PARTIAL HEMOPOIETIC CHIMERISM IN THALASSEMIC RECIPIENTS OF NORMAL BONE MARROW STEM CELLS

Selective advantage of normal erythropoiesis

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PARTIAL HEMOPOIETIC CHIMERISM IN THALASSEMIC RECIPIENTS OF NORMAL BONE MARROW STEM CELLS

Selective advantage of normal erythropoiesis

Correctie van thalassemie in muizen door partieel hemopoëtisch chimerisme na transplantatie van normale beenmergstamcellen

Selectief voordeel van de normale erythropoiese

PROEFSCHRIFT
ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. dr P.W.C. Akkermans M. Lit.
en volgens besluit van het college van decanen

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PROMOTIECOMMISSIE

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Ἐπειδὴ δὲ ἡ βοὴ πλείων τε ἐγίγνετο καὶ ἐγγύτερον καὶ οἱ ἀεὶ ἐπιόντες ἔθεον δρόμῳ ἐπὶ τοὺς ἀεὶ βοῶντας καὶ πολλῷ μείζων ἐγίγνετο ἡ βοή, ὅσῳ δὴ πλείους ἐγίγνοντο, ἐδόκει δὴ μεῖζόν τι εἶναι τῷ Ξενοφῶντι, καὶ ἀναβὰς ἐφ' ἵππον καὶ Λύκιον καὶ τοὺς ἱππέας ἀναλαβὼν παρεβοήθει· καὶ τάχα δὴ ἀκούουσι βοώντων τῶν στρατιωτῶν „Θάλαττα, θάλαττα" καὶ παρεγγυώντων.

Toen het geschreeuw luider werd en dichterbij kwam, en zij, die telkens erna kwamen, in looppas renden naar hen die bleven schreeuwen, en het geschreeuw steeds harder werd, naarmate het er meer werden, meende Xenophon dat er iets nogal belangrijks was, en hij sprong op zijn paard, nam Lucius en de ruiters mee, en snelde te hulp. En weldra hoorden zij de soldaten schreeuwen en elkaar toeroepen: "De zee, de zee".

Xenophon: Anabasis IV 7.
Vertaling: klas 4a, Gymnasium Erasmianum Rotterdam, januari 1994

aan mijn ouders

LIST OF ABBREVIATIONS

ALG	anti-lymphocyte globulin
BM	bone marrow
BSA	bovine serum albumin
C'	complement
CAFC	cobblestone area-forming cell
CD	cluster designation
CFU	colony-forming unit
CFU-BL	blast cell colony-forming unit
CFU-C	colony-forming unit in culture
CFU-E	colony-forming unit - erythrocytes
CFU-Eo	colony-forming unit - eosinophils
CFU-GM	colony-forming unit - granulocytes/macrophages
CFU-GEMM	colony-forming unit - granulocytes/erythrocytes/ macrophages/erythrocytes
CFU-Meg	colony-forming unit - megakaryocytes
CFU-Mix	multilineage colony-forming unit
CFU-S	spleen colony-forming unit
CRU	competitive repopulating-unit
CSF	colony-stimulating factor
GM-CSF	granulocytes/macrophages - colony-stimulating factor
M-CSF	macrophages - colony-stimulating factor
multi-CSF	multi - colony-stimulating factor (= IL-3)
DABCO	1,4-diazobicyclo-(2,2,2)-octane
DFO	deferoxamine
EAE	experimental allergic encephalomyelitis
Ep	erythropoietin
ERA	erythroid repopulating ability
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
FLS	Forward Light Scatter
5-FU	5-fluorouracil
GAM	goat anti-mouse
GARA	goat anti-rat

GvH	Graft-versus-Host
GvHD	Graft-versus-Host Disease
Gy	Gray
Hb	hemoglobin
HGF(s)	hemopoietic growth factor(s)
H&H	HEPES buffered Hank's Balanced Salt Solution
HPFH	hereditary persistence of fetal hemoglobin
HPP-CFC	high proliferative potential colony-forming cell
HSC	hemopoietic stem cell
HvG	Host-versus-Graft
IL(s)	interleukin(s)
KL	kit-ligand (= MGF or SCF)
LCR	locus control region
LD	lethal dose
LD/ER-MP20$^-$	low-density ER-MP20 negative
LD/FU_{6d}BM	low-density day-6 post-fluorouracil bone marrow
LFA-1	lymphocyte function associated antigen-1
Lin$^-$	lineage marker (CD4, CD8, Gr-1, Mac-1, TER-119) negative
LTBMC	long-term bone marrow culture
LTC-IC	long-term culture initiating-cell
LTRA	long-term repopulating ability
LTR-HSC	long-term repopulating hemopoietic stem cell
MACS	magnetic cell separator
MCA(s)	monoclonal antibody/(antibodies)
MGF	mast cell growth-factor (= KL or SCF)
MRA	marrow-repopulating ability
MSCM	mouse spleen conditioned medium
NK	Natural Killer
NBM	normal (unseparated) bone marrow
P	parental
PBS	phosphate buffered saline
PBS-SA	phosphate buffered saline containing 0.01% sodium azide
PHSC	pluripotent hemopoietic stem cell
PMUE	pregnant mouse uteri extract
PRA	platelet repopulating ability

RARa	rabbit anti-rat
RBC	red blood cell(s)
Rh-123	rhodamin-123
RPA	radioprotective ability
RPM	rotations per minute
sAV-PE	streptavidin-conjugated phycoerythrin
Sca-1	stem cell antigen-1
SCF	stem cell factor (= KL or MGF)
SCID	severe combined immune deficiency
s.d.	standard deviation
s.e.	standard error
SEM	standard error of the mean
SPF	specific pathogen free
SSC	standard saline citrate
STRA	short-term repopulating ability
TBI	total body irradiation
TGF-β	transforming growth factor-β
TNF	tumor necrosis factor
WBC	white blood cell(s)
WGA	wheat germ agglutinin

CONTENTS

CHAPTER I
GENERAL INTRODUCTION

1.1 HEMOPOIESIS

1.1.1 Hemopoietic stem cells and progenitor cells

Peripheral blood cells have a limited life span and are continuously replaced. Hemopoiesis is the production of new blood cells, which, in mice, under physiological circumstances, occurs in bone marrow (BM), spleen and thymus. Current knowledge of hemopoiesis originates from the early 50's when it was observed that spleen and BM cell suspensions protected lethally irradiated animals from death due to bone marrow failure.[226,227,282] Although this finding initially prompted a search for a humoral factor, it was soon demonstrated that the production of donor derived blood cells protected the animals from radiation inflicted death.[146,354,526]

Hemopoiesis is maintained throughout life by a population of immature cells, termed *hemopoietic stem cells* (HSC), with the capacity to generate progeny along multiple blood cell lineages. The most immature is thought to be a *pluripotent (or omnipotent) hemopoietic stem cell* (PHSC). (P)HSC are assumed to have the capacity for both self-renewal and differentiation into more mature cells: committed *hemopoietic progenitor cells*. Those hemopoietic progenitor cells are a series of intermediate cells, by classical morphological methods difficult to distinguish from PHSC and HSC. *Precursor cells* are a series of recognizable BM cells, from which the mature cells in the peripheral blood are produced (Figure 1.1). Over the years, a large number of assays has been developed for both hemopoietic stem cells and progenitor cells (Table 1.1).

When syngeneic BM cells are injected into lethally irradiated mice, macroscopically visible colonies of actively proliferating hemopoietic cells will develop in the spleens of those mice.[487] Those spleen colonies are the progeny of single cells called *spleen colony-forming cells*, CFC-S.[36,332] *Spleen colony-forming unit*, CFU-S, is a term which was introduced when it was not known that each colony derived from a single cell. Although the relation between CFU-S and CFC-S is characterized by the seeding efficiency *f* (Table 1.1), the term CFU-S has to date mostly been used to describe the cells that can form spleen colonies. CFU-S comprise only a minority of BM cells.[487] Initially, CFU-S have been considered by some investigators to be equivalent to PHSC, especially when it was demonstrated that a complete hemopoietic system could develop out of the progeny of a single spleen colony.[493]

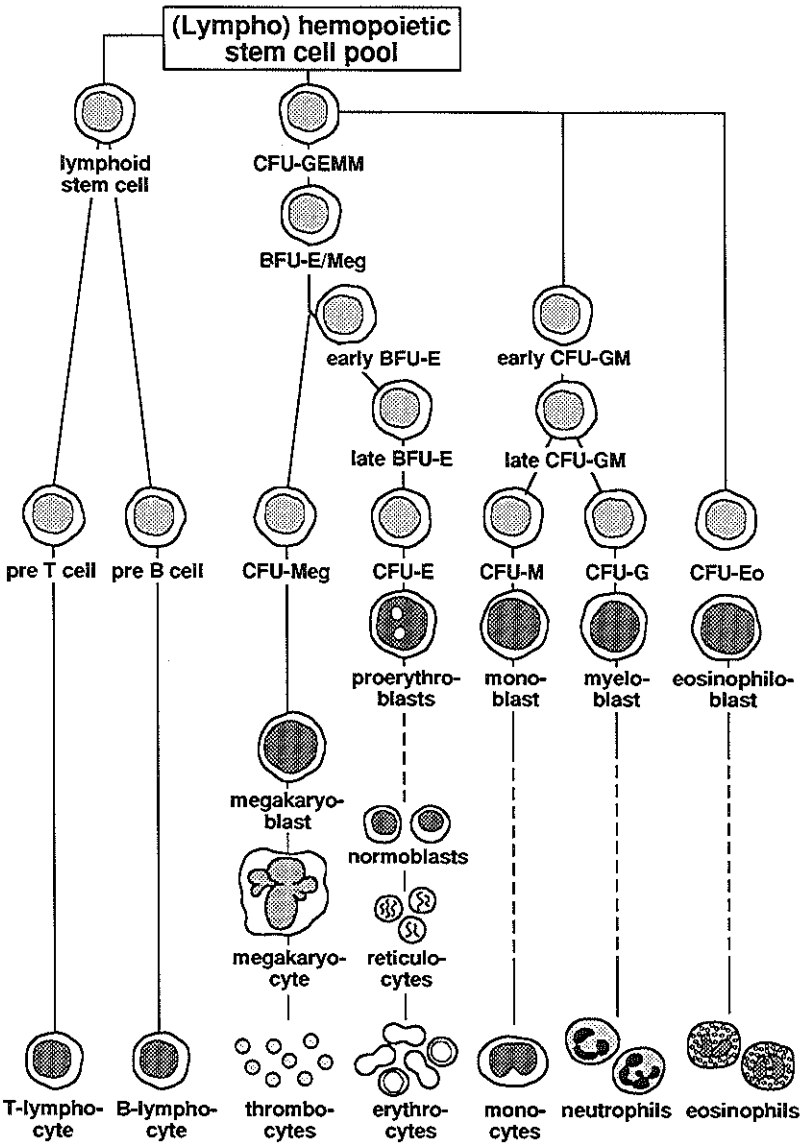


Figure 1.1: Schematic representation of lineage differentiation in the hemopoietic progenitor and precursor cell compartment.

CFU-S, however, were demonstrated to be a cell population with considerable heterogeneity with regard to several different characteristics, including self-renewal capacity and cell cycle status (Table 1.2).[32,48,187,201,300,319,341,377,378,387,570]

The heterogeneity of CFU-S and the failure to unequivocally demonstrate that these cells represented PHSC prompted the search for less lineage-restricted cells, i.e., cell that yield both myeloid and lymphoid progeny.[2,355] Such cells with the capacity to generate HSC and progenitor cells appeared to be less sensitive to the action of the drug 5-fluorouracil (5-FU) than CFU-S.[200,202] These findings led to the development of other assays for (P)HSC (Table 1.1). HSC subsets were (operationally) defined on basis of their ability to repopulate the hemopoietic sites or the peripheral blood of lethally irradiated recipients with HSC, progenitor cells or mature blood cells (repopulating abilities).[48,202,379,380] Radioprotective ability (RPA) defines the ability to rescue irradiated recipients from death due to BM failure in, e.g., the first thirty days after transplantation.[380] Presently, however, the best operational definition of PHSC is based on sustained repopulation of an irradiated recipient, i.e., *in vivo* assays which define cells with *'long-term repopulating ability'* (LTRA-cells). *Competitive repopulating units* (CRU) are LTRA-cells defined on basis of their ability to compete *in vivo* with co-transplanted and residual endogenous stem cells.[476] In this system, 10^5 or $2x10^5$ co-transplanted cells, 'compromised', i.e., depleted for LTRA by two previous rounds of transplantation and regeneration, allow for short-term survival of the recipients.[189,476]

Serial transplantation of BM cells confirmed that long-term repopulation cannot be attributed to CFU-S,[230] and LTRA-cells could be separated from CFU-S by counterflow centrifugal elutriation.[231] (Table 1.3) Using competitive repopulation assays,[189] it was confirmed that LTRA-cells, in contrast to CFU-S, are relatively spared by the action of 5-FU.[192,270] The advance of fluorescence activated cell sorting[197] allowed for characterization of HSC and progenitor cells as cells of an intermediate size and a low degree of structuredness,[33,523] based on, respectively, intermediate forward light scatter (FLS) and low perpendicular light scatter (PLS) properties, and fractionation of HSC subsets on the basis of affinity for lectins or staining with supravital dyes[454,522] (Table 1.3).

Table 1.1: In vivo and in vitro assays for (murine) hemopoietic stem and progenitor cells

Abbreviation	Definition	References (reference number)
LTRA-cell	cell with long-term repopulating ability, perhaps equivalent to PHSC; operational definition depends on what is called repopulation and what is called long-term.	
M/SRA-[cell]	cells that repopulate BM (M) or spleen (S) of lethally irradiated recipients	Hodgson et al., 1982
M/SRA-[CFU-C]	in 13 days with nucleated cells (MRA-[cell]), in vitro clonable	Bertoncello et al., 1985
M/SRA-[CFU-S-12]	progenitors (MRA-[CFU-C]), or spleen colony forming cells (MRA-[CFU-S]).	Ploemacher and Brons, 1988 (379 and 380)
PRA	platelet repopulating ability; defined as platelet content per ml peripheral blood at 13 days after BMT per 10^5 cells transplanted	Bertoncello et al., 1985
ERA	erythroid repopulating ability; defined as reticulocyte content per ml peripheral blood at 13 days after BMT per 10^5 cells transplanted	Bertoncello et al., 1985

Abilities can either be quantified (MRA, PRA, ERA) or compared with that of reference cells, usually normal BM cells (RPA, LTRA)

Table 1.1 continued

Abbreviation	Definition	References (reference number)
RPA	radioprotective ability; the abilty to rescue lethally irradiated mice from death due to BM failure	e.g. Ploemacher and Brons, 1988 (379)
CRU**	competitive repopulating unit; defined as a unit that repopulates recipients with at least 5% BM or thymus cells 5 or 10 weeks after BMT with test cells and 10^5 or 2 x 10^5 cells 'compromised' for LTRA (8-8.5 Gy X-rays irradiated recipients)	Szilvassy et al., 1990
LTC-IC	long-term culture initiating cell; quantified on basis of the number of myeloid, erythroid, and multilineage clonogenic cells present 5 weeks after inoculation on irradiated long-term BM culture stroma	Sutherland et al., 1989
CAFC	cobblestone area-forming cells; qauntified in a miniaturized long-term bone marrow culture system	Ploemacher et al., 1989 and 1991, Ploemacher and Van der Sluijs, 1991

** since the detection efficiency of this assay procedure is also almost certainly <100%, this assay will, analogous to the situation with CFC-S, underestimate the actual number of competitively repopulating cells

Table 1.1 continued

Abbreviation	Definition	References (reference number)
CFU/C-S*	spleen colony forming units/cells; cells that form macroscopically visable colonies in spleens of lethally irradiated recipients	Till and McCulloch 1961
CFU-BL	blast colony assay; enumeration of small colonies of morphologically undifferentiated blast cells, detected after 21 to 28 days of culture	Nakahata and Ogawa, 1982 Koike et al., 1986 Leary and Ogawa, 1987
HPP-CFC	progenitor cells with high proliferative potential, these progenitors form in vitro colonies of at least 5×10^4 cells with appropriate growth factors	Bradley and Hodgson, 1979 Bradley et al., 1980

* CFU = colony forming *unit* ; in the spleen colony assay, the actual number of *cells* capable of forming spleen colonies, i.e., the CFC-S, in a suspension can be calculated on basis of the seeding efficiency *f*. The seeding efficiency *f* indicates the fraction of CFC-S that will eventually produce a spleen colony

Table 1.1 continued

Abbreviation	Definition	References (reference number)
CFU-C	colony forming units in culture; precursor cells that form colonies in semi-solid medium, defined for granulocyte/macrophage precursors (CFU-GM), granulocyte precursors (CFU-G), macrophage precursors (CFU-M), megakaryocyte precursor (CFU-Meg), eosinophil precursors (CFU-Eo), late erythroid precursors (CFU-E), multilineage precursors (CFU-Mix, CFU-GEMM)	Pluznik and Sachs, 1965 and 1966 Bradley and Metcalf, 1966 Stephenson et al., 1971 Tepperman et al., 1974 Metcalf et al., 1974, 1975 (325) and 1979 Nakeff et al., 1975 Fauser and Messner, 1979
BFU-E	burst forming unit - erythroid; an early erythroid progenitor cell	Axelrad, 1974 Iscove and Sieber, 1974 and 1975

Almost all CFU-S and progenitor cells were shown to have a high affinity for the lectin Wheat Germ Agglutinin (WGA). Sorting of cells on basis of light scatter properties as well as WGA-affinity resulted in BM cell fractions highly enriched for CFU-S.[34,379,521] Marrow-repopulating (MRA) cells and LTRA-cells have a lower affinity for WGA than CFU-S.[379,380,382,383] Rhodamine-123 (Rh-123) is a supravital, cationic fluorescent dye that has relatively high affinity for mitochondrial membranes, and is expelled from the cells by the transmembrane protein P-glycoprotein (Pgp).[399]

Table 1.2: Heterogeneity of spleen colony-forming cells

Characteristic	CFU-S-12-14	CFU-S-8	References (reference number)
self-renewal capacity[a]	+++	+[b]	Magli, 1982 Wolf, 1986[b]
progenitor cell content	++	+	Magli, 1982
Qa-m2 expression	71-82% of CFU-S	7% of CFU-S	Harris, 1984
cell cycle status (bromodeoxyuridine incorporation)	low - intermediate	high	Hodgson, 1984
Rh-123 staining	brightly or heterogeneously[c]	brightly	Bertoncello, 1985 Mulder, 1987[c] Ploemacher, 1988 (378) and 1989 (381)
CD45 (T-200) antigen expression	high[d] or low	low	Basch, 1992[d]
radiosensitivity (D_0)	0.94 Gy (X-rays)	0.71 Gy (X-rays)	Meijne, 1991
	0.91 Gy (γ)	0.79 Gy (γ)	Ploemacher, 1992

a) measured as CFU-S in secondary recipients
b) approximately half of the CFU-S-8 persist to day 12, at which time they give rise to approximately the same number of colonies in secondary recipients as late-appearing CFU-S
c) CFU-S-12 staining weakly with Rh-123 showed radioprotective ability
d) high self-renewal capacity is found in CFU-S-12 with high expression of CD45

Table 1.3: Characteristics of LTRA-cells, MRA-cells and CFU-S

Characteristic	LTRA-cell	MRA-cell	CFU-S	References (reference number)
5-FU induced cell kill	very low	low	high	Hodgson, 1979 and 1982 Lerner, 1990
size and density (counterflow elutriation)	smallest cells		intermediate sized cells	Jones, 1990
WGA-affinity	low	low	high	Visser, 1984 Bauman, 1986 Ploemacher, 1988 (397 & 380), 1991 and 1993
Rh-123 uptake		low	high	Bertoncello, 1985 Ploemacher, 1988 (377 &378) and 1989 (381)

Multidrug resistance of tumor cells is often caused by overexpression of Pgp.[399] Immature hemopoietic cells, such as MRA-(CFU-S) cells and cells responsible for the establishment of hemopoiesis in long-term bone marrow cultures, are characterized by relatively low Rh-123 fluorescence.[48,378,381,512]

In 1988, murine PHSC, comprising 0.05% of all BM cells, were selected on the basis of a low expression of Thy-1 (Thy-1^{low}), non-expression of lineage restricted markers (Lin^-), and expression of the stem cell antigen Sca ($Sca\text{-}1^+$).[29,455] However, the selected population, claimed to be almost pure for CFU-S-12,[219,281,455] could be further fractionated for CFU-S, MRA, RPA, and multilineage LTRA on the basis of Rh-123 uptake and *c-kit* receptor expression (Section 1.1.2).[213,277,456,457] Furthermore, clonal *in vivo* analysis revealed that only about 1 in 40 of these cells continued to produce new blood cells for 9 weeks or more.[449,557] The Thy-$1^{low}$$Lin^-$Sca-$1^+$ cell fraction is obviously still a heterogeneous cell population.[220,458] Taken together, these data indicate that, although CFU-S have some characteristics of HSC, probably none, or only a small proportion of these cells are PHSC (Figure 1.2).

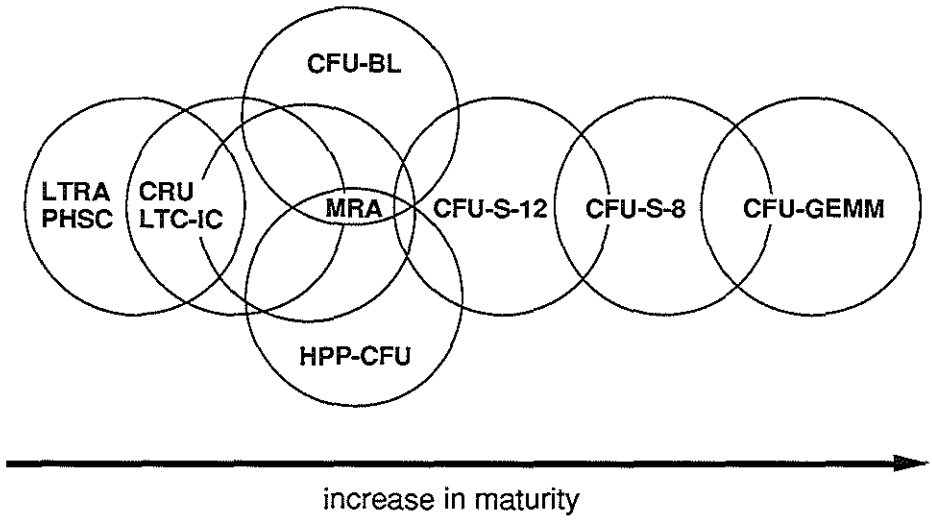


Figure 1.2: Organization of the hemopoietic stem cell compartment, a Venn-diagram illustrates clearly the obvious overlap between the different assayable HSC and progenitor cells, for abbreviations: see Table 1.1 (adapted from Moore, 1991).

Meanwhile, new methods have been applied to purify, enrich, or characterize PHSC or LTRA-cells. Single step sorting on basis of light scatter properties and low Hoechst 33342 uptake has allowed for an approximately 150-fold enrichment for cells with *in vivo* LTRA.[346] Plastic adherence of murine BM cells separates approximately 30% of cells with LTRA from all directly colony-forming cells and the remainder of the LTRA-cells.[242] A recent development in the purification and identification of HSC subsets is sorting on basis of receptor expression for hemopoietic growth factors, a group of stimulatory regulating glycoproteins that will be discussed in the next section.[213,359] These techniques are not solely applied to the purification of murine HSC. Studies aimed at identification of human HSC and progenitor cell subsets revealed that more immature human hemopoietic cells, such as human *long-term culture initiating cells* (LTC-IC; Table 1.1), similar to their murine counterparts, take up relatively low amounts of Rh-123.[465,499]

Transplantation of limited numbers of retrovirally marked HSC clones allows for studies directed at the clonal development of individual immature cells.[268] In the first 3-6 months following transplantation of retrovirally marked cells,

stem cells with both myeloid and lymphoid potential, and stem cells with a restricted potential to myeloid, or to either the T or B lymphoid subset in addition to myeloid differentiation, have been found.[232,269,518,519] In those first few months, sequential analysis revealed stable clones as well as clones undergoing changes in lineage representation.[232,450] With longer follow up, some clones disappeared completely, while others appeared *de novo*. In most of the transplanted mice, however, long-term hemopoiesis, up to 130 weeks after transfection, seemed to be maintained by a few marked clones, which contribute 5 to 50% of blood cell production, as well as unmarked stem cell clones,[79,80,232,240,518,519] which, by serial transplantation, were further shown to be able of *in vivo* expansion.[80,240] However, some clones tended to expand and then diminish upon serial transplantation, indicating that even the clones with the greatest potential may not be potentially immortal, and that the proliferative capacity of an individual HSC clone is unpredictable.[80] Maintenance of hemopoiesis after transplantation of a limited number of HSC was also demonstrated by analysis of the erythrocyte and lymphocyte population in recipients of mixtures of genetically distinguishable cells.[191,331,346] Two of twelve human recipients of allogeneic BMT analyzed by X-linked DNA polymorphism also had donor-derived mono- or oligoclonal hemopoiesis, whereas in the others hemopoiesis was polyclonal,[495] possibly due to transplantation of a relative large number of BM cells. Analysis of erythropoiesis in untransplanted allophenic mice, and whole blood analysis in female mice heterozygous for the X-chromosome linked enzyme phosphoglycerate kinase, indicated that, under physiological circumstances, approximately twenty or even larger numbers of immature HSC may be simultaneously active.[193,330]

The results with marked stem cells, as well as the results obtained in the studies with 5-FU, support the so called generation-age hypothesis, which assumes that more immature HSC have undergone less cell divisions, and consequently have a greater capacity for both self-renewal and formation of progeny than the more mature HSC and committed progenitor cells.[66,405,406] However, this model does not imply self-renewal, since a daughter cell must differ at least with respect to its capacity to form new HSC from its parent.

The observation of continuously active hemopoietic clones both before and after BMT does not support the theory of 'clonal succession'.[237] According to this concept, a cell leaving the stem cell pool produces a clone of maturating offspring, and is replaced by another as soon as the mature cells produced are used up. This model, in accordance with evidence of a limited *in vitro* life span of cells[195] allows, when extended to an age-structured, heterogeneous HSC pool, also for the absence of self-renewal.[238,329] However, it has been argued that the events described in the 'clonal succession' concept of Kay are necessary features of any model for hemopoietic proliferation, which makes the descriptive term 'continuous release of stem cells for differentiation' preferable.[72] Analysis of the expression of the X-chromosome linked enzyme glucose-6-phosphate dehydrogenase by hemopoietic progenitors following transplantation of very low numbers of autologous BM cells in cats revealed fluctuations that would only be explainable on the basis of clonal succession.[1] Transplantation of very low numbers of cells may provide an exceptional situation, in which oligoclonal hemopoiesis may start to resemble 'clonal succession'.

