS signals were used for triggering the measurements. Erythrocytes were electronically excluded from analysis. FITC was measured through a combination of broad band multicavity interference (520-550 nm: Pompfret Research Optics Inc, Stanford, CT) and a 530 nm cut-off filter (Ditric Optics, Hudson, MA) by an S-20 type photomultiplier. PE was measured through a 550 nm cut-off filter (Schott, Mainz, Germany) by photomultiplier R1477 (Hamamatsu, Japan). Logarithmic amplifiers were used to process the signals of fluorescence. A comparison network was used to eliminate the orange emission of FITC from the orange PEchannal[12]. No PE emission could be detected in the green channal.Cells were sorted into 15 ml glass tubes. The wall sides where the sorted cells were deposited were rinsed before sorting with PBS containing 10% fetal calf serum (FCS). The data were collected as a 4-parameter (FLS, PLS, FITC, and PE) list mode on a HP 220 computer for later analysis[13].

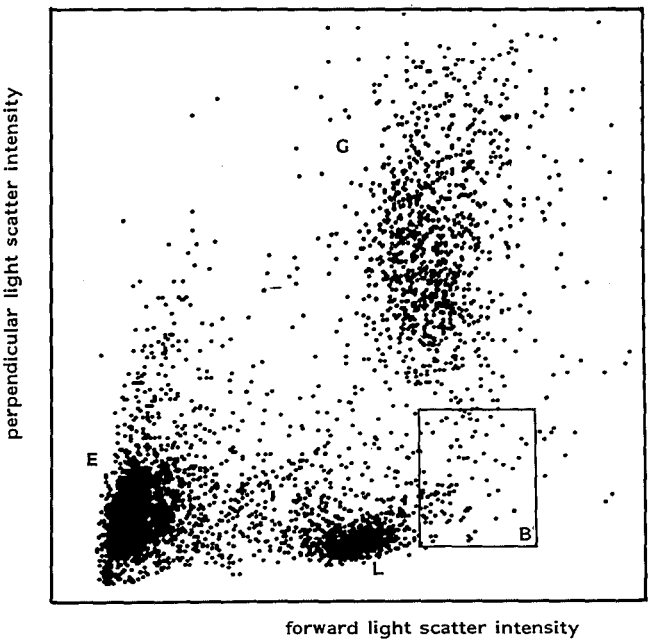

Figure 2.2 **Distribution of scatter parameters of Rhesus monkey bone marrow cells.** Separate clusters of erythrocytes (E), lymphocytes, (L), blasts (B) and granulocytes (G) can be recognized.

2.5. Isolation of CD 34 positive cells by using immunomagnetic beads.

One monkey was transplanted with BM from which the CD34+ cells were removed by binding to immunomagnetic beads. Magnetic microspheres, Dynabeads™ (Dynal, A.S., Oslo, Norway), coated with goat anti-mouse IgG, and a magnetic particle concentrator, Dynal MPC™ were used[14]. 1.25 x 10^9 beads were incubated with 1.25 ml ICH3 for 20 hours at 4°C on a rotating wheel to keep particles in suspension. After washing, the ICH3-carrying beads were in-

cubated with 2.5 x 10^8 LSM isolated mononuclear BM cells in 12 ml PBS, for 20 minutes at 4°C on a rotating wheel. Following this procedure, each CD34 positive cells was bound to a number of immunomagnetic beads. Such a complex was termed a rosette. Rosette forming cells were removed by using the magnetic particle concentrator as illustrated in Figure 2.3. Since pilot experiments had demonstrated the presence of residual CD34 positive cells after this separation, a second separation step was introduced: to remove these cells, the depleted fraction was incubated with ICH3 as described in section 2.2, washed, and incubated with beads (20 minutes, 4°C), coated with goat anti-mouse. Residual rosetting cells were removed, again by use of the magnetic particle concentrator.

For positive selection of ICH3+ cells, a modification of this separation method was developed, since it appeared difficult to remove the beads from the cells if the method described above was used. Briefly, uncoated beads were activated and incubated with protein A, washed four times and incubated with ICH3. By this procedure the immunomagnetic beads were coated with ICH3 and could be used for the isolation of CD34 positive cells by incubation of stem cell concentrates (section 2.2) followed by isolation of rosette forming cells using the magnetic particle concentrator. The beads were removed from the cells by adding an excess bovine IgG, provided by bovine plasma, followed by isolation of the cells by using the magnetic particle separator again (Figure 2.3).

2.6. Culture of *in vitro* colony forming cells (CFU-C).

2.6.1. Agar cultures.

BM cells were cultured using the double-layer agar technique with a feeder layer of human peripheral blood leukocytes, according to Pike and Robinson[15]. Briefly, human leukocytes were suspended in supplemented Dulbecco's modified eagle medium (Gibco Ltd. Paisley, U.K.), containing 20% FCS (Biochrom, Berlin, Germany), and 0.5% agar (Bacto agar, Difco Laboratories, Detroit, MI, U.S.A.. 10^6 cells were plated into 35 mm Petri dishes

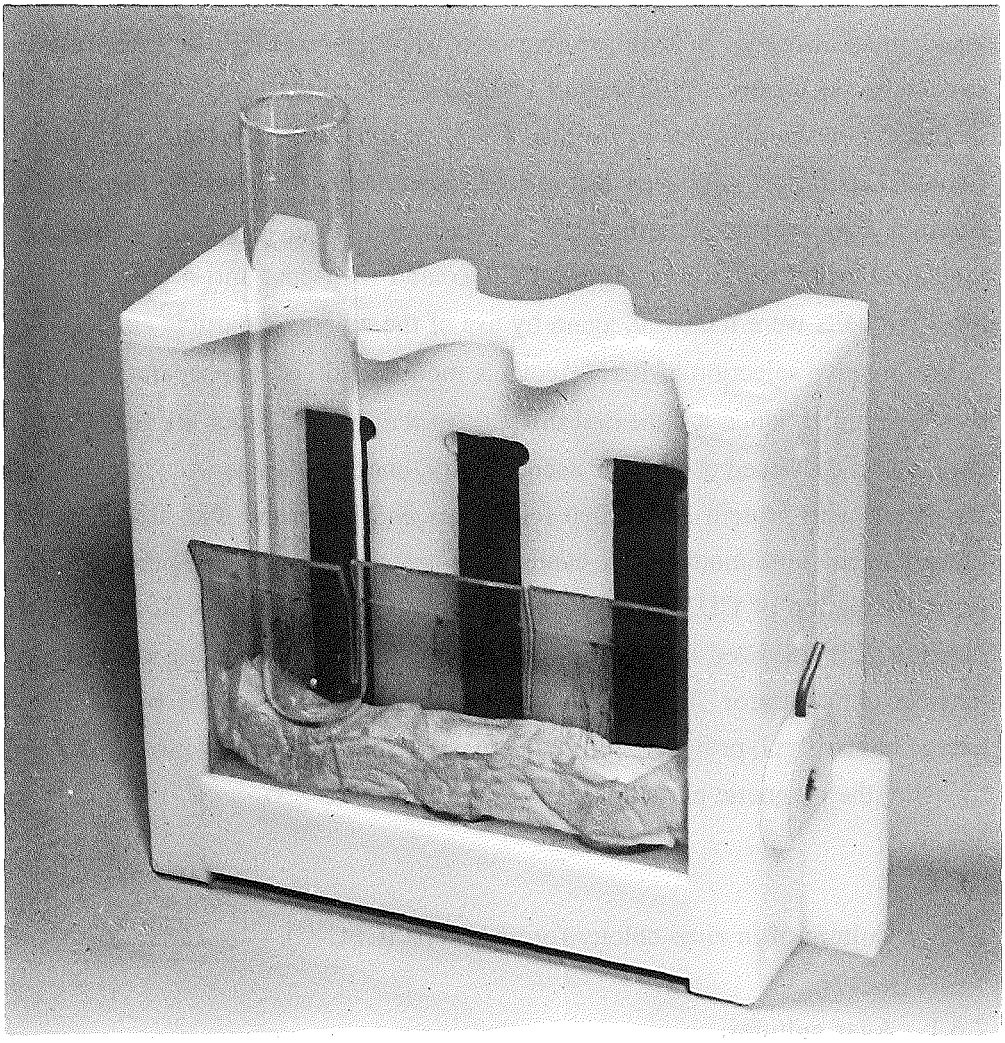

Figure 2.3 **Magnetic cell separator (Dynal^{R}).** A tube, containing a cell suspension is placed against the magnet. Positive cells, linked to immunomagnetic beads, are attracted by the magnet and can be separated from the negative cells by removing the suspension from the tube.

(Falcon, 1008). 1 to 7 days later, appropriate numbers of rhesus monkey BM cells, suspended in a similar medium, were plated on top of these feeder layers. The cultures were incubated for 7 days at 37°C in a saturated humidified atmosphere of 7.5% $C0_2$ in air. Colonies larger than 50 cells were scored on day 7 using an inverted microscope (Zeiss) at a 30-fold magnification.

2.6.2. Methylcellulose cultures.

BM cells were cultured without feeder layers in methylcellulose. This method is a modification of a previously described serum-free culture method for murine BM cells[16]. Briefly, appropriate numbers of BM cells were suspended in supplemented Dulbecco's modified eagle medium (Gibco) containing 0.8% methylcellulose (Methocel A4M Premium grade, Dow chemical), 10% FCS (Biochrom), bovine serum albumin (Sigma, St Louis, MO, U.S.A), iron saturated human transferrin (Behringwerke), Na_2SeO_3 (Merck, Darmstadt, Germany), ß-mercapto-ethanol (Merck), nucleosides (Sigma), linoleic acid, (Merck), and cholesterol (Sigma). Recombinant human GM-CSF was used at supraoptimal concentrations to stimulate the cells as indicated in the results. These cell suspensions were plated into 35 mm Petri dishes (Falcon, 1008). The cultures were incubated for 10 days at 37°C in a saturated humidified atmosphere of 7.5% $C0_2$ in air. Colonies larger than 50 cells were scored on day 10 using an inverted microscope (Zeiss) at a 30-fold magnification.

2.7. Total Body Irradiation (TBI) and bone marrow transplantation.

The monkeys selected for BM transplantation were anaesthetized as described in section 2.3 and conditioned for BM transplantation with 12 Gy bilateral TBI, given in two equal fractions separated by 24 hours. Physical parameters: 6 MV X-rays, average dose rate 0.15 Gy/min., source-to-surface distance 3.60 meters. To avoid radiation pneumonitis, the lungs were shielded by 10 mm lead resulting

in a total lung dose of 8.5 Gy.

For the experiments described in Chapter IV and VII, monkeys were irradiated without BM transplantation. For these experiments, graded, single doses of TBI without lung shielding were given under similar conditions.

Other monkeys were exposed to a single fraction TBI, delivered by 2 opposing X-ray generators. Physical parameters: 300 kV X-rays, average dose rate 0.20 Gy/min., source-to-surface distance 0.8 meter, as described elsewhere[17].

BM, prepared as described in section 2.3 and 2.4, was injected into a peripheral vein 24 hours after TBI. The day of transplantation was designated day 0. In irradiated, untransplanted monkeys the day of TBI was designated day 0.

2.8. Recombinant human GM-CSF.

Recombinant human GM-CSF was kindly provided by Biogen Inc., and later by Glaxo Inc. (Genève, Switzerland), and stored at -20° C until dilution of the daily dose into 1 ml pyrogen free 0.9% NaCl immediately prior to use. A similar culture system whithout the human GM-CSF gene was used for the production of a placebo. The purification procedures of GM-CSF and placebo were similar. Placebo or GM-CSF was injected subcutaneously once daily or continuously iv in different doses and under different conditions as will be described.

2.9. Supportive care.

The animal care after transplantation was a modification of that described before[9,18]. Briefly, acidified (pH3) drinking water and reverse barrier nursing were used for all animals. Selective decontamination of the digestive tract was a-

chieved by oral administration of antimicrobial agents starting at day 0 in transplanted monkeys or at day 1 in irradiated, not transplanted monkeys. The agents were polymixine B, norfloxacin (Noroxin^R, Chibret, Haarlem, The Netherlands) and nystatin (Nystatin Labaz^R Sanofi B.V., Maassluis, The Netherlands) for the transplanted monkeys. The experiments with untransplanted monkeys were part of a preclinical study on the treatment of victims of irradiation accidents. For these monkeys cotrimoxazole (Bactrimel^R, Hoffmann-La Roche, Mijdrecht, The Netherlands) was used instead of polymixine B and norfloxacin because it is easier available in times of emergency. If resistant gram negative bacteria persisted in the faeces, the prophylactic antibiotic treatment was adapted to the antibiogram of these bacteria. Selective decontamination and reverse barrier nursing was continued until leukocyte counts exceeded 1 x 10^9/l. In case of fever (body temperature > 39.5°C), a blood sample was taken for culture and systemic antibiotic treatment (cephamandol in combination with ticarcilin) was started immediately. This treatment was adapted as soon as the antibiogram of a positive blood culture demonstrated the presence of resistant bacteria.

Irradiated platelet transfusions were given whenever thrombocyte counts reached values lower than 40 x 10^9/l. Irradiated packed cells were given if the hematocrit was lower than 20%.

Dehydration and electrolyte disturbances, caused by the gastro-intestinal syndrome, were treated by appropriate parenteral fluid and electrolyte administration.

2.10. Observations following TBI.

The animals were subjected to a thorough inspection twice daily: general condition, appetite, body weight, axillary body temperature, hydration, and production of faeces and urine were recorded. Peripheral blood cell counts were determined daily. Leukocyte counts, red blood cell counts, hemoglobin levels

and hematocrit values were determined with a 7000™ Cell Counter (Baker Instruments, Amersfoort, The Netherlands). Reticulocyte counts were determined by staining with brilliant cresyl blue. Differential counts were done morphologically after May-Grünwald-Giemsa staining. The leukocyte counts were corrected for normoblasts. Thrombocyte counts were determined with an 810™ Platelet Analyzer (Baker Instruments).

Twice weekly, serum protein, sodium, potassium, chloride, CO_2, SGOT, SGPT, LDH, alkaline phosphatase and albumin were determined on a routine base at the laboratory of the cooperating Delft hospitals, Stichting Samenwerkende Delftse Ziekenhuizen, (SSDZ).

Complete autopsy was performed as soon as possible following spontaneous death or immediately following euthanasia of moribund animals. The other monkeys were kept alive for separate studies concerning long term effects of BMT, TBI and treatment with hemopoietic growth factors.

2.11. Statistics.

Mean, standard deviation, and correlation coefficient, and linear regression equations were calculated according to the standard formulas[19]. The student's T test was used to calculate the 95 % confidence limits. The relationship between irradiation dose and mortality in Chapter IV was calculated by probit/logit analysis.

Blood cell counts are subject to random day to day fluctuations, that hamper a proper evaluation. To avoid the influence of those variations and to evaluate the total response of treatment with GM-CSF, a parameter, independent of those fluctuations and covering the whole observation period was used to quantify the influence of this treatment on the production of a specific blood cell type. This parameter, termed cumulated cell count, is the sum of the daily assessed cell counts during a certain observation period.

REFERENCES

1. Vreeswijk, W. van, and Koning, H. (1978). Breeding of macaques and chimpanzees at the Dutch Primate Center. In: Recent Advances in Primatology, Conservation, Vol. 2 (Eds. Chivers, D.J., and Lane-Petter, W.) Academic Press, London, p.279.

2. Vreeswijk, W. van, and Balner, H. (1980). The major histocompatibility complex: matching and other factors influencing skin allograft survival in related and unrelated rhesus monkeys. Transplantation 30:196.

3. Bekkum, D.W. van, and Vries, M.J., de. (1967). Radiation Chimeras. London, Logos, Academic Press.

4. Dicke, K.A., and Bekkum, D.W. van. (1970). Avoidance of acute secondary disease by purification of hemopoietic stem cells with density gradient centrifugation. Exp Hematol. 20:126.

5. Madsen, M., Johnson, H.E., Wendelboe Hansen, P., and Christiansen, S.E. (1980). Isolation of human T- and B- lymphocytes by E-rosette centrifugation. Characterization of the isolated subpopulations. J.Immunol. Methods 33:323-336.

6. Waldman, H., Hale, G., Cividalli, G., Weshler, Z., Manor, D., Rachmilewitz, E.A., Polliak, A., Or, R., Wevss, L., Samuel, S., Brautbar, C., and Slavin, S. (1984). Elimination of graft-versus- host disease by in vitro depletion of alloreactive lymphocytes with a monoclonal rat anti-human lymphocyte antibody (CAMPATH I). Lancet II:483-485.

7. Schaeffer, U.W., Dicke, K.A., And Bekkum, D.W. van. (1972). Recovery of hemopoiesis in lethally irradiated monkeys by frozen allogeneic bone marrow grafts. Rev. Europ. Etudes Clin. et Biol. 17:483-488.

8. Watt, S.M., Karhi, K., Gatter, A.J.W., Furley, F.E., Katz, L.E., Healy, L.J. Atass, N.J., Bradley, N.J., Sutherland, D.R., Levinski, R., and Greaves, M.F. (1987). Distribution and epitope analysis of the cell membrane glycoprotein (HPCA-1) associated with human hemopoietic progenitor cells. Leukemia 1:417-426.

9. Gerritsen, W.R., Wagemaker, G., Jonker, M., Kenter, M., Wielenga, J.J., Hale, G., Waldmann, H., and Bekkum, D.W. van. (1988). The repopulating capacity of bone marrow grafts following pretreatment with monoclonal antibodies against T lymphocytes in rhesus monkeys. Transplantation 45:301-307.

10. Visser, J.W.M, Bauman, J.G.J, Mulder, A.H., Eliason, J.F., and Leeuw, A.M. de (1984). Isolation of murine pluripotent hemopoietic stem cells. J. Exp. Med. 59:1576-1590.

11. Engh, G.J. van den, Visser, J.W.M., and Trask, B. (1979). Identification of CFU-S by scatter measurements on a light activated cell sorter. In: Exp. Hematol. Today. Eds. Baum, S.J., and Ledney, G.D. Springer, New York. p. 19-26.

12. Loken, M.R., Parks, D.R., and Herzenberg, L.A. (1977). Two colour immunofluorescence using a fluorescence activated cell sorter. J. Histochem.

Cytochem. 25:899-908.

13. Engh, G.J. van den, Stokdijk, W., and Peters, D. (1987). A fast data-acquisition system for multiparameter flow analysis and sorting. Cytometry 8, Suppl.:7.

14. Lea, T., Vartdal, F., Davies, C., and Ugelstad, J. (1985). Magnetic monosized polymer particles for fast and specific fractionation of human mononuclear cells. Scand. J. Immunol. 22:207-216.

15. Pike, B.L., and Robinson, W.A. (1970). Human bone marrow colony growth in agar gel. J.Cell. Physiol. 76:77-84.

16. Merchav, S., and Wagemaker, G. (1984). Detection of murine bone marrow granulocyte/macrophage progenitor cells (GM-CFU) in serum-free cultures stimulated with purified M-CSF or GM-CSF. Int. J. Cell. Cloning 2:356-367.

17. Zoetelief, J., de Wit, N.J.P., Wielenga, J.J., Wagemaker, G., and Broerse, J.J. Dosimetry for whole body irradiation of Rhesus monkeys with 300 kV X-rays.in preparation.

18. Broerse, J.J., Bekkum, D.W. van, Hollander, C.F.,Davids, J.A.G. (1978). Mortality of monkeys after exposure to fission neutrons and the effect of autologous bone marrow transplantation. Int. J. Radiat. Biol. 34:253-264.

19. Documenta Geigy Wissenschaftliche Tabellen. (1960). J.R, Geigy, Basel, Schwitzerland.

CHAPTER III
CD34 EXPRESSION ON RHESUS MONKEY HEMOPOIETIC STEM CELLS AND PROGENITOR CELLS.

3.1. Introduction.

Cell surface markers and light activated cell sorting have been used for characterization and purification of murine stem cells[1,2]. By using these techniques, grafts of only 10^2 purified PHSC appeared to be sufficient to protect a lethally irradiated mouse from bone marrow failure. Among the cell surface markers available to isolate immature human hemopoietic cells, the CD34 antigen, identified by the MCAs My-10[3], 12-8[4], BI-3C5[5], ICH3[6], and others is of outstanding interest, since it is mainly expressed on immature hemopoietic cells along various differentiation lineages, including hemopoietic progenitor cells such as CFU-blast, CFU-mix, CFU-GM, and BFU-E. Moreover, it is present only on 1-4 % of human bone marrow cells. The characteristics of the CD34 antigen, also termed the Human Progenitor Cell Antigen 1 (HPCA-1) are summarized in Table 3.1. The monoclonal antibody ICH3 is distinct from the other three in that it is a high avidity antibody of IgG 2a isotype that recognizes a peptide epitope and does not modulate or has any effector function such as complement lysis.

Table 3.1 CELL MEMBRANE GLYCOPROTEIN CD34 (HPCA-1) ASSOCIATED WITH HUMAN HEMOPOIETIC PROGENITOR CELLS

antigen:	
115 kD glycoprotein	
available monoconal antibodies:	
My-10	Civin et al., 1984[3]
12.8	Andrews et a., 1986[4]
BI-3C5	Tindle et al., 1985[5]
ICH3	Watt et al., 1987[6]
selectivity:	
1 - 4 % of all bone marrow cells	
basement membranes	
capillary endothelia	

These properties make ICH3 preferentially suited for positive selection of stem cells for bone marrow transplantation. My-10 and BI-3C5 do not cross react with rhesus monkey bone marrow cells[7]. For My-10 and BI-3C5 a carbohydrate structure of the epitope is involved in antigen recognition, which is not the case for ICH3. Since peptide structures are probably better conserved during evolution than carbohydrate structures, the cross reactivity of ICH3 with rhesus monkey hemopoietic progenitor cells and stem cells was investigated.

In most of the studies in rodents, the enrichment of stem cells was monitored by the quantitative and functional spleen colony assay (SCA). In outbred species, such as rhesus monkeys and humans, the most early detectable cell is the CFU-mix[8]. However, the development of a complete hemopoietic system out of the progeny of a CFU-mix has never been demonstrated. Accordingly, the relationship between CFU-mix and stem cells is less well established than the relationship between CFU-S and stem cells. Studies on the nature of human stem cells have been limited by the lack of a suitable assay and by a restricted possibility for *in vivo* studies. Recently, a

semiquantitative *in vivo* stem cell assay in rhesus monkeys has been developed in our laboratory. This autologous regeneration assay is based on the observation that the peripheral reticulocyte and leukocyte regeneration times after ABMT are directly related to graft size (Figure 3.1). This assay has been used to demonstrate that the regeneration time is independent on the presence of T-lymphocytes in the graft[9]. It also enables a quantitative comparison between enrichment of repopulating cells as calculated from the *in vivo* regeneration time and the enrichment of progenitor cells as calculated from *in vitro* assays.

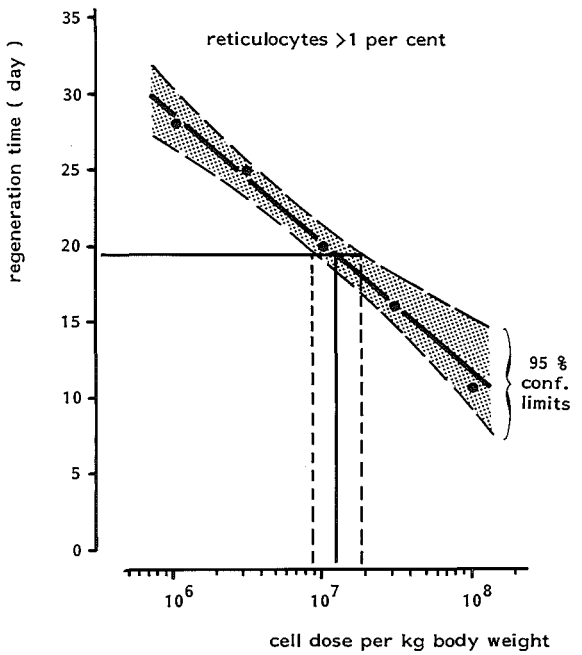

Figure 3.1 **Dependence of the regeneration time of reticulocytes on the number of autologous BM cells grafted.** This is used as the basis for a regenerating stem cell assay *in vivo*[9]. An example is given: if the reticulocyte counts persistingly exceed a value of 1 %, starting from day 19, the graft size is equivalent to 9 x 10^6 to 2 x 10^7 unfractionated BM cells/kg (95 % confidence limits).

MHC Class II antigens have been demonstrated on HPC of human and subhuman primates[10,11,12] and on PHSC which are capable of restoring hemopoiesis *in vivo* in mice and dogs[13,14,15,16]. Previously, the autologous regeneration assay was used to demonstrate the expression of MHC Class II (RhLA-DR) antigens on PHSC[17]. The DR antigen is not exclusively expressed on HPC and PHSC. Therefore, higher HPC concentration factors can be obtained by multiparameter cell sorting, based on the expression of DR and other antigens. Purification of PHSC on the basis of simultaneous expression of the CD34 and the DR antigen has been suggested.

3.2. CD34 expression on Rhesus monkey hemopoietic progenitor cells.

To test the expression of the CD34 antigen on hemopoietic progenitor cells, stem cell concentrates were incubated with ICH3 and stained with anti IgG 2a conjugated to FITC, as described in section 2.3. The fluorescence of the cells was analyzed and cells were sorted on the basis of the fluorescence signal as described in section 2.4. The sorted cells were cultured as described in section 2.6. Figure 3.2 shows the histogram of Rhesus monkey stem cell concentrates labelled with ICH3. About 20% of the cells reacted with ICH3.
Sorting of ICH3 positive cells resulted in a 45-100 fold enrichment of CFU-C, while sorting of ICH3 negative cells resulted in a CFU-C depletion. Sorting of the 3% most bright cells resulted in a higher enrichment, but also in a significant loss of CFU-C, as shown in Table 3.2.

To test the reliability of the sorting procedure, sorted ICH3 positive cells were reanalysed for FITC fluorescence. The histogram of this analysis, shown in Figure 3.3, demonstrates, that more than 95 % of the sorted cells are CD34 positive.

The CD34 expression on a small population of Rhesus monkey bone marrow cells, confined to immature hemopoietic cells, including hemopoietic progenitor cells, is similar to that observed in human bone marrow[18,19].

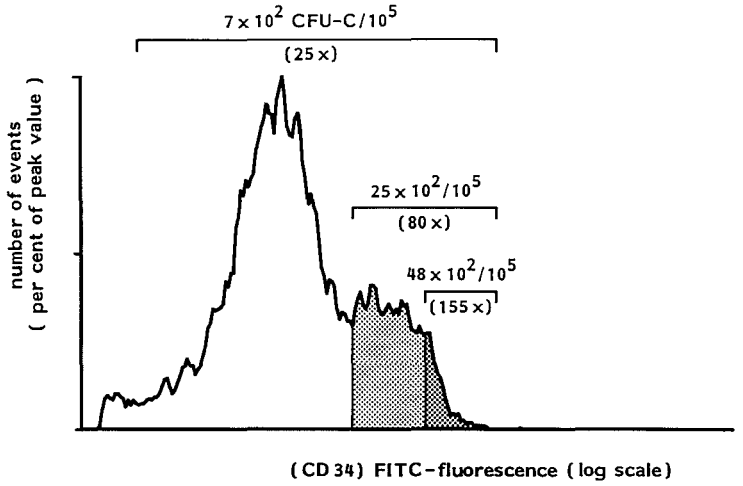

Figure 3.2 **Distribution of Rhesus monkey T-lymphocyte depleted stem cell concentrates according to fluorescence intensity after labeling with ICH3 (anti CD34) and anti IgG 2a/FITC.** The frequency of CFU-C and the CFU-C concentration factor (in parenthesis) is indicated for all cells, all positive cells and the most brightly fluorescent 3 % of the distribution.

Table 3.2 CD34 EXPRESSION ON RHESUS MONKEY CFU-C
(mean of 3 experiments)

BM fraction	CFU-C/10^5cells (mean ± s.d.)	conc. factor* (mean ± s.d)	recovery (mean ± s.d.)
mononuclear cells	59 ± 13	2 ± 0.2	100 ± 19
stem cell concentrates**	736 ± 275	25 ± 4	68 ± 39
sorter control***	759 ± 59	26 ± 4	72 ± 32
CD34-	178 ± 157	6 ± 5	10 ± 10
CD34+	2519 ± 1171	80 ± 30	37 ± 30
CD34++ ****	4788 ± 1019	155 ± 13	8 ± 5

* relative to unfractionated punctured BM, which was set at a value of 1
** prepared as described in section 2.2
*** all cells of stem cell concentrates sorted with label
**** 3% most bright fluorescent cells

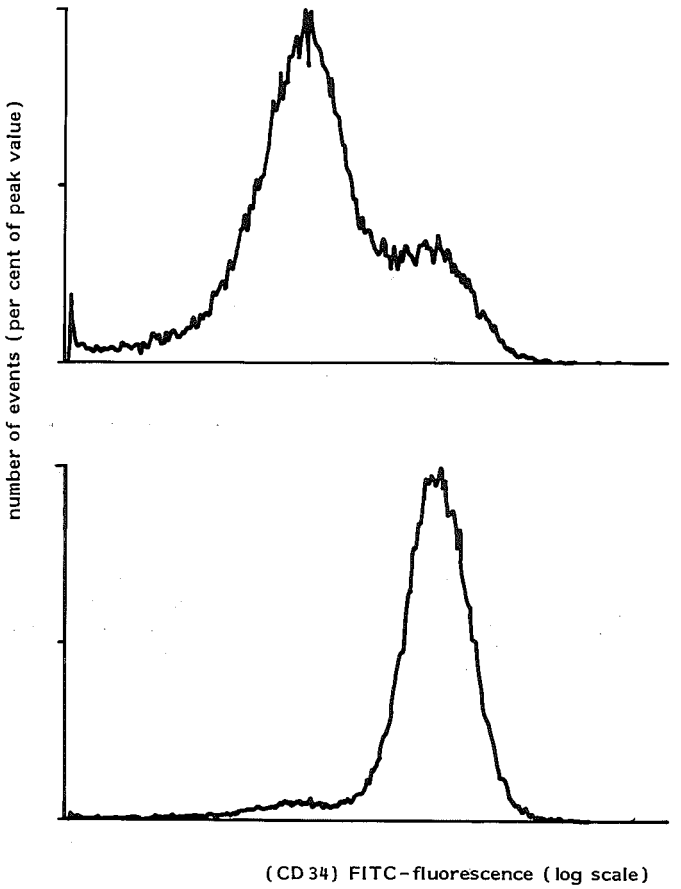

Figure 3.3 **Effect of flowcytometric cell sorting.** Distribution of Rhesus monkey T-lymphocyte depleted stem cell concentrates according to fluorescence intensity after labeling with ICH3 (anti CD34) and anti IgG 2a/FITC before (upper panel) and after (lower panel) sorting of ICH3 positive cells.

3.2.1.Enrichment of Rhesus monkey CFU-C by two colour cell sorting.

In the previous section, the enrichment of Rhesus monkey CFU-C on the basis of the expression of the CD34 antigen was demonstrated. HPC and PHSC can also be enriched on the basis of the expression of MHC class II antigens (DR). To obtain a higher CFU-C enrichment, it was attempted to combine staining with anti-DR and ICH3. Rhesus monkey BM cells were enriched by albumin density centrifugation as described in section 2.2 , incubated with ICH3, washed, and stained with anti-IgG 2a, conjugated to FITC, Subsequently, the cells were washed twice and incubated with anti HLA-DR conjugated to PE, as described in section 2.4. The cells were analyzed and sorted on the basis of red and green fluorescence, and cultured for CFU-C. The results are shown in Figure 3.4, in which three populations can be discerned: CD34-, CD34+/DR bright, and CD34+/DR dull. The percentage of the nucleated cells, the CFU-C concentration factor and the CFU-C recovery are indicated in this Figure. Selection of CD34+/DR+ cells did not improve the enrichment of CFU-C as compared to selection of CD34+ cells alone. This was due to the heterogeneity of DR-expression among CFU-C, which caused a separation of the CD34+ fraction into a brightly and a dull anti-DR stained fraction.

3.3. Repopulating capacity of CD34 positive cells

The expression of the CD34 antigen on hemopoietic progenitor cells has been described in section . To test whether CD34+ BM cells were capable of restoring hemopoiesis *in vivo*, Rhesus monkeys were transplanted with isolated, CD34+ BM cells. For a quantification of the enrichment of PHSC by positive selection on the basis of CD34 expression we used the autologous regeneration

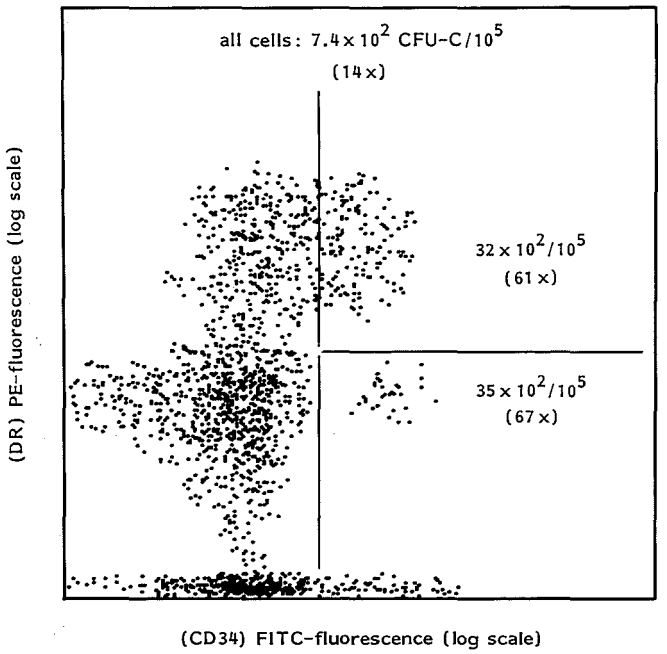

Figure 3.4 **Dot plot of Rhesus monkey T-lymphocyte depleted stem cell concentrates according to red and green fluorescence intensity after labeling with ICH3 (anti CD34), anti IgG 2a/FITC and anti HLA-DR/PE.** The frequency of CFU-C and the CFU-C concentration factor (in parenthesis) is indicated for all cells, for the CD34+/DR+ and for the CD34+/DR- populations.

assay as described in section 3.1. To assess the capacity of CD34+ cells for sustaining chimerism, Rhesus monkeys were transplanted with CD34+ BM cells from RhLA identical, sex mismatched siblings.

3.3.1. Enrichment of repopulating cells by positive selection of CD34+

To quantify the enrichment of repopulating cells by positive selection of CD34 positive BM cells,Rhesus monkeys, irradiated with 2 x 6 Gy TBI, (described in

section 2.7), were transplanted with small standardized numbers of autologous CD34+ cells, selected by sorting ICH3-labelled BM. Two monkeys received a graft of 2 x 10^5 sorted ICH3+ cells/kg body weight. The regeneration time of reticulocytes in one monkey was similar to the regeneration time after transplantation of 8.5-17.2 x 10^6 (95% confidence limits) unfractionated BM cells/kg, while the other regenerated as if it had received 13.5-28.3 x 10^6 unfractionated BM cells/kg body weight (Figure 3.1 and 3.5, Table 3.3). In order to demonstrate, that the regeneration time, expressed as the day at which reticulocyte counts exceed 1% is a representative parameter for the regeneration, in Figure 3.6 the leukocyte regeneration of these monkeys is compared with the regeneration of

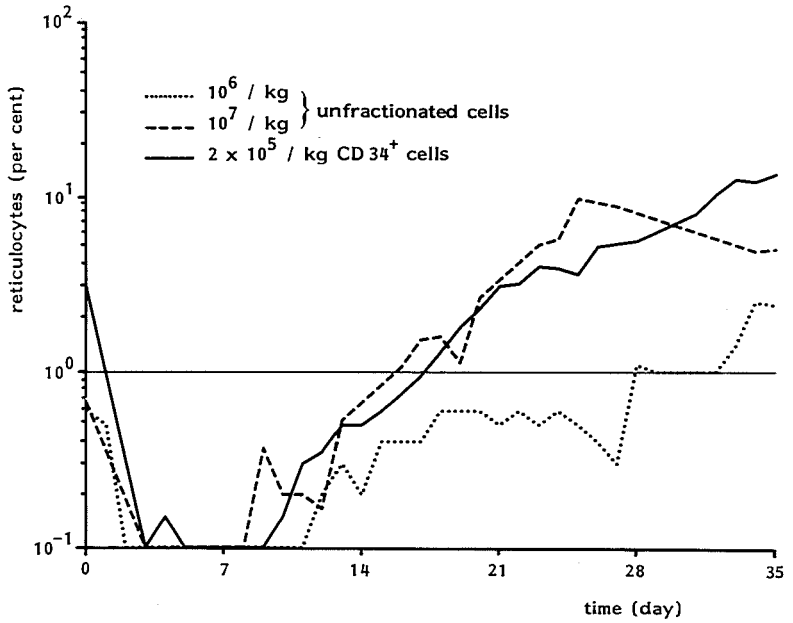

Figure 3.5 **Regeneration of reticulocytes following transplantation of autologous CD34 positive BM cells.** Conditioning with 2 x 6 Gy (6 MV X-rays) TBI.

control monkeys, which received graded numbers of unfractionated bone marrow cells. The enrichment of repopulating cells was not reflected by an early leukocyte regeneration in one of the two monkeys. In this monkey the early phase after transplantation was complicated by sepsis with fever (>40° C) from day 10 - 18, a severe ulcerating gingivitis from day 7 - 20, and blood cultures revealing Streptococcus mitis on days 7, 9, 10, and 14. In the other monkey, which had no infectious complications, the regeneration time of leukocytes indicated a similar stem cell concentration as that of reticulocytes (Figure 3.6).

These results indicate a 40- to 140-fold enrichment of repopulating cells (Table 3.3), which is in the same order of magnitude as the enrichment of CFU-C (Table 3.2).

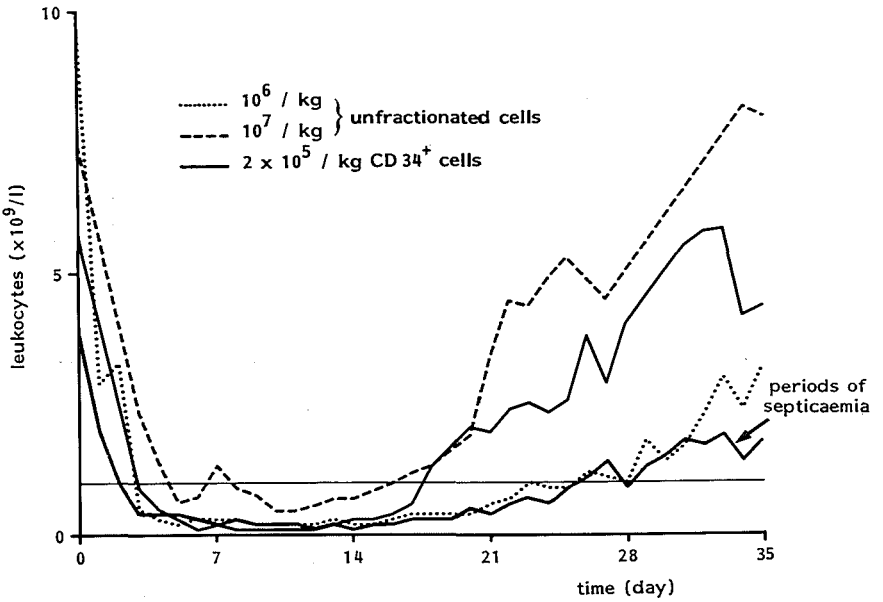

Figure 3.6 **Regeneration of leukocytes following transplantation of autologous CD34 positive BM cells.** Conditioning with 2 x 6 Gy (6 MV X-rays) TBI.

Table 3.3 CD34 EXPRESSION ON RHESUS MONKEY STEM CELLS AS DEMONSTRATED FROM THE REGENERATION RETICULOCYTES AFTER BMT

UMN*	GRAFT size x10^6 /kg	GRAFT popu-la-tion	RETICULOCYTE REGENERATION TIME (day) expected on graft size	RETICULOCYTE REGENERATION TIME (day) observed	BM equiv.** x 10^6/kg (calculated)	STEM CELL conc. factor**
1LG	3	stem cell conc.***	25	18	11-22	4 - 7
M5	0.2	CD34+	>30	19	9-17	42 - 86
D35	0.2	CD34+	>30	17	14-28	67 - 142
1TP	20	CD34-	14	22	4- 9	0.2- 0.4

* Unique Monkey Number
** 95% confidence limits
*** prepared as described in section 2.2

As a negative control, one monkey was grafted with 2 x 10^7 LSM separated cells/kg body weight from which the ICH3 positive cells had been removed by using immunomagnetic beads, as described in section 2.5. This procedure resulted in 90 % CFU-C depletion (table 3.2).

The regeneration time of the reticulocytes in this monkey was similar to a regeneration time following transplantation of 4.1 - 8.5 x 10^6 unfractionated bone marrow cells/kg (95% confidence limits) (Table 3.3). This observation demonstrated a 60 - 80 % depletion of repopulating cells, which is in the same order of magnitude as the CFU-C depletion (Table 3.2). In this monkey, which had no infectious complications, the regeneration time of leukocytes was similar to that following transplantation of 4 x 10^5 - 1 x 10^7 unfractionated cells/kg, indicating a similar depletion of repopulating cells.

3.3.2. Chimerism studies following BM transplantation with CD34+ cells

To study the capacity of CD34+ cells to establish sustained chimerism, two female Rhesus monkeys were transplanted with small numbers of allogeneic CD34+ BM cells/kg, obtained from a RhLA identical male sibling. Allogeneic BM transplantation requires more cells than autologous transplantation. As isolation of sufficient numbers of CD34+ cells for allogeneic BM transplantation by flowcytometric cell sorting requires approximately 30 hours of sorting, the CD34+ cells were isolated by means of immunomagnetic beads, as described in section 2.5. The number of residual T lymphocytes in the CD34+ cell suspensions was determined by flow cytometric analysis after staining with Leu 2a/PE and Leu 3a/PE, (see section 2.3 and 2.4.)

The characteristics of the grafts are summarized in Table 3.4. The monkeys were conditioned by TBI with 9.5 Gy TBI (6 MV X-rays) on day -1, and received supportive care, starting at day 1, as described in section 2.9.

Positive selection of CD34+ cells by immunomagnetic beads following PHSC enrichment by albumin density centrifugation results in a four- to twelve fold further CFU-C enrichment (Table 3.4). Both monkeys were transplanted with 3.5 x 10^6 CD34+ cells/kg, which is the CFU-C equivalent of 2 x 10^8 unfractionated BM cells/kg. (range 1.4 - 2.5 x 10^8).

The regeneration time and chimerism status of these monkeys were compared with a control monkey, which was transplanted with E-rosette depleted, albumin gradient concentrated PHSC, containing the CFU-C equivalent of 2.5 x 10^8 BM cells/kg. This graft was also prepared from the BM of a RhLA identical sibling.

The regeneration patterns following transplantation of CD34+ cells in RhLA identical siblings are shown in Figure 3.7 and 3.8. The regeneration time, expressed as the day at which reticulocyte counts exceed a value of 1 % was similar to the regeneration time of the control animal. Following the dose of TBI used in these monkeys, endogenous regeneration of reticulocytes can be calculated to occur not

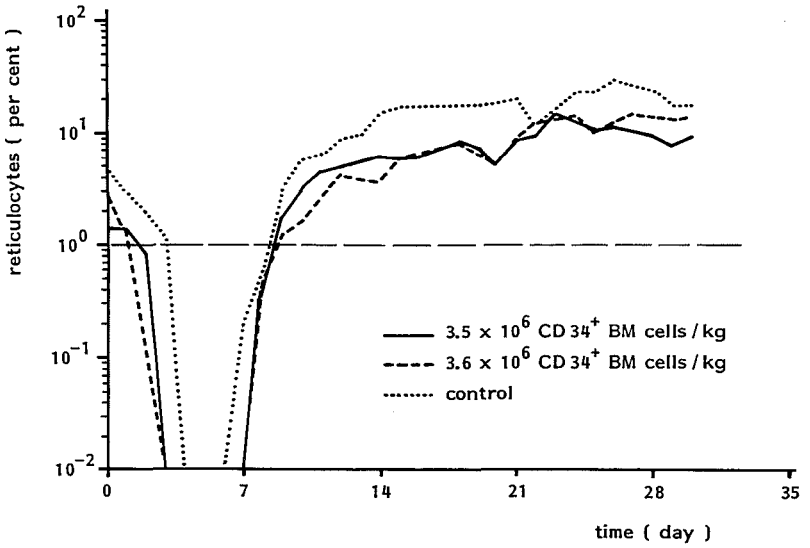

Figure 3.7 **Regeneration of reticulocytes following transplantation of CD34 positive, allogeneic, RhLA identical sibling BM cells.** Conditioning with 9.5 Gy (6 MV X-rays) TBI. The control monkey was transplanted with the CFU-C equivalent of 4 x 10^8 unfractionated BM cells/kg.

earlier than at day 21 ($p < 0.05$), indicating, that allogeneic CD34+ cells have the capacity to repopulate an irradiated recipient.

To investigate the capacity of CD34+ cells to sustain chimerism, the Y chromosome was used as a marker. Other markers for discrimination between recipient cells and RhLA identical donor cells were not available. BM cells were cultured as described in section 2.10., and chromosome preparations were made.

Table 3.4 CHARACTERISTICS OF ALLOGENEIC CD34+ BM GRAFTS

	ficoll	albumin density	ICH3+ (graft)	ICH3- gradient
punctured BM for unique animal no. 8647				
CFU-C/10^5	293	871	10595	101
concentration factor	2.0	6.0	72.4	0.6
number of nucleated cells (x 10^6)	1626	150	9.8 (= 3.5/kg)	53
% of total nucleated cells	100	9.2	0.6	3.2
% CFU-C recovery	100	49.7	39.0	2.0
% T lymphocytes	6.1	14.2	2.7	20.9
absolute number of T lymphocytes (x 10^5)	992	211	2.7 (= 1/kg)	107
pressed BM for unique animal no. 1 ZN				
CFU-C/10^5	104	585	2040	123
concentration factor	2	11.2	39.2	2.4
number of nucleated cells (x 10^6)	2170	150	9.4 (= 3.6/kg)	91.3
% of total nucleated cells	100	6.9	0.4	4.2
% CFU-C recovery	100	46.3	5.8	9.4
% T lymphocytes	9.7	5.6	1.2	7.3
absolute number of T lymphocytes (x 10^5)	210	8.4	1.0 (= 1/kg)	66.6

Cont. Table 3.4

	buffy coat	albumin	Following AET depletion
pressed BM for unique animal no. 1 VL			
CFU-C/10^5	7.3	65	46
concentration factor	1	8.9	6.3
number of nucleated cells (x 10^6)	2192	228	160 (= 40/kg)
% of total nucleated cells	100	10.4	7.3
% CFU-C recovery	100	93.5	46.1
% T lymphocytes	n.d.	n.d.	1.0
absolute number of T lymphocytes (x 10^5)	n.d.	n.d.	16 (= 4/kg)

Both recipients were female and both donors were male, so that the presence of a Y chromosome in a cell indicates the donor origin of that cell, and the absence of a Y chromosome indicates recipient origin (Figure 3.9). The results are summarized in Table 3.5.

The regeneration of these monkeys is much earlier than is expected without allogeneic BM transplantation, indicating the donor origin of the repopulating cells (the repopulating capacity of the allogeneic CD34+ cells). However, complete chimerism was not achieved in these monkeys. Nevertheless, the persistence of donor derived progenitor cells up to 160 days following BM transplantation indicate the stem cell properties of a subpopulation of the CD34+ cells.

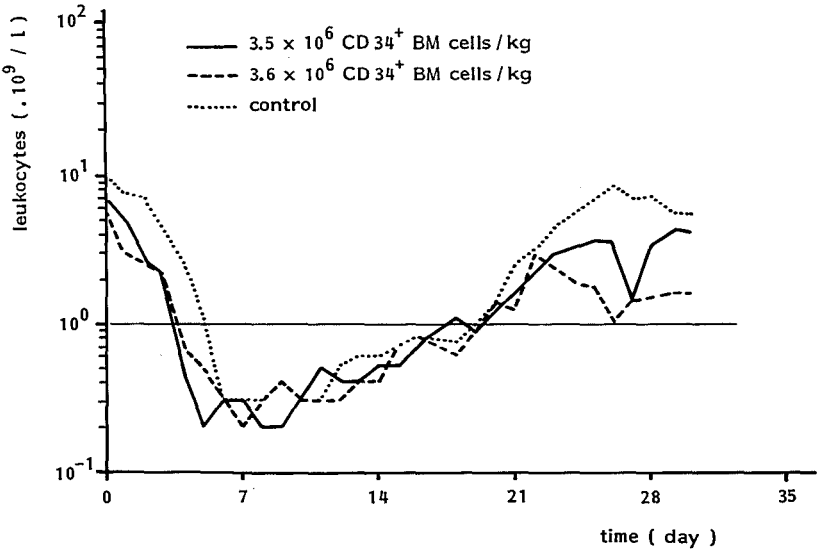

Figure 3.8 **Regeneration of leukocytes following transplantation of CD34 positive, allogeneic, RhLA identical sibling BM cells.** Conditioning with 9.5 Gy (6 MV X-rays) TBI. The control monkey was transplanted with the CFU-C equivalent of 4 x 10^8 unfractionated BM cells/kg.

On the basis of the data which will be described in Chapter IV, the number of residual recipient PHSC following the conditioning TBI was estimated to be the equivalent of approximately 5 x 10^6 unfractionated BM cells/kg body weight. The number of transplanted cells was the equivalent of 1.4 x 10^8 and 2.5 x 10^8 unfractionated BM cells/kg respectively. The ratio between the number of calculated, residual recipient PHSC and the number of transplanted PHSC was different from the ratio between recipient and donor derived progenitor cells after regeneration. A similar, but less pronounced phenomenon was observed in the control monkey. For a definite proof of the capacity of CD34+ cells to sustain long term complete chimerism, additional experiments are required. In such experiments, a more intensive conditioning regimen should be used to reduce endogenous regeneration.

Table 3.5 CHIMERISM FOLLOWING TRANSPLANTATION OF HSC

Time (day)	Unique animal	Number of donor cells	Number of recipient cells	Total number
20	8647	2	8	10
27		0	25	25
33		1	24	25
104		1	8	9
131		4	36	40
19	1 ZN	42	8	50
32		5	20	25
74		5	15	20
103		1	49	50
160		13	12	25
13	1 VL	42	8	50
45		40	10	50
73		30	20	50
101		18	7	25
129		16	9	25
171		25	15	40
200		36	14	50

3.4. Discussion.

Identification and positive selection of early repopulating cells for ABMT was successfully performed in Rhesus monkeys by use of the anti-CD34 antibody, ICH3. The CFU-C assay appeared to be predictive for the enrichment of repopulating cells. This confirms the notion, that ICH3 does not inactivate the labelled cells *in vivo*, which has been described for many other MCA[20,21].

Endogenous regeneration can never be completely ruled out following autologous BM transplantation, but the high regeneration time that was observed following transplantation of ICH3 positive cells excludes an endogenous origin of the *early* repopulating cells.

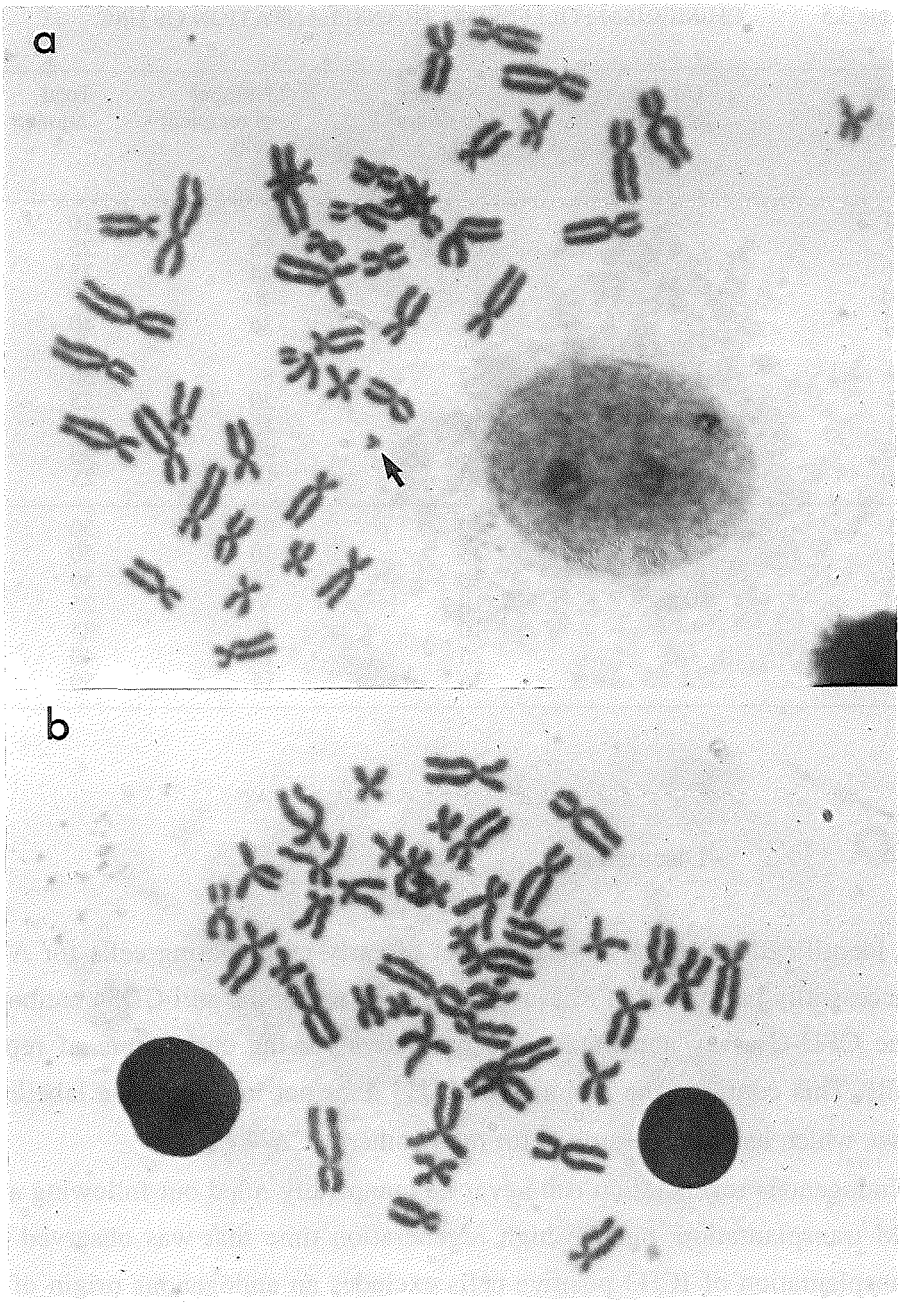

Figure 3.9 **Karyotyping of Rhesus monkey BM cells after transplantation of RhLA-identical male BM in a female recipient.** In this case, the presence of a Y chromosome (arrow) in a cell indicates the donor origin of that cell, and the absence of a Y chromosome in a complete karyogram indicates its recipient origin.

Although the cells measured by the regeneration assay have the capacity for early repopulation, it is by no means certain that these cells also have sufficient self-renewal capacity to sustain blood cell production over a long period of time. This capacity of CD34+ cells was tested in an additional experiment with RhLA identical siblings. In allogeneic bone marrow transplantation, the relationship between graft size and regeneration time has not yet been studied in detail. Therefore the enrichment of repopulating cells cannot be calculated. In addition to the early regeneration after transplantation of CD34+ cells, sustained chimerism was obtained. However, only a minority of the progenitor cells were from donor origin. These results may indicate, that the self-renewal capacity of ICH3+ cells is limited, which means, that the PHSC are not sufficiently enriched by isolation of ICH3+ cells. Alternatively, immunological factors might result in the disappearance of the donor derived cells. The immunological mechanisms involved in mixed chimerism are poorly understood. The probability of graft rejection decreases with the number of transplanted T lymphocytes, and with the immunosuppressive effect of the conditioning regimen (reciprocal interference between graft versus host and host versus graft)[22]. Thus the number of transplanted T-lymphocytes could be too low to allow complete chimerism following a conditioning TBI dose of 9.5 Gy, 6 MV X-rays (equivalent to 8.3 Gy, 300 kV orthovolt) (see Chapter IV). In that case, an immunological reaction rather than insufficient self- replication capacity of the CD34+ cells is the most likely explanation for the low frequency of donor derived progenitor cells following transplantation of allogeneic CD34+ cells. Further experiments, including a more immunosuppressive conditioning regimen, are required to prove this hypothesis.

The data from the present autologous transplantations further indicate, that T lymphocytes in BM grafts are not required as accessory cells for the early regeneration. This is consistent with the observation in mice, that very small numbers of highly purified stem cells can repopulate a lethally irradiated animal, and with observations in Rhesus monkeys, which demonstrate that T lymphocyte depletion

does not jeopardize the regeneration after autologous BM transplantation.

Primate PHSC can also be enriched on the basis of the expression of MHC class II antigens (DR). However, selection of CD34+ cells has obvious advantages over selection of DR+ cells. Firstly, class II MHC antigens are expressed on a wide variety of cell types, including B-lymphocytes, activated T-lymphocytes, and monocytes/macrophages, and on a number of malignant cell types. Therefore, removal of undesirable DR+ cells would still be required to achieve optimal results. This complication does not occur when stem cells are selected on the basis of CD34 expression. Thus, positive selection of CD34+ cells results in a higher degree of CFU-C enrichment than selection of DR+ cells. However, in a number of acute myeloid leukemia patients, the malignant cells express the CD34 antigen. Obviously, in such patients, the CD34 antigen can not be used to separate PHSC from malignant cells. Furthermore, expression of DR- antigens on HSC and HPC appeared to be heterogenous: approximately equal fractions of those cell types were found among the dull and bright DR+ cells. Isolation of cells on the basis of simultaneous CD34 and DR expression did not result in higher degree of CFU-C enrichment than selection of CD34+ cells alone. This is due to the heterogeneous expression of DR among CFU-C. Since a similar heterogeneity of DR expression was earlier observed for repopulating HSC as detected by the autologous regeneration assay, it was not attempted to use two-colour cell sorting for BM transplantation. In contrast to DR expression, the expression of CD34 appeared to be more homogenous, although sorting of the most bright CD34+ cells resulted in a higher CFU-C enrichment than sorting of all CD34+ cells. However, this is possibly not due to heterogeneity of CD34-expression, but to a more complete elimination of CD34- cells from the bright fraction.

Other investigators have studied the repopulating capacity of CD34 positive cells in baboons[23]. However, in this study, the enrichment of PHSC was not quantified, and a 10 fold greater number of CD34+ cells was required to achieve a similar regeneration time as was presented in section 3.3.1. These authors therefore did not

exclude the possibility of inactivation of CD34+ cells by the MCA used. In our study such an inactivation is unlikely, since calculated recoveries and concentration factors for *in vivo* repopulating HSC and for *in vivo* detected HPC appeared to be very similar. Therefore, special precautions to prevent such inactivation *in vivo* are not required if the anti-CD34 MCA ICH3 is used. This considerably simplifies the development of methods applicable to clinical BM transplantation as compared to the other available anti-CD34 MCA.

The frequency of repopulating cells in primate BM is unknown, but positive selection of CD34+ cells does not result in a purification of HSC to homogeneity. Further enrichment might be possible with additional markers such as lectins, rhodamine 123[24,25], or other MCAs directed against surface antigens[26,27]. Although such a further enrichment is interesting, it is not essential for a number of applications of purified stem cells: CD34+ cells can be used to study the direct effects of hemopoietic growth factors on *in vitro* proliferation and differentiation of hemopoietic stem cells and progenitor cells, in the absence of CD34- accessory cells[28], for studies at the molecular analysis of early hemopoietic cell differentiation, including genetic modification of stem cells for possible therapeutic purposes. The current flow cytometric cell sorting techniques are very useful for analytical purposes. However, for the purification of PHSC for clinical BMT a more rapid and aseptic technique is required, which can be performed on a large scale. The immunoabsorption technique with immunomagnetic beads, used for the experiments, described in section 3.3 is safe and easier to perform at a large scale. This development brings positive selection of stem cells as a general applicable method to eliminate T-lymphocytes and/or tumour cells from BM grafts, within the reach of clinical application.

REFERENCES

1. Visser, J.W.M, Bauman, J.G.J, Mulder, A.H., Eliason, J.F., and Leeuw, A.M. de (1984) Isolation of murine pluripotent hemopoietic stem cells. J. Exp. Med. 59:1576-1590.

2. De Vries, P. (1988). Identification of murine haemopoietic stem cells with monoclonal antibodies. Thesis, Erasmus University, Rotterdam.

3. Civin, C.I., Strauss, L.C., Broval, C., Fackler, M.J., Schwartz, J.F., and Shaper, J.H. (1984). Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against Kg-1a cells. J. Immunol. 133:157-165.

4. Andrews, R.G., Singer, J.W., and Bernstein, I.D. (1986). Monoclonal antibody 12-8 recognizes a 115-kd molecule present on both unipotent and multipotent hematopoietic colony-forming cells and their precursors. Blood 67:842-845.

5. Tindle, R.W., Nichols, R.A.B., Chan, L., Campana, Catowski, D., and Birnie, G.D. (1985). A novel monoclonal antibody BI-3C5 recognizes myeloblasts and non-B non-T lymphoblasts in acute leukemias and CGL blast crisis, and reacts with immature cells in normal bone marrow. Leukemia Res. 9:1-9.

6. Watt, S.M., Karhi, K., Gatter, K., Furley, A.J.W., Katz, F.E., Healey, L.E., Atass, L.J., Bradley, N.J., Sutherland, D.R., Levinski, R., and Greaves, M.F. (1987). Distribution and epitope analysis of the cell membrane glycoprotein (HPCA-1) associated with human hemopoietic progenitor cells. Leukemia 1:417-426.

7. Gerritsen, W.R. Personal communication.

8. Fausser, A.A., and Messner, H.H. (1979) Identification of megakaryocytes, macrophages, and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. Blood 53:1023-1027.

9. Gerritsen, W.R., Wagemaker, G., Jonker, M., Kenter, M., Wielenga, J.J., Hale, G., Waldmann, H., and Bekkum, D.W. van. (1988). The repopulating capacity of bone marrow grafts following pretreatment with monoclonal antibodies against T lymphocytes in rhesus monkeys. Transplantation 45:301-307.

10. Fitchen, J.H., LeFevre, C., Ferrone, S., and Cline, M.J. 1982. Expression of Ia-like antigens and HLA- A,B antigens on human multipotential hematopoietic progenitor cells. Blood 59:188-190.

11. Ferrero, D., Gabbianelli, M., Peschle, C., Lange, B., and Rovera, G. 1985. Surface phenotypes of human hemopoietic progenitor cells defined by monoclonal antibodies. Blood 66:496-502.

12. Winton, E.F., Colenda, K.W., McClure, H.M., Chan, W.C., and Gordon, D.S. 1985. Studies on the use of Nonhuman Primates to determine the DR status of the HUman hemopoietic stem cell. Exp. Hematol. 13:869-873.

13. Fitchen, J.H., and Ferrone, S., 1981. Expression of I subregion antigens on murine hematopoietic stem cells. J. Supramol. Struct. Cell. Biochem. 14(suppl. 5):107 (abstr.).

14. Berenson, R.J., Bensinger, W.I., Kalamasz, D., Scheuning, F., Deeg, H.J., Graham, T., and Storb, R. 1987. Engraftment of dogs with Ia-positive marrow cells isolated by avidin-biotin immunoadsorption. Blood 69:1363-1367.

15. Szer, J., Deeg, H.J., Appelbaum, F.R., and Storb, R. 1985. Failure of autologous marrow reconstitution after cytolytic treatment of marrow with anti-Ia monoclonal antibodies. Blood 65:819-822.

16. Scheuning, R., Storb, R., Goehle, S., Meyer, J., Graham, T., Deeg, H.J., and Pesando, J. 1987. Canine pluripotent hematopoietic stem cells and CFU-GM express Ia-like antigens as recognized by two different Class II-specific monoclonal antibodies. Blood 69:165-172.

17. Gerritsen, W.R. 1989. Bone marrow transplantation with T lymphocyte depleted and stem cell enriched bone marrow grafts. Experiments in Rhesus monkeys. Thesis Rotterdam, The Netherlands.

18. Civin, C.I., Banqerigo, M.L., Strauss, L.C., and Loken, M.R. (1987). Antigenic analysis of hematopoiesis. VI. Flow cytometric characterization of My-10 positive progenitor cells in normal human bone marrow. Exp. Hematol. 15:10-17.

19. Strauss, L.C., Rowly, S.D., La Russa, V.F., Sharkis, S.J., Stuart, R.K., and Civin, C.I, (1986). Antigenic analysis of hematopoiesis. V. Characterization of My-10 antigen expression by normal lymphohematopoietic progenitor cells. Exp. Hematol. 14:878-886.

20. Hasthorpe, S., and Rogerson, J. (1982). Tumor-associated surface membrane antigens on CFU-S: characterization using the fluorescent activated cell sorter. Exp. Hematol 10:9-14.

21. Bauman, J.G.J., Mulder, A.H., and Engh, G.J. van den. (1985). Effect of surface antigen labelling on spleen colony formation: comparison of the indirect immunofluorescence and the biotin-avidin methods. Exp. Hematol 13:760-767.

22. Vriesendorp, H.M., Wagemaker, G., and van Bekkum, D.W. (1981). Engraftment of allogeneic bone marrow. Transplant. Proc. 13:643.

23. Berenson, R.J., Andrews, R.G., Bensinger, W.I., Kalamaz, D., Knitter, G., Buckner, C.D., and Bernstein, D. (1988). Antigen CD34+ marrow cells engraft lethally irradiated baboons. J. Clin. Invest. 81:951-955.

24. Bertoncello, I, Hodgson, G.S., and Bradley, T.R. (1985). Multiparameteranalysis of transplantable hemopoietic stem cells homing to marrow and spleen on the basis of Rhodamin-123 fluorescence. Exp. Hematol. 13:999-1006.

25. Mulder, A.H., and Visser, J.W.M. (1987). Separation and functional analysis of bone marrow cells separated by Rhodamine-123 fluorescence. Exp Hematol 15:99-104.

26. Loken, M.R., Shah, V.O., Dattillo, K.L., and Civin, C.I. (1987). Flow cytometric analysis of human bone marrow. II. Normal B lymphocyte development. Blood 70:1316-1324.

27. Askew, D.S., Eaves, A.C., Eaves, C.J., and Takei, F. (1986). Restricted expression of a new leukemia associated antigen (NHL-30.5) on normal progenitor cells. Blood 67:1098-1102.

28. Bot, F.J., Dorssers, L., Wagemaker, G., and Löwenberg, B. (1988) Stimulating spectrum of human recombinant Multi-CSF (IL3) on human marrow precursors: importance of accessory cells. Blood 71:1609-1614.

CHAPTER IV
RADIATION SENSITIVITY OF HEMOPOIETIC STEM CELLS

4.1. Introduction.

The hemopoietic system is highly sensitive to Total Body Irradiation (TBI) and other cytotoxic agents. TBI or chemotherapy eliminates hemopoietic stem cells (HSCs) and progenitor cells, resulting in a reduction in blood cell production and consequently a lack of peripheral blood cells, (pancytopenia). Self-replication and differentiation of the surviving HSCs result in regeneration of the damaged bone marrow (BM) and resumption of normal blood cell production. The regeneration time is dependent on the number of residual HSC. Following a dose of total body irradiation (TBI) that eliminates more than a certain fraction of HSCs, hemopoiesis can only be restored by BM transplantation. Following doses of TBI, that leave a higher number of HSCs, BM transplantation might reduce mortality and morbidity by shortening the duration of the pancytopenic period[1,2]. Therefore, a number of victims of accidental radiation at nuclear power stations at Vinca (Yugoslavia) and Chernobyl (Soviet Union) have been treated by allogeneic BM transplantation[3,4]. However, the conditions for a beneficial effect of BM transplantation in such emergencies, i.e the conditions under which the advantages of a shorter duration of pancytopenia outweigh the risks of allogeneic BM transplantation, are poorly defined.

The duration of radiation induced pancytopenia depends on the number of HSCs that survive irradiation[5]. The number of surviving HSCs is dependent on the initial number of HSC and their radiosensitivity, the radiation dose and the relative biological effectiveness (RBE) of the radiation type. Therefore reliable

information on the radiosensitivity and the frequency of HSCs is essential for the development of a treatment strategy for victims of radiation accidents.

In rodents, survival of HSCs following TBI or irradiation of BM *in vitro* has been determined using the spleen colony assay. The radiation sensitivity has been characterized by a D_0 value between 0.7 and 1.1 Gy and an extrapolation number between 1 and 3 for 200-300 kV X-rays (see section 1.2). For outbred species, such as dogs, Rhesus monkeys and humans, the information on the radiosensitivity and frequency of HSCs is limited. The only available information is an indirect estimate by Vriesendorp and van Bekkum[6]. They investigated the $LD_{50/30days}$ for TBI in several species as well as the BM graft size required for 50 % survival after supralethal TBI (rescue $dose_{50}$) (Table 4.1). They concluded from those data, that the number of Rhesus monkey HSC, surviving 5.25 Gy 300 kV X-rays was the equivalent of a BM graft of 7.5 x 10^6 cells, transplanted after supralethal TBI. On the basis of radioiron incorporation, the total number of bone marrow cells in normal monkeys was estimated at 24 x 10^9/kg[7,8]. On this basis, the D_0 value of Rhesus monkey HSCs was estimated to be 0.6 Gy for 300 kV X-rays. They further observed, that smaller animals have a higher $LD_{50/30\ days}$ for TBI than larger species, and that smaller animals require less bone marrow cells per kg body weight than larger species to survive lethal irradia

Table 4.1 TOXICITY OF TBI (300 kV X-RAYS) AND BONE MARROW CELL DOSE REQUIRED FOR RESCUE

Species	Body weight (kg)	$LD_{50/30days}$ (Gy)	Autologous or syngeneic rescue $dose_{50}$ (BM cells x 10^6/kg)
Mouse	0.025	7.00	2.0
Rat	0.20	6.75	3.8
Rhesus Monkey	2.60	5.25	7.5
Dog	12.0	3.70	17.5

Data from Vriesendorp and van Bekkum 1980.
Adapted with permission from Reference 6.

tion. Since these differences could not readily be explained from differences in the radiation sensitivity of HSC or cell kinetic parameters determining the rate of blood cell production, an inverse relationship between HSC frequency and body weight was postulated.