Long-term bone marrow cultures (LTBMC) are *in vitro* systems in which, under appropriate conditions, BM derived adherent cells will support hemopoiesis for as long as several months.[86,115,116,163,339] Established stroma supports not only hemopoiesis derived from HSC inoculated at the time of culture initiation but also from HSC inoculated after irradiation of the stroma.[583] Based on these LTBMC, an *in vitro* limiting-dilution type assay has been developed, that allows for quantification of different early progenitor and HSC subsets as cobblestone area-forming cells (CAFC).[384-386] Another assay based on the LTBMC techniques is that for long-term culture initiating cells (LTC-IC).[473] In this system, the HSC content of cell suspensions is quantified on basis of the number of clonogenic cells present 5 weeks after inoculation of these suspensions on irradiated LTBMC stroma.[473]

Hemopoietic progenitor cells have been studied extensively *in vitro* (Figures 1.1 and 1.2, Table 1.1). In semi-solid cultures, individual progenitors of particular hemopoietic differentiation lineages proliferate and differentiate into colonies of mature end cells. Progenitor cells giving rise to colonies are called CFU-C, i.e., colony-forming units in culture. An affix or prefix to the term CFU indicates the cells present in the colony, e.g., CFU-GM give rise to colonies

consisting of granulocytes and macrophages.[259] The CFU-GM derived colonies were the first to be described.[69,388,389] Other cultures to be developed were those for early and late erythroid progenitors, termed, respectively Burst Forming Units, BFU-E, and CFU-E,[20,221,222,469,478] eosinophil progenitors,[327] megakaryocyte progenitors,[325,344] and lymphocytes,[328,407] in addition to multilineage colonies.[133,324] The largest colonies are formed by the progeny of cells called HPP-CFC, i.e., high proliferative potential colony-forming cells.[67,68] These HPP-CFC require specific culture conditions with low oxygen content (± 5%) and high CO_2 content (± 10%) and produce cells of the monocyte-macrophage lineage.[67,68] Similar to immature HSC-subsets, these HPP-CFC can be fractionated on the basis of Rh-123 uptake; the most immature, characterized on basis of growth factor requirements, take up the least of the fluorochrome.[47] The blast colony assay allows for enumeration of early progenitors, CFU-BL, which give rise to small colonies of undifferentiated blast cells.[244,261,343]

1.1.2 Humoral regulation of erythropoiesis

Blood cell types of all lineages, all with different life spans, are to be produced daily in large numbers to sustain homeostasis. Disturbances of homeostasis, including infections and bleeding, require flexible expansion of specific blood cell types tuned to demand. These features imply that hemopoiesis must be tightly regulated.[437] Several hormone-like proteins, termed *hemopoietic growth factors* (HGFs), regulate proliferation, differentiation and maturation of hemopoietic cells, and modulate the functions of mature blood cells.[321,338,345] The hemopoietic growth factors include colony stimulating factors (CSFs), Interleukins (ILs) and erythropoietin (Ep), which are produced by different cell types, including lymphocytes[367] and endothelial cells.[431] Further regulation is provided by negative regulators, which include transforming growth factor-β (TGF-β), tumor necrosis factor (TNF), macrophage inflammatory protein 1α (MIP-1α) and interferons, and may be involved in maintenance of a quiescent state of HSC and progenitor cells.[19,338] HGFs act as modulators of the expression of HGF-receptors,[437] and some share receptor subunits.[349]

Erythropoietin (Ep) is a glycoprotein, primarily produced in the kidney[225,252,254,258,313] Under certain circumstances, such as in fetal life and

extreme anemia, Ep is produced by liver cells.[151,152,253,580] Ep gene expression has also been demonstrated in BM macrophages.[524]

In vitro, Ep stimulates not only colony formation by CFU-E[469,478] and maturation of BFU-E derived cells,[221,222] but also acts both as a mitogen and as survival factor for cell line derived erythroid progenitor cells.[251,460] In vivo, Ep is necessary for CFU-E survival.[536] BFU-E, however, do not respond to Ep,[217,536] and regeneration of the erythroid lineage, including CFU-E, after cytotoxic injury is Ep independent.[180,534,538] High numbers of Ep receptors have been found on CFU-E and proerythroblasts. Their numbers decline in parallel with erythroid maturation and decreased dependence on the hormone, orthochromatic erythroblasts no longer possessing Ep receptors.[5,423] Although CFU-E are highly dependent on repeated occupancy of Ep receptors for survival, most erythroblasts no longer require Ep for growth after eight hours in culture.[260] Megakaryocytes and their progenitors have been shown to respond to Ep,[45,113] and Ep receptors have been demonstrated on megakaryocytes.[149]

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein that can be produced by a whole array of cell types, such as T- and B-lymphocytes, monocytes/macrophages and endothelial cells.[164] Initially described as a factor stimulating *in vitro* colony formation of granulocytes and/or macrophages,[350] it is now clear that GM-CSF is an early acting growth factor that stimulates colony growth derived from erythroid progenitors,[438,452] eosinophil progenitors,[323] macrophage progenitors in the presence of macrophage colony-stimulating factor (M-CSF),[81] and megakaryocyte progenitors.[314]

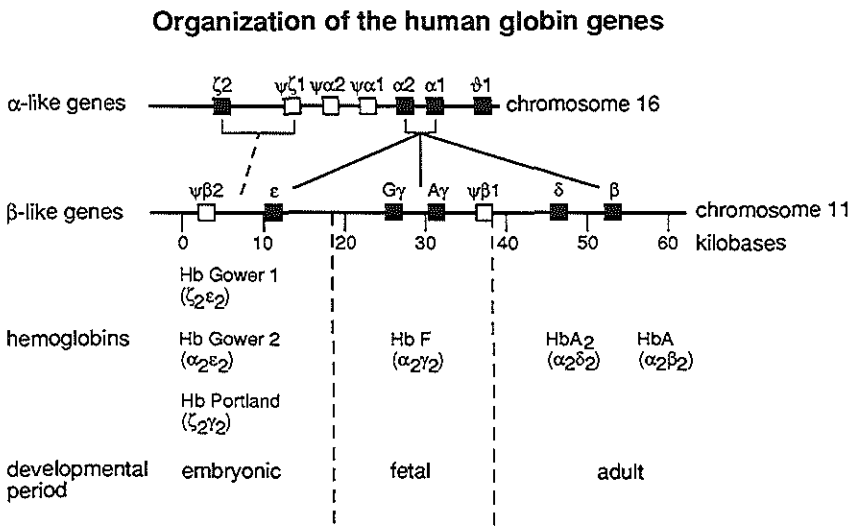
Interleukin-1 (IL-1) is an important mediator of the acute phase response, B- and T-cell activation, and stimulates the production of other cytokines, such as G-CSF (granulocyte colony-stimulating factor) and M-CSF (macrophage colony-stimulating factor), e.g., in BM stromal cells,[137,284,439] as well as hemopoiesis along multiple hemopoietic lineages in normal mice.[337] It acts in synergy with other HGFs, such as granulocyte colony-stimulating factor (G-CSF) to induce the growth of HPP-CFC derived and lineage restricted colonies from 5-FU murine BM.[340] Accelerated regeneration of progenitor and

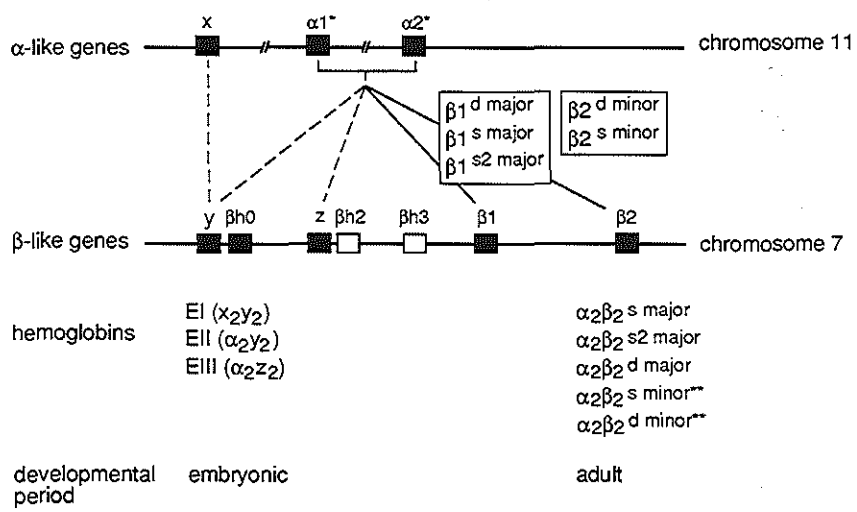
peripheral blood cells was observed when IL-1 was administered to different species treated with 5-FU, such as mice[340] and cynomolgous monkeys.[337]

Interleukin-3 (IL-3) is also termed multilineage colony-stimulating factor (multi-CSF). It has been shown to stimulate *in vitro* growth of BM progenitor cells of most, if not all, hemopoietic lineages, either directly or indirectly via accessory cells,[65,211,427,472] and to act in synergy with other cytokines, such as IL-6.[212,245,573] In vivo administration of IL-3 in different animal species and in humans resulted in a multilineage response with increases in peripheral blood white cell and progenitor cell levels in BM and spleen.[161,322,537]

Stem cell factor (SCF) is a relatively novel HGF, and is also termed mast cell growth factor (MGF) or *kit*-ligand (KL). It has been purified and characterized by conventional protein chemistry, and subsequently produced as a recombinant protein.[306,351,568,582] SCF exists as membrane bound and soluble forms,[7,144] and stimulates, in most cases in synergy with cytokines such as GM-CSF, IL-1, IL-3, IL-6 and erythropoietin, progenitor cell derived colony growth and production of progenitors in suspension cultures.[7,71,306,338,351,582] SCF is encoded by a gene at the steel (*Sl*) locus.[100,144,206,581] In mice, mutations at the steel locus as well as at the dominant spotting (*W*) locus, result in macrocytic anemia.[188,413] It was shown that the *W* locus encodes for the c-*kit* proto-oncogene,[84,167] which is the receptor of SCF. Because of this interaction, SCF has also been termed KL. Mice with *W*-mutations express aberrant forms of the c-*kit* protein.[352] The production of SCF by stromal cells[568] and its membrane-bound expression on the surface of these cells[7,144] stresses the importance of the hemopoietic micro-environment in the regulation of hemopoiesis.[115,116,576,577]

In summary, it can be concluded that erythropoiesis is regulated by an interaction of hemopoietic cells with stromal cells and negatively as well as positively acting regulatory molecules. The most important known stimulatory elements in the developmentally early stages of erythropoiesis are probably KL, IL-1, IL-3, IL-6 and GM-CSF, whereas the later maturation stage is exclusively controlled by erythropoietin.





* numerous haplotypes
** developmental profile resembles that of fetal hemoglobin in other mammals

Figure 1.3: Organization of the human (adapted from Stamatoyannopoulos et al., 1987, and Higgs et al., 1989) and murine globin genes

1.2 THE THALASSEMIAS

1.2.1 Molecular biology of hemoglobin synthesis

Hemoglobin is the molecule responsible for oxygen transport in the body. It is a tetramer made up of two pairs of globin chains, each of them covalently linked to an iron-containing heme group. The thalassemias are characterized by an imbalance in globin chain synthesis. The organization of human and murine globin genes is shown in Figure 1.3. The human β-globin gene cluster is located on the short arm of chromosome 11, with the structural arrangement 5'-ψβ2-ε-^{G}γ-^{A}γ-ψβ1-δ-β-3', the α-globin gene cluster is located on the short arm of chromosome 16, in the arrangement 5'-ζ2-ψζ1-ψα2-ψα1-α2-α1-ϑ1-3'.[198,467,552,553] Production of the embryonal hemoglobins Gower 1 and 2 is found in embryos up to eight weeks of gestation, and Hb Portland is a third normal embryonic hemoglobin. Red cell precursors of the yolk sac first produce ζ and ε chains, while α and γ chain synthesis is initiated later, possibly at about the time of hepatic erythropoiesis (reviewed by Weatherall[552]). Synthesis of HbA is activated early during fetal development, the control of the switch from HbF to HbA synthesis related mainly to gestational age and not to the site of erythropoiesis.[552] Much has been learned about the regulation of human hemoglobin synthesis in transgenic mice (reviewed by Orkin[364] and Ley[275]). High level expression of globin genes in transgenic mice requires linkage in cis to the genes of the locus control region (LCR). The LCR can override stage specific cues of individual γ- and δ-globin genes, that regulate developmentally appropriate erythroid expression, whereas ε-globin gene expression would appear to be autonomously regulated in the presence of LCR. Appropriate stage specific switching of hemoglobin synthesis was observed in LCR-γδβ transgenic mice, suggesting a competition between the γ- and β-globin genes for the strong positive influence of the LCR. However, down regulation of LCR-γ construct expression in transgenic mice according to developmental stage seems to indicate that trans-acting factors may directly silence γ-globin gene expression.

Knowledge about hemoglobin switching is important, since blocking the switch from normal HbF to (defective) adult Hb synthesis may benefit patients with disorders of β-globin chain synthesis, e.g., β-thalassemia or sickle cell anemia.

The murine β-globin genes are located towards the 3' end of the β-globin complex on chromosome 7, with the structural arrangement 5'-y-βh0-z-βh2-βh3-β1-β2-3' of the β-homologous sequences.[132,228,264,555] In the α-globin complex on chromosome 11 the gene coding for the embryonic x-globin and the two adult α-globin genes are closely linked.[563] The α-globin and β-globin loci have been named Hba and Hbb. There are at least 15 naturally occurring Hba haplotypes.[414,560] Hbb^d, Hbb^p, Hbb^s and Hbb^{s2} are the four known β-globin haplotypes.[414] A Hbb^d mouse produces two adult β-globins (β-major and β-minor), and thus two kinds of hemoglobin: $\alpha_2\beta_2^{dmajor}$ and $\alpha_2\beta_2^{dminor}$. The Hbb^p type resembles the Hbb^d, but makes a variant β-minor chain. The Hbb^s allele codes for the β-smajor and β-sminor globins, which are structurally identical to each other, producing $\alpha_2\beta_2^{smajor}$ and $\alpha_2\beta_2^{sminor}$ hemoglobin. The Hbb^{s2} haplotype was discovered during electrophoretic screening of F1 progeny of females treated with ethylnitrosourea.[274] A mutation in the β1-gene results in the production of β-s2major globin.[548] The hemoglobins produced by mice with this Hbb^{s2} haplotype[548] are $\alpha_2\beta_2^{s2major}$ and $\alpha_2\beta_2^{sminor}$. Studies in mice with the Hbb^d and Hbb^{s2} haplotypes show that the two hemoglobin β-chains are expressed independently, with a decrease of the relative level of β-minor globin production through fetal development.[548,549,558] In this aspect β-minor globin resembles the human fetal globins.

Three embryonic hemoglobins, EI-EIII, are produced in mice.[414] EI consists of two x-chains and two y-chains. EII and EIII both have two α-chains, but respectively two y-chains and two z-chains (Figure 1.3). The z-chain in produced predominantly in early embryogenesis, to be replaced later by the y-chain.[132] Expression of the βh0-gene is also found in embryos, where βh0 transcripts were at least 5-fold less abundant than z transcripts.[132] The βh2 and βh3 genes are pseudogenes.[228] The mouse β-globin locus is thus organized from its 5' to 3' end as late and early embryonic, pseudogenes, and adult genes, respectively.[132]

1.2.2. Human thalassemia

Thalassemias are one of the most common groups of monogeneic disorders in humans.[553] In 1983 the WHO estimated that throughout the world there are over 180 million carriers of one of the forms of the disease, whereas approximately 100,000 homozygotes with severe clinical problems are born

annually, 74,000 of which are suffering from severe forms of β-thalassemia.[12] Later data estimated the annual numbers of newborns with severe forms of β-thalassemia to be approximately 50,000.[13,14] In South-east Asia, approximately 14,000 children a year are born with HbH-disease.[15] Thalassemia is particularly common in areas where malaria is or was prevalent,[14,15,280,552] and individuals heterozygous for β-thalassemia were shown to be relatively resistant to malaria infection.[567] The molecular pathogenesis has been extensively reviewed by Weatherall.[550] In the majority of thalassemias, structural abnormalities are not observed in the affected globin chains.[550,552] The α-thalassemias result in most cases from α-globin gene deletions, in contrast to the β-thalassemias, which result in most cases from single nucleotide substitutions in β-globin or regulatory genes.[77,363,553] The molecular basis of β-thalassemia is extremely heterogeneous, and new mutations resulting in β-thalassemia are published at regular intervals. A simplified classification of the groups of genetic defects resulting in β-thalassemia is presented in Table 1.4.[77,363] In addition, non-deletional forms of α-thalassemia as well as deletional forms of β-thalassemia have been described.[363] Other rare forms of both α- and β-thalassemia are those characterized by instable globin chain variants; Hb Indianapolis and HB Quong Sze.[363]

Thalassemias can be classified on basis of a complete absence (α^0/β^0) or a reduced production (α^+/β^+) of globin chains.[363,552] β^+-Thalassemias have been subclassified as type 1, 2, or 3 on the basis of the amount of HbF observed in homozygotes, respectively >80%, 30-50%, and 15-30%.[552] β^0-Thalassemias type 1, 2, and 3 are characterized by, respectively, the absence of β-globin mRNA, the absence of translation of full length β-globin mRNA, and β-globin mRNA with structural abnormalities in its 3' end.[552] Clinically, β-thalassemia exists in the forms thalassemia minor or carrier state, the non-transfusion dependent thalassemia intermedia, and thalassemia major or Cooley's anemia (Table 1.5).

The range in clinical severity of the α-thalassemias depends on the number of normally functioning α-globin genes (Figure 1.4). The absence of α-chains, clinically termed hydrops foetalis, with Hb Bart's (γ_4), is a lethal condition. The silent carrier state (α^+-thalassemia) does not have any hematological abnormality. Hypochromia and microcytosis are found in the α-thalassemia

Table 1.4: Molecular basis of β-thalassemia

Mutant Class	mRNA	Thalassemia form
Promotor mutations	70-80% reduced output of β-globin mRNA	β^+-thalassemia
Nonsense or frameshift mutations producing a stop codon, i.e., premature chain termination	<5% of normal β-globin mRNA	β^0-thalassemia
Processing mutations (a) RNA splicing affected, e.g., via the use of cryptic splice sites or mutations in consensus sequences resulting in less efficient splicing	some or no β-globin mRNA produced	β^0-thalassemia β^+-thalassemia
Processing mutations (b) polyadenylation signal mutations	some β-globin mRNA produced	β^+-thalassemia

traits (heterozygous α^0-thalassemia, and homozygous α^+-thalassemia). HbH disease is the most severe form, in which the imbalance of globin chain synthesis is such that HbH (β_4) is found.[363]

Prenatal diagnosis has become very important for the prevention of thalassemia.[12-15] In each population at risk, a limited number of up to five account for the vast majority (50% to almost 100%) of all molecular defects that can lead to defective β-globin synthesis in that population. This can be advantageously used for both carrier state detection and the prenatal diagnosis of thalassemia.[76-78,210,520] In Sardinia, 95% of the cases of β-thalassemia are caused by the nonsense mutation at codon 39.[76,78,210] A preventive program aimed at the control of β-thalassemia has been shown to be highly effective, resulting in a decline in incidence of thalassemia major in 14 years from 1 in 250 to 1 in 1000 live births.[78] This voluntary program was based on carrier detection, genetic counseling and prenatal diagnosis.[78]

Table 1.5: Different forms of human β–thalassemia

Genetic defect	Consequences	Hemoglobin synthesis	Clinical form
heterozygous, only one abnormal β-globin allele	carrier state	HbA2 ($\alpha_2\delta_2$) >5% of Hb	thalassemia minor
1) homozygous for mild β-thalassemia mutations 2) δβ-thalassemia associated with high HbF 3) coinherited α-thalassemia 4) coinherited nondeletion HPFH* 5) specific β-globin haplotype associated with high HbF production	mild to moderate non-transfusion dependent form of thalassemia	reduced HbA synthesis	thalassemia intermedia
homozygous, two abnormal β-globin alleles	transfusion dependent form of thalassemia	no (β^0-thalassemia) or some HbA (β^+-thalassemia) formed	thalassemia major or Cooley's anemia

* HPFH = Hereditary Persistance of Fetal Hemoglobin

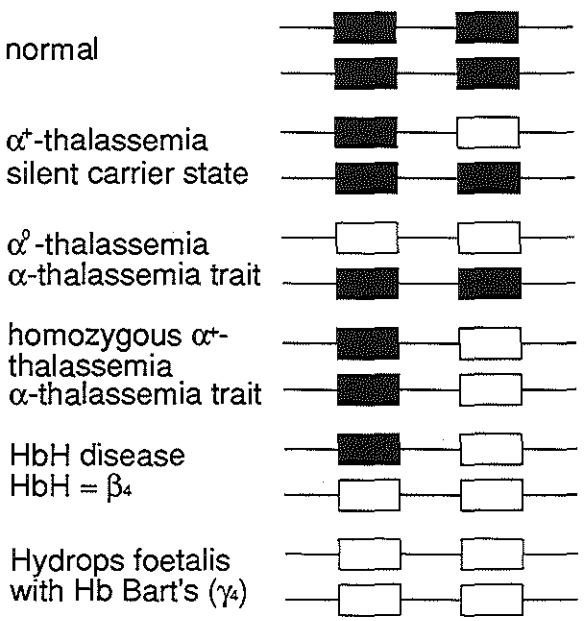


Figure 1.4: Molecular basis of human α-thalassemia. Closed boxes represent normally functioning, open boxes deleted or otherwise non-functioning α-globin genes.

Increased numbers of progenitor cells were observed in the spleens of thalassemic mice as a consequence of partially inappropriate compensatory hemopoiesis.[60,396,540] Similar to these observations in mice, increased numbers of circulating erythropoietic and non-erythropoietic progenitor cells have been observed in patients with hemoglobinopathies, such as thalassemia and sickle cell anemia.[50,89,101,154,229,358] Depletion of adherent mononuclear cells before culture showed that an increase in number or activity of accessory cells was partly responsible for a high frequency of non-erythroid and erythroid progenitors.[101,229]

1.2.3 Murine α- and β-thalassemias

Murine α-thalassemia was first described in the progeny of X-irradiated male mice.[416] Affected offspring suffer from a microcytic hypochromic anemia, with erythrocytosis and reticulocytosis.[393] The life span of α-thalassemic erythrocytes is shortened to 35-37 days, the normal life span being 42 days.[394] Homozygotes for the deletion die in early gestation even before α- and x-chain synthesis is required, suggesting that a deficiency of other closely linked genes may cause death in these homozygotes.[8,392] Chemical analysis of hemoglobin of the progeny of one of the original mutants showed absence of mutant haplotype derived α-globin chain synthesis.[398] This is caused by a deletion of both α-globin genes and the closely linked embryonic x-globin gene in the radiation induced as well as a chemically induced form of murine α-thalassemia.[310,395,397,561,563] Production of α-globin chains is reduced by 20%,[310,564] and reflects the α/β mRNA ratio of approximately 0.8 found in the reticulocytes.[8,310] Electron microscopy studies revealed that excess β-globin chains precipitate as intracytoplasmatic inclusions in erythroid precursors.[564] There is little or no proteolysis of excess β-globin chains, which explains the higher proportion of erythropoietic cells with inclusions observed in α-thalassemic mice, than observed in patients with HbH disease.[564] A high proportion of cells with inclusions is phagocytosed in the BM, since 27% of the non-nucleated erythropoietic cell profiles in the bone marrow contain inclusions, compared to only 5% of the circulating red cell profiles.[564] α-Thalassemic mice show non-transfusional iron overload in spleen, liver and kidney, although in contrast to the human situation the iron is only found in the reticuloendothelial system.[516] High iron absorption rates, however, are only found in the first 10-11 weeks of life, resulting in a non-progressive form of iron overload.[516] In parallel to the situation for humans, α-thalassemic mice are more resistant to lethal and non-lethal forms of rodent malarias.[562]

The increase in erythropoiesis, necessary to maintain red blood cell numbers is accompanied by an increase in the numbers of non-erythroid progenitor cells and CFU-S.[540] However, such a difference is not found between the femoral hemopoietic progenitor cell content of α-thalassemic and normal mice, indicating that the compensatory increase in hemopoiesis is restricted to the spleen.[540] This probably reflects the most appropriate adaptation of the

hemopoietic system to the increased demand for red blood cells, since the spleen is more suitable for erythropoiesis than for granulocytopoiesis.[571] It is possible to alleviate the anemia of α-thalassemic mice by the induction of partial hemopoietic stem cell chimerism, which is accomplished by BMT with small numbers of normal BM cells following low dose total body irradiation (TBI)[27,541] (Section 1.3 of this chapter).

A deletion of 3709 ± 2 bp including regulatory and all coding sequences of the β-major globin gene causes murine β-thalassemia in the homozygous state.[172,443] The mice show a hypocellular, hypochromic, microcytic anemia, with severe anisocytosis, poikilocytosis, extreme reticulocytosis, and leukocytosis.[162,396,443] Leukocytosis and the expansion of splenic CFU-S probably reflect increased hemopoiesis analogous to the situation in α-thalassemic mice.[60,396] The life span of β-thalassemic erythrocytes is reduced to approximately 20 days.[396] A β-minor/α-globin chain synthesis ratio of approximately 0.75 is found.[102,396,424,443] The β-minor/α-globin mRNA ratio is reported to be the same as in normal mice (i.e., 0.2), or only slightly increased to 0.3.[102,424] The observed increase in β-minor globin synthesis is therefore primarily due to preferential translation of β-minor globin mRNA, while modulation at the level of transcription, processing or mRNA stability only plays a minor role.[102] Similar to α-thalassemic mice, excess globin chains are not proteolysed,[102,409] and non-transfusional iron deposition is found in liver, kidney, spleen and bone marrow.[162,396,517] In contrast to α-thalassemic mice, the iron deposition is not restricted to cells of the reticuloendothelial system, but also found in parenchymal cells, such as hepatocytes.[162] This tissue iron overload is probably responsible for the increased risk to infection of homozygous β-thalassemic mice,[6] although, similar to murine α-thalassemia, murine β-thalassemia offers protection against some rodent malaria parasites, such as the Plasmodium chabaudi adami.[408]

The alterations in red cell deformability and density distribution in β-thalassemic mice are comparable to those of human β-thalassemic red blood cells.[411] The degree of rigidity of the β-thalassemic red blood cells correlates with the amount of membrane-skeletal associated α-globin,[409,453] that produces its pathophysiological effects in oxidized form, in conjunction with oxidized RBC membrane specific proteins.[3] In red cells of β-thalassemic mice crossed

with human β^s-containing transgenic mice 10% mouse α/human β^s hybrid hemoglobin is found, resulting in increased deformability and improvement of the hematological indices.[411,412] Murine β-thalassemia can also be improved by administration of high doses of recombinant human erythropoietin.[272] In this case, a further induction of β-minor globin synthesis results in a decrease of the amount of insoluble α-globin chains and membrane protein abnormalities, as well as in normalization of red cell deformability.[272]

1.2.4 Conventional treatment of human thalassemia

Conventional treatment of thalassemia is based on transfusion and iron chelation therapy to mitigate the effects of iron overload.[271] This mode of treatment has dramatically improved survival and quality of life of thalassemic children over the last decades.[62,376] Excess iron absorption and regular transfusions are responsible for iron overload in thalassemia.[83] Iron chelation is usually provided for by subcutaneous infusions of deferoxamine (DFO). Problems associated with DFO chelation therapy are its high costs and the variable compliance rates.[299,533,554] Furthermore, numerous toxic properties of DFO, including auditory and visual neurotoxicity as well as renal toxicity, have been reported.[150] Expectations about compliance must be taken into account when considering therapeutic alternatives such as BMT.[299,533,551] Chelation therapy has unmistakably improved life expectancy for thalassemia patients.[62,63,335,574] Results of two Italian cooperative studies showed that in 1992 survival of patients born after 1970 was 95% at 15 years and 88% at 20 years, whereas in the sixties survival beyond the age of fifteen years was exceptional.[62] However, it has been established that approximately 60% of 20 year old patients are at risk of developing important life threatening complications, such as cardiac complications,[62,63,155] that are still the most frequent cause of death in thalassemia patients, although it has been shown that death due to iron related cardiac disease at least can be delayed.[572] Non-compliant patients are more likely to die than patients compliant with DFO therapy,[271,572] but cardiac status may decrease even in compliant ones.[271] It may, however, be possible to halt or even reverse cardiac complications by more intensive (intravenous) chelation regimens.[98,574] Chelation therapy has been shown to arrest, but only rarely reverse hepatic damage,[312] and growth and sexual development were reportedly

still impaired.[61,62,112,312] Also other endocrinopathies occur during chelation therapy.[61,62,112] In contrast to these data, another study showed that 90% of patients treated with chelation therapy before the age of ten had normal sexual development and reached mean parental height.[74] It thus would seem advantageous to start chelation therapy early in life,[74,312] although care must be taken during the first months of life, since DFO administration before iron overload has been established may adversely affect longitudinal growth.[114]
The recent development of oral chelation therapy may have important advantages over subcutaneous DFO therapy, but long term efficacy still has to be determined.[246-248,488]

1.2.5 Bone marrow transplantation in human thalassemia

The purpose of bone marrow transplantation (BMT) is to provide the recipient with a new functioning hemopoietic system. It has become an important mode of therapy for a variety of neoplastic and non-neoplastic disorders such as hereditary diseases of the hemopoietic system and is presently being evaluated for treatment of other hereditary diseases, such as inborn errors of metabolism.[199,205,356,368,401,404,421,483] Thalassemia is the hereditary disease most frequently treated by BMT. In 1990, clinics reporting to the EBMT (European Group for Bone Marrow Transplantation), performed 117 HLA-identical bone marrow transplantations for thalassemia.[176] However, it was estimated that approximately 1900 children with β-thalassemia major are born each year in Europe.[14] The transplanted patients, therefore, represent only a minor fraction of all thalassemia patients.
The first patient receiving BMT for thalassemia was a 16 months old boy transplanted in December 1981.[484] Since then, several hundreds of patients have received BMT for thalassemia (Table 1.6). Most patients transplanted for thalassemia were of Italian background. In the first reports from Pesaro, Italy, the conditioning regimens used, including 120 or 200 mg/kg cyclophosphamide in combination with TBI, and 16 mg/kg busulphan with 200 mg/kg cyclophosphamide and TBI, resulted in unacceptably high levels of early, transplant related death of approximately 50%.[160,294-298] Reducing the dose of busulphan to a total of 14 mg/kg improved survival of young patients receiving HLA-matched grafts to approximately 80%.[160,290-293]

In the last few years, the Pesaro group has classified its patients according to the risk factors hepatomegaly, portal fibrosis and inadequacy of iron chelation therapy. Patients in class I have no risk factors, those in class II have one or two, whereas class III patients have all three risk factors.[288,291] Most recently, overall survival in class I has been reported to be 97%, with 94% disease free survival.[31,288,289,291,299] Survival was 88% for class II patients, with a disease free survival of 85%, whereas survival and disease free survival were, respectively, 54-61% and 49-53% for class III patients.[168,289,291,299] The conditioning regimen for these patients was 'protocol 6', i.e., 14 mg/kg busulphan and 200 mg/kg cyclophosphamide. The unacceptably high percentage of death with this regimen in class III patients, caused mainly by liver damage or cardiac failure in the early post-transplant period, led to the development of new conditioning regimens: protocols 12 and 16.[11,289,299] Overall survival and disease free survival have increased to respectively 96% and 70% using these protocols with either a lower cyclophosphamide dose (120 mg/kg) and addition of anti-lymphocyte globulin or 16 mg/kg busulphan in combination with 120 mg/kg cyclophosphamide.[11,289,299] Although patients and their parents of the 'good risk' group can expect excellent results, the average rate of rejection of HLA-matched grafts in the other patients still varies between 10 and 30%.[11,168,289,291,299]

Approximately the same rejection rates were reported by the group from Pescara (Italy), with an overall disease-free survival of 85%[118,120,121,489] and by groups from other centers in Taiwan,[278,279,] France,[42,147,148] England,[207,233] Canada[422] and Seatlle, (U.S.A.).[91,482,485] Although graft failures were not reported by groups from Israel and from Cagliari, Italy, the disease free survival is approximately equal to the results of the other Italian and French groups.[99,361,362,446,545] As shown in Table 1.6, small numbers of patients have been transplanted in other centers.[73,223,265,334,528] Successful retransplantation using the same donor after a first BM graft rejection has also been reported by several groups.[119,422]

Conflicting results on the outcome of BMT in adult patients have been reported. Good results were reported by the Pescara group, i.e., 89% disease free survival.[118,121] The Pesaro group was initially less successful, with only 1 of 10 patients surviving without thalassemia for more than a year, and 8 of 10

Table 1.6: Clinical results in BMT for thalassemia

Group conditio-ning/ note	No. patients	dead	% survi-val	alive and well	% disease free survi-val	follow up	GVHD	relapse / (late) rejec-tion	% rejection and engraft-ment failure	references (reference number)
Seattle	10	2/10	80	6/10	60	2 patients 2 to 3 years and 3 patients 5 to 7 years	1/5 chronic GVHD	2/10	20	Thomas et al., 1982 and 1985 Thomas, 1989 Clift, 1993
Pesaro (HLA-matched)	late disease 6	3/6	50					3/6	50	Lucarelli et al., 1983 and 1984 (296)
	early disease 7	3/7	43	2/7	29			2/7	29	
Bu 16 mg/kg & Cy 200 mg/kg	6 (age ≤ 7 years)	3/6	50	3/6	50					Lucarelli et al., 1984 (297), 1985 (295 and 298) Galimberti et al., 1987
Bu 14 mg/kg & Cy 200 mg/kg = protocol 6	24 (age ≤ 7 years)	1/24	96	19/24	79	64 - 624 days	23% acute GVHD (grade≥2) when take	4/24	17	

Table 1.6 continued

Group conditio-ning/ note	No. patients	dead	% survi-val	alive and well	% disease free survi-val	follow up	GVHD	relapse / (late) rejec-tion	% rejection and engraft-ment failure	references (reference number)
Bu 14 mg/kg & Cy 200 mg/kg	40 (8-15 years)	10/40	75		70	26 - 1133 days	initial sustained engraftment: 34% acute GVHD and 9% chronic GVHD		5	Galimberti et al., 1987 Lucarelli et al., 1987 (292)
reviews [1]	222 (< 16 years)		82		75	max. 4 years			13	Lucarelli et al., 1987 (293), 1989, and 1990
Bu 14 mg/kg & Cy 200 mg/kg	116		86		82				6	
reviews [2]	>400 Class I		94-98		94				0	Lucarelli et al., 1990, and 1991 (289) Lucarelli and Weatherall, 1991
	64 [3] (Class I)	2/64	97	60/64	94	upto 9 years	31% acute GVHD 14% chronic GVHD		1/64 1/64 stable mixed chimera	Baronciani et al., 1993 (31) Lucarelli et al., 1993

1) reviews by the same author (Lucarelli) of partially overlapping patients, also (probably) partially overlapping with earlier data

2) a number of reviews by the Pesaro group; results categorized by author of thesis

3) supplementary data from Pesaro group, (probably) partially overlapping with earlier data of Class I patients

Table 1.6 continued

Group conditio-ning/ note	No. patients	dead	% survi-val	alive and well	% disease free survi-val	follow up	GVHD	relapse / (late) rejec-tion	% rejection and engraft-ment failure	references (reference number)
reviews [4]	Class II		80-86		77-83				9	Lucarelli et al., 1990, and 1991 (289) Lucarelli and Weatherall, 1991
	188 [5] (Class II)		88		85		20% acute GVHD 14% chronic GVHD		4	Giardini et al., 1993 (168)
reviews [4]	Class III		54-61		49-53				12-16	Lucarelli et al., 1990, and 1991 (289) Lucarelli and Weatherall, 1991

4) a number of reviews by the Pesaro group; results categorized by author of thesis

5) supplementary data from Pesaro group, (probably) partially overlapping with earlier data of Class II patients

Table 1.6 continued

Group conditio-ning/ note	No. patients	dead	% survi-val	alive and well	% disease free survi-val	follow up	GVHD	relapse / (late) rejec-tion	% rejection and engraft-ment failure	references (reference number)
reviews [6] Bu 14 mg/kg & Cy 120 mg/kg & ALG 10mg/kg (day -5 to +5) = protocol 12	Class III		91		60-70				37	Lucarelli et al., 1991 (289) Lucarelli and Weatherall, 1991
protocol 12 or Bu 16 mg/kg & Cy 120 mg/kg = protocol 16	47 [7]	2/47	96	33/47	70	120-1300 days	9% acute GVHD 1/40 chronic GVHD	12/47	26%	Angelucci et al.,1993 (11)
HLA-mis-matched	18	8/18	56		26	6 months - 10 years	64% (7/11) acute GVHD 44% (4/9) chronic GVHD	9/18	50	Lucarelli et al., 1989 Lucarelli and Weatherall, 1991 Delfini et al., 1985 Galimberti et al., 1991 (159), Polchi et al., 1993

6) a number of reviews by the Pesaro group; results categorized by author of thesis

7) supplementary data from Pesaro group, (probably) partially overlapping with earlier data of Class III patients

Table 1.6 continued

Group conditio-ning/ note	No. patients	dead	% survi-val	alive and well	% disease free survi-val	follow up	GVHD	relapse / (late) rejec-tion	% rejection and engraft-ment failure	references (reference number)
HLA-matched (>16 years)	10	8/10	20	1/10	10			1/10	10	Lucarelli et al., 1989
HLA-matched (17-32 years; 11 class II and 30 class III)	41	6/41 (1/11 class II, and 5/30 class III)	85	33/41	80	upto > 3 years	17% (7/41) acute GVHD 24% (8/33) chronic GVHD	2/41	5	Lucarelli et al., 1991(286), and 1992 Erer et al., 1993 (131)
<u>Pescara</u>										
< 7 years	19	2/19	89	15/19	79	5 - 64 months		2/19	11	Torlontano et al., 1988 Di Bartolomeo et al., 1989, 1991 and *1993 (118)
			92*		82*					
> 7 years	14	0/14	100	14/14	100	4 - 54 months				
			88*		84*					
adults	7	1/7	86	6/7	86					
			89*		89*					

Table 1.6 continued

Group conditio-ning/ note	No. patients	dead	% survi-val	alive and well	% disease free survi-val	follow up	GVHD	relapse / (late) rejec-tion	% rejection and engraft-ment failure	references (reference number)
overall			92		85			1/61 engraft ment failure 2/61 late graft failure	2 (engraft-ment failure) 5*-6 (late graft failure)	Di Bartolomeo et al., 1991 and *1993 (118)
graft fail-ures were retrans-planted (see next row: Di Barto-lomeo et al. 1993)			90*		85*					
second transplant	3	1/3	67	2/3	67	36-50 months				Di Bartolomeo et al. 1993 (119)
Cagliari	10	4/10	60	6/10	60	100-1872 days		0/10		Contu et al 1993
Turin conditio-ning regimen including GM-CSF	4	0/4	100	4/4	100	6-18 months		0/4		Miniero et al. 1993

Table 1.6 continued

Group conditio-ning/ note	No. patients	dead	% survi-val	alive and well	% disease free survi-val	follow up	GVHD	relapse / (late) rejec-tion	% rejection and engraft-ment failure	references (reference number)
France	17	3/17	82	10/17	58	180 - 1125 days	4/12 (33%) low grade GVHD	4/17	24 (partial or complete autolo-gous recovery)	Bergerat et al., 1988 Frappaz et al., 1988, and 1989
T-cell depleted	4	1/4	75	2/4	50			1/4	25	
unmodi-fied	13	2/13	85	8/13	62			3/13	23	
Jeru-salem (T-cell depleted)	12	3/12	75	9/12	75	2 - 50 months (1 x partial chimera at 7 months)				Waldmann et al., 1984 Slavin and Rachmilewitz, 1986 Or et al., 1988, and 1989
London	16 (1 x T-cell depletion)	6/16	63	9/16	56			1/16	6	Joshi et al., 1984 Hugh-Jones et al., 1989

Table 1.6 continued

Group conditio-ning/ note	No. patients	dead	% survi-val	alive and well	% disease free survi-val	follow up	GVHD	relapse / (late) rejec-tion	% rejection and engraft-ment failure	references (reference number)
New York	2			2						Brochstein et al., 1986
Australia	1			1						Vowels et al., 1986
Canada (Toronto)	6		100	3/6	50	upto 4 years			1/3 2/3 stable partial chimeras	Saunders et al., 1993
Taiwan	14	5/14	64	6/14	43			3/14	21	Lin et al., 1986 Lin and Lin, 1989
Thailand	10	1/10	90	5/10	50	104-1429 days		4/10	40	Issaragrisil et al., 1993
Hong Kong	3		100	2/3	67	256-291 days		1/3	33	Lee et al., 1993

patients not surviving the transplantation procedure.[290] More recent data for class II and III patients aged 17 to 26 years, and treated with appropriate conditioning regimens, show better results with a disease free survival in 80% of the patients.[131,286,287]

Other donors than HLA-identical siblings, such as HLA phenotypically identical siblings and parents, have been used by the Pesaro group.[107] Approximately 60% of the thalassemic children do not have an HLA-identical family donor available.[299] Results of bone marrow transplantation using haplo-identical donors with one, two or three loci mismatch, have been very disappointing. Disease free survival of only 25%, with an overall survival rate of less than 60% was reported.[159,290,299,390] It is, however, difficult to interpret these data clearly, since as yet the risk factors in these patient populations have not been reported.

When considering BMT as therapy for thalassemia patients,[299,551] it must be taken into account that the long-term effects of BMT on thalassemia related complications are not known yet. Normal or partial pubertal development can take place when patients are transplanted at an early age, although, perhaps also as a consequence of the agents used for conditioning regimens, only in approximately half of the patients,[111,158,288,299] which is similar to conventionally treated patients.[62] Post-BMT growth depends on the age at transplantation[166] as well as on the development of chronic GvHD,[288] and appears to be still impaired in patients with high serum ferritin levels after BMT.[391] Progression of liver damage may be prevented, depending on the extent of pre-transplant damage.[11,31,168,288,299] Pancreatic β-cell function was improved in a series of transplanted thalassemia patients,[157] as was cardiac function, although for the latter it was necessary to deplete tissue iron stores.[304] Progressive clearance of iron deposits after BMT occurred in class I and the majority of class II patients.[169,288] The effects of removal of excess iron in transplanted thalassemia patients by either regular phlebotomy or DFO therapy are currently being evaluated,[10,169] while the transplantation associated mortality and morbidity, including GvHD, is still significant (Table 1.6).[30,130,333]

Thus, young patients, especially those with none of the risk factors defined by the Pesaro group and with an available HLA-identical family donor, have

excellent prospects of a favorable outcome of the transplantation. The prospects for young patients with risk factors, or older patients are clearly less favorable, and for these cases less toxic conditioning regimens have been developed to improve the clinical outcome of transplantation. Patients without an HLA-identical family donor, i.e., 60% of the thalassemic children, are presently not being considered for transplantation. For these patients, only a conditioning regimen that allows for sustained chimerism using HLA-matched unrelated donor BM or HLA-mismatched BM, but without excessive toxic side effects, will make BMT an acceptable alternative for conventional therapy.

1.3 ENGRAFTMENT OF HEMOPOIETIC STEM CELLS

Graft rejection is one of the major problems in the clinical application of BMT for thalassemia. Whether or not allogeneic BM cells will be able to engraft appeared to be the result of the reaction of immunocompetent cells in the BM allograft, which cause GvH-reactions, and in the recipient, which cause allograft rejection.[110,135,307,501,529] This phenomenon has been called the 'reciprocal interference' of Host-versus-Graft (HvG) and Graft-versus-Host (GvH) reactions.[529,532]

The key role of residual host T-cell in experimental as well as clinical BM allograft rejection has been well documented.[4,40,51,109,110,145,241,283,342] Therefore, these cells represent major targets for manipulative actions aimed at the prevention of BM allograft rejection, e.g., by the use of specific anti T-cell MCAs (Section 1.4),[94,95] or less specifically by increasing the cytoreductive capacity of the conditioning regimen.[542,535] Residual $CD4^+$- as well as $CD8^+$-cells are involved in BM allograft rejection.[94]

Decreased levels of donor type chimerism and graft failure have been observed experimentally as well as clinically in recipients of T-cell depleted grafts as compared to recipients of not depleted grafts.[85,301,308,309,357,400,451,502] In lethally irradiated recipients of mixtures of allogeneic and syngeneic BM cells, T-cell depletion of the allogeneic component led to stable partial chimerism, whereas recipients of a mixture with a non-T-cell depleted allogeneic component became full chimeras.[215] The ability of T-cell subsets to induce Graft-versus-Host Disease (GvHD) may reflect their potential to promote engraftment of allogeneic BM cells. Accordingly, both major T-cell subsets have been shown to

be able to induce GvHD, depending on the kind of histocompatibility mismatch.[57,95,249,250,360,374,463,479] Since the number of transplanted T cells is also an important determinant of GvH reactions,[503] another approach to ensure BM allograft acceptance without causing serious GvHD has been limiting the degree of T-cell depletion in the graft.[285] Thus, BM allograft acceptance is influenced by mutual reactivity of donor as well as residual host T cells, dependent on the genetic disparity of donor and recipient and on the number of BM as well as T cells transplanted. Donor NK-cells do not play a major role in the engraftment of allogeneic BM cells.[56] Other cell types may influence the degree of mutual immunological reactivity of donor and recipient cells. Accessory cells may play a role in the immunogenicity of allogeneic BM grafts,[125] and thereby influence the outcome of allogeneic BMT. Numerous studies have shown that the BM cell dose is another important determinant for acceptance of allogeneic BM cells and subsequent survival of the recipient.[529] Host conditioning is also important for engraftment of allogeneic BM cells,[124,494] since it determines not only residual immunological resistance in the recipients,[124,417] but also the extent to which host immature HSC are ablated, making space for grafted donor type HSC.[124,387,541] As was discussed in Section 1.2.2, the microenvironment plays an important role in the regulation of hemopoiesis.[115] However, it is difficult to evaluate the exact role of BM stromal elements after allogeneic BMT, since they have been shown to be both of donor and recipient origin.[18,41,87,173,239,373,440]

1.4 MONOCLONAL ANTIBODIES FOR PREVENTION OF MARROW GRAFT REJECTION

The role of residual host T cells as important effector cells in allogeneic BM graft rejection makes T cells prime targets for *in vivo* manipulation with MCAs in an attempt to prevent marrow graft rejection.[543,544] T-cell functions have been manipulated *in vivo* with anti-CD4+ MCAs,[117] e.g., for the induction of tolerance to soluble protein antigens.[37,39,175,181,569]

After the discovery of synergistic action of mixtures of polyclonal antibodies directed against different cell surface antigens of guinea pig leukemic lymphocytes in C'-mediated cytotoxicity and *in vitro* antibody-dependent cellular toxicity (ADCC),[129] combinations of MCAs have often been used for

immunomodulation, and synergistic effects have been observed *in vitro* as well as *in vivo*.[52,184,208,209,403,543,544,575]

MCA isotype determines the ability to activate natural effector mechanisms *in vivo*, such as complement and ADCC, and the degree to which MCAs can eliminate their target cells.[543] Only the rat IgG_{2b} MCAs directed against the Thy-1 and other antigens were found to be effective *in vivo*,[52,92,93,96,128,184,185,543] and, although the immunosuppressive capacity also depended on the antigen density of the target cells,[257] the ability to activate these mechanisms *in vitro* has been used to predict the *in vivo* efficacy of MCAs. However, a number of experiments demonstrated later that *in vivo* efficacy of MCAs cannot be predicted solely on the basis of the ability to activate these mechanisms *in vitro*, and also that efficacious MCAs not always activate these mechanisms *in vivo*. Rat IgG_{2c} anti-Thy-1 MCA, although able to bind C', did not prevent GvHD, whereas the IgG_{2b} variant did both.[480] Intrinsic affinity for C1q was shown to be an important factor for the immunosuppressive potency of rat anti-Thy-1 MCAs, and *in vivo* depletion of C3 rendered $rIgG_{2c}$ MCAs unable to prevent GvHD, in contrast to $rIgG_{2b}$ MCAs.[481] These observations indicate that complete complement activation resulting in T-cell lysis is not required for *in vivo* (depleting) efficacy of MCAs.[481] Tolerance to soluble antigens as well as tissue grafts, including allogeneic BM cell grafts, could be induced by the use of MCAs of non-depleting isotypes and even $F(ab')_2$ fragments.[38,82,182,402,544]

The *in vivo* efficacy of murine MCAs appeared to depend on the isotype as well as the target antigens. Murine IgG_{2a} and IgG_1 MCAs were the most effective in the treatment of a murine B-cell lymphoma[234] and of rat EAE.[546] A T-cell lymphoma was most effectively treated with murine IgG_{2b} anti-Thy-1.1 MCAs.[108]

A genetically reshaped human IgG_1 MCA Campath-1H, constructed by transplanting the rat hypervariable regions into normal human immunoglobulin genes, was found to be effective in remission induction of a non-Hodgkin lymphoma[183] and in debulking autoimmune reactive cells in systemic vasculitis.[311] A humanized anti-human-$CD4^+$ MCA, with a human IgG_1 constant and murine variable regions, although partly effective in the treatment of mycosis fungoides, did, on the contrary, not deplete the target cells.[243] Thus,

although cytotoxic lysis of T cells is an important mechanism for immunosuppression, other mechanisms may be equally effective.

MCAs that have been used for prevention of bone marrow allograft rejection are summarized in Table 1.7. Anti-CD4+/CD8+ MCAs, in combination with TBI doses of 6 Gy or higher, allowed for (partial) allogeneic BM chimerism in mice.[94,95] Low percentages of donor type allogeneic chimerism across multiple minor histocompatibility as well as multiple minor histocompatibility barriers plus a class I MHC barrier could be obtained by a combination of anti-CD4+/CD8+ MCAs without TBI.[402] Preliminary data indicated that addition to this protocol of an anti-LFA1+ MCA and 3 Gy TBI allowed for skin graft tolerance across a complete H-2 barrier.[543,544] Anti-CD4+/CD8+ MCAs promoted engraftment of fully allogeneic BM and skin graft tolerance, if combined with 3 Gy (γ) TBI and 7 Gy (X) thymic irradiation.[434,436] When anti-NK1.1+ and anti-Thy-1.2+ MCAs were added to the conditioning regimen, low level engraftment of rat BM cells in mice, with again tolerance to (rat) skin grafts, was observed.[435] Anti-CD3+ MCAs and $F(ab')_2$ fragments promoted the engraftment of completely H-2 mismatched BM in C57BL mice measured between 44 and 63 days after BMT.[55]

Long-term engraftment of fully H-2 mismatched grafts was enhanced by the use of polyclonal anti-asialo-GM1+ antibodies.[486] Enhanced survival, engraftment and hemopoietic recovery was also observed after transplantation of syngeneic BM cells, indicating possibly a generally stimulating effect on hemopoietic recovery by the anti-asialo-GM1+ treatment.[486]

Engraftment of DLA-nonidentical marrow grafts was enhanced by the administration of an anti-Ia MCA directed against a large lymphocyte population, including NK cells,[105,106] as well as by the administration of a $mIgG_1$ MCA directed against CD44, a widespread cell adhesion molecule.[273,420,428]

The lymphocyte function associated antigen-1, LFA-1, is a member of a group of related glycoproteins expressed on leukocytes.[138,464] These cell surface proteins all have a unique α subunit (CD11a-c), share a β subunit (CD18), and play a role in the adherence and function of virtually all white blood cell types.[138,464] Cells expressing LFA-1 are therefore ideal targets for (combined) MCA therapy in the prevention of BM graft rejection. This is illustrated by the

Table 1.7: Combinations of monoclonal antibodies (MCAs) for prevention of BM allograft rejection.

Species	Target cell population	MCA (isotype)	Remarks	References (reference number)
mouse	$CD4^+/L3T4^+$ $CD8^+/Lyt2^+$	YTS191.1 ($rIgG_{2b}$) YTS169.4 ($rIgG_{2b}$)	mixed allogeneic chimerism in combination with TBI, resulting in tolerance to fully allogeneic skin grafts	Cobbold et al., 1986 (94 and 95)
mouse	$CD4^+/L3T4^+$ $CD8^+/Lyt2^+$ $CD8^+/Lyt3^+$	YTS191.1 YTA3.1 ($rIgG_{2b}$) YTS169.4 YTS156.7 ($rIgG_{2b}$)	low percentages of donor type chimerism (no TBI!), allowing for skin graft tolerance across multiple minor plus or minus a class I MHC barrier	Qin et al., 1989
mouse	$CD4^+$ $CD8^+$ $LFA\text{-}1^+$		in combination with low dose TBI (3Gy) resulting in hemopoietic chimerism with skin graft tolerance across complete H-2 barrier	Waldmann, 1989 Waldmann et al., 1989
mouse	$CD4^+$ $CD8^+$	G.K1.5 ($rIgG_{2b}$) 2.43 (r)	mixed allogeneic chimerism (31-79% donor blood cells) achieved by a conditioning regimen of MCA administration, 3 Gy TBI (γ), and 7 Gy thymic irradiation (X) before BMT; also induces skin graft tolerance	Sharabi and Sachs, 1989 Sharabi et al., 1992

Table 1.7 continued

Species	Target cell population	MCA (isotype)	Remarks	References
mouse	CD4+ CD8+ Thy-1.2+ NK1.1+	G.K1.5 ($rIgG_{2b}$) 2.43 (r) 30-H12 (r) PK136 (m)	low levels of mixed xenogeneic chimerism, deminishing gradually over time, with durable acceptance of donor skin grafts.	Sharabi et al., 1990
mouse	CD3+ CD5+/Ly-1+	145-2C11 (hamster IgG) 53-7.313 (rIgG2a)	MCA and $F(ab')_2$ fragments +/- coupled to immunotoxin (ricin toxin A) in combination with 6.5 Gy TBI (X): promotion of short (anti-CD3+/CD5+) and long term (anti-CD3+) engraftment of H-2 mismatched BM	Blazar et al., 1991
mouse	asialo-GM1+	polyclonal rabbit antiserum	increases fraction of mice with allogeneic BM cell engraftment in combibation with 11-12 Gy (γ) split dose TBI	Tiberghien et al., 1990
dog	Ia+	7.2 (IgG_{2b})	promotion of engraftment of DLA-nonidentical BM	Deeg et al., 1985 and 1987
dog	CD44+	S5 ($mIgG_1$)	promotion of engraftment of DLA-nonidentical BM	Schuening et al., 1987 Sandmeier et al., 1990

Table 1.7 continued

Species	Target cell population	MCA (isotype)	Remarks	References
anti-LFA-1 monoclonal antibodies				
man	CD11a	25-3 ($mIgG_1$)	successful in prevention of graft failure in recipients of HLA mismatched BM; patients with immunodeficiencies or osteopetrosis	Fischer et al., 1986 and 1991
man	CD11a	25-3 ($mIgG_1$)	unsuccessful in prevention of HLA-identical BM graft rejection in leukemia patients	Maraninchi et al., 1988, and 1989
man	CD18	M232 (IgG_1)	unsuccessful in prevention of HLA-identical and nonidentical BM graft rejection	Baume et al., 1989
mouse	CD11a	M7/15 ($rIgG_{2b}$)	improved hematological recovery, immunological recovery, and survival in recipients of fully H-2 mismatched BM	Van Dijken et al., 1990

observation that patients with a hereditary defect in the expression of this glycoprotein group could be cured by BMT with both HLA-identical,[139] and HLA-nonidentical BM grafts.[263] An mIgG1 anti-CD11a MCA has subsequently been reported to successfully prevent HLA-mismatched BM graft rejection in children with immunodeficiency and osteopetrosis.[140,141] The engraftment rate in this patient series was also influenced by the method of T-cell depletion, with better results in the E-rosette depleted group than in the Campath-1 plus C' treated group, and possibly by MCA dosage.[140,370] However, proper controls lacked in these clinical studies, since anti-CD11a treated groups were compared with historical controls and patients that received Campath MCAs as additional treatment, without a clear indication of other differences in conditioning between the groups.[140] Furthermore, survival was extremely low in the control groups.[140] The same MCA was unsuccessful in the prevention of HLA-matched BM graft rejection in adult leukemia patients,[302,303] and could not control acute rejection in kidney transplantation.[267] Administration of a mIgG1 MCA directed against the common β-subunit (CD18) did not prevent BM allograft rejection in adult leukemia patients.[35] In a murine study, recipients of fully allogeneic T-cell depleted grafts after conditioning with 11 Gy split dose TBI and anti-CD11a MCA showed improved hematologic and immunologic reconstitution as well as survival compared to animals conditioned with TBI alone, especially at high BM cell doses.[513] However, in this study, neither engraftment, nor degree of partial allogeneic chimerism was evaluated. Nevertheless, the ability of anti-CD11a MCAs to prevent GvHD in murine models demonstrated their *in vivo* efficacy.[186]

1.5 PARTIAL CHIMERISM IN (MURINE) HEMOPOIETIC DISEASES

Incomplete, mixed and partial chimerism are all synonyms for a condition in which the BM recipient has a mixture of recipient and donor type HSC and their descendants.[529] Split chimerism denotes the condition in which donor type hemopoiesis is limited to one or several specific lymphohematopoietic lineages.[529] Some human patients transplanted for severe combined immune deficiency (SCID), are classical examples of split chimeras.[104,165,174,178,527] Partial allogeneic chimerism was first observed in 'experiments of nature'. Dizygotic twins with placental vascular anastomoses in several species have been

shown to develop into stable partial allogeneic chimeras.[43,127,365,470] Recently, it has been recognized that partial chimerism is also a common phenomenon after HLA-matched BMT for hematological malignancies,[46,410,426] and stable partial allogeneic chimerism has been observed in patients transplanted for severe aplastic anemia,[459] as well as thalassemia.[31,288,347,422] In mice, partial allogeneic chimerism has been induced by the application of total lymphoid irradiation (TLI),[447,444] as well as by conditioning regimens that include the use of monoclonal antibodies (reviewed in Section 1.4).