Modern intensive supportive care enables the study of endogenous regeneration of blood cell production following TBI doses that were hitherto assumed to be lethal. By comparing endogenous regeneration patterns to the regeneration patterns after high dose TBI followed by autologous BM rescue, the frequency and radiosensitivity of HSCs was investigated. The irradiations were performed with 300 kV and with 6 MV X-rays. The RBE for the BM syndrome for 6 MV X-rays was assessed to enable a comparison of the data obtained for both types of radiation. Since large numbers of animals are required for such an assessment, this study was performed with mice. There is no reason to assume that the RBE for Rhesus monkey HSCs would differ significantly from that for murine HSC. The assessment of the RBE of 6 MV X-rays for HSCs will be described in section 4.2.1.

Gerritsen et al. have demonstrated, that the regeneration time of peripheral blood cells following autologous BM transplantation is directly related to the number of transplanted cells[9]. This relationship can be characterized by the formula

$$Y = a - b \ln T \qquad (1)$$

in which Y represents the day at which peripheral blood cell numbers exceed a certain value (regeneration time), and T the number of transplanted autologous BM cells.

If we assume, that endogenous hemopoietic regeneration following TBI is the same process as regeneration following autologous BM transplantation, a similar relationship between the number of surviving HSC and the regeneration time can be expected. However, following the injection of a BM graft in a lethally irradiated recipient, only a certain fraction of the injected HSCs reaches an appropriate site for hemopoiesis. This "homing fraction", estimated to be 0.3 in

mice[10], has not been determined in Rhesus monkeys, while the frequency of HSCs in the femoral bone marrow is unknown. It is also unknown, whether the frequency of HSCs in femoral BM is equal to the frequency at other BM sites. Another uncertainty is caused by admixture of the punctured BM with peripheral blood, in the autologous BM transplantation experiments. For all these reasons equation (1) can not be used to calculate the absolute number of HSC that survive a certain dose of TBI. However, the relative number of surviving HSC can be calculated if we assume that:

1. Endogenous regeneration from HSCs that survived TBI is the same process as regeneration from transplanted autologous HSCs.
2. The number of surviving HSCs rather than radiation damage to the BM microenvironment determines the time of endogenous regeneration following TBI.
3. The homing fraction of transplanted BM cells is not dependent on the number of transplanted cells, in the range between 10^6 to 10^8 cells/kg body weight.

On the basis of these assumptions, the number of BM cells (N), that survives a sublethal dose of TBI and results in an endogenous regeneration time Y can be regarded as **proportional** to the number of BM cells transplanted (T) following a marrow ablative dose of TBI that results in a similar regeneration time

$$T = c\,N \tag{2}$$

in which N is the number of surviving HSC, and c is regarded as a constant factor, dependent on the homing fraction, the frequency of HSC among punctured BM cells and among BM cells *in vivo*. Substitution of T in equation (1) by cN (equation 2) results in a relationship between the number of surviving HSC and the regeneration time.

$$Y = a - b \ln N - b \ln c \tag{3}$$

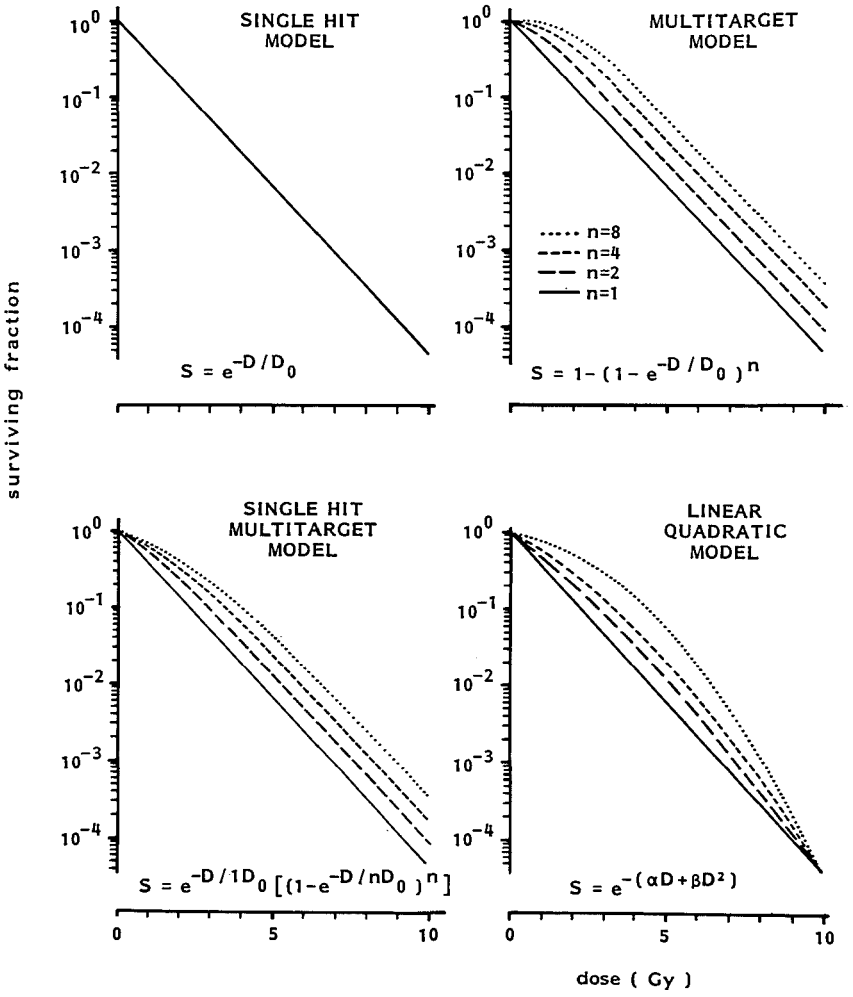

Figure 4.1 **Mathematical descriptions of cell survival following ionising irradiation.**

The relationship between radiation dose and cell survival can be characterized by various mathematical descriptions, based upon the Poisson distribution of the probability of lethal lesions. The most simple mathematical description is the single hit model

$$S = N/N_0 = e^{-D/D_0} \qquad (4)$$

in which N is the number of surviving cells, N_0 the total number of irradiated cells, D the radiation dose and D_0 the dose resulting in an average of one lethal lesion per cell. Since the probability of lethal lesions follows a Poisson distribution, a radiation dose of D_0 Gy results in a surviving fraction e-1, i.e. the fraction of cells with 0 lethal lesions. In most cell types, the dose survival curve shows a shoulder in the lower dose range. This shoulder is not described by equation (4). Other mathematical models, summarized in Figure 4.1 include the description of this shoulder.

The available data on the radiosensitivity of HSCs in a variety of species (see section 1.2) indicate only a small shoulder in the survival curve. In the dose range between 5 and 10 Gy, the survival of HSCs can therefore be approximated by equation (4). This equation can be rewritten as

$$\ln N - \ln N_0 = -D/D_0 \qquad (5)$$

Elimination of N from equation (3) and (5) results in a relationship between the radiation dose and the endogenous regeneration time:

$$Y = \{a - b\,(\ln N_0 + \ln c)\} + \frac{b}{D_0}\,D \qquad (6)$$

This relationship will be fitted to the experimental data in section 4.2.2, and in section 4.2.3 the radiosensitivity of HSC will be calculated under a variety of indicated assumptions.

The slope of the curve described by equation (6) is independent of the uncertain

relationship between T and N (c in equation 2). For an estimate of absolute numbers of surviving cells, we have to assume a certain homing fraction, and HSC distribution over the body. However, the result of a D_0 calculation according to equation (6) is not influenced by such assumptions.

4.2. Results.

4.2.1. The Relative Biological Effectiveness (RBE) of 6 MV X-rays for hemopoietic stem cells determined in mice.

The relative biological effectiveness (RBE) of 6 MV X-rays for hemopoietic HSCs was determined in female (C_{57}Bl/Rij x CBA/Rij)F_1 (BCBA) mice at the age of 12 - 15 weeks. These mice were irradiated with graded doses of 6 MV X-rays or 300 kV X-rays. The relationship between the dose of TBI and 30-day mortality for both types of radiation was established by probit analysis as shown in Figure 4.2. Data of two pooled experiments were used, and the numbers of animals used for each dose are indicated. The RBE for 6 MV X-rays was calculated by dividing the $LD_{50/30\ days}$ for 300 kV X-rays by the $LD_{50/30\ days}$ for 6 MV X-rays, resulting in a value of 0.87. The RBE for ^{60}Co γ radiation was determined in the same experiment, as shown in Figure 4.3. The calculated RBE for ^{60}Co γ radiation was 0.88, which closely approximates the value of 0.85 Gy that was reported earlier[11].

4.2.2. Relationship between dose of TBI and regeneration time of peripheral blood cells in Rhesus monkeys.

The regeneration time of peripheral blood cells following TBI, without transplantation of hemopoietic cells, was thought to be directly related to the number of repopulating hemopoietic cells that survived the irradiation, which is dependent on the absorbed dose of irradiation. To assess this relationship,

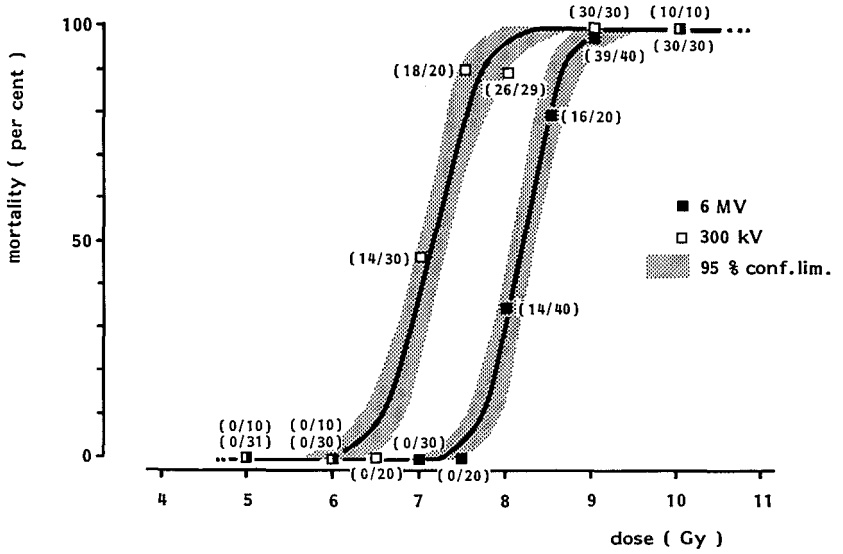

Figure 4.2 **Survival curves used for the calculation of the relative biological effect (RBE) of 6 MV X-rays.** Female CBA x C_{57bl} F1 (BCBA) mice, 12 weeks of age were used. The RBE was calculated by

$$RBE_{6\,MV} = \frac{LD_{50,\,30\,days,\,300\,kV}}{LD_{50,\,30\,days,\,6\,MV}} = 0.87$$

The numbers in parentheses indicate the number of animals that died before day 30/the number of animals used.

monkeys were treated with graded doses of TBI over a range of 4,0 to 10 Gy 6 MV X-rays. Other monkeys were irradiated with 300 kV X-rays, over a dose range of 5.0 to 9.8 Gy. The physical parameters and dosimetry of both types of irradiation are described in section 2.7. For comparison of both radiation types, it was assumed, that the RBE of 6 MV X-rays for Rhesus monkey HSCs is similar to the RBE for murine HSCs, i.e. 0.87 (see section 4.2.1).

All monkeys received full supportive care as described in section 2.9.

In Figure 4.4, the influence of supportive care on mortality rate after TBI is demonstrated. Almost all animals which were submitted to supportive care, survived for more than 30 days, and showed endogenous regeneration, which is not the case for the animals without supportive care. The mortality following TBI

without supportive care have been described earlier[12]. Under full supportive care, only 2 monkeys died before day 30: Monkey 8650 (10 Gy, 6 MV) died from sepsis on day 14. Monkey 1 ZU (10 Gy, 300 kV) suffered from severe diarrhoea starting at day 5. Repeatedly, no pathogenic micro-organisms could be demonstrated in its faeces. In spite of parenteral fluid and electrolyte administration, the monkey lost 40 % of its body weight and died at day 24, after almost 3 weeks of severe diarrhoea. During the observation period, bleeding or infectious problems did not occur. Autopsy revealed a complete villous atrophy of the colon, jejunum and ileum. Four monkeys (2 BU, 1 YK, 1 JB and 1HK) died after

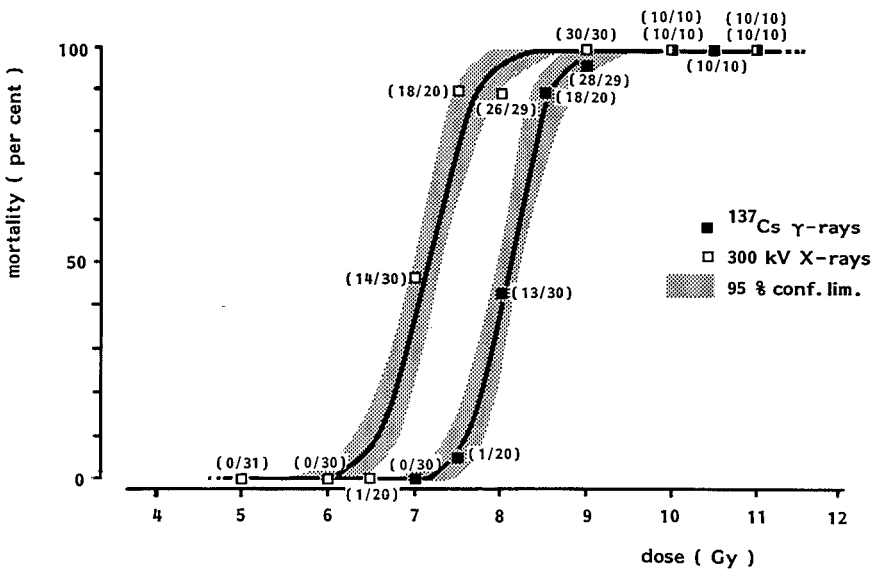

Figure 4.3 **Survival curves used for the calculation of the relative biological effect (RBE) of ^{137}Cs γ–rays.** Female CBA x C_{57bl} F1 (BCBA) mice, 12 weeks of age were used. The RBE was calculated by

$$RBE_{6\ MV} = \frac{LD_{50,\ 30\ days\ 300\ kV}}{LD_{50,\ 30days\ \gamma}} = 0.88$$

The numbers in parentheses indicate the number of animals that died before day 30/the number of animals used.

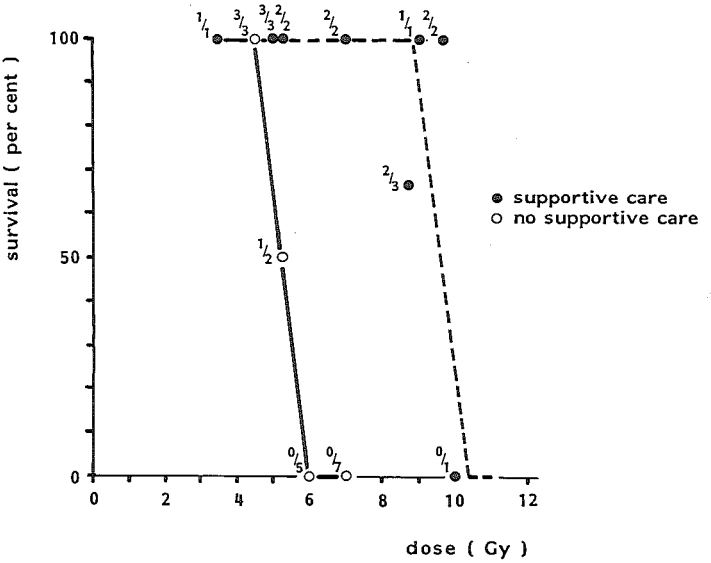

Figure 4.4 **Influence of supportive care on survival$_{30}$ days of Rhesus monkeys following TBI with 300 kV X-rays.** (thrombocyte transfusions, erythrocyte transfusions, selective decontamination, parenteral fluid and systemic antibiotics). Data without supportive care derived from reference 11.

full hemopoietic regeneration had occurred. Two of these monkeys (1 HK and 1 JB) died from sepsis, and two monkeys (2 BU and 1 YK) died from diarrhoea caused by flagellate infections. All other animals are alive and well after a median observation time of 335 days (range 165-508 days). The endogenous regeneration of the peripheral blood cells following TBI is shown in Figure 4.5 for 6 MV X-rays and in Figure 4.6 for 300 kV X-rays. The most essential data of the individual monkeys are summarized in Table 4.2a for 6 MV X-rays and in Table 4.2b for 300 kV X-rays. The regeneration time of peripheral blood cells was defined as the day at which reticulocyte counts exceeded a value of 1%, leukocyte counts a value of 10^9/l, or thrombocyte counts persistently reached values of 40 x 10^9/l or more without transfusions. The three monkeys, which were irradiated with 5 Gy 6 MV X-rays, showed a regeneration time similar to that following transplantation of 10^8 BM cells/kg. This indicates, that the number

Table 4.2a SURVIVAL OF RHESUS MONKEYS AND REGENERATION OF BLOOD CELLS FOLLOWING TBI WITH 6 MV X-RAYS

Unique animal number	dose (Gy)	Reti- > 1% (day)	Leukocytes >10^9/l (day)	Leukocytes penia (days)	Thrombocytes >40 x 10^9/l (day)	Alive* and well (days)	Cause of death
8654	4.0	10	22	5	NA**	> 296	
BB 31	5.0	10	20	12	15	> 365	
BB 40		11	21	11	NA	> 365	
1 UP		9	18	9	NA	> 365	
8667	6.0	13	17	6	NA	> 171	
1 XT		13	21	16	NA	> 365	
C 28	8.0	20	18	12	19	> 365	
1 ZF		20	24	19	23	> 193	
1 YK	10.0	18	21	16	17	103	diarrhoea***
2 BU		24	25	20	24	71	diarrhoea***
8650		> 14	> 14	> 9	> 14		sepsis****

* Survival time October, 1989.
** NA = Not Available, since cell counts remained above threshold value.
***Flagellate enteritis.
****Gram positive rod.

Table 4.2b SURVIVAL OF RHESUS MONKEYS AND REGENERATION OF BLOOD CELLS FOLLOWING TBI WITH 300 kV X-RAYS

Unique animal number	dose (Gy)	6 MV dose equivalent	Reti- > 1% (day)	Leukocytes >10^9/l (day)	Leukocytes penia (days)	Thrombocytes >40x10^9/l (day)	Alive* and well (days)	Cause of death
BB 48	5.0	5.7	NA**	17	10	14	> 365	
1 XS			10	19	14	15	> 212	
1 ZG			13	19	12	14	> 216	
8622	9.0	10.3	29	31	23	50	> 165	
1 JB	9.6	11.0	28	23	18	24	45	sepsis***
1 HK	9.8	11.3	31	26	22	37	42	sepsis****
1 ZU	10.0	11.5	>24	>24	>18	>24	24	gastro-intestinal syndrome

* Survival time on October, 1989
** NA = Not Available, since cell counts remained above threshold value.
*** Proteus mirabilis
****Multiresistent Gram positive coccus.

of HSCs that survives this dose of TBI was the equivalent of 10^8 transplanted BM cells/kg. The relationship between the number of transplanted cells and the regeneration time was previously assessed in the range between 10^6 and 10^8 cells/kg. This means, that the monkey irradiated with 4 Gy 6 MV X-rays, should not be included in the calculation of the D_0 value, since its number of residual HSCs exceeded the maximum numbers of BM cells transplanted in the experiments that were used as a basis for calibration.

Since most animals were irradiated with 6 MV X-rays, the relationship between the regeneration time and the dose of TBI was calculated for this radiation type. The dose of the monkeys irradiated with 300 kV X-rays was converted to an equivalent of a dose of 6 MV X-rays. For this conversion the dose of 300 kV was divided by 0.87, which is the RBE for 6 MV X-rays. The regeneration time of the peripheral blood cells appeared to be directly related to the dose of TBI. In the dose rage between 5 and 11 Gy (6 MV X-rays), this relationship, shown in Figure 4.7, can be characterized by the following regression equations:

For reticulocytes:

$$Y = 3.0\ D - 5.2 \qquad (7)$$

in which Y is the day at which reticulocyte counts exceeded a value of 1% and D is the dose of TBI (6 MV X-rays) in Gy ($r=0.95$, $p < 0.001$).

For leukocytes:

$$Y = 1.1\ D + 12.7 \qquad (8)$$

in which Y is the day at which leukocyte counts exceeded a value of 1 x 10^9/l. ($r = 0.72$, $p < 0.01$). The regeneration time of granulocytes and lymphocytes were not significantly different from each other and from the regeneration time of all leukocytes.

For thrombocytes:

$$Y = 3.3\ D - 4.0 \qquad (9)$$

in which Y is the day at which thrombocyte counts persistently exceeded a value

of 40 x 10^9/l without transfusions. ($r = 0.69$, $p < 0.02$).

Essentially, the relationship between the dose of TBI and the day at which regeneration of peripheral blood cells occurs is not linear over a wide range of doses, since low doses of TBI do not result in a measurable pancytopenia (day of regeneration = 0), and "marrow ablative" doses are not followed by regeneration at all (day of regeneration = ∞). However, in the dose range between 5 and 11 Gy (6 MV X-rays), the regeneration time was in the same range as was described for monkeys transplanted with graded numbers of autologous BM cells between 10^6 and 10^8/kg. In these transplanted monkeys a log-linear relationship between the number of transplanted cells and the regeneration time was demonstrated[9]. Furthermore, in the dose range tested, a log-linear relationship between the fraction of surviving cells and the dose of irradiation can be expected. Therefore, within the dose range tested, the relationship between the dose of TBI and the regeneration time was expected to be linear.

The correlation coefficient for the relationship between the dose of irradiation and the regeneration time was higher for reticulocytes than for leukocytes and thrombocytes. This can be explained by the influence of external factors on the regeneration of peripheral blood cells. The most important external factor is probably the occurrence of infections, which influences leukocyte and thrombocyte counts, but is not expected to affect the reticulocyte counts as long as extensive hemorrhages do not occur.

4.2.3. Radiation sensitivity of hemopoietic stem cells.

In the previous section, a linear relationship between the dose of TBI and the regeneration time of peripheral blood cells was described. Following autologous BM transplantation, the relationship between the number of cells transplanted and the regeneration time of peripheral blood cells was described earlier[9].

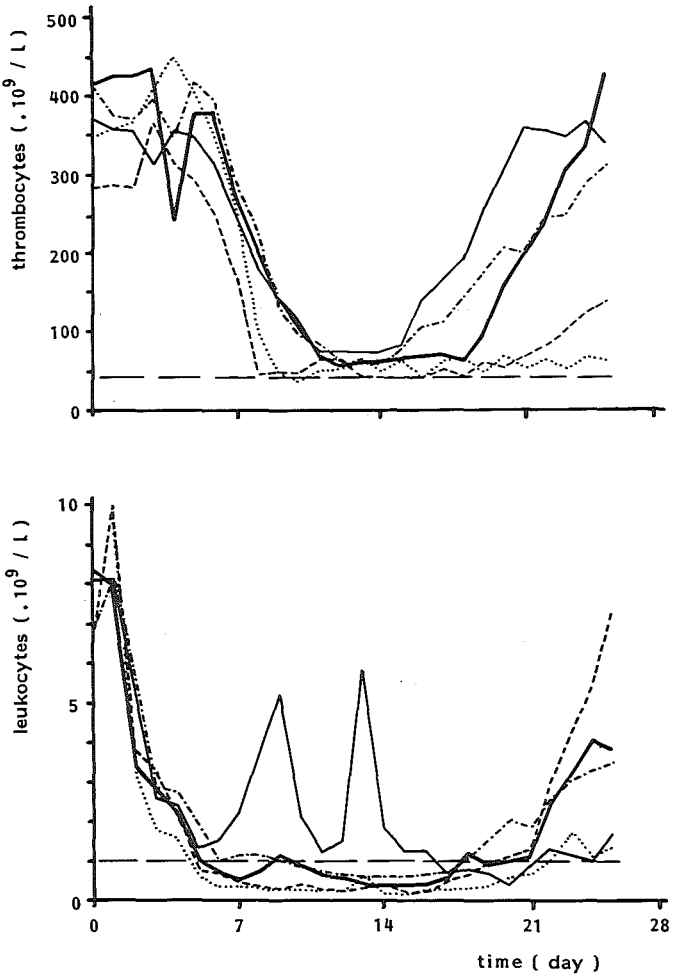

Figure 4.5a **Peripheral blood cell counts following graded doses of TBI with 6 MV X-rays.**

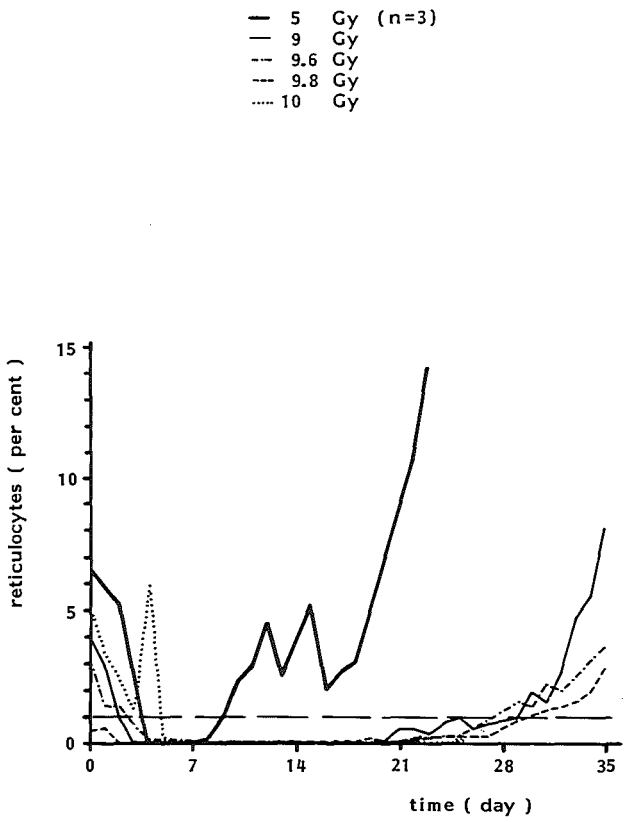

Figure 4.5b **Peripheral blood cell counts following graded doses of TBI with 6 MV X-rays.**

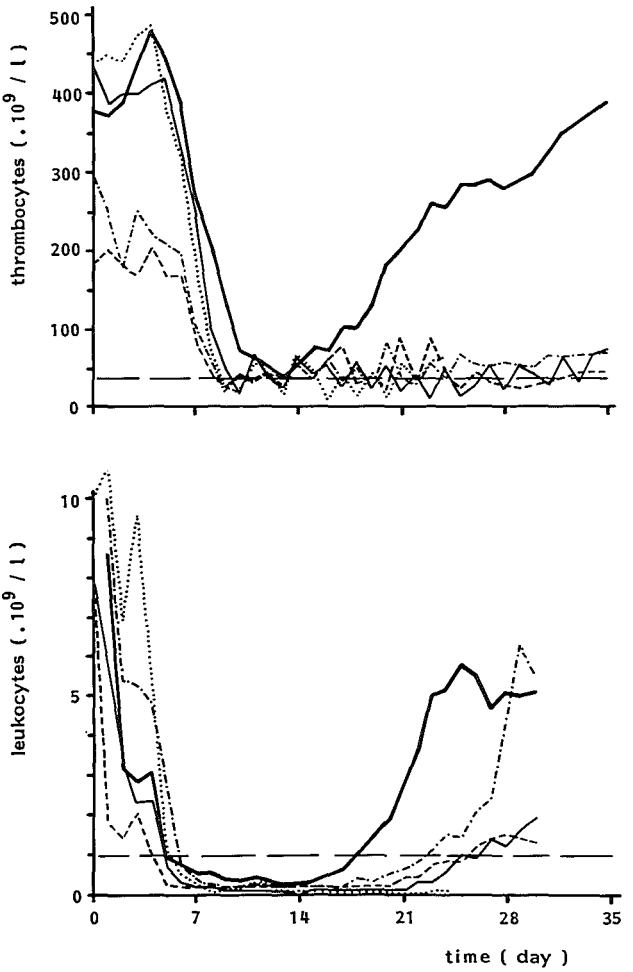

Figure 4.6a **Peripheral blood cell counts following graded doses of TBI with 300 kV X-rays.**

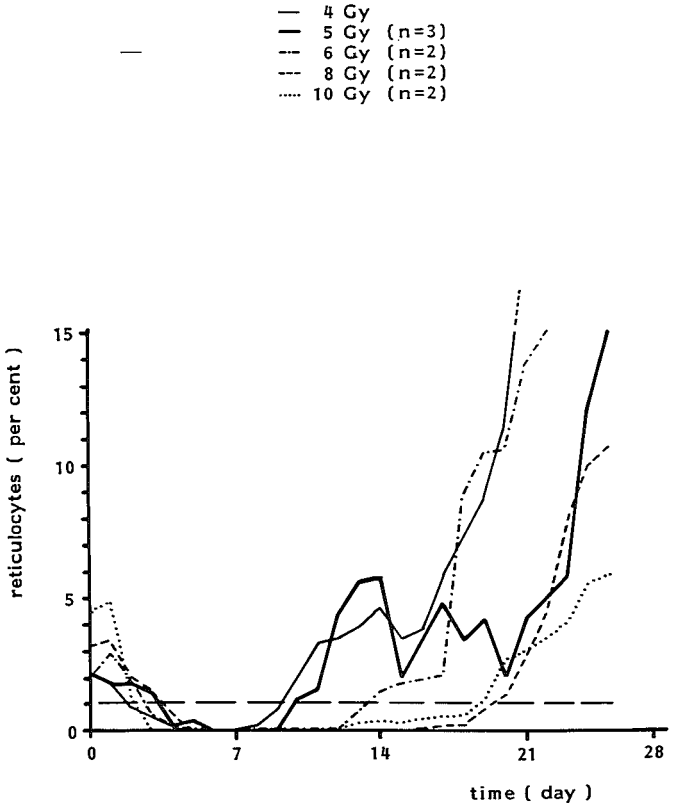

Figure 4.6b **Peripheral blood cell counts following graded doses of TBI with 300 kV X-rays.**

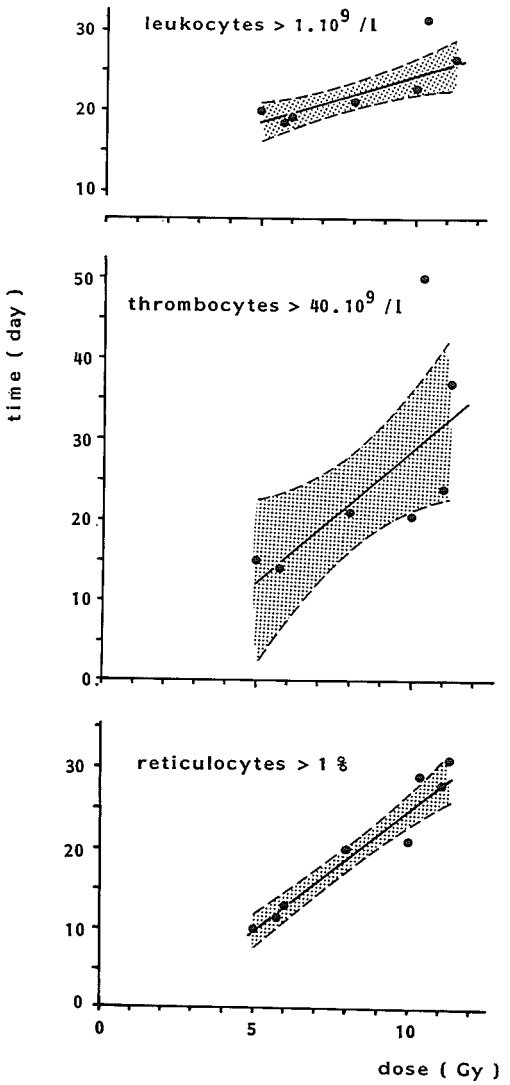

Figure 4.7 **The relationship between the dose of TBI (6 MV X-rays) given and the endogenous regeneration time of peripheral blood cells.** The regeneration times of leukocytes, thrombocytes and reticulocytes were defined as the day at which a level of more than 10^9, 40 x 10^9 and 1 %, respectively was exceeded. Data points indicate the average of 2 to 4 monkeys. Shaded areas represent 95 % confidence limits.

The latter relationship, based on experiments performed by Gerritsen et al.[9] can be rewritten as

$$Y = 83.2 - 4.0 \ln T \qquad (10)$$

in which Y is the day at which the reticulocyte count exceeds a value of 1 % and T is the number of transplanted cells/kg body weight (r=0.98, p=0.0001).

Comparison of the endogenous regeneration times, characterized in section 4.2.1., to the regeneration times following autologous BM transplantation results in a relationship between the dose of TBI and the number of surviving HSCs, as explained in section 4.1. Under the assumptions, discussed in the same section, this relationship enables a calculation of the D_0 value for HSCs, irradiated in vivo. As explained in section 4.2.3, the monkey irradiated with 4 Gy 6 MV X-rays, was not included in the calculation of the D_0 value, since its number of residual HSCs was expected to exceed the maximum numbers of BM cells transplanted in the experiments that were used as a basis for calibration.

Since the leukocyte and thrombocyte counts appeared to be more sensitive to external factors than the reticulocyte counts, calculation of the D_0 value on the basis of the regeneration of leukocytes and thrombocytes results in extremely wide 95 % confidence limits (1.2 - 4.8 Gy for leukocytes and 1.0 -7.3 Gy for thrombocytes). Therefore, the regeneration time of reticulocytes was used only.

From equation (6) and (7) the relationship between b and D_0 can be calculated:

$$b = 3 D_0 \qquad (11)$$

In equation (10) the value of b was found, so that the D_0 value for 6 MV X-rays could be calculated and appeared to be 1.33 Gy (95 % confidence limits 1.16 - 1.61 Gy). The dose/survival curve is shown in Figure 4.8. Multiplication of the D_0 value for 6 MV X-rays by the RBE (0.87) results in a D_0 value of 1.16 Gy (95 % confidence limits 1.0 -1.40 Gy) for 300 kV X-rays. In order to confirm that the RBE value used (0.87), (based on experiments with mice) (section

4.2.1), also applies for Rhesus monkeys, the D_0 value for 300 kV X-rays was calculated separately. In spite of the small number of monkeys irradiated with 300 kV X-rays, the calculated D_0 value appeared to be 1.04 Gy, which is within the 95 % confidence limits of the D_0 value calculated above.