The usefulness of BMT in the treatment of murine hereditary diseases has been reviewed by Barker.[24] Table 1.8 summarizes the diseases that can be completely or partially corrected by induction of partial (or sometimes split) chimerism. The anemia in mice homozygous for mutations at the *W*-locus,[188,413] can readily be corrected by infusion of +/+ cells without prior conditioning or after low dose TBI.[44,415,432] Transplantation of limited numbers of normal syngeneic +/+ cells to anemic and nonanemic *W*-mutants mice revealed that donor erythrocytes repopulated the recipients quicker and often to a greater extent than donor leukocytes and platelets until at least 1 year after BMT, indicating a temporal selective advantage of the normal erythroid lineage.[25,26,190] However, replacement of defective HSC is ultimately responsible for the correction of the anemia in these *W*-mutants. Therefore, the selective advantage exists at the level of HSC, although erythropoiesis is affected to a greater extent than the formation of other peripheral blood cells.[26]

The microcytic anemia in murine α-thalassemia can be (partially) corrected without total eradication of thalassemic HSC, i.e., by BMT with small numbers of syngeneic normal BM cells after sublethal TBI.[27,541] This phenomenon was explained by a selective advantage of the transplanted normal erythroid lineage, attributable to precipitation of excess globin chain[564] and increased cell death among the thalassemic erythroid cells in the hemoglobin synthesizing stages of erythropoiesis.[541] The shortened life span of the α-thalassemic red blood cells (35-37 days[394]) only marginally influenced the development of partial chimerism.[515] Although the studies in α-thalassemic mice were consistent with a selective advantage of the normal (+/+) erythroid lineage, the study by Barker and McFarland[27] did not exclude a stem cell lesion in α-thalassemic,

Table 1.8: Murine genetic diseases than can be completely or partially corrected by partial hemopoietic chimerism (adapted and extended from Barker,1988)

Genetic defect	Mechanism of (partial) cure	Conditioning	References
W-mutations	replacement of defective HSC temporal selective advantage of +/+ erythroid lineage during repopulation	no conditioning or low dose TBI	Russell et al., 1956 Bernstein and Russell, 1959 Seller, 1967 Barker et al., 1988 and 1991 Harrison and Astle, 1991
α-thalassemia	selective advantage of the +/+ erythroid lineage	low dose TBI (e.g. 3 Gy)	Barker and McFarland, 1985 Wagemaker et al., 1986
SCID mice	defective development of host lymphocytes; variable and incomplete chimerism in unirradiated hosts	no conditioning or low dose (4 Gy) TBI	Custer et al., 1985 Fulop and Phillips, 1986
CBA/N mice (x-linked immuno-deficiency)	selective advantage of normal B cells, due to intrinsic defect in *xid* B cell development; split B cell chimerism in unirradiated recipients	no conditioning or lethal TBI	Scher et al., 1975 Volf et al., 1978 Sprent and Bruce, 1984

Table 1.8 continued

Genetic defect	Mechanism of (partial) cure	Conditioning	References
osteopetrosis	partial chimerism, varying in different hemopoietic lineages; sufficient osteoclasts provided to ensure bone resorption	lethal (6-9 Gy) or low dose (4-6 Gy) TBI	Walker, 1975 Marshall et al., 1982
hemolytic anemia (sph^{ha}/sph^{ha})	selective advantage of the +/+ erythroid lineage	low dose (2 or 5 Gy) TBI	Barker and McFarland-Starr, 1989
acatalasemia = catalase deficiency	enzyme provided by donor derived cells	low dose (6 Gy) TBI	Hong et al., 1979
β-glucuronidase deficiency	(partial) correction of enzyme levels in various tissues; minimal in central nervous system; partial correction of storage in central nervous system	34 Gy TLI lethal or low dose (2 or 4 Gy) TBI	Slavin and Yatziv, 1980 Hoogerbrugge et al., 1987 Birkenmeier et al., 1991

whereas the study by Wagemaker et al.[541] did not require such an assumption.[27,541]

SCID mice lack mature lymphocytes.[64,122] Partial cure was reported after injection of syngeneic +/+ cells into unirradiated recipients,[103] whereas pre-transplant conditioning with low dose TBI resulted in normalization of surface-Ig bearing spleen cells and cytotoxic T cell activity.[153] Immunodeficient CBA/N mice can be cured by lethal TBI and injection of +/+ HSC,[425,462] whereas unirradiated recipients will become split B cell chimeras.[525] Since after BMT with normal cells following sublethal TBI in mice with severe hemolytic anemia (*sph*^{ha}/*sph*^{ha}) normal cells are either formed in insufficient numbers or are rapidly destroyed, this procedure resulted in an increase, but not in a normalization of the red blood cell counts.[28] In these mice, as in *W* mutant and α-thalassemic mice, a selective advantage of the normal erythroid lineage was found, in that the degree of red blood cell chimerism exceeded the degree of WBC and platelet chimerism.[28]

Murine osteopetrosis in the grey-lethal and microphthalmic variants is alleviated by BMT after sublethal TBI.[305,547] Catalase deficiency in mice, which resembles Takahara's disease in humans, renders the animals abnormally sensitive to intraperitoneally injected hydrogen peroxide.[203] Conditioning with sublethal TBI followed by injection with +/+ HSC normalized blood catalase levels and resistance to hydrogen peroxide.[203] Beta-glucuronidase deficiency in mice results in murine type VII mucopolysaccharidosis, a severe lysosomal storage disease. Beta-glucuronidase activity in mice with a low level of activity of this enzyme was shown to be increased in serum and various tissues but not in the central nervous system after allogeneic BMT following TLI or TBI.[204,448] Transplantation of +/+ cells after low dose TBI into mutant mice without any β-glucuronidase activity (*gus*^{mps}/*gus*^{mps}), has recently been shown to increase the life span approximately three-fold, approaching that seen in normal mice after BMT following low dose TBI.[53] Storage of glycosaminoglycans was partially corrected in the meninges and perivascular cells in the brain, although enzyme activity in the brain was still low.[53]

Induction of partial chimerism will not always correct a disease. In beige (Chediak-Higashi) mice, the prolonged bleeding time, resulting from a defect in platelet dense granules, can be corrected by lethal TBI and BMT.[316] Partial

chimerism only corrects the defect when over 50-75% normal platelets are present, suggesting that mutant platelets in the chimeric mice may interfere with platelet aggregation.[315] In motheathen mice, which suffer from a combination of immunodeficiency and autoimmunity, BMT with normal cells after 5 Gy TBI will prolong survival, but animals will eventually die because of disease recurrence.[429]

It is still difficult to induce stable partial allogeneic chimerism. Recently, in utero transplantation of preimmune competent fetal lambs, rhesus monkeys, and even mice with fetal HSC or purified xenogeneic human HSC has been shown to result in stable partial allo- and xenogeneic chimerism,[142,194,366,466,579] in which the donor type HSC and their descendants are functionally fully integrated in the hemopoietic system.[126,143] Although clinical experience with this approach is still very limited,[445,490-492] it may, in combination with prenatal diagnosis, become an important treatment modality in the future.

1.6 RADIOSENSITIVITY OF HEMOPOIETIC STEM CELLS

TBI is the most effective single agent used in conditioning regimens for BMT.[529] It creates bone marrow space for engraftment of transplanted (P)HSC, suppresses HvG reactivity, and, if combined with other cytotoxic agents, eradicates tumor cells in case of malignancy.529

When TBI is used as a single agent in BMT conditioning regimens, it determines the number of (P)HSC that survive radiation. The total number of HSC present in various animal species has been estimated by comparison of the LD_{50} for TBI (the dose at which 50% of the irradiated animals will survive) and the BM cell dose required for rescue of 50% of supralethally irradiated animals.[530,531] This method also allowed for estimation of the radiosensitivity of those cells that are important for survival of the animals after acute TBI.[504,530] Recent evidence, however, indicates that the cell populations important for survival of animals after TBI are probably not the cells that allow for long-term donor type hemopoiesis after BMT.[231] Most data on the radiosensitivity of murine HSC were obtained studying spleen colony-forming cells.[487] Table 1.9 summarizes the radiosensitivity data presented in literature of HSC and progenitor cells [indicated by the term D_0, i.e., the dose of irradiation required to reduce survival of the target cell population to 37% (e^{-1}) of control values].

Table 1.9: Radiosensitivity of HSC and progenitor cells

Species	Cell type	Radiation type	In vivo D_0 (s.e.) in Gy	Extrapolation number (s.e.)	In vitro D_0 (s.e.) in Gy	Extrapolation number (s.e.)	References
mouse	femoral CFU-S	γ (^{60}Co)			1.15 (0.08)	2	Till and McCulloch, 1961
	femoral CFU-S	γ (^{60}Co or ^{137}Cs)	1.00 (0.03)	1.4 (0.1)	1.15 (0.08)	1.6 (0.2)	Hendry and Lord, 1983*
	splenic CFU-S			D_0 = 0.93 (0.04), n = 1.0 (0.2)			
	femoral CFU-S	200-300 kV X-rays	0.77 (0.03)	1.7 (0.1)	0.79 (0.07)	2.1 (0.5)	
	splenic CFU-S	250-300 kV X-rays		D_0 = 0.66 (0.03), n = 1.1 (0.7)			
	femoral CFU-S	15 MeV electrons		D_0 = 1.13 (0.10), n = 1.6 (0.5)			
	femoral CFU-S	γ (^{60}Co)	0.62 (0.03)[a] 0.65 (0.02) 0.69 (0.02)	0.65 (0.15) 0.87 (0.08) 0.72 (0.08)			Glasgow et al., 1983

* = average values of the data reviewed by Hendry and Lord, 1983

a = tested with different dose rates, from top to bottom respectively, 1.03 Gy/min, 0.45 Gy/min, and 0.08 Gy/min

Table 1.9 continued

Species	Cell type	Radiation type	In vivo D_0 (s.e.) in Gy	Extrapolation number (s.e.)	In vitro D_0 (s.e.) in Gy	Extrapolation number (s.e.)	References
		γ (^{60}Co)	0.98				Peacock et al.,1986
		γ (^{137}Cs)	0.91 (0.01)[b] 0.79 (0.01)				Ploemacher et al., 1992
		200 kV X-rays	0.81 (0.05)	0.9			Imai and Nakano, 1987
		250 kV X-rays	0.80[c] 0.85				Tarbell et al., 1987
		300 kV X-rays	0.94 (0.03)[b] 0.71 (0.01)	1.25 (0.07) 1.04 (0.06)			Meijne et al.,1991
		1 MeV neutron	0.36[b] 0.31				Meijne et al., 1989
	MRA[CFU-C-12]	300 kV X-rays	1.18 (0.01)	1.39 (0.18)			Meijne et al., 1991
		1 MeV neutron	0.48 (0.05)				Meijne et al., 1990

b = parameters for CFU-S-12 (top) and CFU-S-7 (bottom)

c = parameters for high dose rate (0.8 Gy/min, top) and low dose rate (0.05 Gy/min, bottom), both for CFU-S-8

Table 1.9 continued

Species	Cell type	Radiation type	In vivo D_0 (s.e.) in Gy	Extrapolation number (s.e.)	In vitro D_0 (s.e.) in Gy	Extrapolation number (s.e.)	References
	MRA[CFU-C]	γ (^{137}Cs)	1.25 (0.25)				Ploemacher et al., 1992
		300 kV X-rays	1.13 (0.08)				Meijne et al., 1991
	CFU-C	γ (^{137}Cs)			1.60		Senn and McCulloch, 1970
		γ (^{137}Cs)	1.33 (0.10)				Ploemacher et al., 1992
		300 kV X-rays	1.47 (0.15)	1.28			Meijne et al., 1991
		200 kV X-ray	1.57 (0.11)	1.09			Imai and Nakao, 1987
	CFU-C	γ (^{137}Cs)			1.15 (0.09)[d] 1.29 (0.12) 1.23 (0.05) 0.42 (0.03)		Baird et al., 1990

d = CFU-C responsive to, respectively, rIL-3, rGM-CSF, purified M-CSF, and rG-CSF

Table 1.9 continued

Species	Cell type	Radiation type	In vivo D_0 (s.e.) in Gy	Extrapolation number (s.e.)	In vitro D_0 (s.e.) in Gy	Extrapolation number (s.e.)	References
		γ (^{137}Cs)			1.06 (0.08)[e] 1.42 (0.05) 1.36 (0.07) 1.75 (0.24)		Baird et al., 1991
	CFU-Mix	200 kV X-ray	1.44 (0.30)	1.03			Imai and Nakao, 1987
	BFU-E	200 kV X-ray	0.69 (0.09)	0.85			
	CFU-E	200 kV X-ray	0.53 (0.03)	0.76			
dog	GM-CFC	280 kV X-rays			0.24 (0.01)[f] 0.61 (0.01)		Nothdurft et al., 1983
	CFU-Mix	280 kV X-rays			0.12 (0.02)		Kreja et al., 1991

e = CFU-C responsive to, respectively, rIL-3 plus rIL-1α, (purified) pM-CSF plus rIL-1α, rIL-3 plus pM-CSF, and rIL-3 plus pM-CSF plus rIL-1α

f = parameters for GM-CFC from blood (top) and BM (bottom)

Table 1.9 continued

Species	Cell type	Radiation type	In vivo D_0 (s.e.) in Gy	Extrapolation number (s.e.)	In vitro D_0 (s.e.) in Gy	Extrapolation number (s.e.)	References
	BFU-E	250 kV X-rays			0.26 (0.09)		Schwartz et al., 1986
		280 kV X-rays			0.15 (0.02)		Kreja et al., 1989
					0.16 (0.02)		Kreja et al., 1991
		1 MeV neutrons			0.16 (0.03)		Schwartz et al., 1986
	CFU-E	250 kV X-rays			0.61 (0.05)		Schwartz et al., 1986
		1 MeV neutrons			0.27 (0.01)		
rhesus monkey	LTRA-cell[g]	6 MV X-rays	1.1				Wielenga et al., 1989 Wielenga, 1990
man	CFU-GEMM	γ (^{60}Co)			0.91 (0.07)		Neumann et al., 1981
		γ (^{137}Cs)			0.57		Uckun and Song, 1989

g = calculation based on repopulation kinetics after TBI doses, ranging from 4 to 10 Gy

Table 1.9 continued

Species	Cell type	Radiation type	In vivo D_0 (s.e.) in Gy	Extrapolation number (s.e.)	In vitro D_0 (s.e.) in Gy	Extrapolation number (s.e.)	References
	CFU-C	γ (^{137}Cs)			0.45-1.65[h]	1	Broxmeyer et al.,
					1.65	1.5	1976
		γ (^{137}Cs)			1.22 (0.06)[i]	1	Baird et al., 1989
					1.38 (0.07)	1	
					1.00 (0.08)	1	
					1.08 (0.07)	1	
					1.15 (0.13)	1	
					1.11 (0.03)	1	

h = dependent on different sources of CSA (colony-stimulating activity; top), and D_0 of CFU-C surviving pretreatment with hydroxyurea

i = CFU-C responsive to, respectively, IL-3, GM-CSF, G-CSF, IL-3 plus GM-CSF, IL-3 plus G-CSF, and GM-CSF plus G-CSF

Table 1.9 continued

Species	Cell type	Radiation type	In vivo D_0 (s.e.) in Gy	Extrapolation number (s.e.)	In vitro D_0 (s.e.) in Gy	Extrapolation number (s.e.)	References
		280 kV X-ray			1.46 (0.13)[j] 1.36 (0.09)		Grilli et al., 1982
	CFU-GM	γ (^{137}Cs)			1.37		Senn and McCulloch, 1970
		γ (^{137}Cs)			0.31		Uckun et al., 1989
	BFU-E	γ (^{137}Cs)			0.64		Uckun et al., 1989
		280 kV X-ray			0.93 (0.06)[j] 1.27 (0.11)		Grilli et al., 1982

j = parameters for CFU from blood (top) and BM (bottom)

D_0 values for murine CFU-S between 0.62 Gy and 1.70 Gy for γ-irradiation[171,196,369,487] and between 0.62 Gy and 1.05 Gy for 200-300 kV X-rays[196,216,477] have been reported. Recent data show that CFU-S-12 are not as radioresistant as the more immature HSC subsets, and that CFU-S-7 are more radiosensitive than CFU-S-12.[317-319,387] The relative radioresistance of more immature (P)HSC subsets was also observed when the D_0 for rhesus monkey BM (P)HSC was calculated on basis of repopulation kinetics after graded TBI doses.[565,566]

A comparable hierarchy in radiosensitivity was observed for *in vitro* clonable murine progenitor cells of the granulocyte/macrophage lineage.[21,23] This feature has so far not been confirmed for human progenitor cells.[22,75] Depending on the culture conditions, the reported D_0 values for human CFU-C/GM-CFU vary between 0.31 Gy and 1.65 Gy for γ-irradiation.[22,75,433,497] However, in our view, all D_0 values for γ-irradiation lower than 0.6 Gy must be considered as artifacts. A D_0 value of approximately 1.4 Gy was reported for human CFU-C in response to 280 kV X-rays.[177] Values of 0.91 Gy[348] and 0.57 Gy[498] were reported for the D_0 of human CFU-GEMM. The *in vitro* data are complicated by the observation that preincubation of BM cells with certain HGFs may decrease the radiosensitivity of human progenitor cells.[497] Radiosensitivity of *in vitro* clonable progenitors may depend not only on their primitivity, as inferred from sensitivity to hemopoietic growth factors, but also on their source, i.e., blood or BM.[177,353] D_0 data for canine progenitor cells are characterized by extremely low values between 0.12 and 0.61 Gy.[255,256,353,430]

Little is known about the radiobiological properties of cells capable of long term hemopoietic reconstitution (LTRA-cells). Data on murine HSC subsets, as discussed above, indicate that those cells are probably more radioresistant than CFU-S, and are also, in contrast to CFU-S, capable of some sublethal damage repair.[387]

1.7 RATIONALE AND OUTLINE OF THIS STUDY

The thalassemias represent one of the most common groups of hereditary disorders in humans. Conventional treatment of thalassemia with blood transfusions and iron chelation therapy is expensive and is accompanied by the risks currently related to the use of blood products.[12,551] The predicted growth

of the world population will for the greater part occur in those countries where the β-hemoglobinopathies are particularly common. It is a real challenge, therefore, to develop a treatment modality for thalassemia that can be used in the developing world.[551]

In case of available MHC-identical family donors, allogeneic bone marrow transplantation (BMT) may result in correction of these disorders.[484] BMT, however, requires high-level medical care and transplantation related problems have so far prevented widespread application of BMT in the management of thalassemic patients. Rejection rates of over 20% are still common in patients other than selected 'good-risk' patients, and conditioning regimens are often too toxic for patients with heart and liver disease due to iron overload. Furthermore, approximately 60% of the thalassemic children do not have an HLA-identical family donor.[299]

It had been shown that the microcytic anemia of murine α-thalassemia could be corrected by the induction of partial hemopoietic chimerism, induced by transplantation of small numbers of normal syngeneic BM cells, following a conditioning regimen of sublethal total body irradiation.[27,541] The correction was assumed to result from a selective advantage of the transplanted normal erythroid lineage.[541] Since improvement of the results of allogeneic BMT for thalassemia would require a transplantation procedure that allows for sustained allogeneic partial chimerism, the most important variables determining the outcome of BMT were studied in α- and β-thalassemic mouse models.

Estimation of the degree of selective advantage of normal erythropoiesis in partially chimeric α- and β-thalassemic mice after transplantation of normal syngeneic BM cells was considered a prerequisite to the development of a transplantation procedure that allows for sustained allogeneic partial chimerism. This was done by comparing the degree of peripheral blood red cell chimerism with the degrees of chimerism at the level of immature hemopoietic progenitor cells (CFU-S) and peripheral blood white cells. The methodology to assess peripheral blood red cell chimerism is discussed in Chapter III, and that for assessment of CFU-S chimerism in Chapter V. The results of chimerism analyses in stable partially chimeric α- and β-thalassemic mice are discussed in, respectively, Chapters V and VI. The compensatory splenic hemopoiesis of β-thalassemic mice is reported in Chapter IV.

Induction of appreciable levels of stable partial allogeneic chimerism in outbred species like man will only be obtained if conditioning regimens can be developed that allow for mutual tolerance of donor and recipient type BM derived cells. MCAs directed against T cells and, possibly, other cells that may evoke rejection of BM allografts, are specific immunomodulatory agents which are usually non-toxic for hemopoietic stem cells, while some of them may induce tolerance to allo-antigens. By these features, MCAs may be of key importance in the development of conditioning regimens aiming at sustained allogeneic partial bone marrow chimerism. Anti-CD4/CD8$^+$ MCAs combined with low dose TBI were reported to allow for the development of partial allogeneic chimerism.[94,95] An mIgG1 anti-CD11a MCA has been used successfully to prevent HLA-mismatched BM graft rejection in children with immunodeficiencies and osteopetrosis,[140,141] although its efficacy has so far not been demonstrated in a controlled randomized trial. It was therefore decided to study correction of murine thalassemia by partial allogeneic chimerism after conditioning regimens that combine sublethal TBI with combinations of MCAs directed against CD4$^+$, CD8$^+$ and CD11$^+$ cells. Results of this study are presented in Chapter VII of this thesis.

The actual degree of chimerism that will be obtained in a partial chimera represents a balance between the number of infused pluripotent, LTR-HSC that will seed and function in the recipients hemopoietic system, and the number of residual recipient type LTR-HSC. Since the latter number will be dependent on the radiation sensitivity of these cells and reliable data on the radiosensitivity of LTR-HSC are also important for the development of treatment modalities for radiation accident victims and cancer patients requiring TBI, we used the α-thalassemic mouse model also for a direct estimate of the radiosensitivity of LTR-HSC. The results of these experiments are described in Chapter VIII. In the final Chapter IX, experiments are described to identify the phenotypic properties of the LTR-HSC repopulating α-thalassemic mice, enabling their purification, which is of importance for a direct assessment of the properties ascribed to these cells, as well as for the development of 'engineered' bone marrow grafts and, probably, for genetic modification of diseased stem cells.

CHAPTER II
MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

2.1.1 Thalassemic mice

α-Thalassemic BALB/cAnCrlRij-Hba$^{th/+}$ mice were maintained under specific pathogen free (SPF) conditions at the breeding facilities of ITRI-TNO (Rijswijk, The Netherlands) by repeated backcrossing of thalassemic mice with the BALB/cAnCrlRij mouse strain. They were originally obtained in 1981 from Dr. R.A. Popp (The Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A.) from stock 352HB on a SEC/Re background. SPF C3C-Hbath F1 mice were maintained by mating C3H/LwRij females with BALB/cAnCrlRij-Hba$^{th/+}$ males. Mice were identified as α-thalassemic or normal on basis of their blood smears.[393]

β-Thalassemic mice C57BL/LiARij-Hbb-3th/Hbb-3th were maintained by repeated backcrossing with the C57BL/LiARij mouse strain. They were kindly donated in 1986 by Dr. J.E. Barker (The Jackson Laboratory, Bar Harbor, ME, U.S.A.) on a C57BL/6J background. Hemoglobin phenotypes of these mice were identified by hemoglobin electrophoresis using cystamine.[559]

2.1.2 Other mice

Other mouse strains used in this study are: BALB/cAnCrlRij, C57Bl/LiARij, and BCBA (C57Bl/LiARij x CBA/BrARij) F1. Outbred nude and BALB/cAnCrlRij-nu/nu mice were used for the production of monoclonal antibodies. All mice were bred under SPF conditions.

2.1.3 Animal housing, feeding, and caretaking

As indicated above, mice were bred and kept under under SPF conditions until the initiation of experiments. An exception to this rule were the α-thalassemic mice. They were identified as α-thalassemic or normal as described above, and thereafter housed in conventional animal quarters. In conventional animal quarters, mice were kept in cages with standard bedding (IFFA Credo Broekman B.V., Someren, The Netherlands), and fed with standard laboratory chow (Hope Farms B.V., Woerden, The Netherlands) and acidified water (pH 2.8).

In BMT experiments, mice were kept in a laminar air flow for the first weeks. After complete hematologic recovery, the mice were housed in conventional

animal quarters. Recipient mice for CFU-S determination were kept in laminar air flows for the duration of the experiment, with the exception of recipient mice for determination of CFU-S chimerism, that were housed in laminar air flows one week before TBI and were fed for the duration of the experiment with sterilized standard laboratory chow and water containing 100 mg/l polymixine B (Pfizer, New York, NY, U.S.A.), 100 mg/l ciprofloxacin (Bayer Nederland B.V., Mijdrecht, The Netherlands), and 100 mg/l natamycin (Royal Gist-Brocades, Delft, The Netherlands).

2.1.4 5-Fluorouracil treatment (Chapter IX)

For specific experiments, mice were injected with 150 mg 5-fluorouracil (Sigma, St.Louis, MO, U.S.A.) in phosphate-buffered saline (PBS) per kg body weight. They were killed by carbon dioxide inhalation 6 days after injection.

2.2 HEMOPOIETIC STEM CELL TRANSPLANTATION

2.2.1 Preparation of cell suspensions

Cell suspensions were prepared in ice cold H&H. H&H is Hanks' Balanced Salt Solution (Eurobio, Paris, France) buffered with 10 mmol/l HEPES buffer (Merck, Darmstadt, F.R.G.) at pH 6.9 with an osmolarity of 300-310 mOsm/l, containing 10^5 IU penicillin/l (Royal Gist-Brocades) and 100 mg streptomycin/l (Pharmachemie BV, Haarlem, The Netherlands). To obtain BM cell suspensions, femora of donor mice were flushed using 1 ml syringes (Asik, Rodby, Denmark) adapted with 25G needles (Terumo Europe N.V., Leuven, Belgium). Single cell suspensions were obtained by gentle pipetting and filtration through a nylon sieve. Spleen cell suspensions were obtained by cutting the spleen into pieces and passing these pieces through a nylon sieve. Nucleated cell counts were performed in a Bürker hemocytometer using Türk's (0.005% Crystal Violet, 1% acetic acid, in H_2O) staining solution. Cell concentrations were adjusted using H&H.

2.2.2 Total body irradiation (TBI)

Total body irradiation was given using a two-source 800 Ci ^{137}Cs gamma irradiator (Atomic Energy of Canada, Ottawa, Canada) at a dose rate between 0.75 and 0.69 Gy/min or at a dose rate between 1.04 and 1.06 Gy/min (Chapter

IX; Rotterdam location). A maximum of 25 mice was irradiated at the same time, using specifically constructed animal containers flushed with air. Unless stated otherwise, TBI was performed the day before injection of the BM or spleen cell suspensions.

2.2.3 Injection of cell suspensions

After warming the mice by a lamp, the BM cells were transplanted by injection into the lateral tail vein in 0.5 ml H&H, using a 2 ml syringe (Asik) adapted with a 25G needle.

2.2.4 Preparation of cell suspensions in sorting experiments (Chapter IX)

Preparation of BM cells and buoyant density centrifugation using a discontinuous Ficoll-400 (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient, was performed as previously described.[383,385] Cells with a density of 1.069-1.075 g/ml or 1.069-1.078 g/ml, from untreated or 5-fluorouracil treated mice, respectively, were collected from the interphases, washed in PBS containing 5% fetal calf serum (FCS) and maintained on ice throughout the staining and purification procedures.

2.2.5 Hemopoietic stem cell purification procedure (Chapter IX)*

Low density bone marrow cells were depleted of monocytes and granulocytes by magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) using monoclonal antibody ER-MP20 (rat IgG_{2a}).[266] For magnetic sorting, cells were incubated for 30 minutes with biotinylated ER-MP20 followed by a streptavidin-conjugated phycoerythrin (sAV-PE; Caltag, South San Francisco, CA, U.S.A.) diluted in PBS containing 0.01% sodium azide (PBS-SA). Antibodies and conjugates were titrated for optimal staining of mouse bone marrow. The cells were then washed in PBS-SA containing 5 mM EDTA (Titriplex III; Merck) and incubated for 15 minutes with a 1:100 dilution of biotinylated paramagnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, F.R.G.), at a concentration of 1-2 x 10^8 cells/ml. After washing in PBS-SA containing 5 mM EDTA and 1% (w/v) bovine serum albumin

* Performed by J.C.M. van der Loo at the laboratory of dr R.E. Poemacher, Erasmus University Rotterdam

(Fraction V; Sigma), the cells were separated on the MACS column A2 (Miltenyi Biotec). The flow-rate was set at 0.08-0.10 ml/min using a 27G x 7/8" (0.42 x 22 mm) needle. The non-magnetic ER-MP20 negative population (ER-MP20$^-$) was collected and maintained on ice in PBS containing 5% FCS. For separation by FACS, low density bone marrow cells were stained for 30 minutes with biotinylated ER-MP20 followed by sAV-PE in PBS-SA containing 5% bovine serum albumin. The ER-MP20$^-$ cells, obtained by either FACS or MACS, were further separated by FACS on the basis of Rh123 retention or affinity for WGA. Low density bone marrow cells from 5-fluorouracil treated mice (LD/FU$_{6d}$BM) were not separated using ER-MP20, but were directly labeled with WGA for further sorting. Cells were incubated for 30 minutes in PBS containing 5% FCS and 0.1 µg/ml Rh123 (Eastman Kodak, Rochester, NY, U.S.A.) at 37 °C, or with 0.25 µg/ml fluorescinated WGA (WGA-FITC; Vector, Burlingame, CA, U.S.A.) at room temperature, as previously described.[377,379] After sorting, the WGA-labeled cells were incubated for 30 minutes at 37° C in 0.2 M of the competitive sugar *N*-acetyl-*D*-glucosamine (Sigma) to remove surface-bound WGA. Cell sorting was performed on a FACS II (B-D Systems, Becton Dickinson, Sunnyvale, CA, U.S.A) at a rate of 2500 cells per second using a single argon laser tuned at 488 nm (350 mW).

2.3 HEMATOLOGICAL MEASUREMENTS

Hematocrits were determined using standard hematocrit capillaries (Hirschman, F.R.G.) and centrifuge (Hawksley, Lancing, England). White blood cell counts were performed in a Bürker hemocytometer using Türk's staining solution. Differential white blood cell counts were performed after May-Grünwald-Giemsa staining. Red blood cell counts (Chapter III) were performed in phosphate buffered saline (PBS, NBPI, Emmer-Compascuum, The Netherlands) in a Bürker hemocytometer.

2.4 OSMOTIC FRAGILITY TEST

Normal and thalassemic mice were bled by means of eye extraction under ether anesthesia. Blood was collected in 50% heparin (Thromboliquine®, Organon Technika B.V., Boxtel, The Netherlands) in H&H to avoid clotting. Equal amounts of blood cells were distributed in Falcon 2052 tubes (Becton Dickinson

Labware, Lincoln Park, NY, U.S.A.) and washed once for 5 min (1200-1500 RPM) in a Beckman TJ-6 centrifuge (Beckman Instruments Nederland B.V., Mijdrecht, The Netherlands). Cell pellets were resuspended in a series of sodium chloride solutions, in PBS and in water. Cells were washed again for 5 min (1200-1500 RPM) and absorbance of by the supernatant was determined using an Uvikon 810 spectrophotometer at 416 nm (Kontron Instruments, Zurich, Switzerland).

2.5 MONOCLONAL ANTIBODIES

The rat myeloma Y3/Ag1.2.3.[156] derived hybridomas YTS169.4.2.1, YTS191.1.2 and YTA3.1.2, producing strongly immunosuppressive rat IgG_{2b} MCAs directed against, respectively, the murine CD8, CD4 epitope P, and CD4 epitope Q antigens[39,92,94,95,403] were kindly provided by Dr. H. Waldmann (Department of Pathology, Cambridge University, U.K.). The ATCC cell lines TIB217 and TIB218[418,419] producing rat IgG_{2a} MCAs against, respectively, the murine CD11a and CD18 antigens, were obtained through Dr. L. Nagelkerken (IVVO-TNO, Leiden, The Netherlands). MCAs were harvested as ascites from outbred nude and BALB/c nude mice, that had been treated with pristane (Aldrich-Chemie, Steinheim, F.R.G.) 1 to 3 weeks before inoculation with the hybridoma cell lines. Ascites was centrifuged for 15 min at 12,000 RPM and 4° C in a Sorvall RC5C centrifuge (Du Pont Co., Medical Products, Wilmington, DA, USA), equipped with a Sorvall SA-600 rotor (Du Pont Co., Medical Products) to remove debris. The supernatant was filtrated (0.45 µm, millex-HA, Millipore, Molsheim, France), and affinity purified using a Protein G-Sepharose 4 Fast Flow column (Pharmacia) equilibrated in PBS on an FPLC system (Pharmacia). The MCAs were eluated with 100 mmol/l glycine-HCL pH 3.0 (Merck). The eluate was dialyzed overnight against PBS at 3 kD cut-off. The amount of purified IgG was determined by a colorimetric protein assay (BIO-RAD, München, F.R.G.) with bovine serum albumin (BIO-RAD) as a standard protein. Reactivity of newly purified batches of MCAs were tested by comparing fluorescence intensity on murine thymus and spleen cells, using fluorescinated goat anti-rat serum (GARA-FITC, Nordic, Tilburg, The Netherlands) as a second-layer reagent, with that of a batch of known reactivity on a FACScan flow cytometer equiped with an argon laser emitting 488 nm

light (Becton Dickinson), using the Consort 30 software package for analysis of the results. To obtain prevention of BM allograft rejection, MCAs were administered i.v. in 0.5-0.75 ml H&H, 5 days before BMT in the following doses: YTS169, 200 μg; YTS191, 200 μg; YTA3.1.2, 400 μg; TIB217, 400 μg; and TIB218, 400 μg. To obtain T-cell depletion in allogeneic BMT experiments, donor mice were injected with the same doses of anti-CD4 and anti-CD8 MCAs 4 and 1 days before BMT.

Rat IgG_{2a} anti-murine CD4 (H129.19[372]) and CD8 (53-6.72[262]) MCAs were used for determination of peripheral blood T-cell content in β-thalassemic mice, using fluorescinated rabbit anti-rat serum (RARa-FITC, Nordic) as a second layer reagent. The purified MCAs H129.19 and 53-6.72 were kindly provided by A.C. Knulst (Department of Immunology, Erasmus University, Rotterdam, The Netherlands). Peripheral blood B-cell content was measured with fluorescinated goat anti-mouse serum (GAM-FITC, Nordic).

2.6 THE SPLEEN COLONY ASSAY

This assay was performed as described by Till and McCulloch.[487] Briefly, mice were injected with $5x10^4$ BM cells or $5x10^5$ spleen cells in H&H one day after TBI as described in Section 2.2. Twelve (CFU-S-12) days later, mice were sacrificed, and spleens were excised and fixed in Tellyesniczky's solution (64% ethanol, 5% acetic acid and 2% formaldehyde in H_2O). Macroscopically visible colonies were counted. For determination of CFU-S chimerism in stable partial chimeric mice, the spleens were not fixed, but individual colonies were dissected and processed as will be described in Sections 2.9 and 2.10.

2.7 IN VITRO ASSAYS FOR HEMOPOIETIC PROGENITOR CELLS

Serum free methylcellulose cultures were used in this study.[320,538,534,541] Appropriate numbers of bone marrow or spleen cells were suspended in α300-medium, i.e., Dulbecco's modified eagle medium (Dulbecco's MEM) obtained from GIBCO (Life Technologies LTD, Paisley, Scotland) supplemented with the amino acids L-alanine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid and L-proline (Sigma), vitamin B12, biotin, Na-pyruvate, glucose, $NaHCO_3$ and antibiotics (penicillin and streptomycin) at an osmolarity of 300 mOsm/l. Appropriate numbers of cells in α300 containing 0.8%

methylcellulose (Methocel A4M Premium Grade, Dow Chemical Co., St.Louis, U.S.A.), 1% bovine serum albumin (BSA, Fraction V, Sigma), $4x10^{-6}$ mol/l iron-saturated human transferrin (Behringwerke, Marburg, F.R.G.), 10^{-7} mol/l Na_2SeO_3 (Merck), 10^{-4} mol/l β-mercapto-ethanol (Merck), linoleic acid (Merck) and cholesterol (Sigma) at a final concentration between $7.5x10^{-6}$ mol/l and $1.5x10^{-5}$ mol/l for both, depending on the kind of progenitor cell colony cultured, and 10^{-3} g/l nucleosides (cytidine, adenosine, uridine, guanosine, 2'-deoxycytidine, 2'-deoxyadenosine, thymidine and 2'-deoxyguanosine obtained from Sigma) were plated in 35 mm Falcon 1008 Petri dishes (Becton Dickinson Labware) in 1 ml aliquots.[179]

Granulocyte/macrophage colony formation was stimulated by a saturating concentration of M-CSF,[59,320,509,536] purified from pregnant mouse uteri extract (PMUE) essentially as described before,[70,468] with or without 20% mouse spleen conditioned medium (MSCM), i.e., concanavalin-A stimulated mouse spleen cell culture supernatant.[218,538] GM-CFU colonies were counted after 7 days of culture. The number of $GM\text{-}CFU_{mscm}$ was calculated by subtracting the numbers of $GM\text{-}CFU_{m\text{-}csf}$, detected in cultures stimulated with M-CSF alone, from the numbers of colonies obtained by stimulation with both MSCM and M-CSF.

BFU-E growth was stimulated by 20% MSCM and murine erythropoietin (Ep), purified from the serum of phenylhydrazine treated mice, titrated to an optimal concentration. Colonies were counted after 10 days of culture. CFU-E growth was stimulated by purified erythropoietin alone and colonies were counted after 2 days of culture. The culture medium of the erythroid progenitors also contained hemine (bovine, type I, Sigma) at a concentration of $2x10^{-4}$ mol/l.[336]

Megakaryocyte progenitor cells (CFU-Meg) were cultured in 0.25% agar cultures,[344] using the same supplemented medium as for the other progenitor cells. Colony formation was stimulated by 6.7% 10x MSCM. After 10 days, colonies were dried, stained for acetylcholinesterase positive cells and counted.[224,236] All cultures were grown at 37 °C in a fully humidified atmosphere of 10% CO_2 in air.

2.8. THE CAFC-ASSAY (Chapter IX)**

Long-term bone marrow cultures were established in 96-well plates for limiting dilution analysis of cobblestone area forming cells (CAFC) as previously described.[386,384,385] Stromal layers were irradiated at confluency with a total dose of 20 Gy. After irradiation, the stroma cells were overlaid with 8-12 two-fold dilutions of the sorted or unseparated BM cells, using 15 wells per concentration. The culture medium consisted of α-modified Dulbecco's modified Eagle's medium (Flow, McClean, VA, U.S.A.) at 285 mOsmol/kg, containing 10^{-4} M β-mercaptoethanol, 10^{-5} M hydrocortisone 21-hemisuccinate (Sigma), 2 mM L-glutamine, 10^{-7} M sodium selenite, 100 IU/ml penicillin and 100 μg/ml streptomycin, supplemented with 20% horse serum. The wells were inspected every 2-3 days, between 3 and 35 days after inoculation, using a phase-contrast inverted microscope and scored positively if at least one cobblestone area was observed. The frequency of CAFC was calculated using the maximum likelihood solution.[134,471,511]

2.9 ANALYSES OF CHIMERISM

2.9.1 Assessment of peripheral blood red cell chimerism

Peripheral blood red cell chimerism was assessed using a FACScan flow cytometer. Blood obtained by tail clipping was suspended in hypotonic saline (103 mmol/l) and analyzed by measuring forward light scatter (FLS) distributions. Cytometer settings were specifically adjusted to allow the peak of the distribution curve of the normal red blood cells to be around channel 110 in the pulse height histogram. For the thalassemic red blood cells, the peak was around channel 60. Cytometer data were collected and analyzed using the Consort 30 software package. Ten thousand events were collected per sample. The development of this method will be discussed in detail in Chapter III. RBC chimerism was analyzed quantitatively in α-thalassemic mice, and semi-quantitatively in β-thalassemic mice (Chapter VI).

** The CAFC-assays described were performed by J.C.M. van der Loo and M.R.M. Baert, at the laboratory of dr R.E. Poemacher.

2.9.2 Assessment of peripheral blood white cell chimerism

Peripheral blood white cell chimerism in recipients of sex mismatched bone marrow cells was assessed by fluorescent *in situ* hybridization using a murine Y-chromosome specific probe. This method will be described in detail in Section 2.10. To assess chimerism, duplicate blood smears were prepared from individual chimeric mice and fixated in 100% methanol for 10 min at room temperature. The slides were air-dried and stored at -20 °C until the *in situ* procedure. Percentages of nucleated male blood cells were scored in independently processed duplicate blood smears of individual mice and are expressed as the arithmetic means of the counts. A minimum of 100 cells was scored in each blood smear. Control mice for both sexes were included. Male control mice almost always showed >96% positive cells, whereas female control mice almost always showed <2% positive cells.[511]

2.9.3 Assessment of CFU-S chimerism

To assess CFU-S chimerism, aliquots of $5x10^4$ BM cells or $5x10^5$ spleen cells from individual chimeric mice were injected intravenously in lethally irradiated syngeneic female recipient mice. After 12 days the recipient mice were sacrified and the spleens were removed. Individually dissected colonies were sieved through a nylon filter in 154 mmol/l NaCl and washed once (1500-2000 RPM, 5-7 min). Cells were subsequently washed three times in methanol/acetic acid (3:1). After the last wash, the supernatant was discarded and the cells were resuspended in the remaining methanol/acetic acid and applied to well cleaned slides. Slides were allowed to air-dry and stored at -20 °C. Individual colonies were scored as male or female after fluorescent *in situ* hybridization. All washes for this procedure were performed in a Beckman TJ-6 centrifuge.

2.9.4 Chimerism in other hemopoietic organs and lineages (Chapter IX)

At 9 and 12 months after transplantation, individual recipient mice were studied for the organ distribution and extent of lineage expression of the donor stem cells. Monocytes and granulocytes were sorted from bone marrow within a medium to high forward and perpendicular light scatter window, using antibody ER-MP20. B-lymphocytes were sorted from the spleen using antibody RA3-6B2 (anti-B220)[97] and T-lymphocytes were directly harvested from the

thymus. For FISH, approximately 2 x 10^5 BMC, spleen cells, thymic lymphocytes or sorted cells were transferred onto slides and spread using a drop of serum. In addition, cytospin preparations of the sorted cells were stained with May-Grünwald-Giemsa for differential counting.

2.10 FLUORESCENT *IN SITU* HYBRIDIZATION

In situ hybridization was performed using a modification of an earlier described protocol.[375] The area of cells for hybridization was marked using a glass cutter. The following solutions were applied consecutively as pretreatment: 0.05% Triton X-100 (Merck) in 100 mmol/l HCL under a plastic coverslip for 7.5 min at 37° C in a moisture chamber and 1% formaldehyde (Merck) in phosphate buffered saline (PBS) for 15 min at room temperature. Slides were dehydrated after removal of excess fluid in an ethanol series (70%/90%/100%) at room temperature and allowed to air-dry.

The hybridization mix consisted of (final concentrations) 50% deionised formamide (Merck), 2x SSC (SSC = standard saline citrate, 20x SSC = 3 mol/l NaCl and 0.3 mol/l sodiumcitrate at pH 7), 10% dextran sulfate (Sigma), 0.1% Tween-20 (Merck-Schuchardt, Hohenbrunn bei München, F.R.G.), 0.5 mg/ml carrier herring sperm DNA (Boerhringer-Mannheim, Mannheim, F.R.G.), and 2.5 µg/ml murine Y-specific DNA. The EcoRI fragment 2 of clone M34 in the plasmid pJRD 158 B was used as murine Y-chromosome specific DNA.[441,442] It was kindly provided by Dr. L. Singh (CCMB, Hyderabad, India) through Dr. J.W.M. Visser (ITRI-TNO). The probe DNA was labeled by nick-translation with biotin-16-dUTP (Boehringer-Mannheim) according to the instructions of the supplier (BRL, Gaithersburg, MD, U.S.A.), with the exception that the reaction was allowed to take place for 150 min in stead of 90 min. Total plasmid DNA was used for the in situ hybridization.

The hybridization mix (5 µl) was added under a plastic coverslip and both target- and probe DNA were denatured simultaneously for 5-7 min at 80° C. The slides were transferred directly to a moisture chamber at 37° C and hybridized overnight. After hybridization, the slides were consecutively washed in 2x SSC at room temperature, 50% formamide / 2x SSC (pH 7.0-7.4; final concentrations) at 37° C for 2 x 5 min, and in 2x SSC at 37° C for 2 x 5 min. Slides were placed in 0.1% Triton X-100 in 4x SSC(= wash buffer) at room

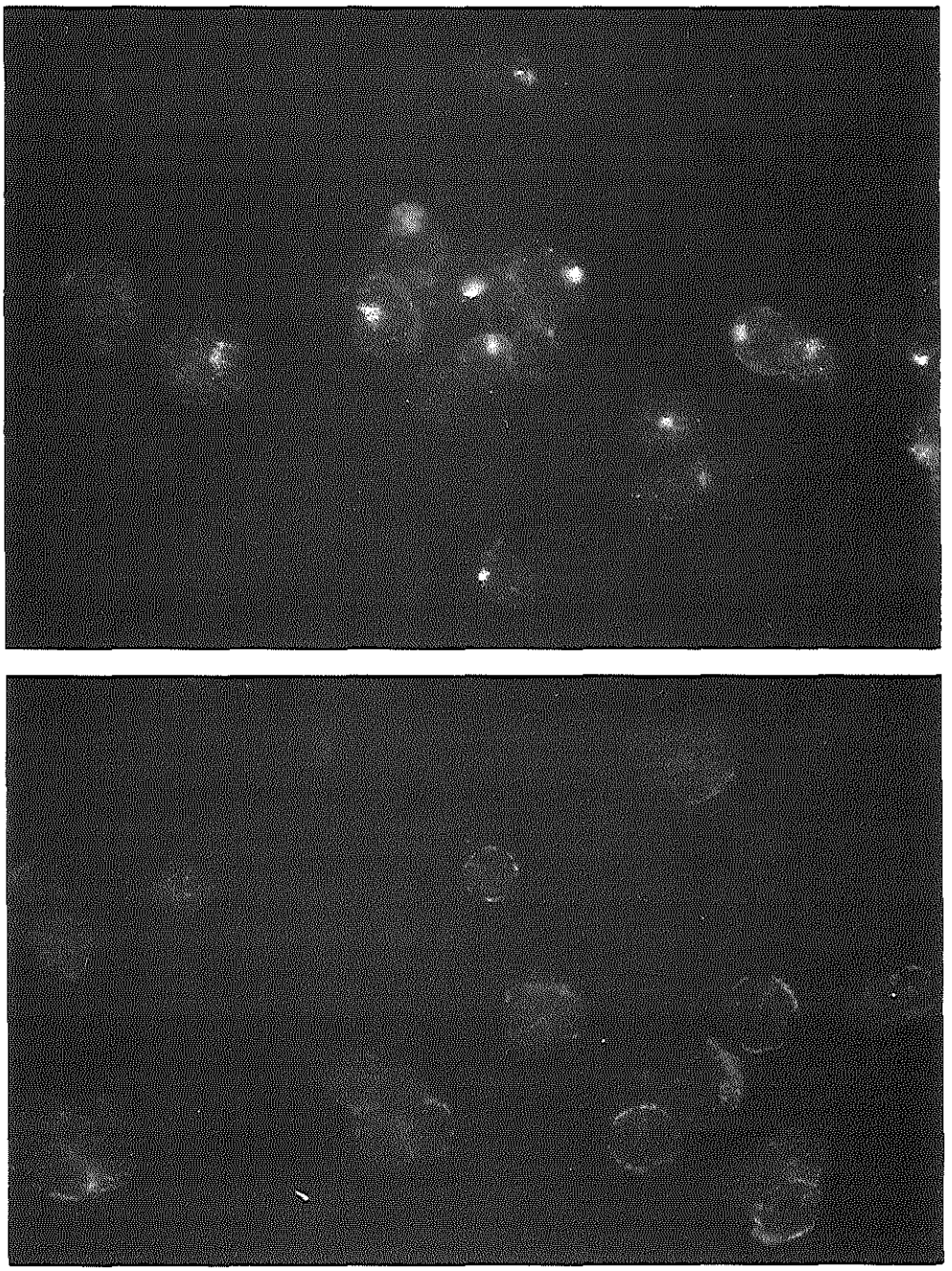


Figure 2.1: Fluorescent in situ hybridization with a murine Y-chromosome specific probe

temperature awaiting cytochemical detection. To this end 20 µl avidin-FITC (Vector) at a concentration of 2 µg/ml in wash buffer containing 3% BSA (Sigma) was added and the slides were placed in a moisture chamber at 37° C for 20 min. After two washes at 37° C for 5 min, 20 µl biotinylated goat anti-avidin (Vector) at a concentration of 5 µg/ml in wash buffer containing 3% BSA was added and slides were again placed in a moisture chamber at 37° C for 20 min. After one more incubation with avidin-FITC, the slides were washed, dehydrated, and allowed to air-dry.

The slides were mounted in antifade medium, consisting of 9 parts glycerol (Merck) containing 2% (w/v) DABCO (Sigma) as an antifading reagent, and 1 part 0.02% NaN_3 in 200 mmol/l Tris-HCl (pH 7.5). Propidium iodide (Sigma) 0.05 µg/ml (final concentration) was added as a DNA counterstain (Figure 2.1).

2.11 STATISTICAL ANALYSES

Mean, standard deviation (s.d.), standard errors (s.e.), and linear regression statistics were calculated using standard statistical formulas. Linear regression lines were compared using F- and Student's t-statistics. Confidence intervals for regression lines and observations were calculated using Student's t-statistics. Means of samples of normally distributed variables were compared using the Student's t-test. The chi-square test and the exact probability test were used to calculate the significance of differences in rates and proportions.[90,170,474]

The results of the colony assays in Chapter IV are expressed as means ± standard deviations (s.d.) per femur or per spleen. Mean values of hemopoietic progenitor cell cultures were weighted on the assumption that crude colony counts are Poisson distributed.[54] In this way, standard deviations reflected all four sources of variation: (i) the Poisson distribution and (ii) the experiment-to-experiment variation of the culture technique; the mouse-to-mouse variation in (iii) progenitor cell frequency and (iv) bone marrow or splenic cellularity. The contribution of the spleen to the total numbers of progenitor cells in Chapter IV was similarly derived on the assumption that one femur contains 8.5% of the total bone marrow.[326] Significance of differences observed was calculated by the nonparametric Mann-Whitney Rank Sum test.

The surviving fractions of CFU-S-12 per femur (Chapter VIII) were calculated on the assumption that colony numbers in the spleens of recipient mice follow a

Poisson distribution as well.[54] Mean surviving fractions of two experiments were calculated using the standard errors of the surviving fractions for calculation of weight factors. The D_0 for CFU-S-12 was calculated according to the single-hit model, using weight factors derived from the standard errors of the mean surviving fractions. The calculation of D_0 values for murine hemopoietic stem cells with long-term repopulating ability will be described in detail in Chapter VIII.

CHAPTER III

FLOW CYTOMETRIC ANALYSIS OF PERIPHERAL BLOOD ERYTHROCYTE CHIMERISM IN α-THALASSEMIC MICE

A rapid and reliable method for longitudinal studies on the degree of red cell chimerism following bone marrow transplantation of α-thalassemic recipient mice is presented. Blood obtained by tail clipping from transplanted mice was analyzed by measuring forward light scatter (FLS) distribution of red cells using a flow cytometer. Amplification and threshold of FLS were specifically adjusted. For flow cytometric analysis, the red cells needed to be suspended in hypotonic saline (103 mmol/l NaCl). Osmotic fragility testing showed that lysis of erythrocytes did not significantly influence the measurements. Flow cytometric measurements allowed for a rapid assessment of the degree of red cell chimerism.

adapted from:

Cor van den Bos, Francis C.J.M. van Gils, Rolf W. Bartstra, and Gerard Wagemaker: Cytometry (1992) 13:659-662

3.1 INTRODUCTION

Mice heterozygous for the deletion of both non-allelic α-genes[416,561] show a microcytic, hypochromic anemia.[393] As was discussed in Chapter I, such α-thalassemic mice can be cured by infusion of low numbers of normal syngeneic bone marrow cells after a sublethal dose of total body irradiation (TBI).[27,541] This procedure results in partial bone marrow chimerism with a preponderance of normal red cells.[541] Red blood cell chimerism was previously determined in peripheral blood smears or by Coulter analysis. The former method is time consuming and neither method allows for quantitative estimation of the degree of peripheral erythroid chimerism, which is needed to study radiation dose, immunosuppression and bone marrow graft size required to correct thalassemia by partial allogeneic chimerism. In the method described in this study, advantage was taken of the smaller average size of the thalassemic erythrocytes as compared to those of healthy litter mates, as can be readily shown by forward light scatter (FLS) distribution. On this basis, we report here a method to determine the degree of peripheral blood erythrocyte chimerism using flow cytometry.

3.2 RESULTS

3.2.1 Assessment of peripheral erythroid chimerism using the FACScan

The high resolution of the FACScan tends to a bimodal FLS distribution for normal red blood cells in isotonic PBS or saline because of the biconcave shape of these cells. As a consequence, only a marginal difference between normal and thalassemic red blood cell distributions was obtained. We therefore attempted to measure the FLS distributions in a hypotonic solution so as to promote swelling and loss of the biconcave shape of the red blood cells. To this end, normal erythrocytes were collected in a series of NaCl solutions ranging from 154 mmol/l to 68 mmol/l. When normal erythrocytes were measured in a 103 mmol/l NaCl solution, an almost symmetrical distribution was obtained (Figure 3.1a). In contrast, the FLS distribution of thalassemic red blood cells suspended in 103 mmol/l NaCl was hardly influenced compared to that in PBS (Figure 3.1b). Figure 3.1c shows FLS distribution curves for an equal mixture of healthy and thalassemic red blood cells suspended in PBS or in 103 mmol/l NaCl. Figure 3.1d shows the same for a partial red blood cell chimera.

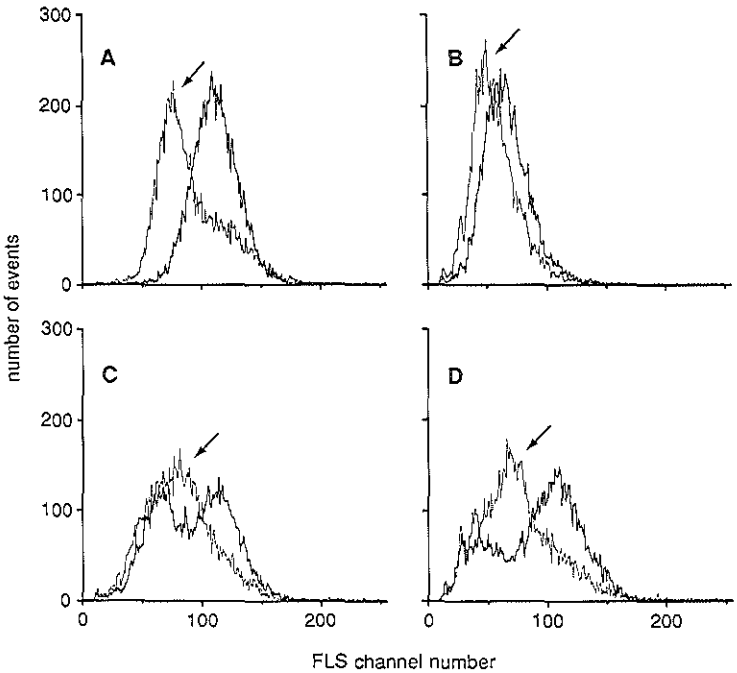


Figure 3.1: Forward light scatter (FLS) distribution curves: A: Normal red blood cells. B: Thalassemic red blood cells. C: A 1:1 mixture of normal and thalassemic red blood cells. D: Red blood cells of a partial chimera.Dotted lines / arrows: measurements in PBS. Solid lines: measurements in 103 mmol/l NaCl.

3.2.2 Lysis studies on normal and thalassemic erythrocytes

The results of osmotic fragility tests are shown in Figure 3.2 (pooled data of two experiments). It is shown that thalassemic red blood cells have a significantly greater tendency to lyse at 103 mmol/l NaCl than the red blood cells of normal litter mates (Student's t test $P<0.01$). However, when suspended in 103mmol/l NaCl, lysis of thalassemic as well as normal erythrocytes is limited and does not significantly influence the degree of red cell chimerism measured.

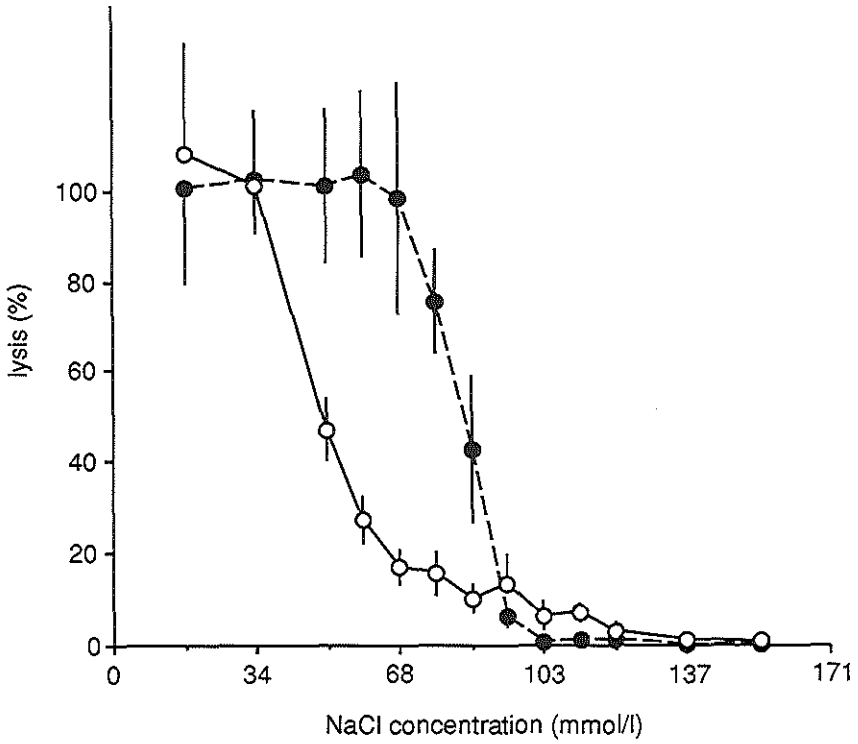


Figure 3.2: Osmotic fragility testing of normal (broken line) and thalassemic (solid line) red blood cells. X-axis: sodium chloride concentration. Y-axis: percentage of lysis measured using an absorbance at 416 nm, compared to the absorbance of a water lysed sample (mean ± s.d)

3.2.3. Development of a quantitative method to determine partial peripheral erythroid chimerism.

To develop a quantitative method for estimation of red blood cell chimerism, normal and thalassemic erythrocytes from unirradiated controls were collected in PBS and, after counting in a Bürker hemocytometer, mixed in 103 mmol/l NaCl to give different percentages of normal erythrocytes in suspension. During single parameter analysis, the markers were set in such a way that the percentage of red blood cells of a normal control mouse outside the high FLS

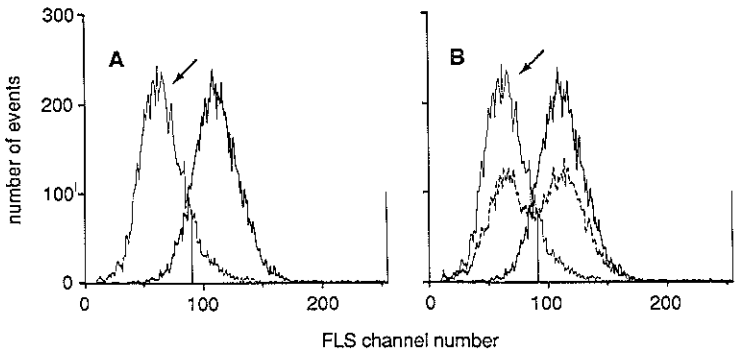


Figure 3.3: **A**: Forward light scatter (FLS) distribution curves of normal (solid line) and thalassemic (dotted line / arrow) red blood cells in 103 mmol/l NaCl; markers set as described in the text. **B**: FLS distribution curves of normal (solid line) and thalassemic (dotted line / arrow) red blood cells. Interrupted line and bimodal curve: a 1:1 mixture of normal and thalassemic red blood cells in 103 mmol/l NaCl.

gate, determined by the markers, was approximately equal to the percentage of red blood cells of a thalassemic control mouse in the high FLS gate, as is shown in Figure 3.3. Figure 3.4 shows the percentage of red blood cells in the high FLS gate as a function of the percentage of normal erythrocytes in the suspension (data from 14 independent experiments). A second order polynomial fit of the data was used as a calibration curve to assess red blood cell chimerism of individual mice. A straight line was not expected due to asymmetry of thalassemic and (less so) normal erythrocyte FLS distributions. This type of FACScan analysis of red blood cell chimerism allows for 100 measurements per hour.

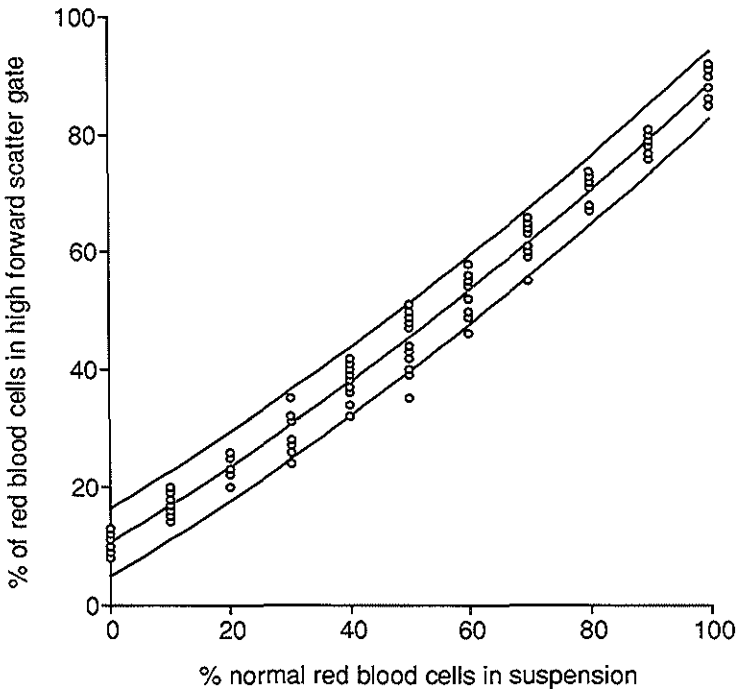


Figure 3.4: FACScan analysis of artificial degrees of partial red blood cell chimerism. X-axis: % normal red blood cells in suspension. Y-axis: % of red blood cells in high forward scatter gate determined by analysis using Consort 30 software package as described in Chapter II. The data points shown represent fourteen independent experiments. *F*-test analysis showed that a straight regression line had to be rejected and that a second order weighted polynomial curve fit the data optimally. The regression coefficients were statistically highly significant ($P < 0.001$; *t*-test). Weights were used since inhomogeneity of variance was expected. The weighted curve, however, did not differ statistically significant from a curve calculated without weights. Tolerance limits (95% confidence limits for the data) were therefore estimated using standard *t*-statistics.