Extrapolation of our data cannot be used for a calculation of the total number of BM cells for two reasons. Firstly, the relationship between N and T in equation (2), which is required for such a calculation, is not known. Secondly, the 95 % confidence limits of the D_0 value are wide, and small variations in D_0 value will result in large variations in the extrapolated numbers. Other investigators have estimated the total number of Rhesus monkey BM cells as 24 x 10^9/kg[7,8]. Inclusion of this total number of bone marrow cells in the calculation requires an assumption on the relationship between N and T in equation (2). The most important factors in this respect are the homing fraction and the

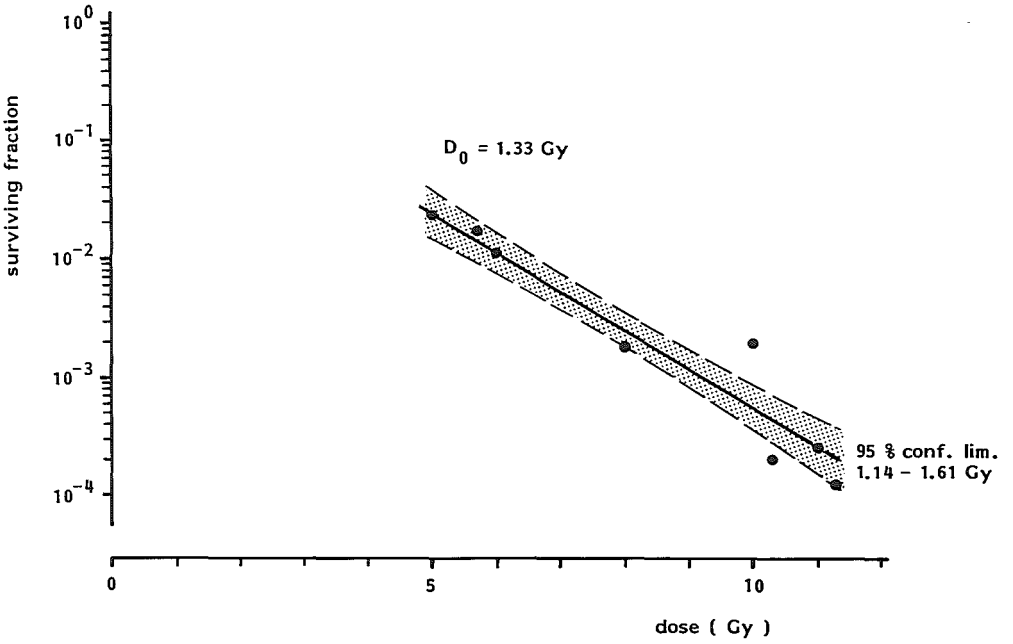

Figure 4.8 **Calculated survival of hemopoietic stem cells after TBI with 6 MV X-rays, using the single hit model.** The data points shown were derived from the regeneration time of reticulocytes and indicate the average of 2 to 4 monkeys.

extrapolation number, (n). If we assume, that the homing fraction of Rhesus monkey HSC is equal to that of murine CFU-S, the numbers of transplanted BM cells should be multiplied by this factor (0.3) to get the number of endogenous HSC according to equation (2). When N_0 is set at 24 x 10^9/kg[7,8] the surviving fraction of cells can be calculated for each dose of TBI, and the best fitting D_0 value can be found. This calculation resulted in a D_0 value of 1.09 Gy for 6 MV X-rays. Assumptions on the extrapolation number, n, in this calculation result in a further change in the D_0 value (Table 4.3), but not in significant changes in the survival curve of HSC (Figure 4.9). For n = 2, which is the median value described in all studies (Table 1.1), the D_0 value is 1.05 Gy (0.88-1.30) for 6 MV X-rays, and 0.91 Gy (0.76-1.13) for 300 kV X-rays.

Table 4.3 INFLUENCE OF THE EXTRAPOLATION NUMBER n ON THE CALCULATED D_0 VALUE OF RHESUS MONKEY HSC

total number of BM cells	extrapolation number (n)	D_0 value (Gy) for 6 MV X-rays (95% conf. lim.)	D_0 value (Gy) for 300 kV X-rays (95% conf. lim.)
not included	1	1.33 (1.14-1.61)	1.16 (0.99-1.40)
	12	1.29 (n.a.)	1.12 (n.a.)
24 x 10^9/kg *)	1	1.09 (0.93-1.33)	0.95 (0.81-1.16)
	2	**1.05 (0.88-1.30)**	**0.91 (0.76-1.13)**
	4	1.01 (0.83-1.27)	0.88 (0.73-1.11)
	12	0.95 (0.77-1.24)	0.83 (0.67-1.08)

*) A homing fraction of 0.3 was assumed.

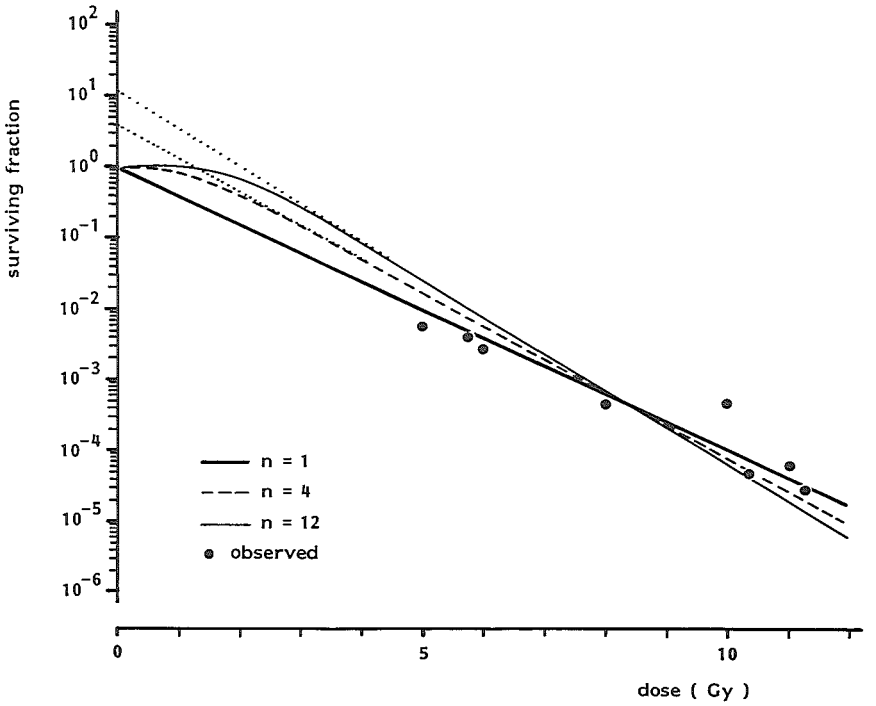

Figure 4.9 **Calculated survival of hemopoietic stem cells after TBI with 6 MV X-rays, using the multi target model, and various assumptions were made for the extrapolation number, n.** (n was set at 1, 4 and 12 respectively). The data points shown are derived from the regeneration time of reticulocytes. In the calculation, the total number of BM cells was assumed to be 24 x 10^9/kg and the homing fraction was assumed to be 0.3.

4.3. Discussion.

Comparison of the endogenous regeneration time after TBI to the regeneration time observed following autologous BM transplantation yields a relationship between radiation dose D and the number of surviving HSCs (N). N can be regarded as the HSC equivalent of a number of unfractionated, transplanted, punctured autologous femoral BM cells. The relationship between N and

the actual number of surviving HSCs is dependent on the frequency of HSCs among femoral BM cells, the frequency of HSCs among other BM cells, the fraction of peripheral blood in punctured BM, and on the homing fraction of intravenously injected HSCs. However, as long as these factors are constant, they do not influence the calculated D_0 value as explained in section 4.1.

In the transplanted monkeys, on which equation (10) was based, the number of endogenous HSCs that survives the conditioning 9.6 Gy TBI, was assumed to be negligible in comparison to the number of transplanted cells. Previous observations indicate, that this cell number is approximately the equivalent of 10^6 transplanted autologous BM cells/kg body weight[9]. Similar D_0 calculations without neglecting this cell number do not result in significant changes.

The D_0 value calculated here is greater than the value of 0.6 Gy, which was calculated earlier[6]. This earlier analysis was also based on a comparison of endogenous regeneration with regeneration after autologous BM transplantation. However, the number of autologous BM cells required to rescue 50 % of the animals after supralethal TBI, was an extrapolation. Furthermore, survival was used as an endpoint. In contrast to the monkeys in which endogenous regeneration was studied, the transplanted monkeys were subjected to supportive care, which was almost identical to the present supportive care and the influence of this treatment on survival was neglected. The influence of supportive care on survival after TBI is demonstrated in Figure 4.4. Although the number of monkeys is small, the dose survival curve for TBI followed by intensive and prophylactic supportive care indicates, that supportive care results in an increase of the $LD_{50/30\ days}$ by at least 3 Gy (Figure 4.4). If this effect is taken into account, also the data used for the earlier calculation are compatible with a D_0 value of about 1 Gy for 300 kV X-rays.

Also for other reasons a D_0 value of 0.6 Gy can be excluded: The endogenous regeneration time following a dose of 9.7 Gy (300 kV X-rays) TBI is not significantly different from regeneration following transplantation of 10^6 BM cells/kg body weight, indicating, that the number of residual HSC following 9.7 Gy TBI is the equivalent of 10^6 BM cells. On the basis of this observation N_0 in equation (5) can be calculated under the assumption of a D_0 value of 0.6 Gy

and an extrapolation number[6] of n = 1

$$\ln (10^6) - \ln N_0 = -9.7/0.6 \qquad (12)$$

Equation (12) results in a value of 10^{13} for N_0, which would indicate, that the total number of HSC in Rhesus monkeys is the equivalent of 10^{13} BM cells/kg body weight, which is physically impossible, since 10^{12} BM cells weigh approximately 1 kg.

The earlier analysis was dependent on a number of assumptions on the relationship between the actual number of surviving HSCs following TBI and the HSC equivalent of a number of unfractionated, transplanted, punctured autologous femoral BM cells (c in equation 2). In addition, an assumption was made for the total number of BM cells in non-irradiated Rhesus monkeys. In the present experimental design, these assumptions could be avoided and, therefore, do not influence the calculated D_0 value, as explained in section 4.1.

The data presented in this chapter can not be used to calculate the frequency of HSC among BM cells. Vriesendorp and van Bekkum have reported, that smaller animals have a higher $LD_{50/30\ days}$ for TBI than larger species, and that smaller animals require less bone marrow cells per kg body weight (BM rescue dose) than larger species to survive lethal irradiation (Table 4.1). On the basis of these differences, an inverse relationship between HSC frequency and body weight among species was postulated. This postulate was based on the assumption, that survival following TBI is dependent only on the number of residual or transplanted HSC. Although the duration of pancytopenia is dependent the number of HSC, survival of an organism following TBI is also determined by external influences, such as the bacteriological status and supportive care (Figure 4.4). Therefore, the lower $LD_{50/30\ days}$ and the lower BM rescue dose for small species in comparison to larger species can also be explained by other factors than HSC frequency, such as the bacteriological status, or the bacteriological barrier function of the mucous membranes against infectious agents. Therefore differences in HSC frequency among species can only be investigated by a comparison of regeneration times, which should be

corrected for possible cytokinetic differences.

As discussed earlier, extrapolation of the data described in this chapter cannot be used for a reliable calculation of the total number of BM cells, but other investigators have estimated the total number of Rhesus monkey BM cells to be 24 x 10^9/kg[7,8]. If we suppose the homing fraction to be 1, which is probably an overestimation, the fraction of peripheral blood admixture in punctured BM to be 0, which is certainly an underestimation, if we further suppose the extrapolation number, n, to be 1.0, the total number of BM cells is between 1.4 x 10^9 and 10^{10}/kg body weight (95 % confidence limits). Even under these extreme assumptions, the extrapolated N_0 is significantly lower than the reported estimate of the total number of BM cells (24 x 10^9/kg[7,8]). A similar problem was observed in earlier D_0 calculations of murine HSC. In these calculations, endogenous regeneration from a shielded femur was compared to regeneration following syngeneic BM transplantation, and spleen weights and BM cellularity were used as end points[13]. The difference between the N_0 value, extrapolated from the present data and the reported total number of BM cells might be explained by systemic errors in the radioiron method that was used and resulted in an overestimation of the total number of BM cells[14,15]. Alternatively, a difference in the frequency of HSCs between femoral BM and other BM localisations might cause that our calculation is an underestimation of the total number of BM cells. Local anoxia in the BM could result in a radioprotective effect on a subpopulation of the HSC[16]. Such an effect could result in a D_0, which is relatively high in the high dose range in which the present data were obtained. However, the data reported by Vriesendorp and van Bekkum were obtained in a lower dose range (0 - 5 Gy) and, as discussed above, also these data indicate a D_0 value of approximately 1 Gy for 300 kV X-rays. Thus, the most likely explanation of the difference between the extrapolated N_0 and the reported total number of BM cells is the unreliability of extended extrapolations as would be necessary here.

As explained above, our method can not be used in the lower dose range. Therefore the extrapolation number, n, can not be calculated on the basis of the obtained data. These data do not predict linearity in the low dose range, but all

available data, reviewed in section 1.2 and more extensively by Hendry[17], indicate a low extrapolation number ($1 < n < 5$), i.e. only a small shoulder in the dose survival curve of HSCs for all species investigated. Figure 4.1 demonstrates that, in the higher dose ranges, the dose survival curves of highly radiosensitive cells, described by different radiobiological models, approach to the curves described by the single hit model. Assumptions on the extrapolation number, n, do not significantly influence the D_0 value: For $n = 12$, the D_0 value was calculated to be only 0.04 Gy lower than for $n = 1$. Roughly, a higher extrapolation number results in an parallel upward shift of the dose survival curve, and in a lower N_0 value.

Under the assumptions mentioned earlier, inclusion of the total number of bone marrow cells in the calculations results in lower D_0 values, which are more influenced by the extrapolation number. However, in the dose range tested, even an unrealistically high extrapolation number ($n = 12$) does not result in a significant change in the fitted dose survival curve (Figure 4.9).

Although the anaesthesia that was used in the monkeys which were irradiated with 6 MV X-rays, does not alter the arterial oxygenation[18], the influence of anaesthesia on the radiosensitivity of HSC *in vivo* cannot be excluded completely. However, the monkeys which were irradiated with 300 kV X-rays and were not anaesthetized, yield a D_0 value of HSC of 1.04 Gy, which is fully compatible with that obtained for 6 MV X-rays. Therefore significant influences of this type of anaesthesia on the radiosensitivity of HSC can be excluded, unless the RBE of 6 MV X-rays, which was based on mouse data, would be significantly higher for Rhesus monkey HSC.

Our measurements of the radiation sensitivity of HSCs have implications for the conditioning for clinical BM transplantation and for the treatment of victims of radiation accidents. Following allogeneic BM transplantation, mixed chimerism frequently occurs[19,20,21]. Apparently, the addition of chemotherapy to the clinically used conditioning doses of TBI does not kill a sufficient number of HSC to prevent endogenous regeneration completely. The D_0 value of leukemic HSCs has been calculated to be 1.0 Gy[22], indicating, that the current conditioning regimens for BM transplantation are insufficient to eradicate 10^{10}

leukemic cells, which is approximately the detection level of human leukemia[23].

BM transplantation following accidental doses of TBI larger than 10 Gy is not effective, since such high doses frequently result in death due to irreversible gastrointestinal damage[12]. Endogenous regeneration following 9.8 Gy TBI has been observed on day 30, which is not significantly different from the regeneration time observed following transplantation of 10^6 autologous BM cells/kg. If we assume, that the total number of Rhesus monkey BM cells is the equivalent of 10^{10} transplanted cells/kg, we can calculate, that the HSC equivalent that survives 9.8 Gy TBI ranges from 5×10^4 to 1×10^7 cells/kg (95% conf. lim.), resulting in a complete hemopoietic reconstitution within 6 weeks. This implies that if optimal supportive care can be provided, the hemopoietic syndrome is not the dose limiting factor for TBI. This is especially true for accidental irradiation which is always inhomogeneous. Endogenous regeneration from a small number of surviving HSCs will take more time than regeneration from a much larger number of transplanted HSCs. The probability to survive the irradiation induced pancytopenia is dependent on the quality of the supportive care and on the duration of this phase. Consequently the aim of BM transplantation in the treatment of victims of irradiation accidents can only be shortening of the pancytopenic phase rather than replacement of destroyed BM. The emerging availability of recombinant hemopoietic growth factors (HGFs) has stimulated trials to shorten the pancytopenic phase after TBI and/or BM transplantation by *in vivo* administration of HGFs. A number of victims of a recent radiation accident has been treated with GM-CSF, which demonstrated the validity of this approach[24].

In spite of full hemopoietic regeneration, a number of monkeys died from infections between day 42 and day 100, suggesting impaired immunological resistance. This was not further investigated. Since these monkeys had normal values for granulocytes, lymphocytes and monocytes, it is highly unlikely, that such events can be prevented by allogeneic BM transplantation.

Our data cannot precisely predict the regeneration time in human victims of radiation accidents, since differences in hemopoietic cell kinetics between humans and Rhesus monkeys have been described[25]. However, it is a time

proven model for most other comparative parameters[26]. Without supportive care, the $LD_{50/30\ days}$ is 5.0 Gy for Rhesus monkeys[12] and estimated to be 4.5 Gy for humans[27], although higher (> 5 Gy[28]) and lower (3 Gy[29] and 3.15 Gy[30]) values have been reported. Therefore, the Rhesus monkey is the best available model for preclinical studies on radiation sensitivity of hemopoietic HSCs. The principles discussed in this chapter are most likely valid for the human situation.

In summary, our data indicate, that primate HSCs are less sensitive for X-rays than was assumed previously. The radiation sensitivity can be characterized by a D_0 value of at least 0.9 Gy instead of 0.6 Gy for 300 kV X-rays. This is based on only two assumptions:

Firstly, repopulating cells can be regarded as a homogeneous cell population. Secondly, endogenous regeneration is the same process as regeneration following autologous BM transplantation, which means, that differences in transplanted cell numbers in the range between 10^6 and 10^8 BM cells/kg do not influence c in equation (2). Consequently, endogenous regeneration would still be expected following doses of TBI which result in 100% gastrointestinal mortality. Shortening of irradiation induced pancytopenia can be achieved by BM transplantation, and, in principle, also by administration of hemopoietic growth factors. The application of these growth factors for the treatment of victims of irradiation accidents is currently under study. The influence of GMCSF on the duration of the pancytopenic phase following different doses of TBI will be discussed in Chapter VII.

REFERENCES

1. Lapidot, T., Singer, T.S., and Reissner, Y. (1988). Transient engraftment of T cell-depleted allogeneic bone marrow in mice improves survival rate following lethal irradiation. Bone Marrow Transplantation 3, 157-164.

2. Monroy, R.L., Vriesendorp, H.M., and MacVittie, T.J. (1987). Improved survival of dogs exposed to fission neutron irradiation and transplanted with DLA identical bone marrow. Bone Marrow Transplantation 2, 375-384.

3. Jammet, H., Mathé, G., and Pendic, B. (1959). Etude de six cas d'irradiation total d'aigue accidentale. Rev. Franc. Etudes Clin. Biol. 4,210-228.

4. Gale, R.P. and Reissner, Y. (1988). The role of bone-marrow transplantation after nuclear accidents. Lancet I, 923-926.

5. MacVittie, T.J., Monroy, R.L., Patchen, M.L., and Darden, J.H. (1984). Acute lethality and radiosensitivity of the canine hemopoietic system to Cobalt-60 gamma and mixed neutron-gamma irradiation. In: Response of different species to total body irradiation. **Eds.:** Broerse, J.J. and MacVittie, T.J. p. 113.

6. Vriesendorp, H.M. and van Bekkum, D.W., p.349. In: Immunobiology of Bone Marrow Transplantation. Springer Verlag, Berlin (1980).

7. Donohue, D.M., Gabrio, B.W., and Finch, C.A. (1958). Quantitative measurement of hemopoietic cells of the marrow. J. Clin. Inv. 37:1564-1576.

8. Pegg, D.E. (1966). p. 8 in: Bone Marrow transplantation. Lloyd-Luke (medical books) Ltd. London.

9. Gerritsen, W.R., Wagemaker, G., Jonker, M., Kenter, M., Wielenga, J.J., Hale, G., Waldmann, H., and Bekkum, D.W. van. (1988). The repopulating capacity of bone marrow grafts following pretreatment with monoclonal antibodies against T lymphocytes in rhesus monkeys. Transplantation 45:301-307.

10. Bekkum, D.W. van, Löwenberg, B., and Vriesendorp, H.M. (1978). Bone marrow transplantation. p 79 In: Immunological engineering MTP Press. Eds: Hirsch, D.W., Lancaster, England.

11. Broerse, J.J., and Zoetelief, J. (1984) The occurrence of radiation syndromes in rodents and monkeys in dependence on dose rate and radiation quality. In: Response of different species to total body irradiation. Eds.: Broerse, J.J. and MacVittie, T.J. Martinus Nijhoff Publishers, Boston, Dordrecht, Lancaster. p. 175-187.

12. Broerse, J.J., Bekkum, D.W. van, Hollander, C.F., and Davids, J.A.G. (1978). Mortality of monkeys after exposure to fission neutrons and the effect of autologous bone marrow transplantation. Int. J. Radiat. Biol. 34:253-264.

13. Bekkum, D.W. van and Schotman, E. (1974). Protection from hemopoietic death by shielding versus grafting of bone marrow. Int. J. Radiat. Biol. 25:361-372.

14. Lord, B.I. (1979). Kinetics of the recognizable erythrocyte precursor cells. Clin. Haematol. 8:335-350.

15. Maillie, H.D., Baxter, R.C., and Lisman, H. (1977). The use of ^{59}Fe uptake in estimating the marrow dose in normal and splenectomized rats exposed to 1000 kVp X-irradiation. Health Phys. 33:425-429.

16. Mottram, J.C. (1936) Factors of importance in radiosensitivity of tumours. Br. J. Radiol. 9:606-614.

17. Hendry, J.H., and Lord, B.I. (1983) The analysis of the early and late response to cytotoxic insults in the hemopoietic cell hierarchy. In: Cytotoxic Insult to Tissue. Effects on cell lineages. Eds.: Potten, C.S., and Hendry, J.H. Churchill Livingstone. Edinburgh, London, Melbourne and New York. p. 1-67.

18. Wielenga, J.J. and Gils, F.C.J.M van. unpublished observations.

19. Hill, R.S., Petersen, F.B., Storb, R., Appelbaum, F.R., Doney, K., Dahlberg, S., Ramberg, R., and Thomas, E.D. (1986). Mixed hematologic chimerism after allogeneic bone marrow transplantation for severe aplastic anemia is associated with a higher risk of graft rejection and a lessened incidence of acute graft-versus-host disease. Blood 67:811-816.

20. Schattenberg, A., De Witte, T., Salden, M., Vet, J., van Dijk, B., Smeets, D., Hoogenhout, J., and Haanen, C. (1989). Mixed hematopoietic chimerism after allogeneic bone marrow transplantation with lymphocyte-depleted bone marrow is not associated with a higher incidence of relapse. Blood 73:1367-1372.

21. Bertheas, M.F., Maraninchi, D., Lafage, M., Fraisse, J., Blaisse, D., Stoppa, A.M., Michel, G., Brizard, C.P., Gaspard, M.H., and Novakovitch, G. (1988). Partial chimerism after T-cell-depleted allogeneic bone marrow transplantation in leukemic HLA-matched patients: a cytogenetic documentation. Blood 72:89-93.

22. Hagenbeek, A., and Martens, A.C.M. (1981). The effect of fractionated versus unfractionated total body irradiation on the growth of the BN acute myelocytic leukemia. Int. J. Oncol. Biol. Phys. 7:1075-1079.

23. Hagenbeek, A. and Martens, A.C.M. (1984). Detection of minimal residual leukemia utilizing monoclonal antibodies and fluorescence activated cell sorting. In: Minimal residual disease in acute leukemia. Eds: B. Löwenberg and A. Hagenbeek. Martinus Nijhof Publishers, Boston, The Hague, Dordrecht, Lancaster p. 45.

24. Butturini, A., Gale, R.P., Lopes, D.M., Cunha, C.B., Ho, W.G., Sanpai, J.M., Souza, P.C.de, Cordiero, J.M., Neto, C., Souza, C.E.P.de, Tabak, D.G., Burla, A., and the Navy Hospital Radiation Team: Use of recombinant granulocyte-macrophage colony in the Brazil radiation accident. Lancet II (1988):471-475.

25. Fliedner, T.M. (1969). A cytokinetic comparison of hematological consequences of radiation exposure in different mammalian species. In: Comparative Cellular and Species Radiosensitivity. Bond, V.P. and Sugara, T. (Editors), Igaku Shoin Ltd. Tokyo, Japan, 89-101.

26. Bekkum, D.W. van. (1978). The Rhesus monkey as a preclinical model for bone marrow transplantation. Transpl. Proc. 10:105-111.

27. Mole, R.H. (1975). Deductions about survival curve parameters from iso-effect radiation regimens: observations on lethality after whole body irradiation of mice. In: Cell survival after low doses of radiation (Alper, T., Editor) Proceedings 6th L.H. Gray Conference

Chicester, Wiley. 229.

28. U.S. NRC. (1975). An assessment of accident risks in US commercial nuclear power plants. US Nuclear Regulatory Commission, WASH-1400, appendix F.

29. Warren, S. and Bowers, J.Z. (1950). The acute radiation syndrome in man. Ann. Int. Med. 32:207-212.

30. National Council on Radiation Protection and Measurements (1971): Basic radiation criteria. NCRP Report 39, NCRP Publications, Washington D.C.

CHAPTER V
THE EFFECT OF HUMAN RECOMBINANT GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTOR (GM-CSF) IN NORMAL MONKEYS

5.1. Introduction.

In the previous chapter, the radiosensitivity of Rhesus monkey hemopoietic stem cells (HSC) was discussed. Of most significance was the finding of a high probability of endogenous regeneration following doses of (accidental) total body irradiation (TBI) which were hitherto assumed to be lethal. Therefore, under conditions of optimal supportive care, bone marrow (BM) transplantation may be not an absolute requirement for hemopoietic regeneration after accidental high doses of TBI. Consequently the role of BM transplantation as a treatment modality for high doses of accidental irradiation is reduced to a shortening of the duration of radiation induced pancytopenia, an intention, which has to be outweighed against the risks of allogeneic BM transplantation. In principle, such a reduction of the pancytopenic period may also be obtained by stimulation of hemopoiesis *in vivo* with hemopoietic growth factors (HGFs).

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), has been used in clinical trials for its benefit following chemotherapy, electively supported by autologous BM transplantation[1,2,3,4]. As discussed in section 1.4, the efficacy of this treatment is difficult to evaluate. The patients, used for these studies, form a heterogeneous group and were not treated under standard conditions. Furthermore, adequate controls were lacking.

Victims of a recent radiation accident have been treated with GM-CSF[5], but again, the efficacy of this treatment is difficult to interpret, as discussed in section 1.4.3.

GM-CSF has a broad range of action on hemopoietic progenitor cells, including multipotent cells. GM-CSF stimulates the formation of colonies derived from progenitors of granulocytes, macrophages[6], and other lineages[7,8]. GM-CSF also stimulates *in vitro* colony formation of several human leukemic cell lines, in some of which GM-CSF also induces differentiation[9].

In addition to the effects of GM-CSF on HPC, GM-CSF induces functional changes in peripheral blood granulocytes and monocytes, a feature which might be important for prevention of infections in immunosuppressed patients[10,11,12,13,14,15,16,17,18].

In the present chapter, pilot experiments concerning the dose of GM-CSF and the route of administration will be described. On the basis of these data the route of administration of GM-CSF for experiments on mitigation of pancytopenia has been selected. Continuous intravenous (iv) administration of GM-CSF resulted in an increase in peripheral blood cell counts in healthy, Cynomolgus monkeys (Macaca fasicularis)[19]. Similar effects have been demonstrated in Rhesus monkeys following daily iv administration over 6 hours or subcutaneous (sc) administration three times daily[20]. However, the activity of GM-CSF in a practical schedule of one sc administration daily, has never been compared to other administration schedules. Therefore, we compared the effects of continuous iv administration of GM-CSF to those of sc administration once daily, in normal Rhesus monkeys.

5.2. Development of a continuous intravenous administration system in Rhesus monkeys.

Continuous intravenous (iv) administration of drugs requires an intravenous access, which can be connected to a pump. Such a system was not only

developed for the experiments with GM-CSF, but also for separate studies on other cytokines such as IL3 and IL2. Rhesus monkeys will spontaneously remove systems for iv administration, unless special precautions are taken. Therefore a jacket was used in combination with a Porth-a-Cath^R system and an electric insulin pump (Dahedi Instruments), which can be carried in the inside pocket of the jacket. A Porth-a-Cath^R system consists of a chamber which is connected to an iv catheter (Figure 5.1). The chamber can be implanted sc and punctured many times with a special fine needle.

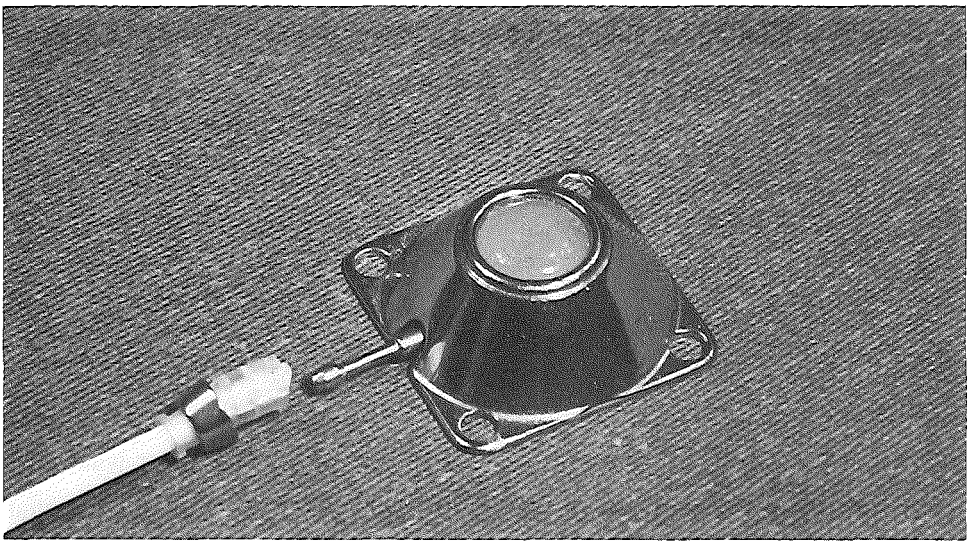

Figure 5.1 **Port-A-Cath^R (Pharmacia) implantable infusion system, used for intravenous GM-CSF administration.** The chamber is implanted subcutaneously and the catheter is introduced into the internal jugular vein. A special needle can be introduced percutaneously into the chamber.

In the electric insulin pump, a 2 ml syringe can be placed and the pump expels on each pulse 12.5 µl from the syringe into a tubing which is connected to a needle (Figure 5.2). This needle is pierced into the chamber of the Port-a-Cath^R system. The pulse frequency can be adjusted in the range from 1.3 to 12 pulses per hour.

5.2.1. Design of a Rhesus monkey jacket.

A jacket was developed to prevent the destruction or removal of the iv administration system and to carry the pump. A perfectly fitting, light cotton jacket was produced for one Rhesus monkey, which was supposed to wear it for several weeks. Unfortunately the jacket was easily destroyed. Another jacket, prepared from canvas resulted in redness and irritation of the skin under the seam of the sleeve, but it took a week for the monkey to destroy this jacket. To prevent irritation of the skin a raglan sleeve and a flannel lining was chosen for the next devise. Furthermore the outside of the jacket was strengthened by a leather stitched covering. This jacket, which was provided with an inner pocket to carry the insulin pump, could be worn for several months (See Figure 5.2).

5.2.2. Introduction of a Porth-a-Cath^{R} systems into Rhesus monkeys.

Rhesus monkeys were anaesthetized as described in section 2.2. The skin of the neck was incised and the jugular vein was released between the sterno-cleidomastoid and the omohyoid muscle. The internal jugular vein was ligated and incised caudally from the ligature (Figure 5.3a). The catheter was introduced into the internal jugular vein, fixed (Figure 5.3b), and tunnelled median from the sternocleidomastoid muscle to the back (Figure 5.3c and d). The sternocleidomastoid and the omohyoid muscle were attached to each other with catgut sutures, and the skin was closed with nylon sutures. Just below the scapula a second skin incision was made and the chamber was fixed subcutaneously and connected to the catheter. Unfortunately, a few hours after the operation the monkey removed the sutures in the neck as well as the catheter. To prevent this problem, in the subsequent monkeys, a horizontal skin incision was made two cm above the right mammilla. This incision was shoved up to the neck to enable the release of the internal jugular vein. The further procedure was not changed. The result of this modification was that

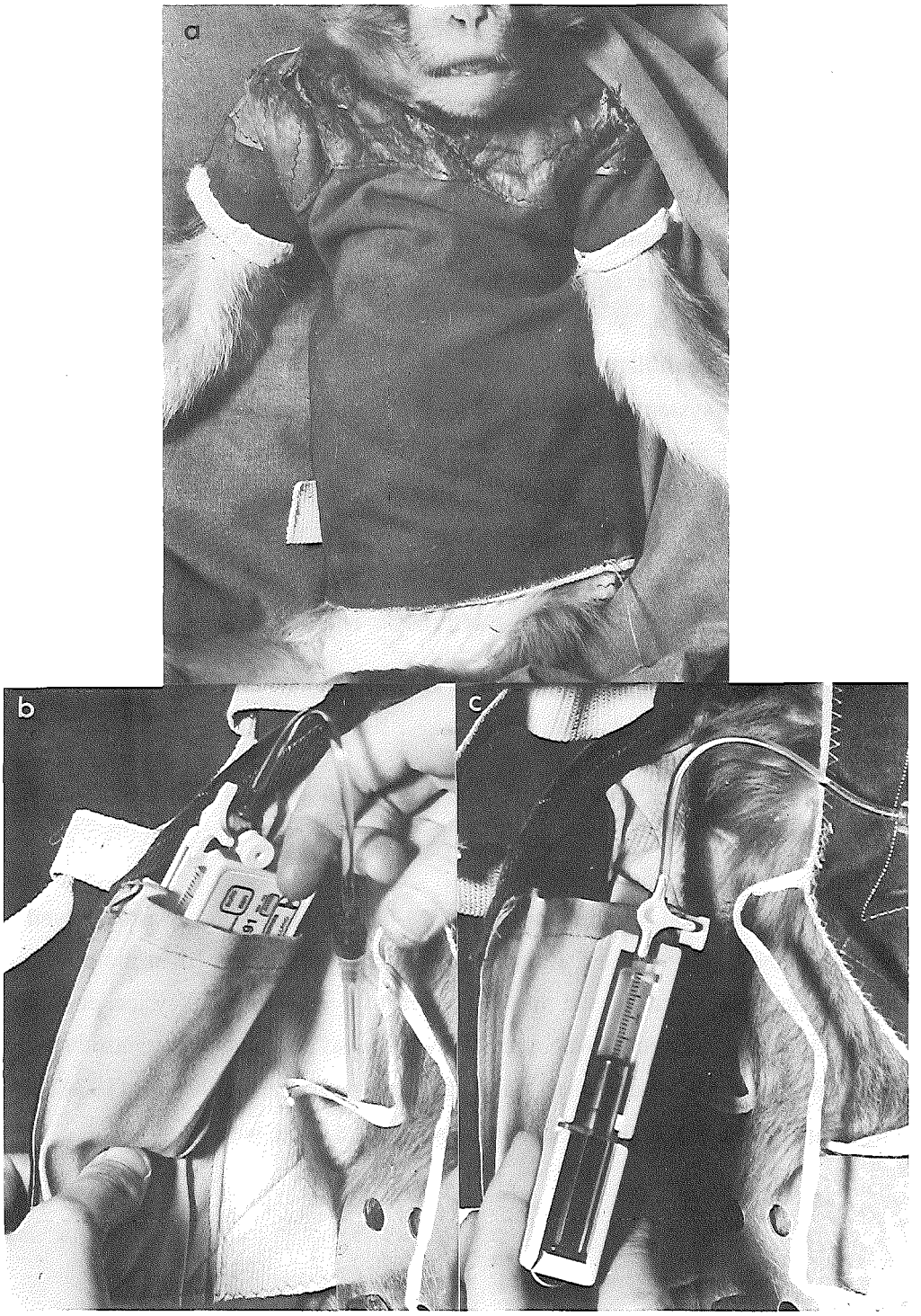

Figure 5.2 **Canvas Rhesus monkey jacket and insulin pump.**

the two skin wounds were fully covered by the jacket, so that it was impossible for the monkey to remove the system. However, during the weeks after the operation, these monkeys scrubbed their back against the wall of the cage continuously. In spite of the protection by the jacket a small skin laceration developed, which resulted in a local infection in 3 out of 3 monkeys. Systemic antibiotic treatment in combination with local measures could not prevent the spread of this infection. Therefore removal of the system was inevitable.