3.3 DISCUSSION

A rapid method to determine the degree of red cell chimerism in murine α-thalassemia is presented. FLS measurements by FACScan, performed in hypotonic saline (103 mmol/l), allowed for a quantitative assessment of the ratio of normal and thalassemic erythrocytes. The conditions chosen did not result in preferential lysis of either of the two types red blood cells, as shown by osmotic fragility. The measurement by FACScan of red blood cell chimerism required the use of a specific calibration curve. Wickramasinghe et al.[564] also showed that red cell volume distribution curves determined with a Coulter counter (model Fn) could discriminate between thalassemic mice and normal litter mates after four weeks of age. Previous work from our lab used an Elzone Particle Channelyzer to discriminate between healthy and thalassemic size distribution curves.[541] This method is not suitable to perform classification studies. Although it discriminated clearly between healthy and thalassemic red cell distributions, it did not sufficiently quantify degrees of partial chimerism. The method described here is highly reproducible and can be easily performed, allowing measurements on a large number of samples in a relatively short time.

CHAPTER IV
COMPENSATORY SPLENIC HEMOPOIESIS IN β-THALASSEMIC MICE

β-Thalassemic mice, homozygous for the deletion of the β^{major}-globin gene, were investigated for compensatory hemopoiesis in bone marrow and spleen. Apart from characteristic severe anemia, these mice have a marked granulocytosis, monocytosis and lymphocytosis. A large compensatory expansion of late (CFU-E) erythroid progenitor cells was demonstrated, predominantly in the spleen. Immature hemopoietic cells (CFU-S) were also expanded, as were early progenitor cells of erythroid (BFU-E) as well as granulocyte/macrophage (GM-CFU) and megakaryocytic (CFU-Meg) lineages. It is concluded that the persistent erythropoietic stress results in a selective expansion of immature hemopoietic cells and inappropriate production on nonerythroid cells from excess production of progenitor cells.

adapted from:

Cor van den Bos, Dorinde Kieboom, Trudi P. Visser, and Gerard Wagemaker: Exp Hematol (1993) 21:350-353

4.1 INTRODUCTION

As discussed in Chapter I, a 3.7 kilobase deletion including regulatory and all coding sequences of the β^{major}-globin gene at its locus on chromosome 7 leads to the complete absence of the β^{major}-globin polypeptide in homozygous mice.[172,443] Such mice suffer from a hypocellular, hypochromic and microcytic anemia.[396,443] As a consequence, reticulocyte counts may rise to numbers as high as 40% of all red cells.[162,443] Heterozygous litter mates do not differ significantly in red blood cell parameters from unaffected mice.[162,396,443] The nucleated white cell numbers in the peripheral blood of homozygous β-thalassemic mice appeared to be significantly increased compared to those of normal mice.[396,443] This phenomenon has been explained by assuming a generalized increase in hemopoiesis, necessary to sustain a modest number of thalassemic erythrocytes.[396] The increased hemopoiesis is also reflected by marked splenomegaly, as well as by substantial increases in numbers of spleen colony-forming units (CFU-S) in the spleen of β-thalassemic mice.[60,396] In this chapter the influence of β-thalassemia on various hemopoietic differentiation pathways in both bone marrow and spleen was further analyzed.

4.2 RESULTS

Elevated white blood cell counts with an essentially normal differential distribution were observed in β-thalassemic mice (Tables 4.1 and 4.2), in agreement with those reported.[396] Flow cytometric analysis showed that among the peripheral blood cells with the light scatter characteristics of lymphocytes, 20% were $CD4^+$, 9% $CD8^+$, while 50% stained positively with GAM-FITC (n=3).

The results of the progenitor cell determinations in BM and spleen are shown in Tables 4.3 and 4.4. Femoral numbers of GM-CFU_{mscm}, BFU-E, and CFU-Meg in β-thalassemic male and female mice were not significantly different from those of normal control mice. Femoral GM-$CFU_{m\text{-}csf}$ numbers were significantly lower in β-thalassemic female mice, but not in male mice. In male β-thalassemic mice, femoral CFU-E numbers were moderately but significantly increased. All hemopoietic progenitor cells tested were significantly increased in number in β-thalassemic spleens as compared to spleens of normal controls (Table 4.4).

Table 4.1: Differential counts of nucleated blood cells of female β-thalassemic (Hbb^{th}/Hbb^{th}) C57BL mice and normal congenic controls (n = 2 for each category)

		females			
		Hbb^{th}/Hbb^{th}	% of cells	+/+	% of cells
nucleated blood cells ($10^9/l \pm$ s.d.)		15.2 ± 2.5		4.4 ± 0.8	
differential ($10^9/l \pm$ s.d.)					
granulocytes	early	0.02 ± 0.02	0.1	n.d.	0
	band	0.05 ± 0.07	0.4	n.d.	0
	segment	0.9 ± 0.07	6	0.50 ± 0.04	12
	eosinophilic	0.16 ± 0.01	1	0.06 ± 0.01	1.4
normoblasts		0.04 ± 0.01	0.3	n.d.	0
monocytes		0.6 ± 0.2	4	0.3 ± 0.08	7.3
lymphocytes		13.5 ± 2.3	88	3.5 ± 0.7	79

n.d. = not detected

The developmentally early hemopoietic progenitors, GM-CFU_{mscm}, BFU-E, and CFU-S, were approximately two- to fourfold increased, whereas the more mature progenitors, GM-$CFU_{m\text{-}csf}$ and CFU-E, were approximately eightfold expanded in female thalassemic mice. In male mice, greater expansions of both early and late hemopoietic progenitors were observed than in female animals. Calculation of the contribution of the spleen to the total numbers of progenitor cells per mouse (Table 4.5), demonstrated that roughly half of the late erythroid progenitor cells (CFU-E) of thalassemic mice are present in the spleen as opposed to only a small percentage in the spleens of normal mice.

4.3 DISCUSSION

The results demonstrate compensatory erythropoiesis in the spleen rather than in the bone marrow of β-thalassemic mice. Late erythroid progenitor cells, CFU-E, were dominant among progenitor cells found in the spleen and were found to be twice the splenic contribution to CFU-E numbers observed earlier in α-thalassemic female mice.[540] The difference is undoubtedly attributable to the more severe anemia in β-thalassemic mice, in accordance with the shorter

Table 4.2: Differential counts of nucleated blood cells of male β-thalassemic (Hbb^{th}/Hbb^{th}) C57BL mice and normal congenic controls (n = 2 for each category)

		males			
		Hbb^{th}/Hbb^{th}	% of cells	+/+	% of cells
nucleated blood cells (10^9/l ± s.d.)		12.7 ± 0.1		4.8 ± 0.1	
differential (10^9/l ± s.d.)					
granulocytes	early	n.d.	0	n.d.	0
	band	0.3 ± 0.1	2.2	n.d.	0
	segment	2.2 ± 1.9	17	0.7 ± 0.1	14
	eosinophilic	0.13 ± 0.01	1	0.01 ± 0.02	0.3
normoblasts		n.d.	0	n.d.	0
monocytes		0.7 ± 0.5	5.7	0.3 ± 0.1	5.8
lymphocytes		9.3 ± 2.1	74	3.7 ± 0.1	79

n.d. = not detected

life span (20 days) of β-thalassemic erythrocytes than the approximately 35 days observed in α-thalassemic mice,[396,394] as is also reflected by the higher reticulocyte counts in β-thalassemic mice (40%[162,443] vs. 3-7% in α-thalassemic mice[540]). It is unlikely that the lack of a numerical increase of CFU-E in bone marrow is compensated for by an accelerated cell cycle time, since the high percentage of S-phase cells of normal bone marrow CFU-E[217,539] does not increase in response to anemia,[217] while duration of G_1 and G_2/M phases is already minimal.[539] We do not exclude, however, that the greatly expanded splenic hemopoiesis requires perpetual replenishment from immature bone marrow cells, which, in that case, would have an increased turnover as well as an increased migration.

Similar to the α-thalassemic mouse,[540] compensatory hemopoiesis was not restricted to the erythroid lineage. Immature hemopoietic progenitor cells (CFU-S) were found to be increased in the spleen, but not in the bone marrow.[60,396]

Table 4.3: Progenitor cell content of bone marrow of β-thalassemic female and male mice

cell type	mean number per femur (x 10^3 ± s.d.)							
	n*	Hbb^{th}/Hbb^{th} female	n*	+/+ female	n*	Hbb^{th}/Hbb^{th} male	n*	+/+ male
CFU-E	27	35 ± 25	10	52 ± 46	10	108 ± 31 [b]	6	53 ± 28
GM-$CFU_{m\text{-}csf}$	25	12 ± 8 [a]	10	39 ± 12	10	26 ± 10	6	30 ± 5
GM-CFU_{mscm}	25	6 ± 7	10	18 ± 16	10	18 ± 11	6	24 ± 14
BFU-E	27	4 ± 4	10	10 ± 3	10	3 ± 5	6	13 ± 3
CFU-S-9	4	2 ± 1	4	4 ± 2	4	2 ± 1	4	3 ± 1
CFU-Meg	27	0.4 ± 0.5	10	0.9 ± 1.2	10	1 ± 1	6	2 ± 1
cellularity**	27	2.7 ± 1.1	10	2.4 ± 0.6	10	3.4 ± 1.0	6	3.1 ± 1.2

* = number of mice individually analyzed
** = average number of nucleated cells ± s.d. (x 10^7) / femur
significance of difference in progenitor cell content between thalassemic and normal femurs was calculated using the Mann-Whitney Rank Sum test: a = $p < 0.001$; b = $p < 0.02$

Table 4.4: Progenitor cell content of spleens of β-thalassemic female and male mice

cell type	mean number per spleen (x 10^3 ± s.d.)							
	n*	Hbb^{th}/Hbb^{th} female	n*	+/+ female	n*	Hbb^{th}/Hbb^{th} male	n*	+/+ male
CFU-E	27	491 ± 486 [a]	10	60 ± 67	10	675 ± 55 [b]	6	34 ± 17
GM-$CFU_{m\text{-}csf}$	27	16 ± 21 [a]	10	2 ± 3	10	45 ± 23 [b]	6	0.7 ± 0.6
GM-CFU_{mscm}	27	14 ± 13 [c]	10	4 ± 7	10	34 ± 27 [c]	6	5 ± 3
BFU-E	27	10 ± 13 [a]	10	5 ± 5	10	30 ± 15 [b]	6	7 ± 5
CFU-S-9	4	10 ± 2 [d]	4	3 ± 1	4	8 ± 3 [d]	4	1 ± 1
CFU-Meg	25	4 ± 3 [a]	10	2 ± 1	10	3 ± 4 [c]	6	0.4 ± 0.4
cellularity**	27	6.9 ± 3.0	10	2.2 ± 0.7	10	7.5 ± 1.6	6	2.1 ± 0.3

* = number of mice individually analyzed

** = average number of nucleated cells ± s.d. (x 10^8) / spleen

significance of difference in progenitor cell content between thalassemic and normal spleens was calculated using the Mann-Whitney Rank Sum test: a = $p < 0.001$; b = $p < 0.002$; c = $p < 0.005$; d = $p < 0.05$

Table 4.5: Calculated contribution of the spleen (% of total) to the total number of progenitor cells per mouse

cell type	Hbb^{th}/Hbb^{th} female	+/+ female	Hbb^{th}/Hbb^{th} male	+/+ male
CFU-E	59 ± 18	3 ± 4	37 ± 17	4 ± 2
$GM\text{-}CFU_{m\text{-}csf}$	13 ± 8	<1	11 ± 5	<1
$GM\text{-}CFU_{mscm}$	11 ± 7	4 ± 2	13 ± 6	1 ± 1
BFU-E	8 ± 9	3 ± 3	15 ± 9	4 ± 2
CFU-S-9	19 ± 8	4 ± 1	20 ± 4	3 ± 1
CFU-Meg	22 ± 15	6 ± 3	9 ± 11	1 ± 1

$$\text{splenic contribution} = \frac{\text{(number of progenitor cells / spleen)}}{\left(\left(\frac{100}{8.5} \times \text{number of progenitor cells / femur}\right) + \text{(number of progenitor cells / spleen)}\right)} \times 100$$

This indicates that the adaptive production of blood cells to the sustained erythropoietic stress is primarily located in the spleen, while the increases in GM-CFU and CFU-Meg numbers demonstrate its lack of specificity. The granulocytosis observed[396,443] can now be explained on the basis of the observed increase in splenic GM-CFU. The origin of the observed lymphocytosis remains to be elucidated.

CHAPTER V

SELECTIVE ADVANTAGE OF NORMAL ERYTHROCYTE PRODUCTION AFTER BONE MARROW TRANSPLANTATION OF α-THALASSEMIC MICE

Anemia resulting from α-thalassemia in mice was corrected by transplantation of normal bone marrow cells following sublethal total body irradiation, resulting in partial hemopoietic chimerism with a preponderance of normal peripheral blood red cells. Peripheral blood red cell chimerism in recipients of graded numbers of bone marrow cells from sex mismatched donors, determined by cytometric analysis, was directly compared with immature hemopoietic cell (CFU-S) chimerism and peripheral blood white cell chimerism. The latter two were assessed by fluorescent *in situ* hybridization using a murine Y-chromosome specific probe. The degree of donor type red cell chimerism uniformly exceeded that of peripheral blood white cell chimerism, which corresponded more closely to immature hemopoietic cell chimerism, thus emphasizing the selective advantage of normal red cell production in partially chimeric α-thalassemic mice.

adapted and extended from:

Cor van den Bos, Dorinde Kieboom, Johannes P. van der Sluijs, Miranda R.M. Baert, Rob E. Ploemacher and Gerard Wagemaker: submitted for publication

5.1 INTRODUCTION

Among hereditary diseases, the thalassemias represent a major global health problem.[12] Due to the hemopoietic nature of the genetic defect, bone marrow transplantation (BMT) offers a possibility of cure for selected patients with an available donor identical for major histocompatibility antigens.[368,484] The necessity to completely replace the hemopoietic system of the recipients has never been established.[199] Correction of the characteristic hypochromic, microcytic anemia in murine α-thalassemia,[8,310,393,416,562] has been shown to result from infusion of relatively low numbers of normal bone marrow (BM) cells following a sublethal dose of total body irradiation (TBI).[27,541] Subsequently, this has been similarly shown in murine β-thalassemia (Chapter VI[507]). It was assumed that the procedure had resulted in partial hemopoietic stem cell chimerism, in which a minority of normal stem cells produce a preponderance of normal red cells.[541]

To evaluate the hypothesis of a selective advantage of normal erythropoiesis in sublethally irradiated α-thalassemic recipients, stable chimeric α-thalassemic mice were analyzed for the degree of donor type chimerism of immature hemopoietic cells (CFU-S), as well as peripheral blood red and white cells. Sex mismatched donor-recipient combinations were used to assess donor and recipient origin of nucleated cells by fluorescent *in situ* hybridization (FISH) with a murine Y-chromosome specific probe. To exclude a possible intrinsic stem cell defect in α-thalassemic mice, chimerism was compared in normal recipients of normal and α-thalassemic BM cells. A possible difference between regeneration of endogenous and transplanted stem cells was analyzed by transplantation of a mixture of thalassemic and normal bone marrow cells into lethally irradiated thalassemic recipients. Promotion of engraftment of transplanted stem cells due to microenvironmental changes resulting from compensatory erythropoiesis in α-thalassemic mice was assessed by a comparison of peripheral blood white cell chimerism levels in normal and α-thalassemic recipients of normal BM cells.

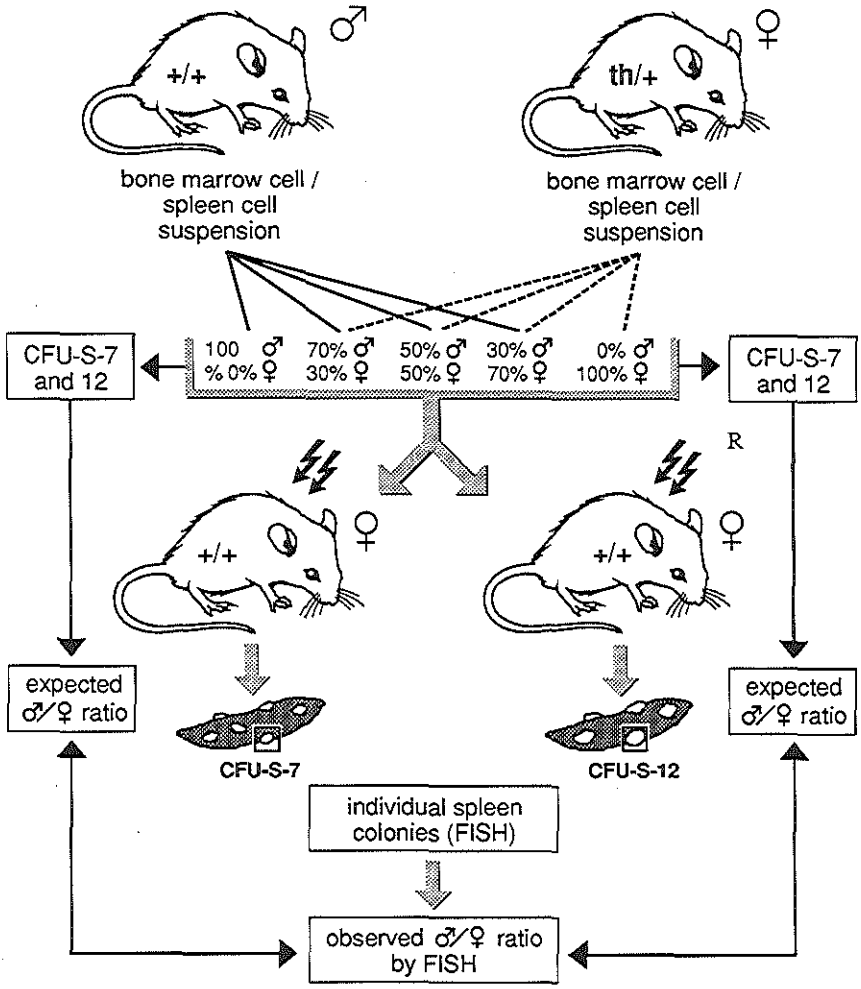


Figure 5.1:
Experimental design to evaluate the accuracy of CFU-S-12 chimerism determination. CFU-S content of suspensions of normal male and α-thalassemic female cells (left and right hand arrows) was determined using standard techniques. This allowed for calculation of the expected male/female ratios in mixtures prepared from these BM and spleen cell suspensions. Male or female origin of colonies dissected from the spleen of recipients of these mixtures was determined by FISH, and observed male/female ratios were compared directly with the expected ratios (CFU-S-7 data not shown in this chapter).

5.2 RESULTS

5.2.1 Accuracy of CFU-S chimerism assay

To test the accuracy of the chimerism assay for CFU-S, individual spleen colonies from recipients of mixed cell suspensions were analyzed for male or female origin by FISH. The observed proportions of male colonies were compared directly with those expected, which were calculated on the basis of CFU-S numbers in the original BM and spleen cell suspensions (Figure 5.1). Experimental data for CFU-S-12 were directly proportional to the expected values (Figure 5.2).

5.2.2 Chimerism in α-thalassemic recipients of normal bone marrow

CFU-S chimerism was determined in α-thalassemic mice after sustained red cell chimerism had been established by transplantation with normal BM cells (Table 5.1). The proportion of donor type red blood cells was larger than that of donor type CFU-S-12 in BM and/or spleen of all mice analyzed, whereas peripheral blood white cell chimerism corresponded closely to BM CFU-S-12 chimerism (Figure 5.3).

Peripheral blood red and white cell chimerism was assessed in 34 female α-thalassemic recipient mice transplanted with normal syngeneic male BM cells (Figure 5.4). The degree of red cell chimerism was found to exceed the degree of white cell chimerism in all mice tested by, on average, 20 to 30%.

5.2.3 Chimerism in normal recipients of thalassemic and normal bone marrow

Six normal female BALB/c mice transplanted with α-thalassemic male BM cells were analyzed. The proportion of donor type CFU-S-12 was larger than that of donor type peripheral blood red cells in three of these mice (Table 5.2). In the vast majority of normal female BALB/c mice transplanted with syngeneic α-thalassemic male BM cells, the proportion of donor type peripheral blood white cells exceeded the proportion of donor type red cells (Figure 5.4).

In normal female mice transplanted with normal male BM cells, the proportion of donor type CFU-S-12 was similar to that obtained in normal-to-thalassemic or thalassemic-to-normal donor-recipient combinations. After 3 Gy TBI, BMT with 10^6 normal cells resulted in 33% donor type CFU-S-12, similar to the

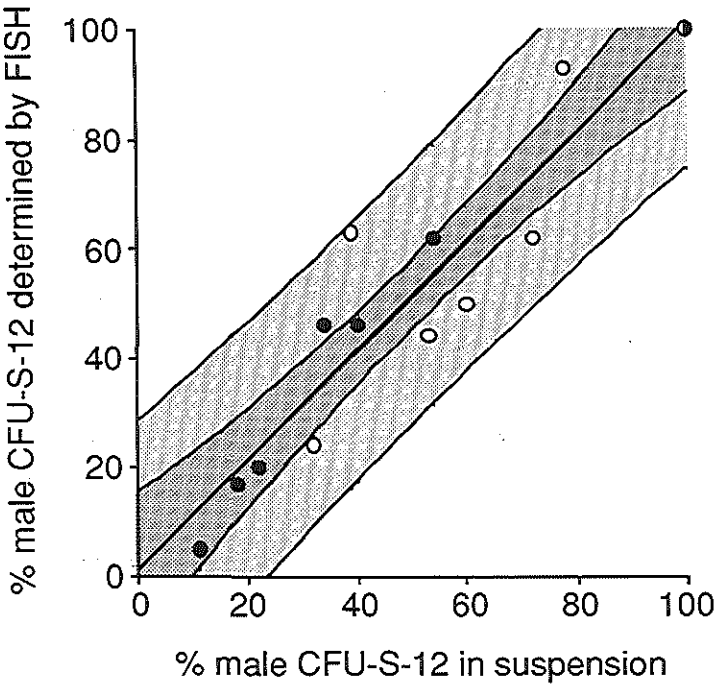


Figure 5.2: Accuracy of CFU-S-12 chimerism determination in BM or spleen cell suspensions. Individual data points represent percentages male type colonies calculated from analysis of on the average 31 spleen colonies (range 8-47). Data from spleen (closed symbols) and BM (open symbols) cell suspensions were pooled, since their regression lines did not differ significantly. Correlation coefficient (r) for the line is 0.94. The darkly shaded area represents the 95% confidence interval for the regression line. The lightly shaded areas represent the confidence for the individual observation, i.e., the total variability that arises from the variation of the individual data points about the line of means, and the uncertainty in the location of that line (as represented by the darkly shaded areas). See also reference 170.

average level of donor type CFU-S-12 in thalassemic recipients of normal cells (mean ± SEM: 33 ± 14%). Similar proportions of donor type CFU-S were found after transplantation of 10^7 normal BM cells in 2 Gy irradiated thalassemic recipients and after transplantation of 10^7 thalassemic BM cells in 2 Gy irradiated normal recipients, i.e., respectively, 32% and 38%.

Since peripheral blood leukocyte chimerism corresponded closely to immature hemopoietic cell chimerism (Figure 5.3), it may be inferred from Figure 5.5

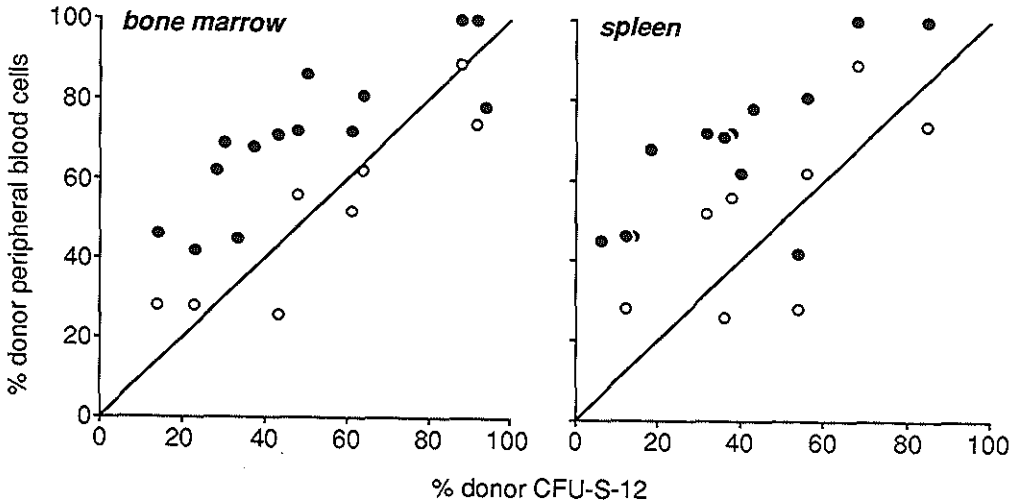


Figure 5.3: Relationship between CFU-S-12 chimerism in BM or spleen (as determined by FISH on individually dissected colonies) and peripheral blood red (closed symbols) and white (open symbols) cell chimerism (determined, respectively, by flow cytometry and by FISH) in stable partial chimeric α-thalassemic mice (8 to 19 months after BMT; TBI doses between 2 and 5 Gy, BM cell doses between 10^6 and 10^7).

that in all donor-recipient combinations similar proportions of repopulation with donor derived immature BM cells were obtained at similar TBI doses and BM cell numbers.

5.2.4 Chimerism in lethally irradiated recipients of a mixture of normal and thalassemic bone marrow cells

After lethal TBI (8.25 Gy), female α-thalassemic mice were transplanted with a mixture of female α-thalassemic and normal syngeneic male BM cells (total number of cells transplanted 10^7). The relationship between peripheral blood white and red cell chimerism in these recipients was closely similar to that obtained in α-thalassemic mice transplanted with normal BM cells after low dose TBI (triangles in left side of Figure 5.4)

Table 5.1: Chimerism in stable male (+/+) to female α-thalassemic (th/+) partially chimeric mice (between 8 and 19 months after BMT)

TBI dose (Gy)	cell dose	percentage donor			
		bone marrow CFU-S-12*	spleen CFU-S-12*	red blood cells	white blood cells
2	3×10^6	33 (15/45)	6 (2/36)	45	n.a.
2	1×10^7	28 (8/29)	40 (8/20)	62	n.a.
		37 (10/27)	18 (2/11)	68	n.a.
3	1×10^6	-	14 (1/7)	46	n.a.
		23 (7/30)	54 (7/13)	42	28
		14 (5/36)	12 (4/34)	46	28
		61 (22/36)	32 (6/19)	72	52
3	3×10^6	30 (7/23)	n.a.	69	n.a.
		50 (10/20)	n.a.	86	n.a.
		94 (33/35)	43 (10/23)	78	n.a.
		43 (6/14)	36 (5/14)	71	26
		48 (14/29)	38 (5/13)	72	56
		64 (23/36)	56 (14/25)	81	62
5	1×10^6	92 (24/26)	85 (17/20)	100	74
		88 (22/25)	68 (13/19)	100	89

n.a. = not analyzed; * = between brackets: number of donor type colonies / total number of colonies analyzed

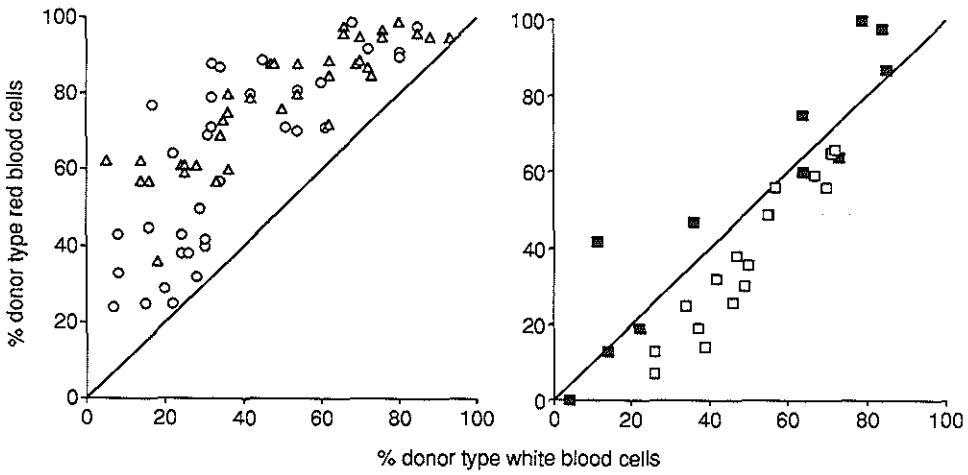


Figure 5.4: Peripheral blood red and white cell chimerism in partially chimeric mice. *Left hand side:* Circles: α-thalassemic female mice at 7 (n = 17), 16 (n = 14), and 18.5 (n = 3) months after transplantation of normal syngeneic male BM cells. TBI doses varied between 2 Gy and 5 Gy, BM cell numbers between 10^6 and $3x10^6$. Triangles: lethally irradiated (8.25 Gy) α-thalassemic female mice, transplanted with mixtures of α-thalassemic female and normal male BM cells, 4 months after transplantation. *Right hand side:* normal recipients of α-thalassemic BM cells at 2 (n=16; open squares) and 18 months (n=11; closed squares) after BMT (different experiments). TBI doses varied between 2 Gy and 5 Gy, BM cell numbers between $3x10^5$ and 10^7.