To prevent this problem, in all other monkeys (n = 7), the chamber of the Porth-a-Cath^R system was tunneled to the lateral side of the thoracic wall (Figure 5.3e, f and g). In 6 out of 7 monkeys this procedure resulted in uncomplicated wound healing. The final surgical procedure is illustrated in Figure 5.3. An X-ray photograph of the Porth-a-Cath^R system, introduced into a Rhesus monkey is shown in Figure 5.4. The system was flushed every four weeks with 500 U heparin in NaCl 0.9%. The system was not used until the wounds were completely healed.

5.2.3 Procedure for continuous administration of GM-CSF to Rhesus monkeys.

For the intravenous administration of GM-CSF the daily dose was dissolved in 1.8 ml NaCl 0.9% in a 2 ml syringe, which was introduced in the pump. The pump was fixed in the inside pocket of a Rhesus monkey jacket, and the syringe was connected with a needle, which was percutaneously introduced into the Port-a-Cath^R chamber. Each day a new syringe filled with GM-CSF was put into the pump and the needle was replaced twice weekly.

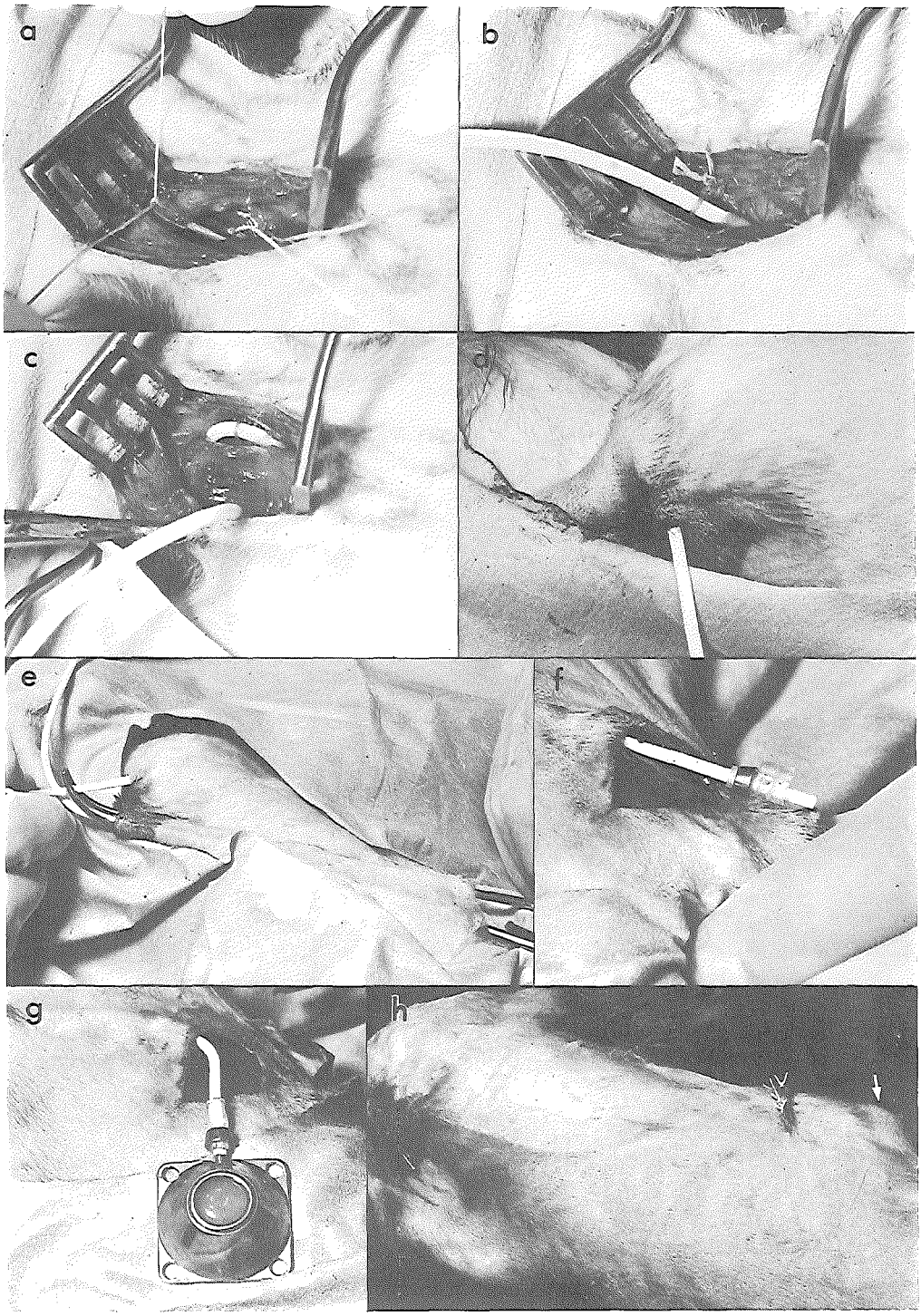

Figure 5.3 **Surgical procedure for the implantation of a Port-a-Cath in a Rhesus monkey.** For explanation see text.

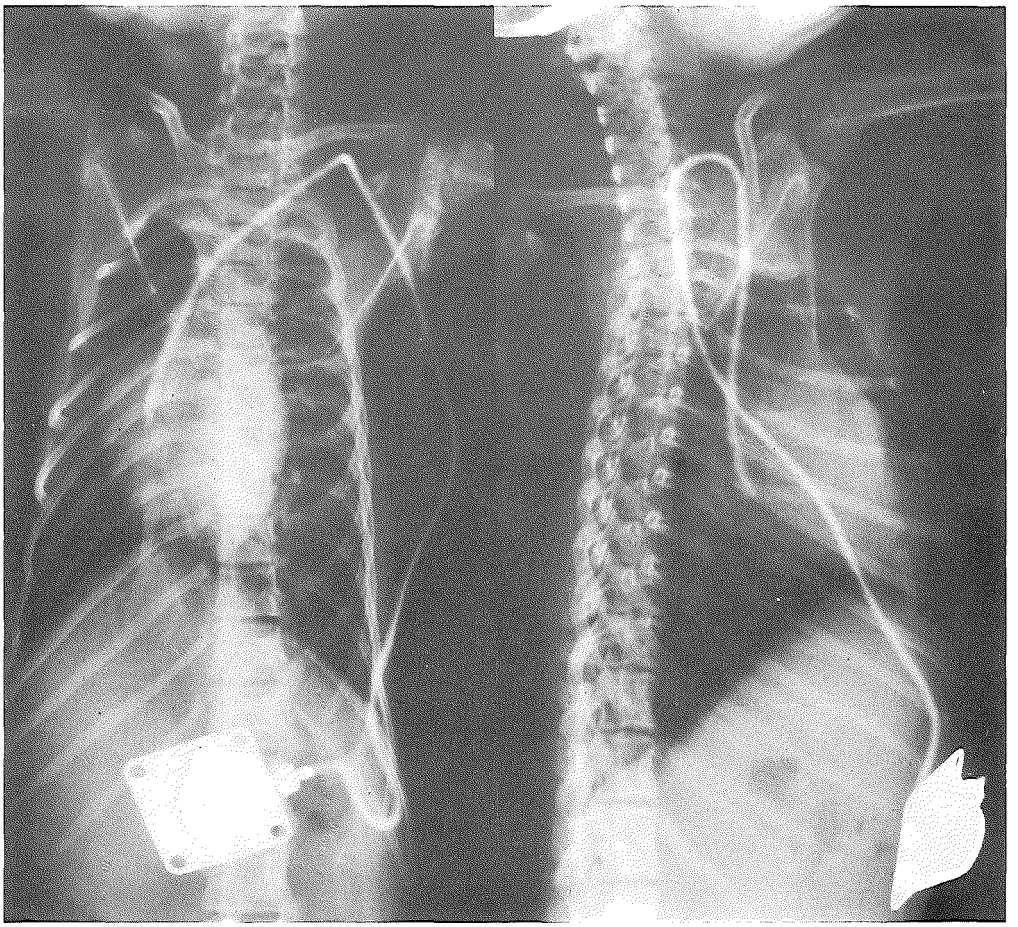

Figure 5.4 **X-ray photograph of a Port-A-Cath inplanted in a Rhesus monkey.**

5.3. Results

A dose of 10 μg/kg/day was administered iv for 14 days and the effects were compared to those of subcutaneous administration of 10 or 30 μg/kg GM-CSF/day. One monkey received 3 μg/kg GM-CSF/day for 7 days. On day 2 of the intravenous administration, a tube came untied from the syringe in the

pump, resulting in the loss of an unknown quantity of GM-CSF.

The influence of GM-CSF on the production of white blood cells is shown in Figure 5.5. Continuous iv administration of 10 μg GM-CSF resulted in a tenfold increase in white blood cell counts, while sc administration once daily resulted only in four- to fivefold rise, even when a higher dose (30 μg/kg/day) was used. The effect of 3 μg/kg/day sc for 7 days is only marginal. The leukocytosis is mainly due to a rise in neutrophils and eosinophils.

Continuous iv treatment with GM-CSF resulted in a marked thrombocytosis. During sc administration of 30 μg/kg/day, a temporary rise in thrombocyte counts was observed. Lower sc doses did not result in changes in thrombocyte counts (Figure 5.6). Also in the placebo treated monkey, the reticulocyte and thrombocyte counts increased, probably induced by the daily procedure of taking blood samples and giving sc injections. The effects of GM-CSF administration on reticulocyte counts were not significantly different from the placebo treated control. (Figure 5.7).

At pharmacological doses, GM-CSF does not only stimulate the production of granulocytes and eosinophils, but also affects other cell lineages. To quantify the differential effects of the treatment with GM-CSF we used the cumulated cell counts (see section 2.11). In Figure 5.8 these effects of GM-CSF are shown. Treatment with GM-CSF predominantly influences the counts of granulocytes and eosinophils. The normoblastosis during treatment with 30 μg/kg and to a lesser extent with 10 μg/kg s.c. was not reproduced in the other monkeys. The absence of a significant effect of GM-CSF on monocyte counts was not expected.

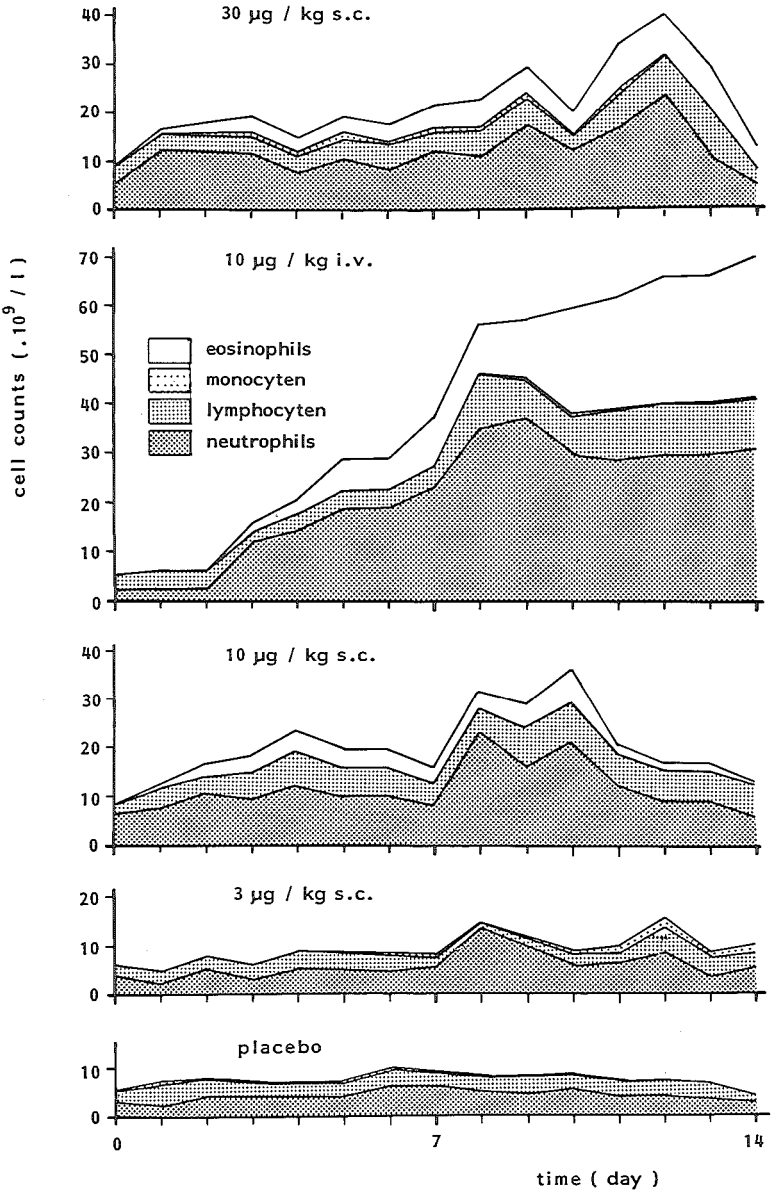

Figure 5.5 **Peripheral blood cell counts during intravenous and subcutaneous administration of GM-CSF.** GM-CSF was administered from day 1 to day 14 except for the dose of 3 µg which was given from day 7 to 14.

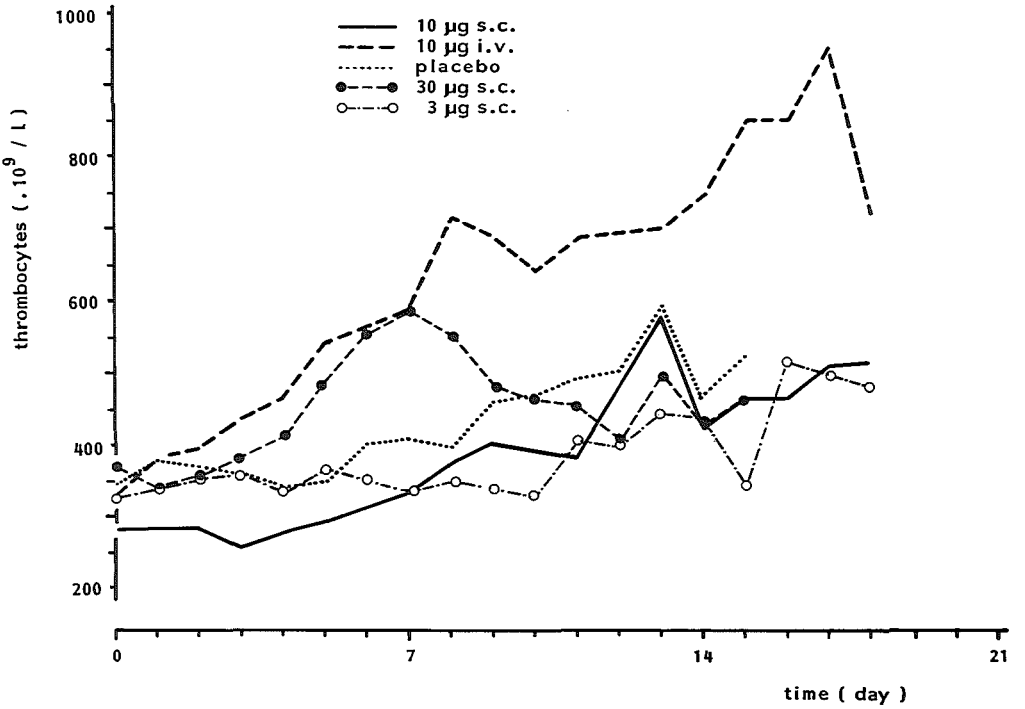

Figure 5.6 **Thrombocyte counts during intravenous and subcutaneous administration of GM-CSF.** Duration of GM-CSF administration was as given in the legend of Figure 5.5.

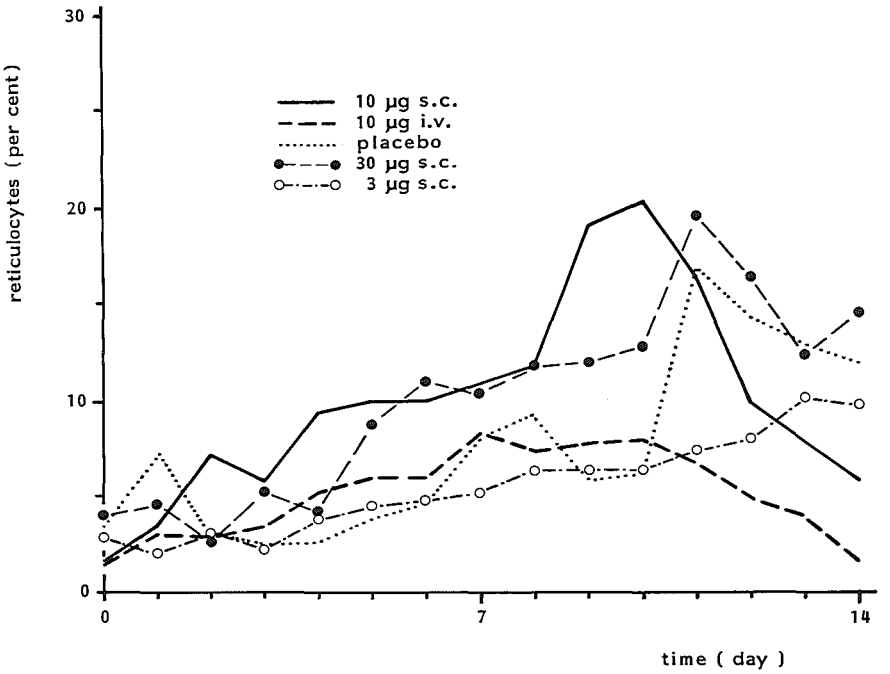

Figure 5.7 **Reticulocyte counts during intravenous and subcutaneous administration of GM-CSF on reticulocytes.** Duration of GM-CSF administration was as given in the legend of Figure 5.5.

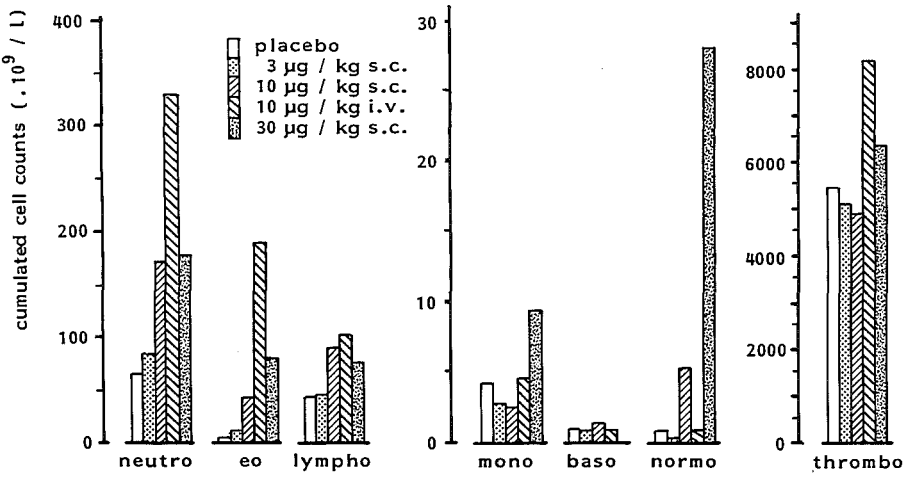

Figure 5.8 **Differential counts of blood cells during administration of GM-CSF.** Duration of GM-CSF administration was as given in the legend of Figure 5.5. Cell counts cumulated from day 2 to 15.

5.4. Discussion

Continuous iv administration of 10 µg/kg GM-CSF/day results in a tenfold increase in leukocytes, and a two- to threefold rise in thrombocyte counts. The absence of an effect during the first days of administration can partially be explained by the disconnection of a tube, which resulted in the loss of an unknown quantity of GM-CSF at day 2.

As expected from the *in vitro* characteristics of GM-CSF, treatment with GM-CSF predominantly influences the counts of granulocytes and eosinophils[7,8,9,21,22,23,]. This *in vivo* effect was also found by other investigators[19,20]. The normoblastosis, which was observed in two monkeys was not reported earlier. This effect will be further discussed in Chapter VI. A rise in monocyte counts, which was observed by others, was only observed in one monkey. A possible explanation for this discrepancy could be the inaccurate quantification of monocytes, which is caused by the low frequency of monocytes among peripheral blood leukocytes. Only the dose schedules that resulted in the largest numbers of granulocytes (10 µg/kg/day iv and 30 µg/kg/day s.c) resulted in an increase in thrombocyte counts. GM-CSF has also been found to stimulate thrombopoiesis *in vitro*. The effects of GM-CSF on thrombocytes *in vivo* will be discussed in more detail in Chapter VI and VII.

Administration of GM-CSF once daily sc resulted also in a marked leukocytosis, but this effect was less pronounced than that of continuous iv treatment. Other investigators who compared iv and sc administration have reported, that sc was more effective than iv[20]. However they compared a dose schedule of 3 dd sc to iv infusion that lasted only 6 hours a day. After a single sc adminstration, serum GM-CSF levels peak at 4 to 6 hours and persist for 14 to 24 hours[24]. After a single iv bolus injection, GM-CSF is cleared from the circulation with a half life of seven minutes during the initial α phase and 85 minutes during the subsequent β phase[19]. Consequently, cessation of the iv

infusion after six hours results in rapidly decreasing and very low GM-CSF levels for a large part of the day, which is not the case for sc administration thrice daily.

Although continuous iv administration of GM-CSF resulted in higher leukocyte counts than sc administration once daily, the latter schedule also effectively induced a leukocytosis, and its efficacy could be augmented by elevation of the dose. For clinical applications, especially in out-patients, sc administration once daily is more practical than continuous iv administration or sc administration thrice daily. Therefore, sc administration once daily was used for the experiments on mitigation of pancytopenia, which will be described in Chapter VI and VII.

REFERENCES

1. Brandt, S.J., Peters, W.P, Atwater, S.K., Kurtzberg, J., Borowitz, M.J., Jones, R.B., Shpall, E.J., Bast, R.C., Gilbert, C.J., and Oette, D.H. (1988) Effects of recombinant human granulocyte-macrophage colony-stimulating factor on hemopoietic reconstitution after high dose chemotherapy and autologous bone marrow transplantation. N. Engl. J. Med 318:869-876.

2. Nemutis, J., Singer, J.W., Buckner,C.D., Hil, R., Storb, R., Thomas, E.D., and Appelbaum, F.R. (1988) Use of recombinant Granulocyte-Macrophage Colony-Stimulating Factor in autologous marrow transplantation for lymphoid malignancies. Blood 72:834-836.

3. Antin, J.H., Smith, B.R., and Rosenthal, D.S. (1987) Phase I/II study of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) in bone marrow failure. Blood 70:Suppl 1:129a.

4. Antman, K.S., Griffin, J.D., Elias, A., Socinski, M.A., Ryan, L., Cannistra, S.A., Oette, D., Whitley, M., Frei, E. III, and Schnipper, L.E. (1988) Effect of recombinant human granulocyte-macrophage colony-stimulating factor on chemotherapy-induced myelosuppression. N. Engl. J. Med. 319:593-598.

5. Butturini, A., Gale, R.P., Lopes, D.M., Cunha, C.B., Ho, W.G., Sanpai, J.M., Souza, P.C. de, Cordiero, J.M., Neto, C., Souza, C.E.P. de, Tabak, D.G., Burla, A., and the Navy Hospital Radiation Team. (1988). Use of recombinant granulocyte-macrophage colony stimulating factor in the brazil radiation accident. Lancet II:471-475.

6. Nicola, N.A., Metcalf, D., Johnson, G.R., Burgess, A.W. (1979) Separation of functionally distinct human granulocyte-macrophage colony-stimulating factors. Blood 54:614-627.

7. Sieff, C.A., Emerson, S.G., Donahue, R.E., Nathan, D.G., Wang, E.A., Wong, G.G., and Clark, S.C. (1985) Human recombinant granulocyte-macrophage colony-stimulating factor: a multilineage hemopoietin. Science 230:1171-1173.

8. Metcalf, D. (1986) The molecular biology and function of the granulocyte-macrophage stimulating factors. Blood 67:257-267.

9. Tomonaga, M., Golde, D.W., and Gasson, J.C. 1986. Biosynthetic (recombinant) human granulocyte-macrophage colony-stimulating factor: Effect on normal bone marrow and leukemia cell lines. Blood 67:31-36.

10. Fleischmann, J., Golde, D.W., Weisbart, R.H., and Gasson, J.C. (1986). Granulocyte-macrophage colony stimulatory factor enhances phagocytosis of bacteria by human neutrophils. Blood 68:708-711.

11. Gasson, J.C., Weisbart, R.H., Kaufman, S.E., Clark, S.C., Hewick, R.M., Wong, G.G., and Golde, D.W. (1984). Purified human granulocyte-macrophage colony stimulating factor: direct action on neutrophils. Science 226:1339-1342.

12. Weisbart, R.H., Golde, D.W., and Gasson, J.C. (1986). Biosynthetic human GM-CSF modulates the number and affinity of neutrophil f-met-leu-phe receptors. J. Immunol. 137:3584-3592.

13. Weisbart, R.H., Golde, D.W., Clark, S.C., Wong, G.G., and Gasson, J.C. (1985). Human granulocyte macrophage stimulating factor is a neutrophil activator. Nature 314:361-363.

14. Weisbart, R.H., Kwan, L., Golde, D.W., and Gasson, J.C. (1987). Human GM-CSF primes neutrophils for enhanced metabolism in response to the major physiological chemoattractants. Blood 69:18-21.

15. Grabstein, K.H., Urdal, D.L., Tushinski, R.J., Mochizuki, D.Y., Price, V.L., Cantrell, M.A., Gillis, S., and Conlon, P.J. (1986). Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. Science 232:506-508.

16. Coleman, D.L., Chodakewitz, J.A., Bartiss, A.H., and Mellors, J.W. (1988). Granulocyte-macrophage colony-stimulating factor enhances selective effector functions of tissue de-rived macrophages. Blood 72:573-578.

17. Socinski, M.A., Cannistra, S.A., Sullivan, R., Elias, A., Antman, K., Schnipper, L., and Griffin, J.D. (1988). Granulocyte-macrophage colony-stimulating factor induces the expression of the CD11b surface adhesion molecule on human granulocytes in vivo. Blood 72:691-697.

18. Sanchez-Madrid, F., Nagg, J.A., Robbins, E., Simon, P, Springer, T.A. (1983). A human leukocyte differentiation antigen family with distinct ‡-subunit and a common ß-subunit: The lymphocyte-function-associated antigen (LFA-1), the C3b complement receptor (OKM1/Mac-1), and the p150,95 molecule. J. Exp. Med. 158:1785-1794.

19. Donahue, R.E., Wang, E.A., Stone, D.K., Kamen, R., Wong, G.G., Seghal, P.K., Nathan, D.G., and Clark, S.C. (1986) Stimulation of hematopoiesis in primates by continuous infusion of recombinant GM-CSF. Nature 321:872-875.

20. Mayer, P., Lam, C., Obenhaus, H., Liehl, E., and Besemer, J. (1987) Recombinant human GM-CSF induces leukocytosis and activates peripheral blood polymorphonuclear neutrophils in nonhuman primates. Blood 70:206-213.

21. Burgess, A.W., Begley, C.G., Johnson, G.R., Lopez, A.F., Williamson, D.J., Mermod, J.J., Simpson, R.J., Schmitz, A., and DeLamarter, J.F. (1987) Purification and properties of bacterially synthesized human granulocyte-macrophage colony stimulating factor. Blood 69:43-51.

22. Metcalf, D., Begley, C.G., Johnson, G.R., Nicola, N.A., Vadas, M.A., Lopez, A.F., Williamson, D.J., Wong, G.G., Clark, S.C., and Wang, E.A. (1986) Biologic properties in vitro of a human recombinant granulocyte-macrophage colony-stimulating factor. Blood 67:37-45.

23. Metcalf, D., Johnson, G.R., and Mandel, T. (1979). Colony formation in agar by multipotential hemopoietic cells. J. Cell. Physiol. 98:401-420.

24. Thompson, J.A., Lee, D.J., Kidd, P., Rubin, E., Kaufmann, J., Bonnem, E.M., and Fever, A. (1989). Subcutaneous granulocyte-macrophage colony-stimulating factor in patients with myelodysplastic syndrome: toxicity, pharmacokinetics, and hematological effects. J.Clin. Oncol. 7:629-637.

CHAPTER VI
MITIGATION OF BONE MARROW TRANSPLANTATION RELATED PANCYTOPENIA BY TREATMENT WITH GM-CSF

6.1. Introduction.

As discussed in Chapter IV, high dose chemotherapy and/or total body irradiation (TBI), electively followed by BM transplantation results in a (temporary) lack of peripheral blood cells termed pancytopenia. Thrombopenia may result in haemorrhagic complications, and leukopenia increases the risk for serious infectious complications, particularly when neutrophil counts fall below 0.5 x 10^9/l[1]. Therefore, if treatment with HGFs results in a mitigation of the neutropenia, serious infectious complications might be prevented.

Other investigators have described, that treatment with GM-CSF result in a reduction of leukopenia following autologous BM transplantation in Rhesus monkeys[2,3]. In those studies, large numbers (0.5 to 5 x 10^8/kg) of low density autologous bone marrow cells were transplanted. Since a reduction of pancytopenia is especially of clinical relevance under conditions associated with a long duration of pancytopenia, (if the number of HSC is limited), the influence of GM-CSF on hemopoietic regeneration after transplantation of a small number of autologous BM cells was investigated. T-cell depleted stem cell concentrates were used to exclude any production of HGFs by grafted lymphocytes. This approach provides a highly critical experimental setting to evaluate the influence of GM-CSF on BM transplantation related

pancytopenia.

The dose-effect relationship of GM-CSF so far has not been studied systematically under such critical conditions. Therefore, we undertook the study of graded doses of GM-CSF, ranging from 3 to 100 μg/kg/day, on hemopoietic regeneration after transplantation of limited numbers of autologous, T-cell depleted, concentrated HSC.

6.2. Effects of different doses of GM-CSF on peripheral blood cells following transplantation of autologous T-cell depleted stem cell concentrates.

Rhesus monkeys were conditioned by two equal fractions of 6 Gy TBI as described in section 2.7 on day -1 and day -2. On day 0, an autologous T-cell depleted stem cell concentrate, prepared as described in section 2.2, was injected into a peripheral vein. This graft was prepared two weeks before irradiation, stored in liquid nitrogen, and thawed by stepwise dilution. In all monkeys, the graft size was the CFU-C equivalent of 10^7 unfractionated BM cells/kg body weight. Peripheral blood cell counts were determined daily after autologous BM transplantation. All monkeys received supportive care as described in section 2.9. Recombinant human GM-CSF was administered sc once daily from day 1 to day 30. Graded doses, ranging from 3 to 100 μg/kg body weight were used. For each dose level of GM-CSF, one monkey was used, except for the dose of 10 μg/kg which was studied in three monkeys. Table 6.1 gives specific details of the transplanted monkeys.

Table 6.1 SURVIVAL OF RHESUS MONKEYS FOLLOWING GM-CSF TREATMENT AFTER TRANSPLANTATION OF AUTOLOGOUS T-CELL DEPLETED STEM CELL CONCENTRATES

Unique animal number	GM-CSF dose (µg/kg/day)	Alive and well (days)	Cause of death
M 19	3	126	diarrhoea*
1 TZ	10	> 365	
1 YN		33	sepsis
K 25 (hyper-transfusion)		> 365	
1 WE	30	> 365	
1 ZB	100	31	CMV
1 YA		24	sepsis
D 29	placebo (equivalent of 30 µg/kg)	56	sepsis
BB 33	placebo (equivalent of 100 µg/kg)	129	diarrhoea*
BB 34	no HGF	> 365	

* Flagellate enteritis

Figure 6.1 shows the maximum effects of GM-CSF on the regeneration patterns of leukocytes, reticulocytes, and thrombocytes. These maximal effects were observed at different doses. As expected, the effect on leukocytes was more pronounced than the effect on thrombocytes and reticulocytes. A considerable leukocytosis was observed during the third and fourth week of GM-CSF administration, demonstrating the effectiveness of the treatment. However, an actual shortening of the leukopenic phase, (defined as the day at which the leukocyte counts exceeded a value of 10^9/l) was restricted to 2-7 days as compared to placebo treated or untreated

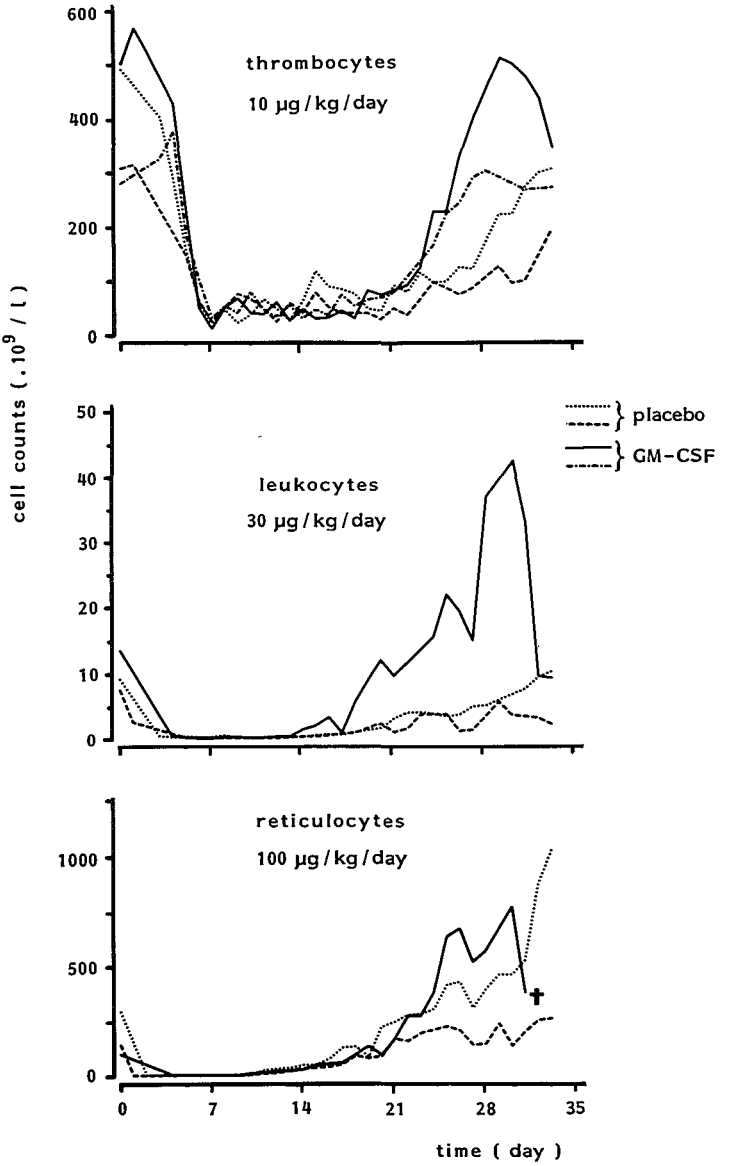

Figure 6.1 **Peripheral blood cell counts during administration of GM-CSF after TBI and transplantation of autologous stem cell concentrates.** For each cell type the monkey in which the highest cell counts were observed was selected. GM-CSF was administered once daily s.c. for 30 days after TBI (2 x 6 Gy 6 MV X-rays) and transplantation of autologous T-lymphocyte depleted concentrated stem cells, equivalent to 10^7 unfractionated cells/kg.

control monkeys. This is illustrated in Table 6.2, in which the peak value of the leukocytosis and the duration of the leukopenia are shown for all doses of GM-CSF in comparison to untreated and placebo treated control monkeys. To avoid the influence of random day to day fluctuations and to evaluate the

Table 6.2 INFLUENCE OF GM-CSF ON DURATION OF LEUKOPENIA AND THE REBOUND LEUKOCYTOSIS AFTER TRANSPLANTATION OF AUTOLOGOUS T-CELL DEPLETED STEM CELL CONCENTRATES

Unique animal number	GM-CSF dose (µg/kg/day)	Number of septic episodes*	Leukocytes > 10^9/l (day)	Maximum observed value of leukocytes	
				cell number (x 10^9/l)	reached at day
D 29	placebo (equivalent of 30 µg/kg)	0	20	5.9	29
BB 33	placebo (equivalent of 100 µg/kg)	1	21	2.7	25
BB 34	no HGF	3	18	5.3	30
mean			20	4.6	28
M 19	3	0	14	9.0	30
1 TZ	10	2	15	19.2	29
1 YN		2	16	23.4	26
K 25 (hyper-transfusion)		0	14	15.5	30
mean			15	19.3	28
1 WE	30	0	14	42.6	30
1 ZB	100	1	14	22.3	25
1 YA		1	11	26.4	21
mean			13	24.3	23

* Axillary temperature > 40° C and positive blood culture.

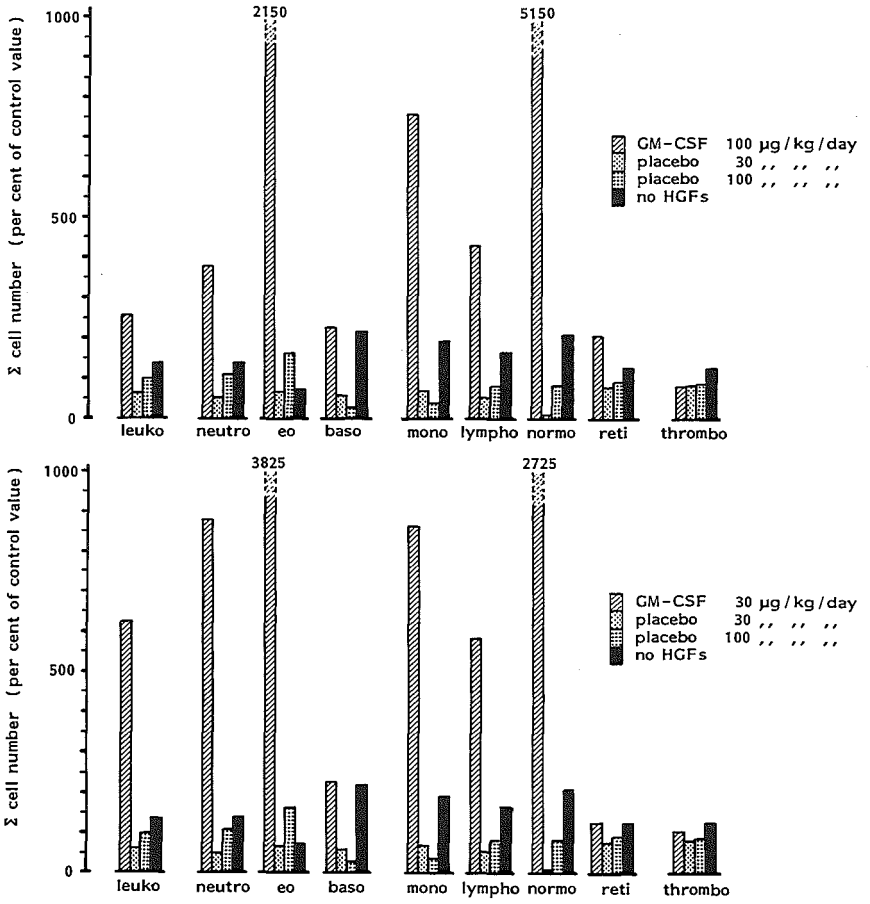

Figure 6.2a

Differential counts of peripheral blood cells during administration of graded doses of GM-CSF. GM-CSF administration was as given in the legend of Figure 6.1. Cell counts cumulated from day 2 to 31.

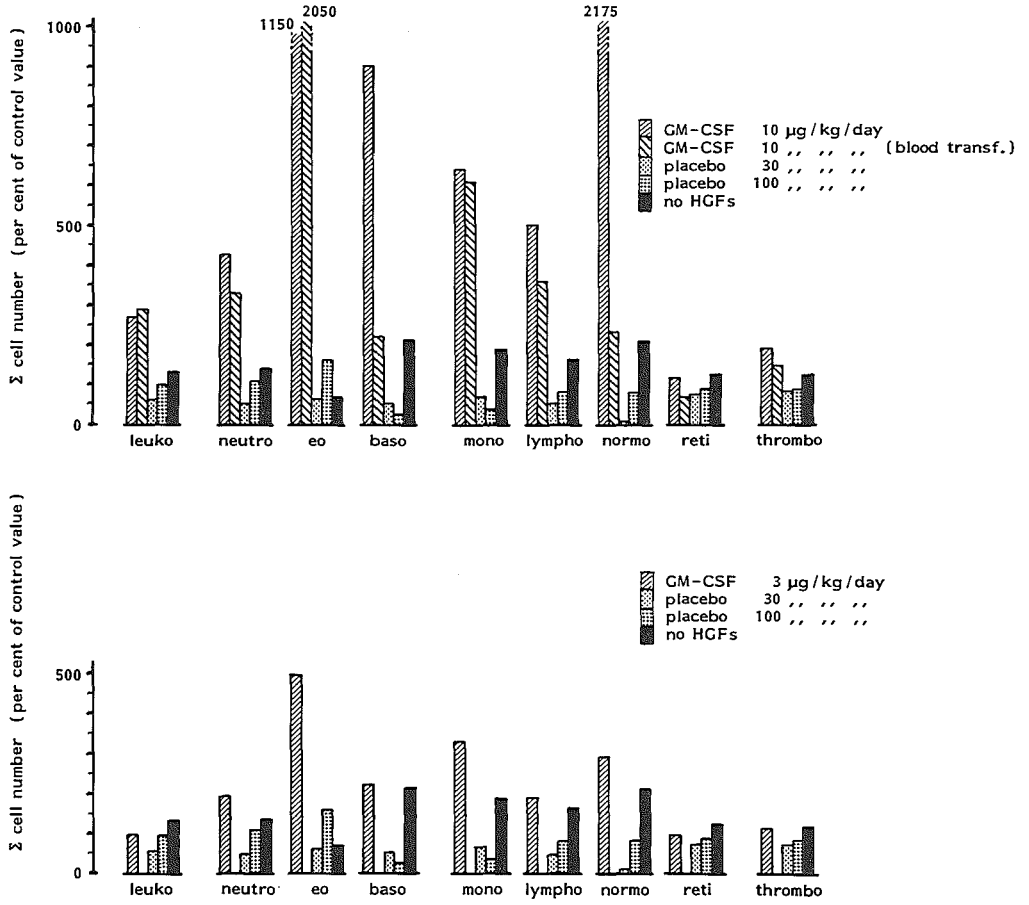

Figure 6.2b **Differential counts of peripheral blood cells during administration of graded doses of GM-CSF.** GM-CSF administration was as given in the legend of Figure 6.1. Cell counts cumulated from day 2 to 31.

total response of the treatment with GM-CSF, cumulated cell counts were used to quantify the effects of GM-CSF on the production of different blood cell types. Figure 6.2 depicts the relationship between cumulated cell counts and GM-CSF dose administered for leukocytes, neutrophils, eosinophils, basophils, monocytes, lymphocytes, normoblasts, reticulocytes and thrombocytes, thus giving a fair account of the total GM-CSF stimulated output of blood cells and the differential dose-effect relationships for each of these cell types. Table 6.2 and Figure 6.2 demonstrates that the effect of the GM-CSF treatment is clearly apparent in the whole dose range. The dose effect relationship for the leukocytes, as deduced from the maximum cell counts (Table 6.2), was confirmed by analysis of the cumulated cell counts (Figure 6.2). Increasing the dose up to 30 μg/kg/day resulted in an increased effect of GM-CSF on the counts of all types of leukocytes. Increasing the dose to 100 μg/kg/day did not exert a greater effect, but tended to lower leukocyte values of all types. This effect was not apparent for normoblasts and reticulocytes which exhibited the highest values upon stimulation with the highest dose (100 μg/kg/day) of GM-CSF administered. In contrast, thrombocyte peak values were highest at the 10 μg/kg/day dose in one of the monkeys and did not significantly exceed the control values at the dose of 30 and 100 μg/kg/day. In Figure 6.2 it is shown, that the effect of GM-CSF on the production of blood cells is predominantly an effect on granulocytes, eosinophils, normoblasts and monocytes. The effects on other cell types are significant, but much less pronounced. The effect of GM-CSF on peripheral blood normoblasts and reticulocytes was largely or completely abolished when the transfusion of thrombocyte concentrates was replaced by whole blood transfusions, as shown in Figure 6.3.

Compared to the large effects of GM-CSF on maximum cell counts (for instance about 7-fold more granulocytes and monocytes, and about 150-fold more normoblasts, at the maximum counts achieved), the effect on the duration of the pancytopenic phase (Table 6.2) was limited: an average leukopenic phase of 19.7 ± 1.2 (s.d.) days was reduced to 14.0 ± 1.4 days.

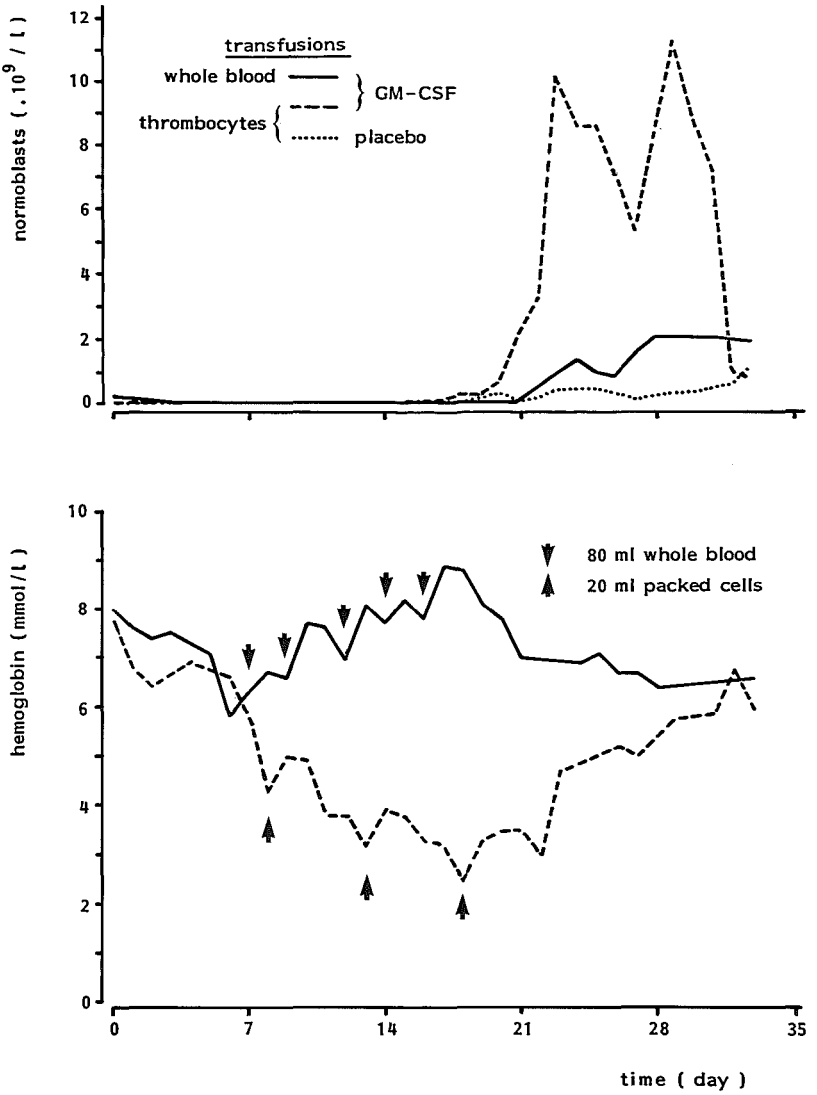

Figure 6.3 **Suppression of GM-CSF induced normoblastosis by prevention of anemia.** GM-CSF administration was as given in the legend of Figure 6.1. The placebo treated monkeys received thrombocyte transfusions.

GM-CSF. The number of animals might be not sufficiently large to detect a reduction of the occurrence of sepsis. This subject will be discussed in more detail in section 7.4

6.3. Infectious complications during treatment with GM-CSF.

Leukopenia is associated with an increased risk of serious infectious complications. Therefore, prevention of these complications is one of the major objectives of treatment with GM-CSF. In the present experimental setting, sepsis caused by multiresistent streptococci is the most common infectious complication. The frequency of sepsis during leukopenia and regeneration is indicated in table 6.2. In spite of a shortening of the pancytopenia, the frequency of sepsis was not reduced in the monkeys treated with GM-CSF. The number of animals might be not sufficiently large to detect a reduction of the occurrence of sepsis. This subject will be discussed in more detail in section 7.4.

6.4. Adverse effects of GM-CSF.

GM-CSF was well tolerated in the dose range tested. As shown in Figure 6.4, body weight, serum protein, liver enzymes, renal function and axillary body temperature in the animals treated with GM-CSF were not different from control animals. Four animals died from unrelated causes. One animal died on day 31 after autologous BM transplantation from a cytomegalovirus infection. Another monkey died on day 60 from Lamblia enteritis, which is endemic in the Rhesus monkey colony. Two animals died on day 24 respectively on day 29 from sepsis, caused by multiresistent streptococci, an endemic problem in the Rhesus monkey colony. The other animals are alive and well after an observation period of more than a year.

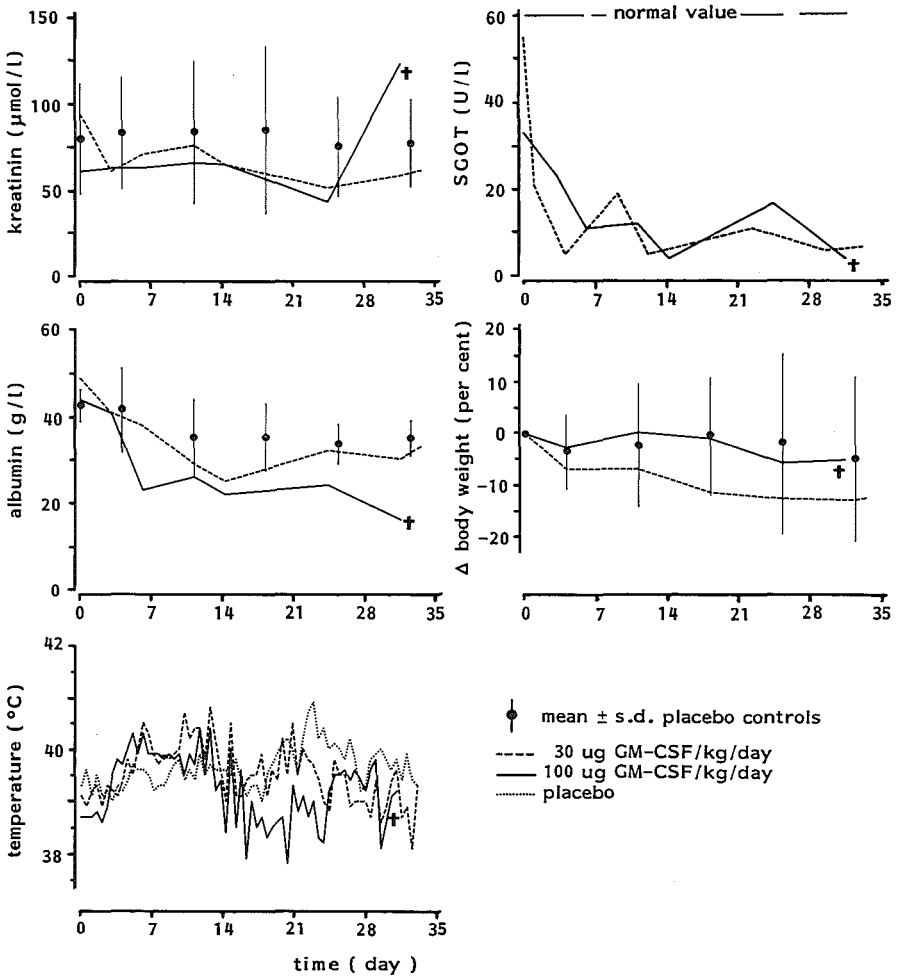

Figure 6.4 **Evaluation of toxic side effects of GM-CSF in Rhesus Monkeys.** GM-CSF administration was as given in the legend of Figure 6.1.

6.5. Discussion.

GM-CSF administration after autologous BM transplantation resulted in a dose dependent rise in peripheral blood cell counts. This was most

pronounced during the fourth week of administration.

Adverse effects of GM-CSF were not observed. The most commonly described adverse effects of GM-CSF include fluid retention, rash, myalgia, arthralgia, bone pain and fever[4,5,6,7] Some of these effects, such as myalgia can not be appropriately evaluated in monkeys. Furthermore, the experiments were designed as an efficacy study rather than a toxicity study. The toxicity of the conditioning TBI and the subsequent pancytopenia, might have masked the relatively mild toxic effects of GM-CSF.

As expected on the basis of the *in vitro* stimulating properties of GM-CSF[8,9,10], treatment with GM-CSF mainly influenced the numbers of granulocytes, eosinophils and monocytes, rather than thrombocytes and reticulocytes. To observe a stimulating effect of GM-CSF on the production of all types of leukocytes, 30 µg/kg/day appeared to be the optimal dose. Increasing the dose to 100 µg/kg/day did not result in a further increase in leukocyte production. However, the maximal effect on the numbers of normoblasts and reticulocytes was observed during treatment with the highest dose of GM-CSF (100 µg/kg/day). This may indicate, that stimulation of the red cell series requires higher doses of GM-CSF than stimulation of the white cell series and thrombocytes. The effect of GM-CSF on peripheral blood normoblasts and reticulocytes was largely, or completely abolished when the transfusion of thrombocyte concentrates was replaced by whole blood transfusions. The latter type of transfusions resulted in a more pronounced prevention of transplantation related anaemia. This demonstrates that the GM-CSF stimulated normoblastosis was co-dependent on erythropoietin, indicating increased erythropoiesis. GM-CSF stimulated erythropoiesis appeared to be largely ineffective, in that the huge rise of normoblasts was disproportionate to the quite moderate increase in reticulocyte numbers. The GM-CSF induced normoblastosis has not been described in clinical studies. This can be explained by the higher frequency of red cell transfusions in the patients, compared to the Rhesus monkeys described in this chapter.

Compared to the pronounced effects of GM-CSF on maximum cell counts and on the cumulated cell counts, the effect on the duration of the leukopenic

phase was restricted to a shortening by 5.7 ± 1.5 days, while the duration of the leukopenic period was 19.6 ± 1.2 days in monkeys not treated with GM-CSF. Such a shortening could also be obtained by transplanting 5 to 10 times more HSC[11], indicating, that during the first 2 weeks after transplantation the treatment with GM-CSF resulted in a production increase equivalent to an average of 3 additional cell divisions along the granulocyte pathways. Apparently, the lowest dose of GM-CSF (3 µg/kg/day sc)is already saturating for this effect. Other authors have described a more promising shortening of the pancytopenic phase[2,3]. This may be explained by a 20-fold larger number of transplanted cells than in the present study. Furthermore, as discussed in paragraph 6.1, the experiments described in this chapter were purposely designed with low numbers of concentrated stem cells, which were T-cell depleted. The present observations indicate that, under these conditions, the effect of GM-CSF is limited, and suggests that GM-CSF, without the addition of other HGFs, does not exert much effect on the early phase of exponential growth after BM transplantation. Therefore it was postulated, that early after transplantation the number of GM-CSF sensitive cells is the limiting factor. Later after transplantation, when more GM-CSF sensitive cells are present, the response is much more dramatic, and increase with the dose of GM-CSF, with a maximum at 30 µg/kg. To test this hypothesis prospectively, we made use of the known relationship between dose of TBI and residual stem cell numbers (Chapter IV), and studied the effect of GM-CSF on endogenous regeneration following graded doses of TBI as is described in the next chapter.

REFERENCES

1. Pizzo, P.A. (1984). Granulocytopenia and cancer therapy. Past problems, current solutions, future challenges. Cancer 54:2649-2661.

2. Nienhuis, A.W., Donahue, R.E., Karlsson, S., Clark, S.C., Agricola, B., Antinoff, N., Pierce, J.E., Turner, P., Anderson, W.F., and Nathan, D.G. (1987). Recombinant human granulocyte-macrophage colony-stimulating factor shortens the period of neutropenia after autologous bone marrow transplantation. J. Clin. Invest. 80:573-577.

3. Monroy, R.L., Skelly, R.R., MacVittie, Davis, T.A., Sauber, J.J., Clark, S.C., and Donahue, R.E. (1987). The effect of recombinant GM-CSF on the recovery of monkeys transplanted with autologous bone marrow. Blood 70:1696-1699.

4. Brandt, S.J., Peters, W.P, Atwater, S.K., Kurtzberg, J., Borowitz, M.J., Jones, R.B., Shpall, E.J., Bast, R.C., Gilbert, C.J., and Oette, D.H. (1988) Effects of recombinant human granulocyte-macrophage colony-stimulating factor on hemopoietic reconstitution after high dose chemotherapy and autologous bone marrow transplantation. N. Engl. J. Med 318:869-876.

5. Nemutis, J., Singer, J.W., Buckner,C.D., Hil, R., Storb, R., Thomas, E.D., and Appelbaum, F.R. (1988) Use of recombinant Granulocyte-Macrophage Colony-Stimulating Factor in autologous marrow transplantation for lymphoid malignancies. Blood 72:834-836.

6. Antin, J.H., Smith, B.R., and Rosenthal, D.S. (1987) Phase I/II study of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) in bone marrow failure. Blood 70:Suppl 1:129a.

7. Antman, K.S., Griffin, J.D., Elias, A., Socinski, M.A., Ryan, L., Cannistra, S.A., Oette, D., Whitley, M., Frei, E. III, and Schnipper, L.E. (1988) Effect of recombinant human granulocyte-macrophage colony-stimulating factor on chemotherapy-induced myelosuppression. N. Engl. J. Med. 319:593-598.

8. Nicola, N.A., Metcalf, D., Johnson, G.R., Burgess, A.W. (1979) Separation of functionally distinct human granulocyte-macrophage colony-stimulating factors. Blood 54:614-627.

9. Sieff, C.A., Emerson, S.G., Donahue, R.E., Nathan, D.G., Wang, E.A., Wong, G.G., and Clark, S.C. (1985) Human recombinant granulocyte-macrophage colony-stimulating factor: a multilineage hemopoietin. Science 230:1171-1173.

10. Metcalf, D. (1986) The molecular biology and function of the granulocyte-macrophage stimulating factors. Blood 67:257-267.

11. Gerritsen, W.R., Wagemaker, G., Jonker, M., Kenter, M., Wielenga, J.J., Hale, G., Waldmann, H., and Bekkum, D.W. van. (1988). The repopulating capacity of bone marrow grafts following pretreatment with monoclonal antibodies against T lymphocytes in rhesus monkeys. Transplantation 45:301-307.

1. Pizzo, P.A. (1984). Granulocytopenia and cancer therapy. Past problems, current solutions, future challenges. Cancer 54:2649-2661.

2. Nienhuis, A.W., Donahue, R.E., Karlsson, S., Clark, S.C., Agricola, B., Antinoff, N., Pierce, J.E., Turner, P., Anderson, W.F., and Nathan, D.G. (1987). Recombinant human granulocyte-macrophage colony-stimulating factor shortens the period of neutropenia after autologous bone marrow transplantation. J. Clin. Invest. 80:573-577.

3. Monroy, R.L., Skelly, R.R., MacVittie, Davis, T.A., Sauber, J.J., Clark, S.C., and Donahue, R.E. (1987). The effect of recombinant GM-CSF on the recovery of monkeys transplanted with autologous bone marrow. Blood 70:1696-1699.

4. Brandt, S.J., Peters, W.P, Atwater, S.K., Kurtzberg, J., Borowitz, M.J., Jones, R.B., Shpall, E.J., Bast, R.C., Gilbert, C.J., and Oette, D.H. (1988) Effects of recombinant human granulocyte-macrophage colony-stimulating factor on hemopoietic reconstitution after high dose chemotherapy and autologous bone marrow transplantation. N. Engl. J. Med 318:869-876.

5. Nemutis, J., Singer, J.W., Buckner,C.D., Hil, R., Storb, R., Thomas, E.D., and Appelbaum, F.R. (1988) Use of recombinant Granulocyte-Macrophage Colony-Stimulating Factor in autologous marrow transplantation for lymphoid malignancies. Blood 72:834-836.

6. Antin, J.H., Smith, B.R., and Rosenthal, D.S. (1987) Phase I/II study of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) in bone marrow failure. Blood 70:Suppl 1:129a.

7. Antman, K.S., Griffin, J.D., Elias, A., Socinski, M.A., Ryan, L., Cannistra, S.A., Oette, D., Whitley, M., Frei, E. III, and Schnipper, L.E. (1988) Effect of recombinant human granulocyte-macrophage colony-stimulating factor on chemo-therapy-induced myelosuppression. N. Engl. J. Med. 319:593-598.

8. Nicola, N.A., Metcalf, D., Johnson, G.R., Burgess, A.W. (1979) Separation of functionally distinct human granulocyte-macrophage colony-stimulating factors. Blood 54:614-627.

9. Sieff, C.A., Emerson, S.G., Donahue, R.E., Nathan, D.G., Wang, E.A., Wong, G.G., and Clark, S.C. (1985) Human recombinant granulocyte-macrophage colony-stimulating factor: a multilineage hemopoietin. Science 230:1171-1173.

10. Metcalf, D. (1986) The molecular biology and function of the granulocyte-macrophage stimulating factors. Blood 67:257-267.

11. Gerritsen, W.R., Wagemaker, G., Jonker, M., Kenter, M., Wielenga, J.J., Hale, G., Waldmann, H., and Bekkum, D.W. van. (1988). The repopulating capacity of bone marrow grafts following pretreatment with monoclonal antibodies against T lymphocytes in rhesus monkeys. Transplantation 45:301-307.

CHAPTER VII
MITIGATION OF RADIATION INDUCED PANCYTOPENIA BY TREATMENT WITH GM-CSF

7.1. Introduction

From the results, described in Chapter VI it was concluded that white blood cell production following autologous bone marrow transplantation could be accellerated by treatment with GM-CSF. The effect became apparent near the end of the leukopenic period and lead to a marked leukocytosis which peaked between day 25 and day 30. Increasing the dose of GM-CSF resulted in increased responses up to 30 μg/kg/day, which appeared to be the optimal and saturating dose. In spite of the clear cut effects of GM-CSF, in this experimental setting the duration of the leukopenia was reduced by only 4 to 8 days. In the dose range between 3 and 100 μg/kg, the duration of the leukopenia was not related to the dose of GM-CSF given. This indicates an increased leukocyte production rate, which is equivalent to 3 additional cell divisions during the first 2 weeks of hemopoietic regeneration. Other investigators who have transplanted larger numbers of BM cells have reported a more pronounced reduction of the leukopenia.[1,2] We postulated that early after transplantation the number of GM-CSF sensitive cells (dependent on the number of transplanted stem cells) was the limiting factor. The postulated relationship between the GM-CSF effect and stem cell numbers was studied by measuring the endogenous of peripheral blood cells following graded doses of Total Body Irradiation (TBI). This approach is based on the established relationship between the number of residual stem cells and the dose of TBI (Chapter IV). In addition, this approach would yield direct data on the

maximum irradiation dose, for which GM-CSF would still be effective as a treatment of pancytopenia.

Accidental irradiation is often not a homogeneous exposure, resulting in damage to the hemopoietic system, which is difficult to predict. In case of a relationship between the number of HSC and the response to HGFs, the latter could be used to estimate the fraction of surviving HSC following inhomogeneous TBI and/or in situations of uncertainty on the irradiation dose.

Pancytopenia following TBI can be regarded as a general model for cytotoxic insult to the hemopoietic system. Toxicity to the hemopoietic system is dose limiting for many chemotherapy schedules in cancer patients. Individual variations in pharmacokinetic parameters of cytotoxic drugs influence the effects of chemotherapy and thereby hamper an adequate evaluation of the influence of treatment with GM-CSF or other HGFs on chemotherapy induced pancytopenia. Such factors do not play a role in sensitivity to TBI. Therefore, TBI can be employed to study the conditions under which treatment with GM-CSF reduces pancytopenia following cytoreductive treatment.

7.2. Dose scheduling of GM-CSF following TBI

To determine the most effective dose schedule, monkeys were irradiated with 5 Gy TBI (6 MV or 300 kV X-rays), and treated with 30 μg/kg GM-CSF/day (see section 6.3). The day of TBI was designated day 0; full supportive care was provided from day 1 until leukocyte counts exceeded 10^9/l. Blood cell counts were determined daily. Four different dose schedules were used, as summarized in Table 7.1. The peripheral blood cell counts are shown in Figure 7.1 and 7.2.

Daily treatment with 30 μg GM-CSF/kg, initiated on the first day post TBI, almost completely prevented leukopenia. A later onset of the treatment with GM-CSF was less effective and resulted only in a moderate reduction of leukopenia. Although the delayed treatment with GM-CSF resulted in a significant increase in white blood cell counts (Table 7.2), this increase occurred too late to mitigate the leukopenia (Figure 7.2).

Table 7.1 INFLUENCE OF VARIOUS SCHEDULES OF GM-CSF TREATMENT* ON LEUKOPENIA, INDUCED BY 5 GY TBI

unique animal number	irradiation source	HGF treatment	duration of leukopenia (days)
1 UP	6 MV X-rays	PLACEBO	9
BB 40			11
BB 31			12
BB 47	6 MV X-rays	GM-CSF day 1 to 14	2
1 XK			0
8607	6 MV X-rays	GM-CSF day 1 to 7	1
BB 52			0
1 ZM	6 MV X-rays	GM-CSF day 7 to 21	7
BB 50	300 kV X-rays	PLACEBO	10
1 XS		no HGF	14
1 ZG		no HGF	12
BB 48	300 kV X-rays	GM-CSF day -14 to -1	15

* 30 μg/kg/day sc.

Pretreatment with GM-CSF did not result in a later onset or an earlier ending of the leukopenic period. In the pretreated monkey, there was even a slightly earlier onset of the leukopenia and a slightly delayed regeneration. Although only one monkey was studied, the absence of any beneficial effect contrasts strongly to the post irradiation treatment schedules and an unfavourable effect of pretreatment with GM-CSF can not be excluded.

Treatment with GM-CSF during days 1-14 after TBI resulted in higher WBC than treatment from day 1-7. Figure 7.2 suggests, that this difference was already present during the first week of observation. Comparison of the

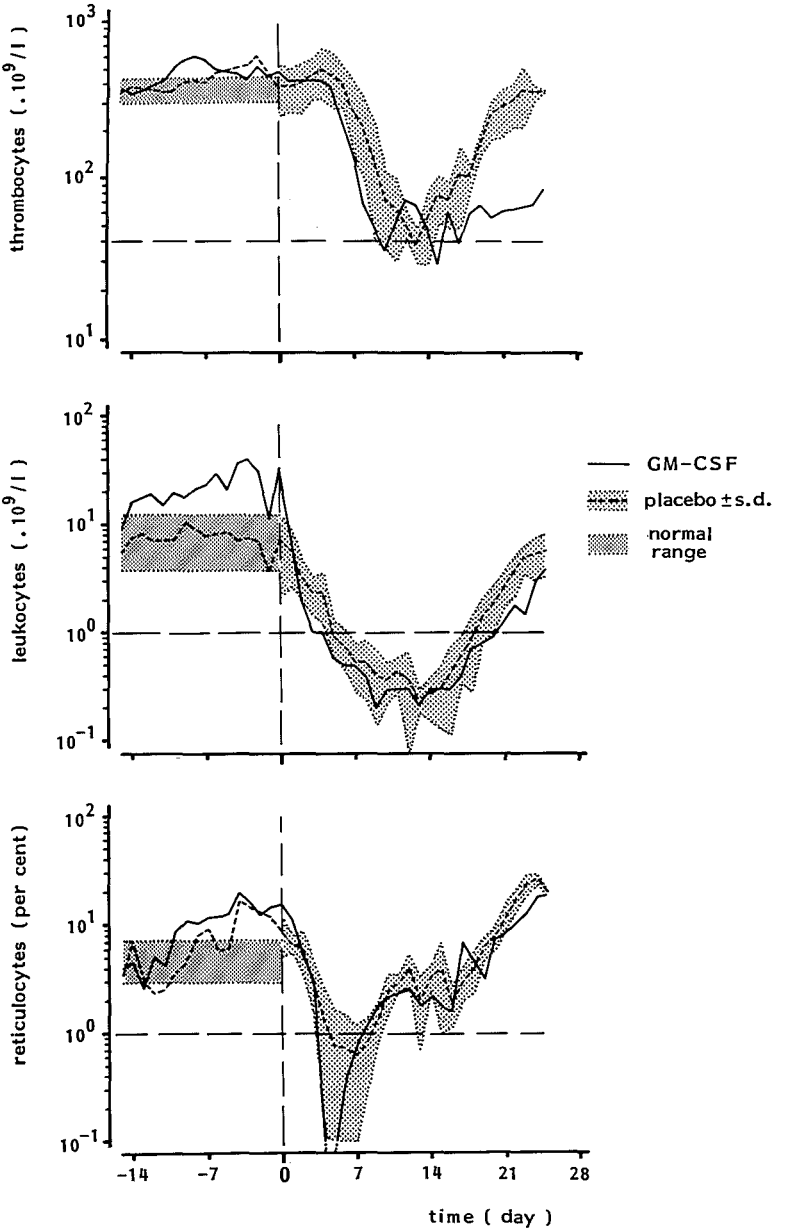

Figure 7.1 **Peripheral blood cell counts following treatment with GM-CSF prior to TBI.** 30 microgram GM-CSF was administered once daily s.c. from day - 14 to day -1. The monkeys were irradiated on day 0 (5 Gy 300 kV K-rays).