5.3 DISCUSSION

Transplantation of normal, syngeneic bone marrow cells in α-thalassemic recipients following sublethal TBI resulted uniformly in partial red blood cell chimerism. At TBI doses lower than 5 Gy, transplantation of 10^6 to 10^7 BM cells resulted in a minority of immature hemopoietic cells (CFU-S-12) in bone marrow and spleen, which produced a preponderance of normal peripheral blood red cells. In contrast, peripheral blood white cell chimerism was in line

Table 5.2: Chimerism in stable female (+/+) partially chimeric mice, transplanted with α-thalassemic (th/+) or normal (+/+) male BM cells (between 7.5 and 20 months after BMT)

donor/recipient combination	TBI dose (Gy)	cell dose	percentage donor			
			bone marrow CFU-S-12*	spleen CFU-S-12*	red blood cells	white blood cells
(th/+) / (+/+)	2	1×10^7	50 (10/20)	95 (20/21)	46	n.a.
			27 (6/22)	48 (10/21)	47	n.a.
	3	3×10^5	4 (1/28)	14 (3/21)	27	18
	3	3×10^6	66 (21/32)	83 (39/47)	64	n.a.
	3	1×10^7	58 (18/31)	53 (10/19)	48	44
	4	3×10^6	59 (27/46)	45 (14/31)	73	56
(+/+) / (+/+)	3	1×10^6	33 (14/43)	12 (5/42)	§	36
	3	3×10^6	-	39 (9/23)	§	n.a.
			14 (5/36)	13 (2/15)	§	n.a.
			61 (22/36)	81 (22/27)	§	56
			44 (17/39)	69 (27/39)	§	26

n.a. = not analyzed
* = between brackets: number of donor type colonies / total number of colonies analyzed
§ = not applicable

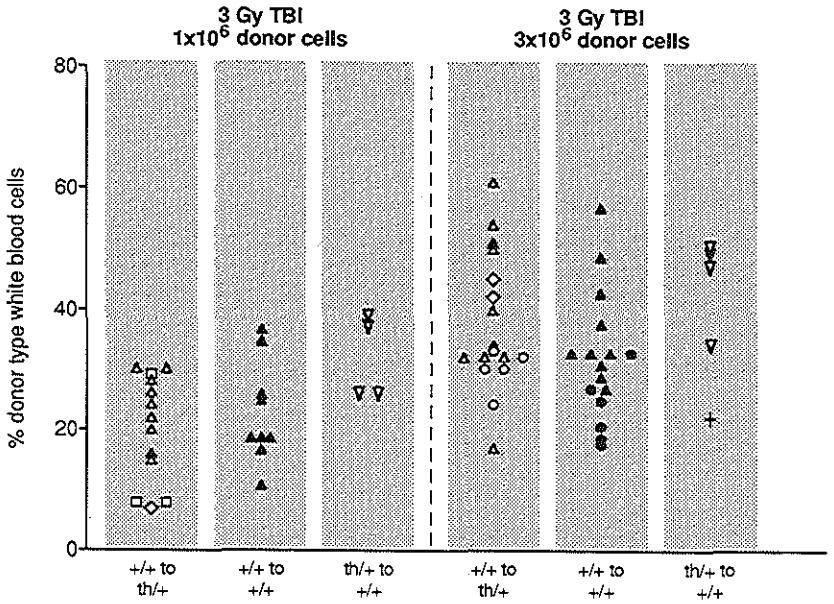


Figure 5.5: Peripheral blood white cell chimerism in female mice after transplantation with 10^6 or $3x10^6$ male BM cells following 3 Gy TBI. Donor/recipient combinations are indicated at the bottom of the figure (+/+ = normal, th/+ = α-thalassemic). Different symbols represent separate experiments. All measurements were performed on blood smears obtained more than 2 months after BMT.

with CFU-S-12 chimerism. Reverse transplantation, i.e., α-thalassemic bone marrow into normal recipients, led to similar levels of bone marrow CFU-S-12 and peripheral blood white cell chimerism, which did also not differ from results obtained in normal recipients of normal bone marrow. On this basis an intrinsic defect of α-thalassemic hemopoietic stem cells as well as micro-environmental differences between normal and α-thalassemic recipients were excluded. Since transplantation of a mixture of α-thalassemic and normal bone marrow cells in lethally irradiated mice yielded similar results, a difference between exogenous and endogenous regeneration could also be excluded. Since the difference in life-span between normal and α-thalassemic erythrocytes is too small to explain the observed preponderance of normal red cells in partially chimeric α-thalassemic recipients,[394,515] it is concluded that the preponderance

of normal red cells results from a selective advantage of the normal red cell lineage in the hemoglobin synthesizing stages of erythropoiesis, presumably on the basis of increased death of thalassemic cells as a result of precipitation of excess β-globin chains.[564]

The selective advantage appeared to be considerable smaller than previously predicted on the basis of the radiation sensitivity of hemopoietic stem cells [196,541] or experimental results.[27] The results obtained in this study can be explained by assuming that the hemopoietic stem cells of BALB/c mice are more radiosensitive than assumed on the basis of data for other mouse strains. This would result in an overestimation of the number of residual hemopoietic stem cells in our earlier study,[541] explaining the discrepancy between the results obtained here and those predicted.

Although allogeneic bone marrow transplantation in human thalassemia patients convincingly has been shown to correct anemia,[484] BM graft rejection remains an important complication,[120,148,278,291,299] as the maximum doses of cytoreductive agents tolerated are limited due to tissue damage related to iron overload. The present experimental study demonstrates that as little as 20 - 30% donor type immature hemopoietic cells may produce far over 50% normal peripheral blood red cells. The α/β-globin mRNA ratio in murine α-thalassemic reticulocytes[8,310] is approximately equal to that reported for humans with α-thalassemia trait.[550] α-Thalassemia trait is characterized by hypochromia and microcytosis, and carriers of the trait are not transfusion dependent.[363] In our view, this implies that those α-thalassemic patients with severe forms of the disease, i.e., HbH disease,[363] will benefit even more from the selective advantage of normal erythropoiesis in case of partial hemopoietic stem cell chimerism.

We propose that the approach of partial chimerism may also be advantageously used in clinical BMT for thalassemia, if a conditioning regimen that allows for sustained partial allogeneic chimerism can be developed, e.g., by the use of specific immunosuppressive agents such as monoclonal antibodies,[55,95,94,436] and if patients are transplanted sufficiently early to avoid hypersensitization due to blood transfusions and iron storage problems.

CHAPTER VI

CORRECTION OF MURINE β-THALASSEMIA BY PARTIAL BONE MARROW CHIMERISM: SELECTIVE ADVANTAGE OF NORMAL ERYTHROPOIESIS

β-Thalassemic mice were transplanted with normal congeneic bone marrow cells after sublethal total body irradiation, which resulted in partial red blood cell chimerism and correction of anemia. Enumeration of donor type early hemopoietic progenitor cells (CFU-S) demonstrated that the correction of anemia originated from a minority of normal immature bone marrow cells. It is concluded that successful bone marrow transplantation in β-thalassemia does not necessarily require ablation of endogenous bone marrow.

adapted and extended from:

Cor van den Bos, Dorinde Kieboom, and Gerard Wagemaker: Bone Marrow Transplant (1993) 12:9-13

6.1 INTRODUCTION

β-Thalassemia is one of the most frequent genetic disorders in humans. Over 50,000 children are born yearly with severe forms of the disease, and its treatment is a health care burden for most countries where it has a high prevalence.[12,14] Bone marrow transplantation (BMT) offers the possibility of cure for selected patients with an available donor identical for major histocompatibility antigens,[484] but has found only limited application due to the risks involved, i.e., graft rejection and graft-versus-host disease.

In a murine model for β-thalassemia, a 3.7 kilobase deletion including regulatory and all coding sequences of the β^{major}-globin gene leads to the complete absence of the β^{major}-globin polypeptide in homozygous mice.[172,443] Such mice suffer from a hypocellular, hypochromic and microcytic anemia, with an extreme reticulocytosis.[162,396,443] As a consequence of compensatory hemopoiesis, immature hemopoietic progenitor cells are increased in the spleen, while white cell counts in the peripheral blood are significantly increased and marked splenomegaly is found.[60,396,443] (Chapter IV[506])

Based on our earlier studies in α-thalassemic mice,[540,541] we hypothesized that a minority of transplanted normal BM stem cells may be sufficient to correct thalassemia. In the present report, this hypothesis was tested by transplantation of small numbers of normal BM cells following sublethal total body irradiation (TBI). Chimerism of early hemopoietic progenitor cells detected as CFU-S-day-12 was compared directly with peripheral blood red and white cell chimerism.

6.2 RESULTS

6.2.1 Assessment of red blood cell chimerism

Red blood cell (RBC) chimerism in transplanted β-thalassemic mice was assessed using a FACScan® flow cytometer, based on the method described for α-thalassemic mice (Chapter III[508]). Briefly: a small amount of blood obtained by tail clipping was suspended in 2 ml hypotonic (103 mmol/l) saline and the FLS distribution of the red blood cells of each sample was determined. Mice were classified as (Figure 6.1): (1) thalassemic, when normal RBC were present as only a minor tail to the FLS distribution or not at all, (2) partial chimeras, when both normal and thalassemic RBC were clearly present with the peak incidence of the thalassemic cells higher than 25% of the peak incidence of the

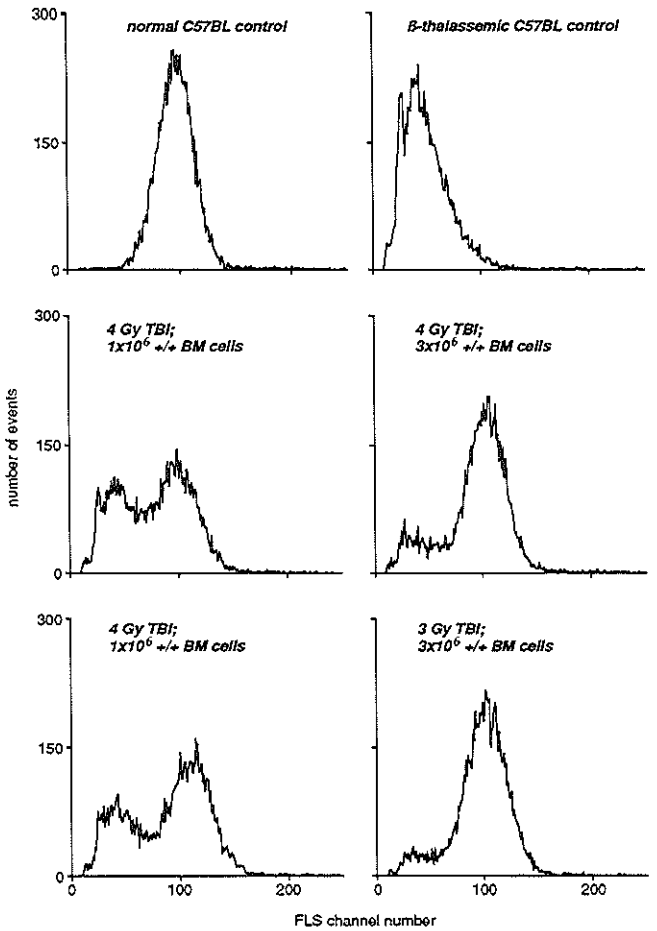


Figure 6.1: Flow cytometric determination of peripheral RBC forward light scatter (FLS) distribution curves of partial chimeric β-thalassemic mice. β-Thalassemic recipient mice were transplanted with small numbers of normal congeneic BM cells following sublethal TBI.

normal cells, or (3) predominantly or completely normal (PCN). A more precise estimate, as has been developed for α-thalassemic mice (Chapter III[508]), could not be made, since in some mice a significant proportion of the β-thalassemic erythrocytes was located below the FLS threshold.

Table 6.1: Peripheral RBC chimerism in β-thalassemic mice, following BMT with limited numbers of normal congeneic bone marrow cells after low dose TBI.

TBI dose (Gy)	cell dose (x 10^6)	donor sex (m/f)[d]	recipient sex (m/f)	PCN[a]	Partial[b]	time after BMT
3	1	m	m	1/1	0/1	14 mo
		m	f	0/1[c]	0/1[c]	
		f	f	1/3	2/3	1 - 6 mo
	3	f	m	2/2	0/2	6 mo
		f	f	1/1	0/1	
	5		m	1/2	1/2	21 wks
	10	f	m	3/3	0/3	6 mo
4	1	f	m	0/3	3/3	5 mo
		f	f	0/2	2/2	
	3	f	m	0/2	2/2	
		f	f	0/2	2/2	3 wks - 5 mo
	10	f	f	3/3	0/3	5 mo
8	1	f	f	1/1	0/1	5.5 mo

[a]Predominantly or completely normal; [b]Partial RBC chimeras
[c]Recipient mice remained thalassemic
[d]m =male; f = female

6.2.2 Chimerism in β-thalassemic recipients of normal bone marrow

In pilot studies, stable partial chimerism developed from transplantation of 10^6 and 10^7 congeneic normal BM cells after TBI doses of 3 and 4 Gy (Table 6.1). Longitudinal follow-up showed stability of chimerism for an observation time as long as 70 weeks after BMT. The development of sustained chimerism took approximately 2-3 months. To assess competition between transplanted normal cells and residual thalassemic cells more precisely, two different cell doses (10^6 and $3x10^6$) of normal congeneic female BM cells were transplanted into male or female β-thalassemic recipient mice after graded TBI doses ranging from 2 to 7 Gy. The degree of RBC chimerism appeared to be directly related to the radiation dose given to the recipients (Table 6.2), as was expected from competition between grafted normal cells and residual thalassemic BM cells.

Table 6.2: Relationship of radiation dose, BM cell dose, and RBC chimerism in male and female β-thalassemic recipients of congeneic normal female BM cells (30 weeks after BMT).

	1 x 10^6 donor cells		3 x 10^6 donor cells	
TBI dose (Gy)	PCN[a]	Partial[b]	PCN[a]	Partial[b]
2		ND	1/6[c]	0/6[c]
3	1/5[c]	3/5[c]	5/6[c]	0/6[c]
4	2/6	4/6	3/6	3/6
5	1/4	3/4	3/6	3/6
6	5/5	0/5	4/4	0/4
7	4/4	0/4		ND

[a]Predominantly or completely normal
[b]Partial RBC chimeras
[c]All other mice remained thalassemic
ND = not done

Hematocrits, in turn, were directly related to the proportion of donor type RBC (Figure 6.2). In these sets of experiments, 29 mice reached red cell chimerism that was characterized as predominantly or completely normal (PCN), and 16 mice were partial chimeras. These mice were followed for a period of at least 10 months up to a period of 20 months. In all mice, red cell chimerism established at 3 months remained stable throughout the observation period.
The proportion of donor type early hemopoietic progenitor cells (CFU-S-12) in BM and spleen was determined in a number of partial chimeric β-thalassemic mice, between 12 and 16 months after BMT with sex mismatched donor cells (Figure 6.3). The results demonstrated that a majority of normal RBC originated from a minority of immature hemopoietic cells (CFU-S-12) in BM and spleen.

6.3 DISCUSSION

Transplantation of small numbers of normal congeneic BM cells following low-dose TBI readily resulted in partial RBC chimerism in β-thalassemic mice. Mice classified by flow cytometry as complete or as partial red blood cell chimeras showed a marked improvement of their hematocrit values. As in α-thalassemia,[541] lasting improvement of the anemia associated with β-thalassemia can be obtained by the induction of stable partial hemopoietic stem cell chimerism. A lower proportion of donor type immature hemopoietic progenitor cells (CFU-S-12) than donor type peripheral RBC was uniformly observed. This clearly shows that correction of anemia by partial chimerism in thalassemic mice results from a selective advantage of normal RBC production.
In contrast to α-thalassemic recipients (Chapter V), the peripheral blood white cells display a significantly ($p<0.01$: chi-square test) larger number of donor cells than would be predicted on the basis of the number of donor type CFU-S. This feature is reminiscent of the leukocytosis in untransplanted β-thalassemic mice, that was explained by an excess production of splenic GM-CFU from immature cells as a consequence of severe erythropoietic stress (Chapter IV[506]).

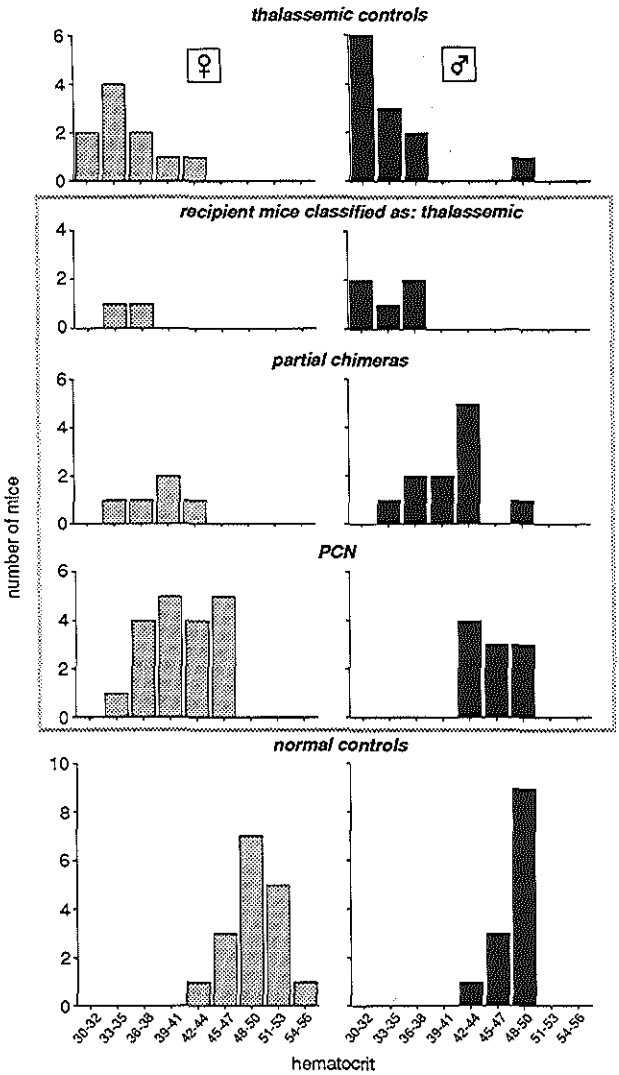


Figure 6.2: Hematocrit values in stable partial chimeric β-thalassemic recipients of normal congeneic female BM cells 30 weeks after BMT. Average hematocrits ± s.d.for female (F) and male (M) recipient mice are shown between brackets. Rows from top to bottom: thalassemic controls (F, 35 ± 3%; M, 35 ± 5%), BM recipients classified, respectively, as thalassemic (F, 34 ± 2%; M, 34 ± 2%,), mixed chimeras (F, 38 ± 2%; M, 41 ± 4%), and predominantly or completely normal (F, 41 ± 4%; M, 46 ± 3%), and normal control mice (F, 49 ± 2%; M, 48 ± 2%).

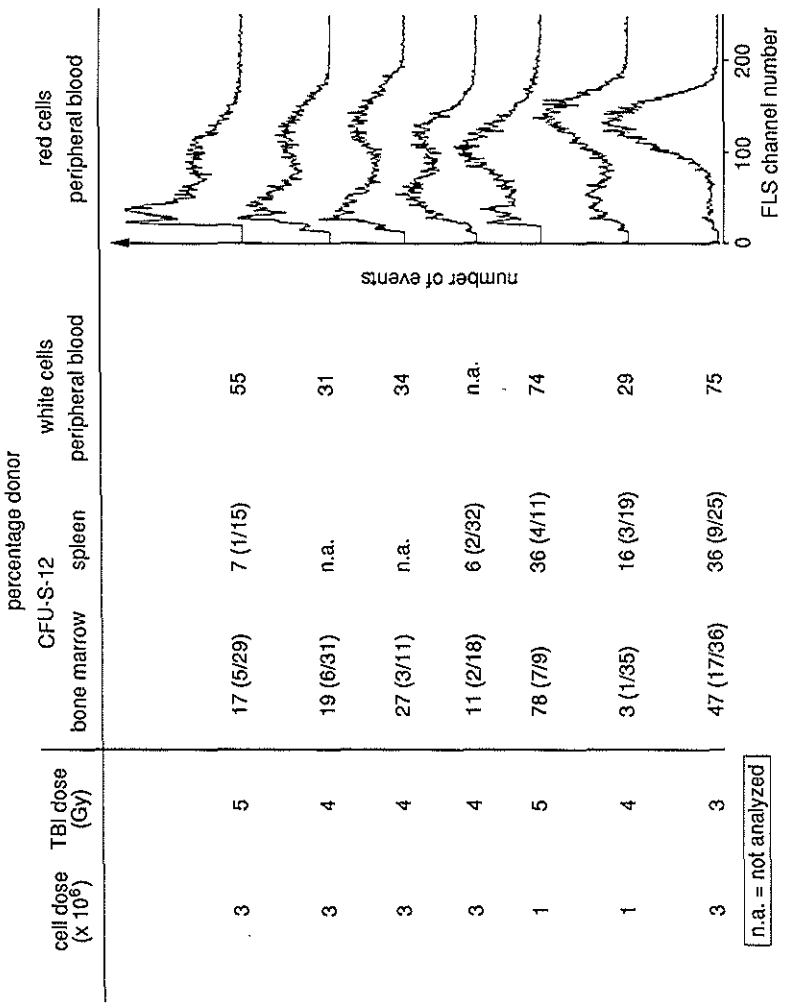


Figure 6.3: Hemopoietic chimerism in male β-thalassemic (Hbb^{th}/Hbb^{th}) mice transplanted with small numbers of normal congeneic (+/+) female BM cells, following sublethal TBI. Peripheral blood white cell chimerism was determined by FISH on blood smears using a murine Y-chromosome specific probe. RBC chimerism was determined flow cytometrically. CFU-S chimerism was determined by analysis of donor or recipient origin of individual colonies dissected from the spleens of secondary recipients by FISH.

Such an excess production of splenic progenitor cells was also observed in α-thalassemic mice.[540] It is conceivable, therefore, that the accelerated normal red cell production in the initial phase after transplantation resulted similarly in an excess production of donor type GM-CFU, which may have been sustained after the establishment of stable partial chimerism.

Congeneic donors are not available for human thalassemia patients. However, stable partial allogeneic chimerism and mutual tolerance of donor- and recipient-type cells have been achieved in mice with conditioning regimens that combine TBI with specific immunosuppressive agents, such as anti T-cell MCAs.[55,94,95,436]

TBI in BMT conditioning regimens is applied to create space for engraftment of donor hemopoietic cells as well as to provide immunosuppression to avoid allograft rejection.[529,541] In conditioning regimens for human thalassemia patients, TBI is generally replaced by cyclophosphamide and busulphan. Combinations of these agents with specific immunosuppressive agents might very well allow for sustained partial allogeneic chimerism in human patients similar to the murine studies.

Presently, clinical data are available from several hundreds of thalassemic patients subjected to BMT for thalassemia major. A significant proportion of failures due to early death or graft failure has been reported.[292,295] More recently, the probability of event-free survival of 'good risk' patients without evidence of hepatomegaly or portal fibrosis was shown to be over 90% with a 3% probability of graft rejection.[289,291,299] In general, however, BM allograft rejection is still frequently observed.[120,148,278,291,299] Based on our earlier data[541] as well as the results reported here, we conclude that correction of thalassemia does not necessarily require complete ablation of thalassemic BM. Therefore, the significant numbers of failures of clinical BMT following marrow 'ablative' conditioning regimens should be attributed to graft rejection rather than to an inability of the transplanted stem cells to compete successfully with residual thalassemic stem cells. Partial chimerism has indeed been reported in a number of patients transplanted for thalassemia.[9,361]

Iron overload related to thalassemia limits the maximum tolerated dose of cytoreductive agents.[299] We propose that improvement of the results of allogeneic BMT for thalassemia would require an immunosuppressive regimen that allows for sustained partial chimerism rather than a more intensive BM ablative regimen. It is recognized that such a regimen is presently not available and difficult to develop. We note that future attempts to correct thalassemia by insertion of normal globin genes and regulatory elements in thalassemic hemopoietic stem cells may profit from the selective advantage of normal erythropoiesis introduced in thalassemic recipients.

CHAPTER VII

STABLE PARTIAL CHIMERISM IN α-THALASSEMIC RECIPIENTS OF ALLOGENEIC NORMAL BONE MARROW CELLS AFTER CONDITIONING WITH SUBLETHAL TBI AND IMMUNOSUPPRESSIVE ANTIBODIES

Transplantation of small numbers of normal syngeneic bone marrow (BM) cells readily resulted in partial hemopoietic chimerism and corrected anemia in both α- and β-thalassemic mice after a conditioning regimen of sublethal doses of total body irradiation (TBI). Since only allogeneic donors are available for human thalassemia patients, we studied conditions that allow for induction of stable allogeneic partial bone marrow chimerism, using the murine model for α-thalassemia. To prevent allograft rejection, additional immunosuppression was provided by pretreatment with anti-CD4, anti-CD8, anti-CD11a (LFA-1α), and anti-CD18 (LFA-1β) MCAs four days before TBI. TBI alone resulted uniformly either in rejection of bone marrow grafts or in full engraftment, depending on the dose used. However, bone marrow transplantation after pretreatment with MCAs resulted in stable and sustained partial chimerism at TBI doses of 4 and 5 Gy. Of the anti-LFA-1 MCAs, only anti-CD11a was effective in combination with anti-CD4 and anti-CD8 MCAs in parental recipients of F1 donor cells. In recipients of sex and MHC-mismatched BM cells, pretreatment with anti-CD11a MCA did not increase the rate of engraftment achieved by use of anti-CD4 and anti-CD8 MCAs alone. Partial chimerism occurred in red blood cells, white blood cells and immature bone marrow cells (CFU-S-12).

Larger percentages of donor type peripheral blood red cells than immature BM cells and white cells demonstrated a selective advantage of normal erythropoiesis in the α-thalassemic recipients. It is concluded that stable allogeneic partial chimerism can be achieved following transplantation of bone marrow cells in numbers considered feasible for clinical bone marrow transplantation in human patients by use of a conditioning regimen supplemented with immunosuppressive MCAs.

adapted from:

Cor van den Bos, Francis C.J.M. van Gils, Dorinde Kieboom, Yvonne Westerman and Gerard Wagemaker (submitted for publication)

7.1 INTRODUCTION

Thalassemia belongs to the most frequent hereditary disorders in humans, and the management of thalassemic patients is a health care burden for those countries where it has a high prevalence.[12] In case of available MHC-identical donors, allogeneic bone marrow transplantation (BMT) may result in permanent correction of these disorders.[484] However, BMT has not found widespread application in the management of thalassemic patients due to the risks of allograft rejection as well as GvHD and the possible complications of the procedure, which require high-level medical care.