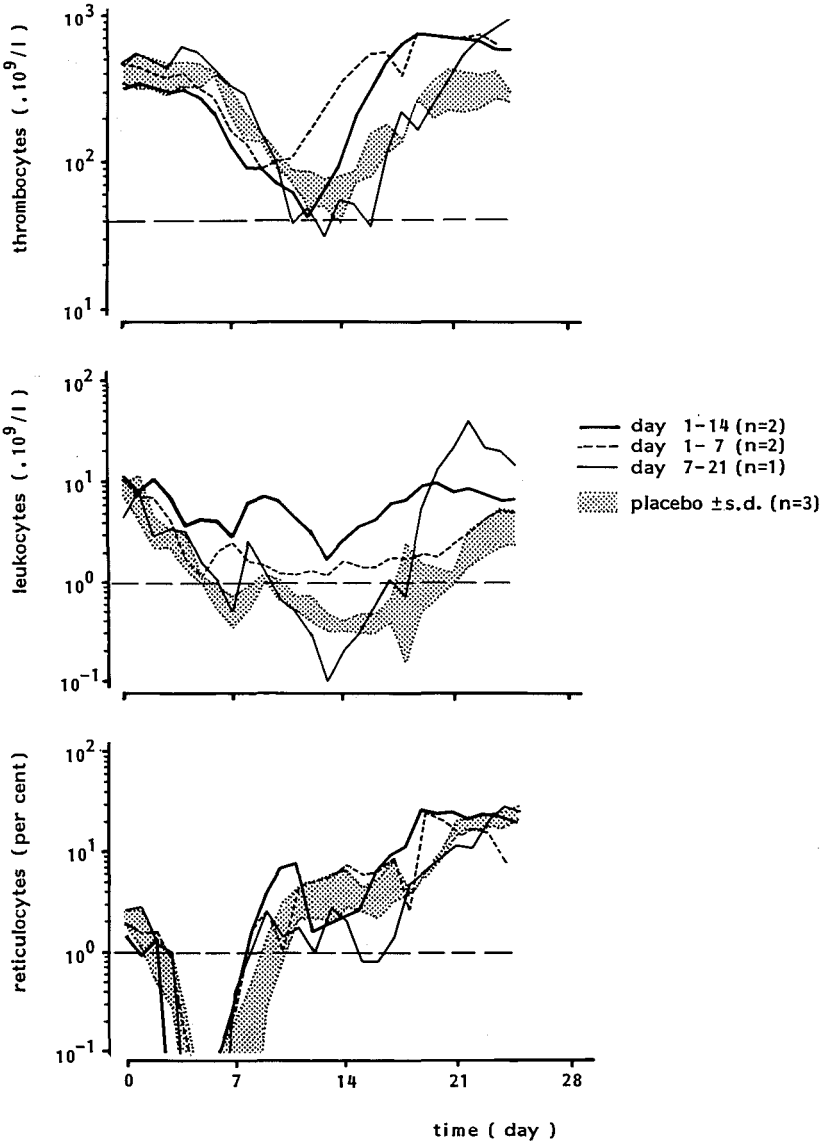

Figure 7.2 **Peripheral blood cell counts under different treatment schedules with GM-CSF after 5 Gy TBI with 6 MV X-rays.** 30 microgram GM-CSF was administered once daily s.c. Treatment schedules as indicated in the figure. Placebo treated and untreated controls not indicated (see Figure 7.5 to 7.7 and 4.5).

Table 7.2

THE EFFECTIVENESS OF DIFFERENT TREATMENT SCHEDULES OF GM–CSF* CUMULATED LEUKOCYTE COUNTS FOLLOWING TBI WITH 5 GY

HGF treatment		CUMULATED LEUKOCYTE COUNTS day 2 - 8	day 2 - 25
placebo	(a)	11.9	34.3
GM-CSF day 1-7	(b)	20.9	63.1
GM-CSF day 1-14	(c)	42.6	146.6
GM-CSF day 7-21	(d)	15.6	159.7
p value (Student T)	b - a	< 0.05	< 0.01
	c - a	< 0.05	< 0.005
	d - a	N.S.	< 0.01
	b - c	N.S.	< 0.02
	d - b	N.S.	N.S.

* 30 μg/kg/day sc

cumulated cell counts of these two treatment groups demonstrates, that this difference is not significant during the first week (day 2 to 8), ($p > 0.1$). However, the GM-CSF treated groups showed significantly higher cumulated cell counts (day 2 to 8) than the control group. For the whole observation period (cell counts cumulated from day 2 to 25) a significantly different effect between treatment for 7 days and treatment for 14 days was found (Table 7.2).

At pharmacological doses, GM-CSF does not only stimulate the production of granulocytes, but it also activates other cell lineages[3]. The cumulated cell counts were used for a quantification of effects of GM-CSF on the production of a number of different blood cell types. In Figure 7.3 these effects are shown separately for neutrophils, eosinophils, basophils, monocytes, lymphocytes, reticulocytes and thrombocytes, thus giving an overall impression of the total GM-

CSF stimulated output of blood cells for each of these cell types. As was expected on the basis of the *in vitro* characteristics of GM-CSF, treatment with GM-CSF predominantly influences the counts of granulocytes, eosinophils and monocytes. The effect on basophil counts is probably due to the representation as a percentage of the average control value, since basophils were found only rarely in the control monkeys. Treatment with GM-CSF resulted also in an increased output of lymphocytes. However, these cells were only characterized on the basis of morphology, which is not always reliable during early regeneration. Also slight effects of treatment with GM-CSF on reticulocytes and thrombocytes were found. Thrombocyte transfusions were only rarely required, and thus hardly influenced the thrombocyte counts.

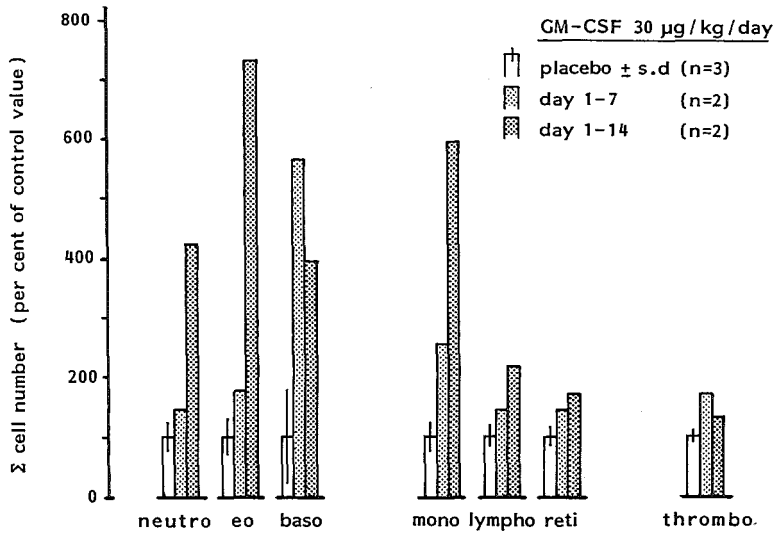

Figure 7.3 **Cumulated differential counts of peripheral blood cells under GM-CSF administration after 5 Gy TBI with 6 MV X-rays.** GM-CSF administration was as given in the legend of Figure 7.2. Cell counts cumulated from day 2 to 25.

The data described above (Table 7.2 and Figures 7.1 and 7.2) demonstrated that treatment with GM-CSF from day 1 - 14 was more effective than the other schedules. Therefore, this schedule was used for the subsequent experiments on the effects of treatment with GM-CSF following graded doses of TBI.

7.3. Relationship between GM-CSF effects and the number of residual stem cells and progenitor cells.

To determine the relationship between the GM-CSF effect and the number of residual stem cells (HSC), Rhesus monkeys were irradiated with graded doses of TBI covering the range from 4 to 8 Gy 6 MV X-rays. For each dose of TBI, two monkeys were used, except for the doses of 4 and 7 Gy for which one monkey was used. The day of TBI was designated day 0, and full supportive care was provided from day 1 until leukocyte counts exceeded 10^9/l. Blood cell counts were determined daily. A daily dose of 30 μg/kg GM-CSF was administered from day 1 to day 14, which appeared to be the most effective dose schedule (see paragraph). The regeneration curves of these monkeys are shown in Figure 7.4.

As pointed out in Chapter IV, the relationship between the residual fraction of HSC and the dose of TBI can be calculated:

$$\ln S = -D/1 \qquad (1)$$

in which S is the surviving fraction, D the dose of TBI in Gy, and 1 the D_0 value for 6 MV X-rays as calculated in Chapter IV.

To express the GM-CSF effects in relation to the irradiation dose, the cumulated cell counts were used. This relationship is shown in Figure 7.5. for leukocytes. The difference between the GM-CSF regression line and the placebo regression line is considered to be the GM-CSF effect. It will be clear from Figure 7.5, that the GM-CSF effect decreases with increasing doses of TBI, which means with decreasing numbers of residual HSC. Following a dose of

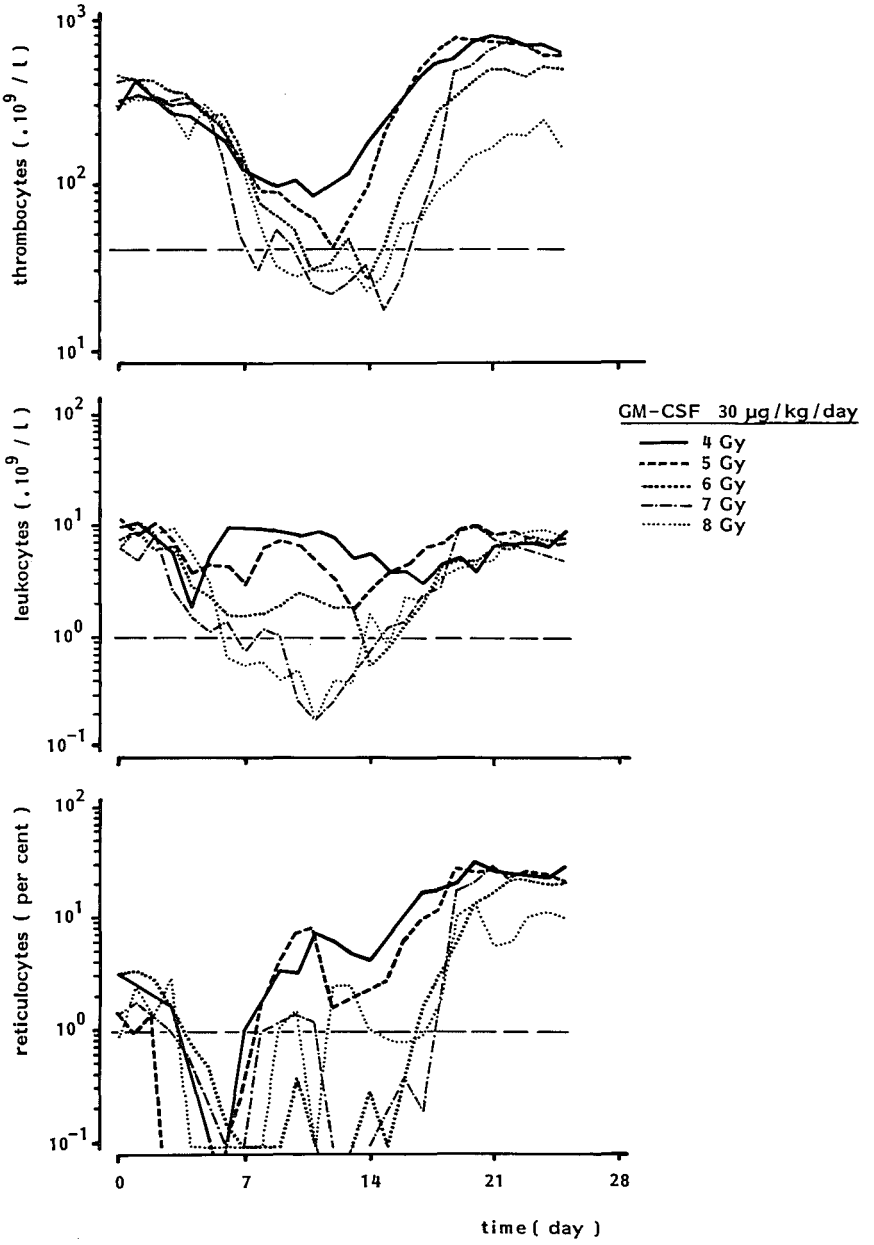

Figure 7.4 **Peripheral blood cell counts after graded doses of TBI with 6 MV X-rays followed by treatment with GM-CSF.** Once daily, 30 microgram GM-CSF was administered s.c. from day 1 to 14.

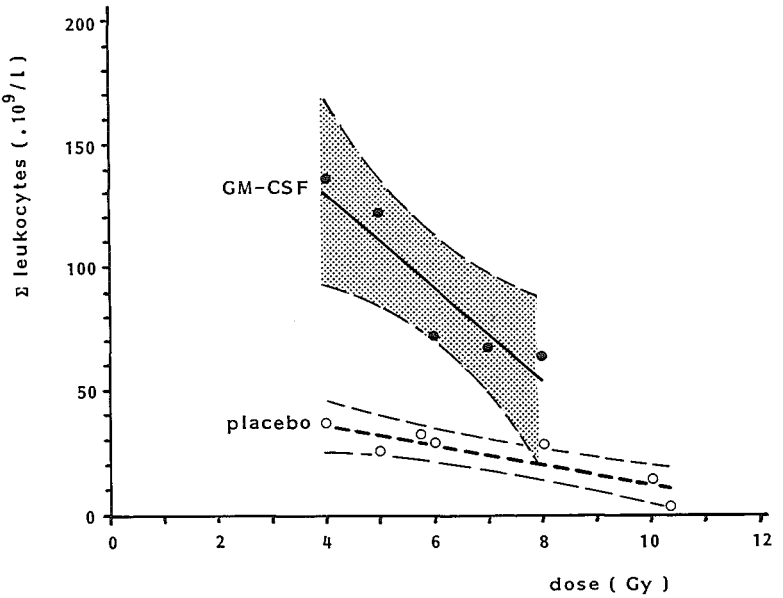

Figure 7.5 **Cumulated leukocyte counts, related to the dose of TBI (6 MV X-rays).** Effect of GM-CSF administration as given in the legend of Figure 7.4. Cell counts cumulated from day 6 to 25.

8 Gy TBI the effect is statistically not significant and extrapolation of the GM-CSF regression line shows, that following a dose of 10 Gy an effect of GM-CSF can not be expected any more. In Figure 7.6, a similar effect is demonstrated for the cumulated thrombocyte counts. As expected, the effect of GM-CSF on thrombocyte production is moderate as compared to the leukocyte response, but the same principle is valid: a low dose of TBI results in a relatively high GM-CSF effect. The effect of GM-CSF on reticulocytes is marginal and only statistically significant for doses of TBI < 6 Gy, as shown in Figure 7.7.

The effectiveness of GM-CSF is dependent on the dose of TBI and can be characterized by subtracting the regression equation for placebo treated animals from that for GM-CSF treated animals. By use of equation (1), this can be linked to the fraction of surviving HSC. This is shown in Table 7.3. The data, presented in this chapter did not demonstrate a significant effect of GM-CSF on leukocytes following TBI at a dose of 8 Gy or more, i.e. following more than 3 log stem

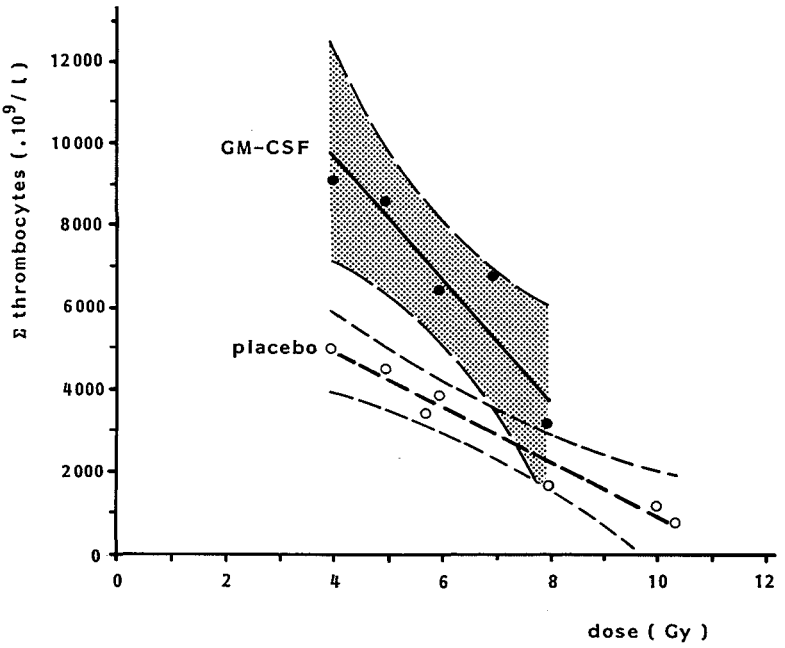

Figure 7.6 **Cumulated thrombocyte counts, related to the dose of TBI (6 MV X-rays).** Effect of GM-CSF administration as given in the legend of Figure 7.4. Cell counts cumulated from day 6 to 25.

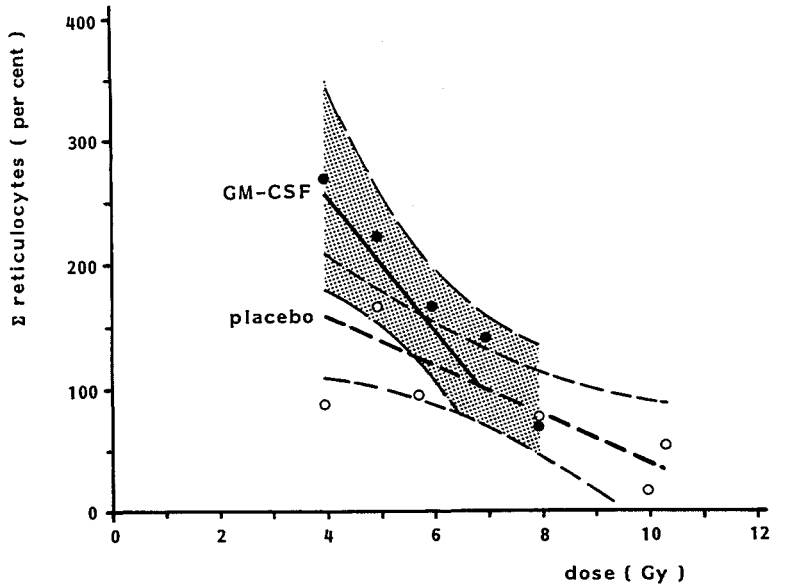

Figure 7.7 **Cumulated reticulocyte counts, related to the dose of TBI (6 MV X-rays).** Effect of GM-CSF administration as given in the legend of Figure 7.4. Cell counts cumulated from day 6 to 25.

cell kill. The data further indicate, that GM-CSF has no effect at all on leukocytes following 10 Gy, on thrombocytes following 9.5 Gy and on reticulocytes following 7 Gy, i.e. at a residual HSC fraction of 5 x 10-5 for leukocytes and higher for the other cell types.

Table 7.3 GM-CSF EFFECTIVENESS RELATED TO THE IRRADIATION DOSE

cell type	significant effect observed		no effect expected at all	
	dose of TBI	corresponding fraction of surviving stem cells	dose of TBI	corresponding fraction of surviving stem cells
leukocytes*	< 8 Gy	> 3 x 10^{-4}	10 Gy	5 x 10^{-5}
thrombocytes	< 7 Gy	> 10^{-3}	9.5 Gy	7 x 10^{-5}
reticulocytes	< 6 Gy	> 3 x 10^{-3}	7 Gy	10^{-3}

* representing neutrophils, eosinophils, monocytes and lymphocytes

7.4. GM-CSF response as an *in vivo* assay for repopulating cells.

In victims of radiation accidents, the rate of hematological regeneration is dependent on the number of residual HSC. Dosimetric problems[4,5,6] and inhomogeneity of the irradiation hamper a reliable estimate of the residual HSC number in such individuals[7]. In the previous section, the relationship between the dose of TBI and the effects of treatment with GM-CSF has been demonstrated and quantified in relation to the number of residual HSC. In principle, this relationship can be used as an *in vivo* HSC assay for victims of radiation accidents (biological dosimetry) and for cancer patients receiving chemotherapy (estimate of BM reserve). Measurement of the cumulated WBC (day 6 - 25) following an unknown dose of TBI and treatment with GM-CSF (day 1 - 14) enables an estimate of the dose of TBI (i.e. the number of residual HSC). We further analyzed the time point after TBI at which the GM-CSF response can be used to assess the radiation damage. For that purpose, the effects of GM-CSF on the cumulated WBC were also analyzed for shorter observation periods (Figure 7.8). During the first five days following TBI, the WBC decrease, and the

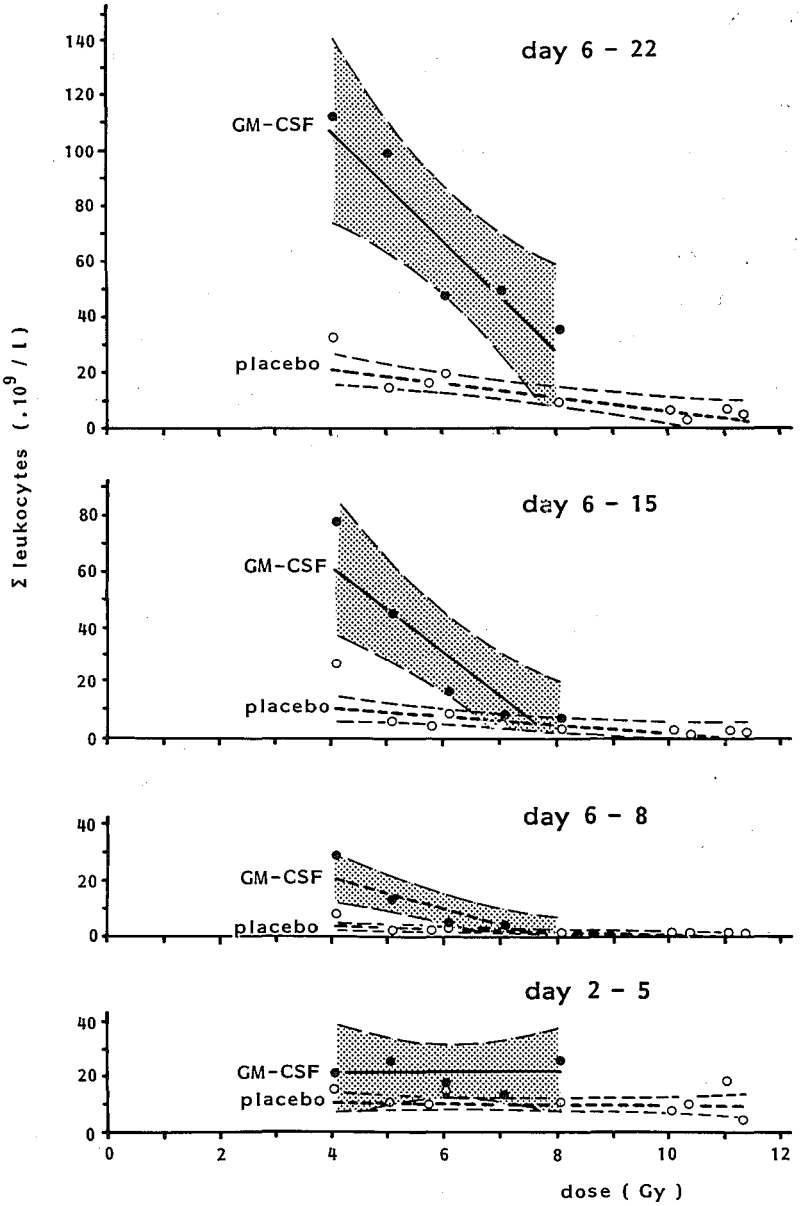

Figure 7.8 **Leukocyte counts cumulated over various time intervals, related to the dose of TBI (6 MV X-rays).** Effects of GM-CSF administration as given in the legend of Figure 7.4. Cell counts cumulated as indicated.

cumulated WBC were not related to the radiation dose. Although treatment with GM-CSF resulted in an increase of the cumulated WBC during this episode, this effect was not related to the dose of TBI. Hence the cumulated WBC during the first five days following TBI can not be used for biological dosimetry. In GM-CSF treated monkeys, the cumulated leukocytes in the period between day 6 and day 8 were correlated weakly to the dose of TBI. This was not the case in the control group. Therefore, the cumulated leukocytes in the period between day six and day eight, allow for only a very rough estimate of the dose of TBI (Table 7.4). Longer observation periods (day 6 - 15 or day 6 - 22) result in a slight increase of the accuracy of the dose estimate. Without GM-CSF, the cumulated cell counts can only be used for biological dosimetry following 3 weeks of observation, and the estimate of the irradiation dose is even less accurate.

Table 7.4 BIOLOGICAL DOSIMETRY BASED UPON THE EFFECT OF GM-CSF ON CUMULATED WBC FOLLOWING TBI

		GM-CSF		PLACEBO	
observation period (days)	dose (Gy)	cumulated wbc (calculated)	dose range (95 % conf lim)	cumulated wbc (calculated)	dose range (95 % conf lim)
6 - 22	4	112	< 5.0	21	< 5.6
	6	68	4.6 - 7.1	16	< 7.2
	8	28	> 6.9	11	> 6.4
6 - 15	4	61	< 5.1	10	
	6	30	4.9 - 7.0	8	
	8	0	> 7.0	5	
6 - 8	4	21	< 5.1	3	
	6	10	4.8 - 7.0	3	
	8	0	>6.8	2	

7.5. Prevention of leukopenia related sepsis.

In section 7.3 a profound effect of treatment with GM-CSF on radiation induced leukopenia was reported. Reduction of the leukopenia is especially important since it is associated with an increased risk of infectious complications,

especially sepsis. Sepsis with gram negative bacteria can be prevented by selective decontamination of the digestive tract, as described in section 2.9. However, the digestive tract of the majority of our Rhesus monkeys is colonized with low numbers of streptococci, which are resistant to the commonly used antibiotics. Selective decontamination of the digestive tract therefore results in a selective growth of these streptococci. Consequently, during leukopenia, the frequency of sepsis caused by these streptococci is high. Therefore, our Rhesus monkeys provide an extremely sensitive model to test the efficacy of prevention of bacterial infections by treatment with HGFs.

The frequency of sepsis in the GM-CSF treated animals and in the placebo treated control animals is summarized in Table 7.5. Sepsis was defined as an axillary temperature > 40° C and a positive blood culture. In spite of a reduction of leukopenia in GM-CSF treated monkeys, the number of septicemic episodes was not reduced. In fact a tendency towards a negative effect was observed. Despite reduction of leukopenia by treatment with GM-CSF, 11 septic episodes were observed in 9 GM-CSF treated monkeys versus 4 septic episodes in 10 control monkeys. If the transplanted monkeys, described in Chapter VI are included, 16 episodes of sepsis were observed in 15 GM-CSF treated animals, versus 8 episodes in 13 control monkeys.

Table 7.5 INFLUENCE OF GM-CSF ON THE FREQUENCY OF SEPSIS* AFTER TBI IN RHESUS MONKEYS

Dose (Gy)	GM-CSF		PLACEBO OR NO HGFs	
	unique animal number	number of septic episodes	unique animal number	number of septic episodes
TBI				
4 (6 MV)	1 YQ	0	8654	1
5	1 XK	0	BB 31	0
	BB 47	3	BB 40	1
	1 ZM	1	1 UP	1
6	8605	2	1 XT	0
	8681	1	8667	1
7	8602	2		
8	1 YE	1	C 28	0
	1 ZQ	1	1 ZF	0
5 (300 kV)			1 XS	0
			1 ZG	0
TBI FOLLOWED BY AUTOLOGOUS BM TRANSPLANTATION				
2 x 6	M 19	0	D 29	0
(6 MV)	1 TZ	2	BB 33	1
(+ ABMT)	1 YN	2	BB 34	3
	K 25	0		
	1 WE	0		
	1 ZB	1		
Total	15	16	13	8

* Axillary temperature > 40° C and positive blood culture.

7.6. Discussion.

Treatment with 30 µg/kg GM-CSF per day from day 1 to day 14 appeared to be highly effective to prevent leukopenia, induced by 5 Gy TBI. Other schedules were less effective.

Treatment with GM-CSF before TBI resulted in a leukocytosis, but, as expected, not in a later onset of the leukopenia. The regeneration time of leukocytes was not earlier in the GM-CSF pretreated monkey than in the control monkeys, indicating, that treatment with GM-CSF did not result in a significant increase in HSC numbers. Thus, the mechanisms by which pretreatment with HGFs could mitigate pancytopenia, visualised in Figure 1.3, could not be demonstrated for GM-CSF. In the pretreated monkey, there was even a slightly delayed regeneration. Although only one monkey was studied, the absence of any beneficial effect contrasts strongly to the other treatment schedules. A negative effect of pretreatment with GM-CSF can not be excluded. An unfavourable effect of GM-CSF might be due to a decrease in HSC numbers before TBI as a result of differentiation induced by GM-CSF. Such an unfavourable effect of GM-CSF has not been reported earlier. Additional experiments, involving a longer duration of the pretreatment might demonstrate this effect.

Treatment with GM-CSF from day 1 to 7 after TBI results in a reduction of the leukopenia which is less pronounced than treatment from day 1 to 14. Although the duration of the leukopenia (defined as leukocyte counts $<10^9/l$) was similar for both schedules, the monkeys treated for 1 week had lower the leukocyte counts than the monkeys treated for 2 weeks.

Treatment from day 7 to 21 results in a leukocytosis at the end of the treatment, but occurred too late to reduce the leukopenia effectively.

As expected, treatment with GM-CSF had the most pronounced effects on granulocytes, eosinophils and monocytes, and minor effects on thrombocytes and reticulocytes. These effects are dependent on the intensity of the cytoreductive treatment. This conclusion was quantified in relation to the dose of TBI, and the effect on leukocytes appeared only significant ($p < 0.05$) at irradiation doses lower than 8 Gy. The doses of TBI following which a slight effect on thrombocytes and reticulocytes was observed were lower. This was linked to the numbers of residual HSC, which could be calculated on the basis of the relationship between the dose of TBI and the surviving fraction of HSC as described in Chapter IV. A significant effect of GM-CSF was only observed if the stem cell kill did not exceed 3 log for leukocytes and less for thrombocytes

and reticulocytes. In Chapter VI it was demonstrated that the reduction of BM transplantation related pancytopenia by treatment with GM-CSF was limited if low numbers of concentrated stem cells were transplanted. Other investigators described more pronounced effects of GM-CSF following transplantation of large numbers of unfractionated BM cells[1,2]

The hypothesis, that the difference between the results presented in Chapter VI and the data reported by the other investigators could be attributed to differences in graft size, was confirmed by the demonstration of a relationship between the effect of treatment with GM-CSF and the number of PHSC present at the start of this treatment. This does not necessarily mean that PHSC are GM-CSF responsive cells, since PHSC have the capacity to differentiate into GM-CSF responsive cells.

The data described in this chapter permit an assessment of the therapeutic window of GM-CSF for mitigation of pancytopenia induced by acute cytotoxic insults to the hemopoietic system. The maximum effect of GM-CSF treatment is observed following an approximately 2 log stem cell kill, and the effect is only significant if the stem cell kill does not exceed 3 log. A response to GM-CSF is completely absent at more than 4 log stem cell kill, although the residual PHSC number is sufficient for endogenous regeneration. In clinical studies in which GM-CSF treatment significantly reduced chemotherapy induced neutropenia, the mean duration of neutropenia without GM-CSF was only 7.4 days, indicating that the chemotherapy did not kill more than 3 log of stem cells[8]. On the other hand, in 20 patients with solid tumors, transplanted with 2.8 to 7.9 x 107 autologous BM cells/kg body weight, the reduction of leukopenia by treatment with GM-CSF was not significant. In those patients a leukocyte count >10^9/l was achieved on day 15.0 ± 6.6 (mean ± sd) in patients treated with GM-CSF and on day 16.7 ± 3.4 in historical controls[9]. Failure of GM-CSF to reduce cytotoxic treatment related pancytopenia of long duration is probably due to a lack of GM-CSF sensitive cells in the early regeneration phase, as was the case in the transplanted monkeys in Chapter VI.

Victims of a recent radiation accident have been treated with GM-CSF[3]. On the basis of the relationship between the number of surviving PHSC and the GM-

CSF effect we may conclude that this approach can only be effective following relatively low doses of accidental irradiation. However, our doses of TBI can not be directly extrapolated to accidental irradiation, since the latter is, in general, inhomogeneous, resulting in an unpredictable surviving HSC fraction.

The response to GM-CSF might also be used to estimate the number of residual HSC following cytotoxic treatment or accidental irradiation. The data presented in this chapter indicate, that GM-CSF can be used for such a purpose in the range between (the equivalent) of 4 - 8 Gy TBI (6 MV X-rays). However, the 95 % confidence limits are wide, and at least 8 days of observation are required to obtain interpretable results. A longer observation period results only in a slight increase of the accuracy. However, the other methods of biological dosimetry are also not very accurate, especially in the high dose range[4,10,11]. Additional experiments including other HGFs, alone and in combination with GM-CSF, shall be performed and may result in a more useful *in vivo* assay for PHSC, i.e. a type of biological dosimetry, that estimates the radiation damage of the hemopoietic system.

The reduction of leukopenia by treatment with HGFs is aimed at a reduction of infectious complications. In spite of the reduction of leukopenia by the GM-CSF treatment there was a tendency of an **increased** frequency of sepsis in those animals as compared to the placebo treated or untreated controls. Although the incidence of sepsis was not significantly increased in this limited number of monkeys, it is unlikely, that a reduction of infectious complications will be found in a larger series. GM-CSF has been reported to stimulate the function of granulocytes in vivo[12,13,14,15,16]. A possible explanation for the increased frequency of infections could be a premature activation of granulocytes by pharmacological doses of GM-CSF, resulting in an exhaustion of granulocyte function. Alternatively, a reduced capacity of granulocytes to migrate to an inflammatory site, during treatment with GM-CSF has been described[17]. This could hamper the defense against bacterial invasion into tissues, and increase the risk of infections.

The data presented lead to the following conclusions:

In Chapter I, three possible mechanisms, by which treatment with HGFs could

mitigate the TBI induced leukopenia were postulated (figure 1.2 and 1.3): Only the first mechanism (stimulation of regeneration) could be confirmed for GM-CSF. The other two mechanisms (pretreatment resulting in higher numbers of peripheral blood cells and a later onset of pancytopenia, and pretreatment resulting in an increase in numbers of PHSC and thereby in an earlier regeneration) could not be demonstrated for GM-CSF.

Treatment with GM-CSF prevents the leukopenia induced by 5 Gy (6 MV X-rays). This is mainly an effect on granulocytes, eosinophils and monocytes.

The effectiveness of GM-CSF to mitigate leukopenia is dependent on the number of PHSC during the early regeneration phase. The maximum effects can be expected following 2 log stem cell kill, the effects were not significant following 3 log stem cell kill, and can be expected to be absent following 4 log stem cell kill.

Surprisingly, mitigation of the leukopenia by treatment with GM-CSF did not result in a decreased incidence of infectious complications. This finding suggests additional studies concerning the influence of GM-CSF on the function of granulocytes *in vivo*.

The quantitative and qualitative characterization of the effects of GM-CSF, as discussed above, establishing the therapeutic window of this HGF, should be similarly done for all other HGFs. Furthermore, these data are a basis for studies on synergistic effects of hemopoietic growth factors *in vivo*.

REFERENCES

1. Nienhuis, A.W., Donahue, R.E., Karlsson, S., Clark, S.C., Agricola, B., Antinoff, N., Pierce, J.E., Turner, P., Anderson, W.F., and Nathan, D.G. (1987) Recombinant human granulocyte-macrophage colony-stimulating factor shortens the period of neutropenia after autologous bone marrow transplantation. J. Clin. Invest. 80:573-577.

2. Monroy, R.L., Skelly, R.R., MacVittie, Davis, T.A., Sauber, J.J., Clark, S.C., and Donahue, R.E. (1987) The effect of recombinant GM-CSF on the recovery of monkeys transplanted with autologous bone marrow. Blood 70:1696-1699.

3. Sieff, C.A., Emerson, S.G., Donahue, R.E., Nathan, D.G., Wang, E.A., Wong, G.G., and Clark, S.C. (1985) Human recombinant granulocyte-macrophage colony-stimulating factor: a multilineage hemopoietin. Science 230:1171-1173.

4. Delafield, H.J. (1988). Nuclear accident dosimetry - an overview. Rad. Prot. Dosimetry 23:143-149.

5. Butturini, A., Gale, R.P., Lopes, D.M., Cunha, C.B., Ho, W.G., Sanpai, J.M., Souza, P.C. de, Cordiero, J.M., Neto, C., Souza, C.E.P. de, Tabak, D.G., Burla, A., and the Navy Hospital Radiation Team. (1988) Use of recombinant granulocyte-macrophage colony stimulating factor in the brazil radiation accident. Lancet II:471-475.

6. Langlois, R.C., Bigbee, W.L., Kyoizumi, Nakamura, N., Bean, M.A., Jensen, R.H. (1987). Evidence for increased somatic cell mutations at the glycophorin A locus in atomic bomb survivors. Science 236:445-448.

7. Jones, T.D., Auxier, J.A., Cheka, J.S., and Kerr, G.D. (1975). In vivo dose estimates for A-bomb survivors shielded by typical japanese houses. Health Physics 28:367-381.

8. Antman, K.S., Griffin, J.D., Elias, A., Socinski, M.A., Ryan, L., Cannistra, S.A., Oette, D., Whitley, M., Frei, E. III, and Schnipper, L.E. (1988) Effect of recombinant human granulocyte-macrophage colony-stimulating factor on chemotherapy-induced myelosuppression. N. Engl. J. Med. 319:593-598.

9. Brandt, S.J., Peters, W.P, Atwater, S.K., Kurtzberg, J., Borowitz, M.J., Jones, R.B., Shpall, E.J., Bast, R.C., Gilbert, C.J., and Oette, D.H. (1988) Effects of recombinant human granulocyte-macrophage colony-stimulating factor on hemopoietic reconstitution after high dose chemotherapy and autologous bone marrow transplantation. N. Engl. J. Med 318:869-876.

10. International Atomic Energy Agency. (1986). Biological Dosimetry: Chromosomal aberrations for dose Assessment, Technical report series No 260 IAEA, Vienna.

11. Fliedner, T.M., Nothdurft and Steinbach, K.H. (1988). Blood cell changes after radiation exposure as an indicator for hemopoietic stem cell function. Bone Marrow Transplantation 3:77-84.

12. Fleischmann, J., Golde, D.W., Weisbart, R.H., and Gasson, J.C. (1986) Granulocyte-macrophage colony stimulatory factor enhances phagocytosis of bacteria by human neutrophils. Blood 68:708-711.

13. Gasson, J.C., Weisbart, R.H., Kaufman, S.E., Clark, S.C., Hewick, R.M., Wong, G.G., and Golde, D.W. (1984) Purified human granulocyte-macrophage colony stimulating factor: direct action on neutrophils. Science 226:1339-1342.

14. Weisbart, R.H., Golde, D.W., and Gasson, J.C. (1986). Biosynthetic human GM-CSF modulates the number and affinity of neutrophil f-met-leu-phe receptors. J. Immunol. 137:3584-3587.

15. Weisbart, R.H., Golde, D.W., Clark, S.C., Wong, G.G., and Gasson, J.C. (1985) Human granulocyte macrophage stimulating factor is a neutrophil activator. Nature 314:361-363.

16. Weisbart, R.H., Kwan, L., Golde, D.W., and Gasson, J.C. (1987) Human GM-CSF primes neutrophils for enhanced metabolism in response to the major physiological chemoattractants. Blood 69:18-21.

17. Peters, W.P., Stuart, A., Affronti, M.L., Kim, C.S., and Coleman, R.E. 1988. Neutrophil migration is defective during recombinant human granulocyte-macrophage colony-stimulating factor infusion after autologous bone marrow transplantation in humans. Blood:72:1310-1315.

CHAPTER VIII
GENERAL DISCUSSION

The hemopoietic system is highly sensitive to cytotoxic insults such as those induced by chemotherapeutic drugs or total body irradiation (TBI). These agents result in a dose dependent kill of hemopoietic stem cells (HSC) and hemopoietic progenitor cells (HPC). As a consequence, a lack of peripheral blood cells, termed pancytopenia, may occur. Some of the more serious complications include an increased susceptibility to infections and bleeding. Surviving HSC have the capacity to proliferate and differentiate. This process eventually results in the reconstitution of the hemopoietic system. The duration of the pancytopenia is directly related to the available number of repopulating cells[1].

Blood cell production and, hence, also regeneration of blood cell production is controlled by a number of glycoproteins, termed hemopoietic growth factors (HGFs). Recombinant DNA technology allows for the production and purification of these factors on a large scale, enabling *in vivo* manipulation of the hemopoietic system by administration of these compounds. Shortening of irradiation induced pancytopenia can be achieved by BM transplantation, and, in principle, also by *in vivo* manipulations of the hemopoietic system with HGFs.

In humans, it is impossible to perform lethal experiments under standard conditions. This hampers the study of human hemopoiesis *in vivo*. For nearly 30 years, the Rhesus monkey (Macaca mulatta) has been used as a model for human hemopoiesis and bone marrow transplantation. This model has been used successfully, to test many techniques in BM transplantation before its introduction into clinical practice[2,3,4,5,6,7,8,9].

8.1. Characteristics of repopulating cells.

The current concept of hemopoiesis includes the existence of PHSCs, which have the capacity of both self renewal and differentiation into specific lineages of committed hemopoietic progenitor cells, which eventually differentiate into mature blood cells. Consequently, a PHSC has the capacity to repopulate a lethally irradiated recipient, i.e. to grow out to a complete hemopoietic system[10]. In rodents, a quantitative functional assay, the spleen colony test can be used for HSC characterization[11]. In outbred species, such as rhesus monkeys and humans, the most immature cell detectable is CFU-mix[12]. Although CFU-S have been shown to be a heterogeneous group of cells[13], not necessarily capable of repopulating a lethally irradiated recipient, the relationship between CFU-mix and PHSC is less well established than the relationship between CFU-S and PHSC. Studies on the nature of human PHSCs have been limited by the lack of a suitable assay and restricted by the impossibility of *in vivo* studies. Recently an *in vivo* HSC assay in rhesus monkeys has been developed in our laboratory. This autologous regeneration assay is based on the observation that the peripheral reticulocyte and leukocyte regeneration rates after autologous BM transplantation are directly related to graft size[1]. The relationship between PHSCs and the early repopulating cells, which are measured by this assay has not been proved. In outbred species, it is impossible to study PHSC and early repopulating cells separately. The early repopulating cells have clinical importance, since the complications of pancytopenia, can be largely prevented by early regeneration.

Cell surface markers and light activated cell sorting have been used for characterization and purification of murine stem cells[14,15]. Among the cell surface markers available to isolate immature human hemopoietic cells, the CD34 antigen, identified by a number of monoclonal antibodies (MCAs)[16,17,18,19] is of outstanding interest. It is mainly expressed on immature hemopoietic cells along various differentiation lineages, including hemopoietic progenitor cells such as CFU-blast, CFU-mix, CFU-GM, and BFU-E. Moreover, it is present only on 1-4% of human bone marrow cells. In CHAPTER III the

autologous regeneration assay was used to measure the HSC enrichment that could be obtained by selection of CD34 positive cells. For this purpose the anti-CD34 MCA ICH3[20], was used. Isolation of ICH3 positive BM cells resulted in a 80 - 155 fold enrichment of colony forming units (CFU-C). The enrichment of HSC appeared to be in the same order of magnitude as the enrichment of CFU-C. The early repopulating capacity of CD34 positive cells was confirmed in RhLA identical, sex mismatched, sibling donor recipient combinations. The capacity of at least a subpopulation of these cells to sustain chimerism was demonstrated by using of the Y chromosome as a marker. However, only a minority of the progenitor cells were of donor origin. This mixed chimerism might indicate, that the PHSC are not sufficiently enriched by isolation of ICH3+ cells. Alternatively, immunological factors might result in the disappearance of donor derived cells. The immunological mechanisms and the cytokinetics involved in mixed chimerism are poorly understood. Further investigations, including a more intensive conditioning regimen are required for a more convincing proof of the capacity of CD34 positive cells to sustain long term chimerism. Such a conditioning might eradicate the recipient derived stem cells more effectively and suppress the possibly involved immunological reaction. This development brings positive selection of stem cells as a generally applicable method to eliminate T-lymphocytes and/or tumour cells from BM grafts, within the reach of clinical application.

Characterization and purification of HSCs is further of importance for investigations on the influence of hemopoietic growth factors on self replication and/or differentiation of HSCs, and necessary for studies on the genetic control of stem cell differentiation, which needs to be elucidated to make gene therapy in hemopoietic stem cells feasible. Finally, specific cell surface markers could be used for the development of an *in vitro* HSC assay. However, the relatively weak fluorescence signal of ICH3 labelled cells, stained with anti IgG 2a/FITC, partially overlaps the aspecific signal of the negative cells. This hampers a quantitative detection of rare events. Furthermore, the CD34 antigen is not exclusively expressed on HSC. Combination of ICH3 with other markers, such as lectins[14] or antibodies directed against other surface antigens might solve both

problems[21]. However, whether the appropriate additional markers for the development of a specific *in vitro* HSC assay will be developed remains questionable in view of numerous unsuccessful attempts to find stem cell specific markers. Also growth factor receptors will probably not add to a solution, since these are not exclusively expressed on HSC.

8.2. Effect of cytotoxic agents on the hemopoietic system.

Radiation induced cell kill can be described by several mathematical models. As discussed in CHAPTER IV, in the higher dose ranges, the dose survival curves of highly radiosensitive cells described by different radiobiological models approach the curve described by the single hit model. Although the hemopoietic cells are more heterogeneous in their responses to cytotoxic drugs than to irradiation, dose-effect curves of many cytotoxic drugs can be described by similar mathematical models[22,23]. This heterogeneity is especially important for phase specific cytotoxic agents such as ARA-C and hydroxyurea. The effect of a cytotoxic drug is influenced by its distribution over the various body compartments, by protein binding, metabolism, and by the clearance of the drug from the body[24]. After a certain time the drug will be effectively cleared from the body and hemopoietic regeneration may occur. This is essentially similar to regeneration following TBI. Therefore, radiation induced pancytopenia can be used as a simple, general model to study the prevention and treatment of these types of pancytopenia. Reliable information on the radiosensitivity of HSC is the starting-point for such studies.

The autologous regeneration assay was used to measure the radiation sensitivity of Rhesus monkey HSC. In these experiments, described in CHAPTER IV, the D_0 value of HSC appeared to be 1.33 Gy for 6 MV and 1,16 for 300 kV X-rays. The method used does not allow for a highly accurate estimate of the D_0 value, which is reflected in wide confidence limits. Inclusion of an estimate of the total number of BM cells results in a somewhat lower D_0 value, but this value is significantly higher than the value that was assumed hitherto (0.6 Gy for 300 kV

X-rays). The measurements of the radiation sensitivity of hemopoietic stem cells have implications for the treatment of victims of radiation accidents. BM transplantation following doses of TBI larger than 10 Gy is useless, since such high doses frequently result in death due to irreversible gastrointestinal damage. Endogenous regeneration following 9.8 Gy TBI (300 kV) has been observed on day 30. On the basis of these data it was calculated, that the stem cell equivalent surviving 9.8 Gy TBI is in general sufficient for a complete hemopoietic reconstitution within 6 weeks. Consequently, the aim of BM transplantation in the treatment of victims of irradiation accidents is shortening of the pancytopenic phase rather than replacement of destroyed BM.

8.3. *In vivo* manipulation of the hemopoietic system with hemopoietic growth factors (HGFs).

In vivo manipulations of the hemopoietic system with HGFs could result in a shortening of the pancytopenic period, induced by cytotoxic damage to the hemopoietic system. This principle has been investigated for Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) in the experiments described in CHAPTER V, VI and VII. Three mechanisms were postulated: Firstly, the stimulation of hemopoietic regeneration by treatment with HGFs following the cytotoxic treatment. Secondly, treatment with HGFs before the cytotoxic treatment could increase the number of mature blood cells, resulting in a later onset of the pancytopenia. Finally, treatment with HGFs before the cytotoxic treatment could increase the number HSC, resulting in the survival of a larger number of HSC and, consequently, in an earlier regeneration. The studies performed demonstrate that the latter two mechanisms do not apply to GM-CSF. The increased numbers of peripheral blood cells, induced by the treatment with GM-CSF prior to TBI, subsequently rapidly decrease, due to a short survival time of these mature cells. Also, pretreatment with GM-CSF does not result in an earlier

regeneration following TBI, indicating, that GM-CSF does not induce a significant increase of HSC numbers. Under certain conditions, the first mechanism, stimulation of hemopoietic regeneration, appeared to result in a significant reduction of leukopenia. It was possible to define these conditions: Following high doses of TBI the number of HSC available for repopulation appeared to be a limiting factor for the effectiveness of treatment with GM-CSF. If only small numbers of HSC are available, treatment with GM-CSF is apparently not additive to the endogenous production of HGFs. Therefore a significant effect on leukocyte counts was only observed if the HSC kill did not exceed 3 log.

As expected, GM-CSF mainly influences the numbers of granulocytes, eosinophils and monocytes.

Surprisingly, mitigation of the leukopenia by treatment with GM-CSF did not result in a decreased incidence of infectious complications. This finding urges additional studies concerning the influence of GM-CSF on the function of granulocytes *in vivo*.

The quantitative and qualitative characterization of the effects of GM-CSF, as discussed above, established the therapeutic window of this HGF. A similar analysis is currently in progress for other HGFs, as well as for synergistic effects of different HGFs on hemopoietic regeneration.

REFERENCES

1. Gerritsen, W.R., Wagemaker, G., Jonker, M., Kenter, M., Wielenga, J.J., Hale, G., Waldmann, H., and Bekkum, D.W. van. (1988). The repopulating capacity of bone marrow grafts following pretreatment with monoclonal antibodies against T lymphocytes in rhesus monkeys. Transplantation 45:301-307.

2. Schaeffer, U.W., Dicke, K.A., And Bekkum, D.W. van. (1972) Recovery of hemopoiesis in lethally irradiated monkeys by frozen allogeneic bone marrow grafts. Rev. Europ. Etudes Clin. et Biol. 17:483-488.

3. Bekkum, D.W., (1984) Conditioning regimens for marrow grafting. Seminars in hematology 21:81-90.

4. Muller-Bérat, C.N., Putten, L.M., and Bekkum, D.W. van (1966) Ann. N.Y. Acad. Sci. 129:340.

5. Bekkum, D.W. van, Balner, H., Dicke, K.A., Berg, F.C van den, Prinsen, G.H., and Hollander, C.F. (1972) The effect of pretreatment of allogeneic bone marrow graft recipients with antilymphocytic serum on the acute graft-versus-host reaction in monkeys. Transplantation 13:400-407.

6. Merrit, C.B., Darrow, C.C., Vaal, L., and Rogentine Jr., G.N. (1972) Bone marrow transplantation in Rhesus monkeys following irradiation. Transplantation 14:9-13.

7. Bekkum, D.W. van, Dicke, K.A., and Balner, H. (1970) Exp. Hematol. 20:126.

8. Wagemaker, G. (1986) Problems and prospects of histoincompatible bone marrow transplantation studied in rhesus monkeys. In: Minimal residual disease in acute leukemia. Proc. Second Int. Symp. on detection and treatment of minimal residual disease in acute leukemia. Eds.: Hagenbeek, A., and Löwenberg, B. Rotterdam, Dordrecht, Martinus Nijhoff, p. 363-373.

9. Gerritsen, W.R., Jonker, M., Wagemaker, G., and Bekkum, D.W. van. (1986). The influence of T-cell depletion by monoclonal antibodies on repopulating hemopoietic stem cells and their efficacy in preventing GvHD in rhesus monkeys. In: Experimental hematology today - 1985. Eds.: Baum, S.J., Pluznik, D.H., and Rozenszajn, New York, Springer, p.128-132.

10. Trentin, J.J., and Fahlberg, W.J. (1963) An experimental model for studies of immunological competence in irradiated mice repopulated with "clones" of spleen cells. In: Conceptual Advances in Immunology and Oncology, 16th Annual Symposium (1962). Texas, Harper & Row, New York, p. 66-74.

11. Till, J.E., and McCulloch, E.A. (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Rad. Res. 14:213-222.

12. Fausser, A.A., and Messner, H.H. (1979) Identification of megakaryocytes, macrophages, and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. Blood 53:1023-1027.

13. Magli, M.C., Iscove, N.N., and Odartchenko, N. (1982) Transient nature of early hemopoietic spleen colonies. Nature 295:527-529.

14. Visser, J.W.M, Bauman, J.G.J, Mulder, A.H., Eliason, J.F., and Leeuw, A.M. de (1984) Isolation of murine pluripotent hemopoietic stem cells. J. Exp. Med. 59:1576-1590.

15. De Vries, P. (1988). Identification of murine haemopoietic stem cells with monoclonal antibodies. Thesis, Erasmus University, Rotterdam.

16. Civin, C.I., Strauss, L.C., Broval, C., Fackler, M.J., Schwartz, J.F., and Shaper, J.H. (1984). Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against Kg-1a cells. J. Immunol. 133:157-165.

17. Andrews, R.G., Singer, J.W., and Bernstein, I.D. (1986). Monoclonal antibody 12-8 recognizes a 115-kd molecule present on both unipotent and multipotent hematopoietic colony-forming cells and their precursors. Blood 67:842-845.

18. Tindle, R.W., Nichols, R.A.B., Chan, L., Campana, Catowski, D., and Birnie, G.D. (1985). A novel monoclonal antibody BI-3C5 recognizes myeloblasts and non-B non-T lymphoblasts in acute leukemias and CGL blast crisis, and reacts with immature cells in normal bone marrow. Leukemia Res. 9:1-9.

19. Watt, S.M., Karhi, K., Gatter, K., Furley, A.J.W., Katz, F.E., Healey, L.E., Atass, L.J., Bradley, N.J., Sutherland, D.R., Levinski, R., and Greaves, M.F. (1987). Distribution and epitope analysis of the cell membrane glycoprotein (HPCA-1) associated with human hemopoietic progenitor cells. Leukemia 1:417-426.

20. Watt, S.M., Karhi, K., Gatter, K., Furley, A.J.W., Katz, F.E., Healey, L.E., Atass, L.J., Bradley, N.J., Sutherland, D.R., Levinski, R., and Greaves, M.F. (1987). Distribution and epitope analysis of the cell membrane glycoprotein (HPCA-1) associated with human hemopoietic progenitor cells. Leukemia 1:417-426.

21. Herweijer, H. (1988). Photodamage cell selection. Development of a high speed cell selection method based on flow cytometry. Thesis. Leiden.

22. Hendry, J.H., and Lord, B.I. (1983) The analysis of the early and late response to cytotoxic insults in the hemopoietic cell hierarchy. In: Cytotoxic Insult to Tissue. Effects on cell lineages. Eds.: Potten, C.S., and Hendry, J.H. Churchill Livingstone. Edinburgh, London, Melbourne and New York. p. 1-67.

23. Martens, A.C.M. (1988). Normal and leukemic stem cells during minimal residual disease. Thesis Leiden.

24. Melmon, K.L., Morelli, H.F. (1978). Clinical Pharmacology. Basic principles in therapeutics. Macmillan Publishing Co., Inc. New York, Toronto, London.

SUMMARY

Peripheral blood cells have a limited life span. Therefore, in humans a daily production of approximately 10^{11} blood cells is required to replace the lost cells. This process, termed hemopoiesis, is maintained from a small population of pluripotential hemopoietic stem cells (PHSC). PHSCs are primitive, archetypal round cells, which have the capacity of both self-renewal and differentiation into specific lineages of committed hemopoietic progenitor cells (HPC), which eventually differentiate into mature blood cells.

Characterization and positive selection of HSC, is essential for the development of a generally applicable method to remove unwanted cells, such as T-lymphocytes or malignant cells, from bone marrow grafts. Furthermore, characterization of HSC could result in the development of a technique for HSC enumeration. Among the cell surface markers available to isolate immature human hemopoietic cells, the CD34 antigen, identified by several monoclonal antibodies (MCAs) is of outstanding interest, since it is mainly expressed on immature hemopoietic cells, including hemopoietic progenitor cells such as CFU-mix, CFU-GM, and BFU-E. Moreover, it is present only on 1-4% of human bone marrow (BM) cells and does not co-express with most differentiation antigens. ICH3, one of the anti CD34 MCAs was used for positive selection of HPCs and HSCs. These experiments have been described in CHAPTER III. Isolation of ICH3 positive BM cells resulted in a 80 - 155 fold enrichment of colony forming units (CFU-C). A recently developed autologous repopulation assay for Rhesus monkey HSC was used to measure the repopulating capacity of autologous CD34 positive cells. The enrichment of repopulating cells appeared to be in the same order of magnitude as the enrichment of CFU-C. The repopulating capacity of CD34 positive cells was confirmed in allogeneic transplantations, and mixed chimerism was found. Further investigations are required to confirm the capacity of CD34+ cells to grow out to a complete hemopoietic system.

BM transplantation, therapeutic or accidental TBI, and chemotherapy are

associated with a lack of peripheral blood cells, termed pancytopenia. The duration of this pancytopenia is dependent on the number of HSC. Radiation induced pancytopenia can be used as a simple, general model to study the prevention and treatment of this type of pancytopenia. Therefore, reliable information on the radiosensitivity of HSC is essential.

The autologous regeneration assay was used to measure the radiation sensitivity of Rhesus monkey HSC. In these experiments, described in CHAPTER IV, the D_0 value of HSC appeared to be 1.33 Gy for 6 MV and 1,16 for 300 kV X-rays. This is higher than the value that was assumed hitherto (0.6 Gy for 300 kV X-rays). The measurements of the radiation sensitivity of hemopoietic stem cells have implications for the treatment of victims of radiation accidents. BM transplantation following doses of TBI larger than 10 Gy is useless, since such high doses frequently result in death due to irreversible gastrointestinal damage. Endogenous regeneration following 9.8 Gy TBI (300 kV X-rays) has been observed on day 30. On the basis of these data it was calculated, that the number of stem cells surviving 9.8 Gy TBI is in general sufficient for a complete hemopoietic reconstitution within 6 weeks. Consequently, the aim of BM transplantation in the treatment of victims of irradiation accidents can only be to shorten the pancytopenic phase rather than replacement of destroyed BM.

Hemopoiesis is tightly regulated by a number of glycoproteins, termed hemopoietic growth factors (HGFs). Recombinant DNA techniques allow for the production and purification of these factors on a large scale, enabling in vivo manipulation of the hemopoietic system by administration of these compounds. Shortening of irradiation induced pancytopenia can be achieved by BM transplantation, and, in principle, also by in vivo manipulations of the hemopoietic system with HGFs. The influence of Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), one of the HGFs, on the duration of the pancytopenic phase following different doses of TBI, electively followed by BM transplantation have been described in CHAPTER V to VII.

In CHAPTER V, a number of pilot experiments on the route of administration of GM-CSF have been described. Although intravenous administration of GM-CSF resulted in higher leukocyte counts than subcutaneous administration, the

latter, also highly effective route of administration was chosen for the subsequent experiments.

In CHAPTER VI, the influence of graded doses of GM-CSF on hemopoietic regeneration after autologous BM transplantation has been described. Treatment with GM-CSF from day 1 to 30 after autologous BM transplantation resulted in a marked leukocytosis, which reached a maximum in the fourth week of administration. Increasing the dose of GM-CSF up to 30 μg/kg/day resulted in an increase of the observed leukocyte counts. Further dose escalation up to 100 μg/kg/day did not result in a further increase in leukocyte counts. In spite of the induction of leukocytosis, the shortening of leukopenic phase was limited to a few days to at maximum a week. The reduction of leukopenia after transplantation of larger numbers of (unfractionated) BM cells has been reported to be more pronounced. Therefore it was thought, that in the early exponential growth phase after BM transplantation, the number of GM-CSF sensitive cells was a limiting factor for the effectiveness of treatment with GM-CSF.

This hypothesis was tested in CHAPTER VII. In this CHAPTER the experiments on the effects of GM-CSF after graded doses of TBI were described. Treatment with GM-CSF from day 1 to 14 after 5 Gy TBI almost completely prevented the radiation induced leukopenia. Increasing the dose of TBI resulted in a significant decrease in the effect of the GM-CSF administration. This observation was linked to the number of stem cells that survive the TBI.

In spite of the observed reduction of leukopenia, the frequency of sepsis in the GM-CSF treated group, was not lower than in the placebo treated control group. There was even a tendency of a higher frequency of infections in the GM-CSF treated group. This phenomenon requires further investigation, since the prevention of infections is the ultimate aim of the mitigation of leukopenia by treatment with HGFs.

SAMENVATTING

Bloedcellen hebben een zeer beperkte levensduur. Daarom is het noodzakelijk dagelijks een groot aantal van deze cellen te produceren. Voor een volwassen mens komt dit neer op de aanmaak van ongeveer 10^{11} cellen per dag. Dit proces, hemopoiese genaamd, vindt plaats in het beenmerg (BM). Hierbij speelt de pluripotente hemopoietische stamcel (PHSC) een belangrijke rol. Kenmerkende eigenschappen van de PHSC zijn "self-renewal", d.w.z. het vermogen zich te delen in twee dochtercellen die beide alle eigenschappen van de stamcel behouden, en het vermogen tot differentiëren in de richting van een van de verschillende soorten bloedcellen.

Onderzoek naar de eigenschappen van HSC en de zuivering van HSC zijn van belang voor de ontwikkeling van een algemeen toepasbare methode voor het verwijderen van ongewenste cellen, zoals T-lymphocyten of kwaadaardige cellen, uit een BM transplantaat. Verder zou meer informatie over stamcellen kunnen leiden tot de ontwikkeling van een techniek om stamcellen quantitatief te kunnen meten.

Van de oppervlakte antigenen die hiervoor gebruikt kunnen worden is het CD34 antigen erg interessant, omdat het voorkomt op hemopoietische voorlopercellen, zoals de CFU-mix, de CFU-GM, en de BFU-E. Bovendien komt dit antigen slechts op 1 - 4 % van alle BM cellen voor en is er geen co-expressie met de meeste differentiatie antigenen. Tegen dit antigen zijn verscheidene monoclonale antilichamen (MCAs) geproduceerd. Eén van de monoclonale antilichamen, gericht tegen CD34, het ICH3, werd gebruikt voor positieve selectie van hemopoietische progenitor cellen (HPCs) en HSCs. Deze experimenten zijn beschreven in HOOFDSTUK III. Isolatie van ICH3 positieve BM cellen leidde tot een 80 tot 155 voudige verrijking van CFU-C. Een recent ontwikkelde, autologe repopulatie test voor Rhesus apen HSC werd gebruikt om te testen of deze CD34 positieve cellen het vermogen hebben een bestraalde ontvanger te repopuleren. De verrijking van repopulerende cellen bleek van dezelfde orde van grootte te

zijn als de CFU-C verrijking. Het repopulerend vermogen van CD34 positieve cellen werd bevestigd in allogene transplantaties, waarna gemengd chimerisme ontstond. Nader onderzoek is nodig om te bevestigen, dat CD34+ cellen het vermogen hebben uit te groeien tot een compleet hemopoietisch systeem.

Therapeutische of accidentele totale lichaamsbestraling (TBI) en chemotherapie, al dan niet gevolgd door BM transplantatie, veroorzaken een tekort aan periphere bloedcellen, pancytopenie genaamd. De duur van deze pancytopenie is afhankelijk van het aantal HSC. Door straling geinduceerde pancytopenie kan gebruikt worden als een algemeen model om bovengenoemde pancytopenieën te bestuderen. Hiervoor is betrouwbare informatie over de stralingsgevoeligheid van HSC van belang. De autologe regeneratietest voor HSC werd gebruikt om de stralingsgevoeligheid van HSC te meten. In deze experimenten, beschreven in HOOFDSTUK IV, bleek de D_0 waarde van HSC 1.33 Gy te zijn voor 6 MV en 1.16 voor 300 kV röntgenstralen. Dit is hoger dan tot dusver werd aangenomen (0.6 Gy). Dit heeft consequenties voor de behandeling van slachtoffers van stralingsongevallen. BM transplantatie na TBI dosis van meer dan 10 Gy is waarschijnlijk zinloos, omdat zulke hoge doses dikwijls dodelijk zijn door onherstelbare schade aan het maagdarmkanaal. Endogene regeneratie na 9.8 Gy TBI (300 kV röntgenstraling) is waargenomen op dag 30. Op grond van deze gegevens werd uitgerekend, dat het aantal stamcellen dat een dosis van 9.8 Gy TBI overleeft in het algemeen voldoende is voor een herstel van de hemopoiese binnen 6 weken. Daarom is het doel van beenmergtransplantatie bij de behandeling van slachtoffers van stralingsongevallen vooral verkorting van de duur van de pancytopenie in plaats van het vervangen van een vernietigd BM.

Hemopoiese wordt gereguleerd door een aantal glycoproteïnes, hemopoietische groeifactoren (HGFs) genaamd. Recombinant DNA technieken maken het mogelijk deze factoren in zuivere vorm te produceren op een schaal die groot genoeg is om er in vivo de hemopoiese mee te stimuleren. De door straling geïnduceerde pancytopenie kan bekort worden door BM transplantatie, en, in beginsel ook door behandeling met HGFs. De invloed van Granulocyte Macrophage colony stimulating factor, GM-CSF, een van die groeifactoren, op

de pancytopenie na verschillende doses TBI en na BM transplantatie wordt besproken in HOOFDSTUK V t/m VII. In HOOFDSTUK V worden een aantal pilot experimenten beschreven betreffende de wijze van toediening van GM-CSF. Hoewel continue intraveneuze toediening van GM-CSF tot hogere leukocyten aantallen in het perifere bloed leidde dan subcutane toediening, werd voor het laatste toedieningsschema gekozen voor de volgende experimenten. Immers, subcutane toediening 1 dd is ook zeer effectief en bovendien praktischer.

In HOOFDSTUK VI is de invloed van verschillende doses GM-CSF op de hemopoietische regeneratie na BM transplantatie beschreven. Behandeling met GM-CSF van dag 1 tot 30 na autologe BM transplantatie leidde tot een forse leukocytose die een maximum bereikte in de vierde week van de behandeling. Hogere doses, tot 30 μg/kg/dag leidden tot hogere aantallen leukocyten. Nog verdere dosisverhoging, tot 100 μg/kg/dag leidde niet tot een toename van het effect. Ondanks het optreden van die leukocytose, bleek de verkorting van de leukopenie slechts enkele dagen tot maximaal een week te zijn. Anderen hadden een sterkere verkorting van de leukopenie na autologe BM transplantatie beschreven; in die studies waren echter grotere transplantaten gebruikt. Daarom werd gepostuleerd, dat tijdens de exponentiële groei kort na een BM transplantatie het aantal GM-CSF gevoelige cellen bepalend is voor het GM-CSF effect.

Deze hypothese werd getoetst in HOOFDSTUK VII. In dit hoofdstuk zijn de experimenten beschreven betreffende de effecten van GM-CSF na verschillende stralingsdoses. Behandeling met GM-CSF van dag 1 tot 14 na 5 Gy TBI bleek de leukopenie vrijwel geheel te kunnen voorkomen. Na hogere stralingsdoses nam het GM-CSF effect sterk in betekenis af. Dit werd in verband gebracht met het aantal stamcellen, dat de bestraling overleeft.

Ondanks het waargenomen gunstige effect op de aantallen leukocyten, was er, vergeleken met de placebo behandelde controle groep, in de met GM-CSF behandelde groep geen lagere frequentie van infectieuze complicaties. Er was zelfs een tendens tot een hogere infectiefrequentie. Dit fenomeen dient nader onderzocht te worden, aangezien juist het voorkomen van infecties het uiteindelijke doel is van de verkorting van de leukopenie door behandeling met HGFs.

CURRICULUM VITAE

Jenne J. Wielenga was born in Rotterdam on January 19, 1953. He completed his secondary education (Gymnasium β) in 1971. After studying chemistry for one year, he enrolled in the Free University Medical School in Amsterdam in 1972. After graduation in 1979 he started his specialization in internal medicine at the Municipal Hospital Leyenburg, The Hague (head: Dr. J.C.M. van de Vijver). His interest in experimental hematology was stimulated during practical work at the Department of Hematology (head: Dr. H.L. Haak) of this hospital in 1981. After registration as a specialist in internal medicine he worked from 1984 to 1986 at the Department of Hematology of the Daniel den Hoed Cancer Center in Rotterdam. From 1986 to 1989, the experiments described in this thesis were performed at the Department of Radiobiology, Erasmus University, Rotterdam, located in the Radiobiological Institute TNO (director: Prof. Dr. D.W. van Bekkum) under the inspiring supervision of Dr. G. Wagemaker (Department of Experimental Hematology and Bone Marrow Transplantation).

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