BM allograft rejection is frequently encountered in patients treated for thalassemia and other hemoglobinopathies.[16,120,148,278,291,299] Tolerated doses of the usual cytoreductive agents given to prepare patients for bone marrow allograft acceptance, such as TBI, cyclophosphamide and busulphan, are limited due to tissue damage related to iron overload. Therefore, relatively non-toxic supplementary immunosuppressive agents should be used to avoid BM allograft rejection in thalassemic patients.[16,299]

Monoclonal antibodies directed against the major T-cell markers CD4 and CD8 have been used previously to provide additional immunosuppression in mice receiving BM allografts.[94,95] In these studies, however, TBI doses of 6 Gy or higher were necessary to allow for sustained engraftment of allogeneic BM cells and skin graft tolerance.[94] Sustained allogeneic partial hemopoietic chimerism after a lower TBI dose of 3 Gy in combination with anti T-cell MCAs could only be achieved at the expense of adding thymic irradiation to the conditioning regimen.[436] Anti-LFA-1 MCAs, both anti-CD11a and anti-CD18, have been used in adult and pediatric patients receiving BMT for, respectively, malignancies and immunodeficiencies or osteopetrosis.[35,140,141,303] The addition of these MCAs marrow ablative conditioning regimens was supposed to successfully promote engraftment of HLA-mismatched BM cells in pediatric patients with immunodeficiencies or osteopetrosis,[140,141] but did not increase the engraftment in adult leukemia patients receiving either HLA-matched or HLA-mismatched BM cells.[35,303] Murine thalassemias[393,416,443] and other hereditary hemopoietic diseases can be corrected by induction of syngeneic partial hemopoietic chimerism[24,27,541] (Chapters V[505] and VI[507]) and offer suitable models for the search of regimens that comply with the requirements of

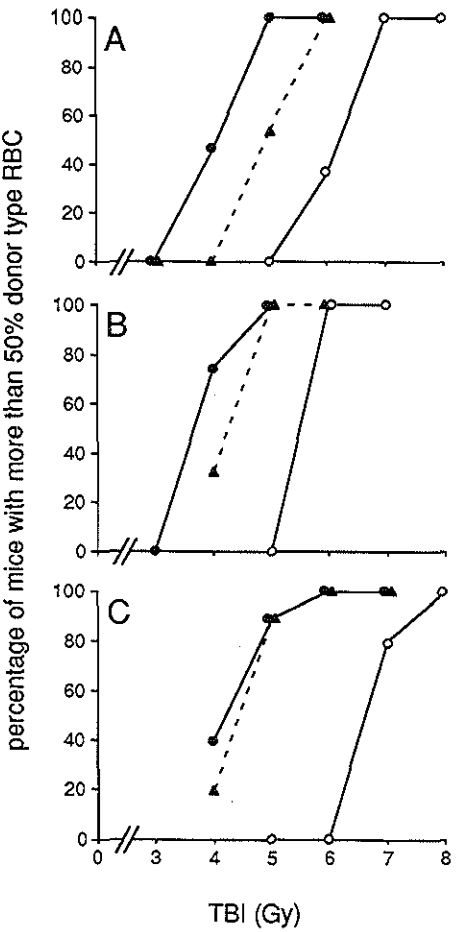


Figure 7.1:
Correction of α-thalassemia in female recipients of $3x10^6$ normal bone marrow (BM) cells. Symbols; open circles: no MCA or control rat IgG; closed triangles: anti-CD4/CD8 MCAs; closed circles: anti-CD4/CD8 MCAs + anti-CD11a MCA. A: 12 weeks after BMT with female C3C F1 BM cells, pooled data from 4 independent experiments. Total number of 266 mice analyzed (mean number of mice (± s.d.) per data point 12 (6), range 4-26). Follow-up for periods between 18 and 51 wks after BMT showed no changes in the chimeric state. B: 17 weeks after BMT with male C3C F1 BM cells, 3-4 mice per data point. C:12 weeks after BMT with male allogeneic BCBA F1 BM cells, pooled data from 2 independent experiments. Total number of 116 mice analyzed (mean number of mice (± s.d.) per data point 6 (2), range 2-9). Follow-up between 18 and 29 wks after BMT showed no changes in the chimeric state.

relatively low toxicity and sufficient immunosuppression to allow for sustained allogeneic bone marrow chimerism and mutual tolerance of donor and recipient type immunocompetent cells.

Red cell chimerism in α-thalassemic mice can be easily monitored by a flow cytometric method based on the size difference of normal and thalassemic erythrocytes.[508] In the present study, these mice were used to test the efficacy of anti-CD4, anti-CD8 and anti-LFA-1 MCAs as a supplement to doses of TBI which are insufficient for uniform acceptance of a bone marrow allograft. Transplantation of C3C (C3H x BALB/c) F1 BM cells enabled assessment of the efficacy of MCAs uncomplicated by possible immunosuppression due to GvH reactions, whereas transplantation of BCBA F1 bone marrow cells into BALB/c recipients represented a fully MHC-mismatched combination. Red cell chimerism was used to monitor the stability of the chimeric state and white blood cell and immature bone marrow cell chimerism were measured by in situ hybridization using a Y-chromosome specific probe to assess the degree of chimerism achieved in female recipients of male bone marrow cells.

7.2 RESULTS

7.2.1 Prevention of normal C3C F1 BM allograft rejection by MCAs

Recipient mice received a conditioning regimen of different combinations of MCAs and graded doses of TBI, before transplantation with $3x10^6$ sex matched F1 BM cells (Figure 7.1a). Supplementing the conditioning TBI with anti-CD4/CD8 MCAs resulted in approximately 1 Gy reduction in the TBI dose required to obtain sustained correction. Anti-CD4/CD8 and anti-CD11a MCAs allowed for a 2 Gy lower dose than after conditioning with TBI alone. The combination of all four MCAs was as effective as that of the anti-CD4/CD8 and anti-CD11a MCAs (Table 7.1). By adding anti-CD18 MCA to a conditioning regimen of TBI and anti-CD4/CD8 MCAs, correction of α-thalassemia, defined as > 50% normal RBC, was obtained as frequently as after conditioning with TBI and anti-CD4/CD8 MCAs alone (P-values at 4 and 5 Gy respectively 0.65 and 0.24). Anti-LFA-1 (anti-CD11a/CD18) pretreatment alone was not effective (Table 7.1).

Long-term stable partial allogeneic hemopoietic chimerism was observed after conditioning with 4 and 5 Gy TBI and anti-CD4/CD8 MCAs or both anti-T-cell

Table 7.1: Efficacy of different combinations of MCAs and TBI in the promotion of engraftment of $3x10^6$ female C3C F1 BM cells in female α-thalassemic recipient mice

MCAs (anti-)	4 Gy TBI	P-value	5 Gy TBI	P-value	6 Gy TBI	P-value
-	not done		0/12‡	0.28	4/11	0.73
CD11a/CD18			3/14		3/5	
CD4/CD8 CD11a	6/13	0.08	26/26§		5/5§	
CD4/CD8 CD11a/CD18	10/14		22/22§		5/5§	

‡ number of mice with >50% donor type RBC / total number of mice transplanted
§ in both groups all mice >50% donor type RBC; P-value therefore not evaluated

and anti-LFA-1 MCAs. At TBI doses higher than 5 Gy, either 100% normal donor type RBC were found, or donor marrow was completely rejected. The results obtained in recipients of sex mismatched C3C F1 BM cells closely resemble those obtained in recipients of sex matched C3C F1 BM cells (Figure 7.1b). Similar to recipients of sex matched C3C F1 BM cells, an additional immunosuppressive effect of the anti-CD11a MCA when combined with anti-CD4/CD8 MCAs was found.

7.2.2 Prevention of fully MHC-mismatched normal BM allograft rejection by MCAs

In vivo treatment of recipients with anti-CD4/CD8 MCAs allowed for approximately 2 Gy reduction in the TBI dose required for sustained correction of thalassemia. However, an additional immunosuppressive effect of either anti-CD11a MCA alone, or in combination with anti-CD18 MCA was not observed (Figure 7.1c).

7.2.3 Selective advantage of normal red cell production

Comparison of RBC and WBC chimerism in recipients of fully MHC- and sex mismatched BM cells (Table 7.2) demonstrated a selective advantage of normal red cell production, in that in all analyzed groups, RBC chimerism exceeded WBC chimerism. After conditioning with 5 Gy TBI and anti-CD4/CD8 MCAs, the lowest level of donor type WBC was found. These recipients were partial hemopoietic stem cell (HSC) chimeras, but engraftment was obviously sufficient to allow for correction of thalassemia. WBC chimerism observed in recipients conditioned with 5 Gy TBI and all MCAs reached somewhat higher levels than observed for the anti-CD4/CD8 treated mice, indicating that anti-LFA-1 MCAs may contribute to the engraftment of allogeneic cells.

Table 7.2: RBC and WBC chimerism in female α-thalassemic mice conditioned with TBI and anti-CD4/CD8 as well as anti-CD11a/CD18 MCAs before transplantation with $3x10^6$ normal male BCBA F1 BM cells (8 wks after BMT). Chimerism appeared to be stable in these mice for a period up to 29 weeks after BMT.

conditioning regimen		rejections	mean percentage donor type (s.d.)	
TBI (Gy)	MCAs (anti-)		WBC*	RBC‡
7	-	0/3	89 (4)	99 (2)
5	CD4/CD8	1/3	71§	95§
6	CD4/CD8	0/5	84 (4)	99 (1)
5	CD4/CD8/CD11a/CD18	0/5	81 (2)	98 (3)

* determined by FISH on peripheral blood smears; ‡ measured by flow cytometry; § rejection not included

BM CFU-S-12 chimerism was analyzed in four mice 13 weeks after BMT following a conditioning regimen of 5 Gy TBI and various combinations of MCAs (Table 7.3). The degree of chimerism of these immature BM cells

Table 7.3: Hemopoietic chimerism in female α-thalassemic recipients of $3x10^6$ normal male BCBA F1 BM cells (13 wks after BMT)

TBI dose (Gy)	MCAs (anti-)	percentage donor type: peripheral blood white cells*	peripheral blood red cells‡	BM CFU-S-12§
5	CD4/CD8	31	not evaluated	53 (10/19)
5	CD4/CD8	54	90	27 (8/30)
5	CD4/CD8 CD11a/CD18	70	97	63 (12/19)
5	CD4/CD8 CD11a/CD18	70	87	95 (20/21)

* determined by FISH on peripheral blood smears; ‡ measured by flow cytometry; § between brackets: number of donor type colonies / total number of colonies analyzed

appeared to be significantly lower than RBC chimerism, with the exception of one mouse that reached almost complete chimerism.

7.3 DISCUSSION

In this study, immunosuppression by a combination of anti-CD4 and anti-CD8 MCAs allowed for a 1 to 2 Gy reduction in the TBI dose required to obtain sustained correction of murine α-thalassemia after transplantation of allogeneic BM cells. When these MCAs were further supplemented with anti-CD11a MCAs, an additional immunosuppressive effect was observed after transplantation of C3C F1 BM cells, but not for completely MHC-mismatched BM cells. Anti-CD18 MCA was not effective in promoting the engraftment in any of the two donor-recipient combinations used. In this study, determination of chimerism at different hemopoietic stages confirmed the selective advantage of normal erythropoiesis observed previously in stable syngeneic partial chimeras (Chapters V[505] and VI[507]).

Stable allogeneic partial chimerism was obtained after pretreatment with MCAs and TBI doses of 4 and 5 Gy. Sustained allogeneic partial chimerism was induced at lower TBI levels than after the conditioning regimens developed previously that used either anti-CD4 and anti-CD8 MCAs,[94,95] or anti-CD3 MCAs.[55] In addition, it was not necessary to use thymus irradiation.[436] Strain differences in the radiosensitivity of BM allograft rejecting cells have been reported.[417] Evaluation of the relative efficacy of these conditioning regimens using sublethal TBI and MCAs would therefore require parallel testing in the same mouse strain.

In previous studies, anti-LFA-1 MCAs have been used as additional immunosuppressive agents in conditioning regimens that were already totally marrow ablative.[35,140,141,303,513] An anti-CD11a MCA has been reported to be effective in promoting engraftment of allogeneic BM cells in pediatric patients with immunodeficiencies or osteopetrosis,[140,141] although evaluation of the results is difficult due to the fact that a prospective randomized trial is still lacking. In adult leukemia patients neither anti-CD11a MCAs, nor anti-CD18 MCAs prevented MHC-matched or MHC-mismatched BM allograft rejection.[35,303] The absence of an engraftment promoting potential of anti-CD18 MCA was confirmed in our study. Experimentally, anti-CD11a MCAs have been reported to promote hemato-immunological reconstitution after transplantation of allogeneic BM cells, although the degree of donor type engraftment was not evaluated.[513] To our knowledge, this is the first study to show that addition of anti-CD11a MCAs to a conditioning regimen of sublethal TBI and anti-CD4/CD8 MCAs allows for engraftment at lower TBI doses as well as for higher levels of donor type chimerism.

Additional immunosuppression of anti-CD11a MCAs was not observed in the donor-recipient combination that was fully MHC-mismatched. Dependence of engraftment of allogeneic BM cells on factors such as BM cell dose, pretransplant conditioning regimen, and donor-recipient combination, has been observed by others.[55,136,500,501,514] It is conceivable that the observed difference of efficacy of anti-CD11a MCA in both donor-recipient combinations used in this study has been caused by the different degrees of histoincompatibility.

We propose that combinations of anti-human anti-CD4, anti CD8 and anti-CD11a MCAs may not only be clinically useful in the prevention of BM

allograft rejection, but also for the induction of stable allogeneic partial chimerism, which may be especially suitable for thalassemia patients, as such patients may be expected to benefit from the relatively low toxicity of the conditioning regimen, as well as from the selective advantage of normal erythropoiesis replacing the affected endogenous red cell lineage.

CHAPTER VIII

MURINE HEMOPOIETIC STEM CELLS WITH LONG-TERM REPOPULATING ABILITY AND HIGH RADIOSENSITIVITY

Radiosensitivity of murine long-term repopulating hemopoietic stem cells (LTR-HSC) was determined *in vivo*, using competition between residual endogenous and transplanted LTR-HSC. α-Thalassemic mice and normal litter mates were transplanted with graded numbers of normal or α-thalassemic bone marrow cells after total body irradiation with doses varying between 2 and 7 Gy (γ). Peripheral blood red cell chimerism, measured between 24 and 32 weeks after bone marrow transplantation was used to calculate D_0 values for LTR-HSC. The D_0 values were 0.68 ± 0.08 Gy, and 0.59 ± 0.10 Gy for, respectively, α-thalassemic and normal LTR-HSC. The D_0 value for normal bone marrow derived day-12 spleen colony-forming cells (BM CFU-S-12) in the same mouse strain was 0.67 ± 0.15 Gy. In addition to recent publications, which showed that cells with long-term repopulating ability are less radiosensitive than CFU-S-12, the results obtained in this study indicate the existence of a population of murine hemopoietic stem cells of long-term repopulating ability with a radiosensitivity similar to that of CFU-S-12 of the same mouse strain. We propose that the population of hemopoietic stem cells with long-term repopulating ability is heterogeneous with respect to radiosensitivity.

adapted and extended from:

Cor van den Bos, Rolf W. Bartstra, Dorinde Kieboom and Gerard Wagemaker (submitted for publication)

8.1 INTRODUCTION

Total body irradiation (TBI) is one of the most important single agents used in conditioning regimens for bone marrow transplantation (BMT).[529] It creates space for engraftment of transplanted hemopoietic stem cells (HSC), suppresses host-versus-graft reactivity, and it eradicates, when present, tumor cells.[529] The radiosensitivity of endogenous HSC determines the fraction that will survive a certain dose of TBI. Spleen colony-forming-units (CFU-S)[487] have for a long time been held equivalent to HSC, and their radiobiological properties have been studied extensively.[196] D_0 values for murine CFU-S have been estimated to vary between 0.62 and 1.70 Gy for γ-irradiation,[171,196,369,487] and between 0.62 and 1.05 Gy for 200-300 kV X-rays.[196,216,477] However, CFU-S have more recently been subdivided in several subsets,[201,300,570] and evidence has accumulated for the existence of more immature cells, which are not readily detected by the spleen colony test and, therefore, considered as pre-CFU-S.[200,202,230] Furthermore, CFU-S can be physically separated from more immature HSC[48,231,380,381,383] (Chapter IX[510]). Current knowledge about the radiobiological characteristics of those more immature pre-CFU-S cells is limited. Recent data obtained with *in vitro* assays suggest that these cells are less radiosensitive than CFU-S.[319,387]

α-Thalassemic mice suffer from a hypochromic, microcytic anemia,[393,416] that can be corrected by the induction of partial hemopoietic chimerism.[27,541] Peripheral blood red cell chimerism reflects the degree of HSC chimerism (Chapter V[505]), and can easily be quantified longitudinally (ChapterIII[508]). In this study, radiosensitivity of murine LTR-HSC, was determined by analysis of endogenous repopulation in comparison to repopulation by graded numbers of transplanted bone marrow (BM) cells.

8.2 CALCULATION OF D_0 VALUES AND α/β RATIOS

D_0 values for long-term repopulating stem cells were calculated on the assumptions that (i) the homing fraction of transplanted HSC is independent of the number of BM cells injected in the dose range used in this study, (ii) radiation damage to the hemopoietic microenvironment in the TBI dose range used in this study, equally influences proliferation and differentiation of residual endogenous HSC and transplanted HSC, and (iii) the ratio of

thalassemic to normal LTR-HSC determines the degree of peripheral blood red cell chimerism.

According to the standard single-hit model, the fraction (S) of surviving target cells that survives an irradiation dose (D) can be mathematically expressed as:

$S_{(D)} = e^{-D/D0}$ (1)

The number of LTR-HSC which reach the hemopoietic sites (Nh) can be expressed, on basis of the first assumption, as a fraction (C_1) of the number of cells transplanted (Nt):

$Nh = C_1 \times Nt$ (2)

Assumption 3 states that the residual number of LTR-HSC after a TBI dose (D50/Nt), i.e., the dose that leads to partial chimeras with 50% donor type RBC after transplantation of Nt cells, is equivalent (C_2) to the number of cells reaching hemopoietic sites (Nh). If the total number of LTR-HSC in a mouse equals N, this means that:

$N \times S_{(D50/Nt)} / Nh = C_2$ (3a) or:

$N \times S_{(D50/Nt)} = C_2 \times Nh$ (3b)

Equation 3b can be rewritten to:

$N \times e^{-D50/Nt/D0} = C_2 \times C_1 \times Nt$ (4)

and:

$\ln (Nt) = -1/D_0 \times D50/Nt + C_3$ (5)

The D_0 values can be calculated by solving equation 5 (C_3 is a constant calculated as equation 4 is rewritten to equation 5):

$\ln (Nt) = -1/D_0 \times D50/Nt$ (6)

For different numbers of donor BM cells transplanted, the D50/Nt was calculated by linear regression analysis according to the least squares method in the linear parts of the dose effects curves. The 95% confidence limits for the D50/Nt calculated using standard *t*-statistics, were used to derive weights in the calculation of the D_0 of LTR-HSC on basis of equation 2.

Recent radiobiological models have focused on a linear-quadratic approach to fit cell kill as a function of radiation dose.[123]. Accordingly, target cell survival is represented by the equation:

$S_{(D)} = e^{-(\alpha D + \beta D^2)}$ (7)

Substitution in equation 3 results in:

$N \times e^{-(\alpha D_{50/Nt} + \beta D^2_{50/Nt})} = C_4 \times Nt$ (8)

and:

$\ln (Nt) = -(\alpha D_{50/Nt} + \beta D^2_{50/Nt}) + C_5$ (9)

Values for α and β were obtained by fitting a second order polynomial regression line for this function using Apple Macintosh personal computers and StatView^{TM} software.

8.3 RESULTS

Sublethally irradiated (2-7 Gy) α-thalassemic recipient mice were injected with normal syngeneic BM cell numbers ranging from 3×10^4 to 3×10^6 per mouse. Identical dose-effect relations were found in three independent experiments (Figure 8.1). Mean levels of donor type red blood cells found in normal mice (pooled data of two experiments) after transplantation of α-thalassemic BM cells are also shown in Figure 8.1. $D_{50/Nt}$ values were calculated for each cell dose. Figure 8.2 shows $D_{50/Nt}$ as a function of the number of BM cells transplanted for, respectively, α-thalassemic recipients of normal BM cells, and normal recipients of α-thalassemic BM cells. The D_0 values calculated were 0.68 ± 0.08 Gy and 0.59 ± 0.10 Gy for, respectively, α-thalassemic and normal LTR-HSC.

Radiosensitivity of BM CFU-S-12 was determined for BALB/c-Hba$^{+/+}$ and BCBA mice (Figure 8.3). BALB/c CFU-S-12, with a D_0 value of 0.67 ± 0.15 Gy and an extrapolation number of 1.65, were found to be more radiosensitive than BCBA CFU-S-12, with a D_0 value of 0.98 ± 0.14 Gy and an extrapolation number of 1.31.

Target cell survival was also analyzed according to the linear-quadratic (LQ) model (Table 8.1). For the LTR-HSC and BALB/c BM CFU-S-12, β values were found to be either negative or not significantly different from zero. The α- and β-values obtained for BCBA BM CFU-S-12 were, respectively, 0.77 ± 0.01 Gy^{-1}, and 0.037 ± 0.02 Gy^{-2}, with an α/β ratio of 20.73 ± 1.17 Gy.

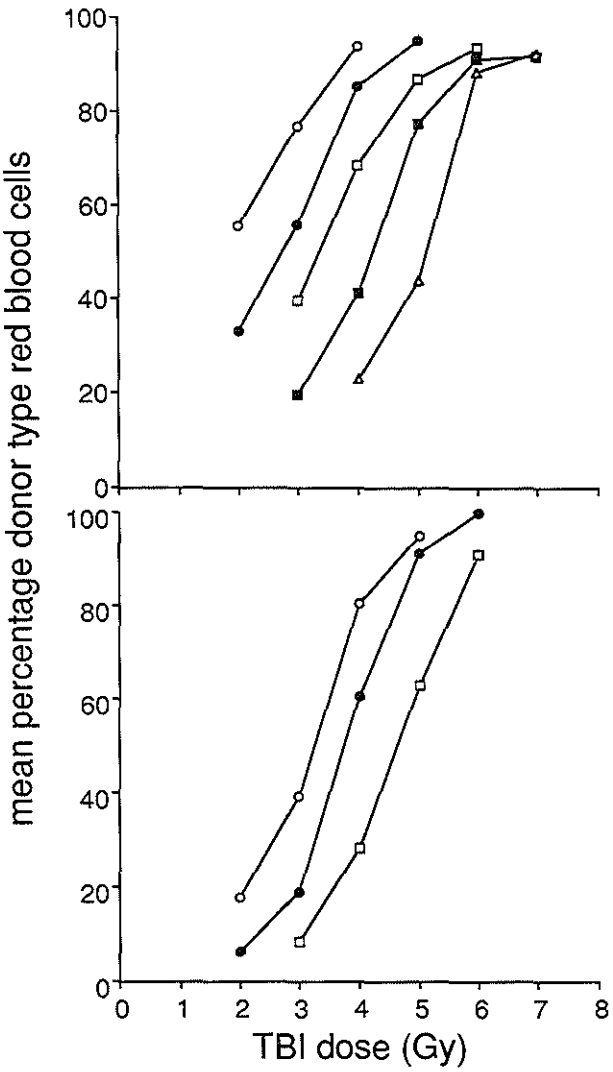


Figure 8.1:
Top; Mean percentage donor type peripheral blood red cell chimerism in α-thalassemic recipients of normal BM cells (pooled data from three independent experiments; total number of 126 mice analyzed between 24 and 30 weeks after BMT). Bottom; Mean percentage donor type peripheral blood red cell chimerism in normal recipients of α-thalassemic BM cells (pooled data from two independent experiments; total number of 74 mice analyzed 30 to 32 weeks after BMT). Symbols represent BM cell doses: open circles, 3 x 10^6 cells; closed circles, 10^6 cells; open squares, 3 x 10^5 cells; closed squares 10^5 cells; open triangles, 3 x 10^4 cells.

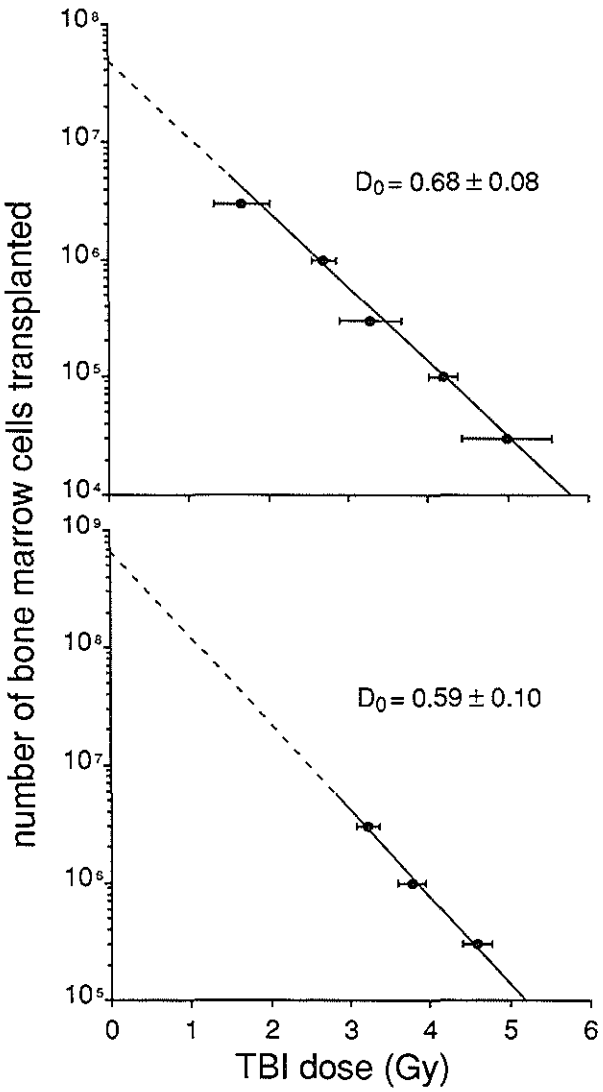


Figure 8.2:
Determination of D_0 value for LTR-HSC on basis of the estimated TBI doses leading to chimeras with 50% donor type peripheral blood red cells ($D_{50/Nt}$). Top: α-thalassemic LTR-HSC; bottom: normal LTR-HSC.

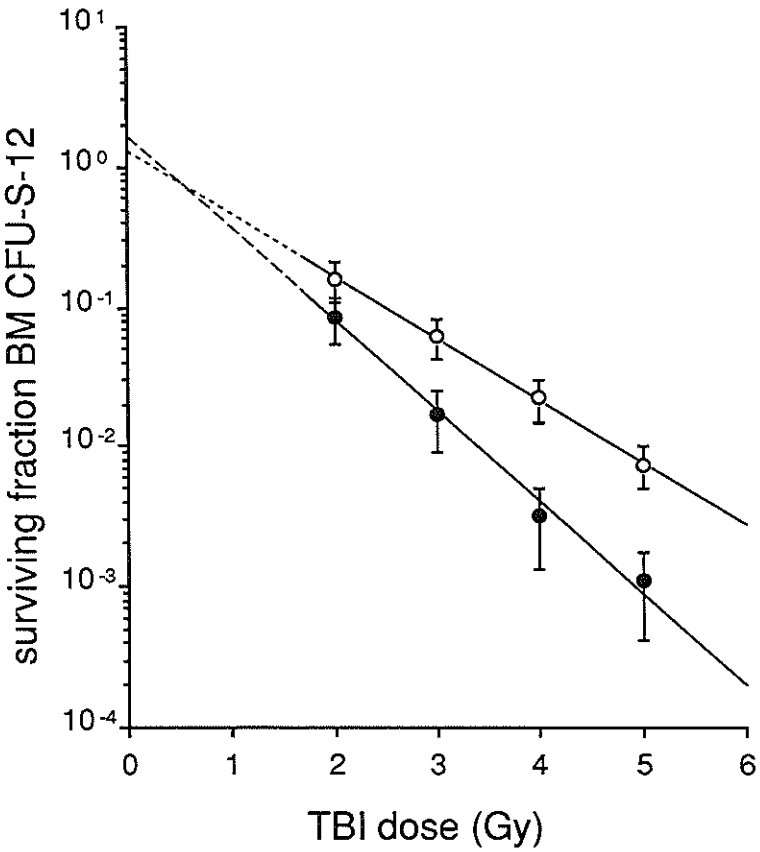


Figure 8.3: Radiosensitivity of BM CFU-S-12 in female BALB/c-Hba$^{+/+}$ and BCBA mice. Pooled data of two independent experiments for both mouse strains, based on colony counts in nine to twelve recipient mice per data point per experiment. The same number of recipients was used for determination of femoral CFU-S-12 content in unirradiated control mice. Bars represent standard errors of the mean surviving fractions. Open circles: BCBA mice, closed circles BALB/c-Hba$^{+/+}$ mice

8.4 DISCUSSION

Radiosensitivity for γ-irradiation of HSC with long-term repopulating ability, i.e., those capable of producing erythroid progeny for at least 6 months after BMT, was determined. The D_0 values found for BALB/c LTRA, 0.68 ± 0.08 Gy and 0.59 ± 0.10 Gy, are similar to the D_0 value for BALB/c BM CFU-S-12, i.e., 0.67 ± 0.15 Gy, and considerably lower than the 0.98 ± 0.14 Gy for BCBA BM CFU-S-12. The D_0 values for LTR-HSC are also lower than those reported for pre-CFU-S cells of BCBA mice, determined by a number of *in vitro* assays,[387] whereas the value for BCBA CFU-S-12 is very similar to the 0.91 Gy reported.[387] In different mouse strains irradiated with various radiation qualities, identical observations have been made, i.e., pre-CFU-S cells always had higher D_0 values than CFU-S-12.[317-319,387] Although strain differences in the radiosensitivity of HSC subsets[578] have been reported, such differences do not explain the observed discrepancy between the *in vivo* and *in vitro* data, since D_0 values determined *in vivo* for LTR-HSC were similar to that for CFU-S-12.

Therefore, these data can be explained by heterogeneity of HSC with long-term repopulating ability, i.e., a population with a high radiosensitivity, measured by the *in vivo* method applied in this study and a population with a lower radiosensitivity, measured by the *in vitro* methods.[385-387] It is not feasible to assay the latter with the competitive *in vivo* method used in this study, since it would require doses of TBI close to full ablation of endogenous hemopoietic cells to minimize repopulation by LTR-HSC with a low D_0 (Figure 8.4). However, since the radiosensitivity of LTR-HSC of BCBA mice has not been determined in this study, a BALB/c-strain specific high radiosensitivity of LTR-HSC can also explain the observations of this study.

Negative β-values or values not significantly different from 0 were found for LTR-HSC when the data were fitted to the LQ model. Although evidently based on only a limited number of data points, these observations are compatible with the existence of two subpopulations of LTR-HSC with different radiosensitivities (Figure 8.4, composite curve).

Partial peripheral blood red cell chimerism in α-thalassemic mice resulted not only in coexistence of thalassemic and normal LTR-HSC, but also demonstrated a selective advantage of the normal erythroid lineage[541] (Chapter V[505]).

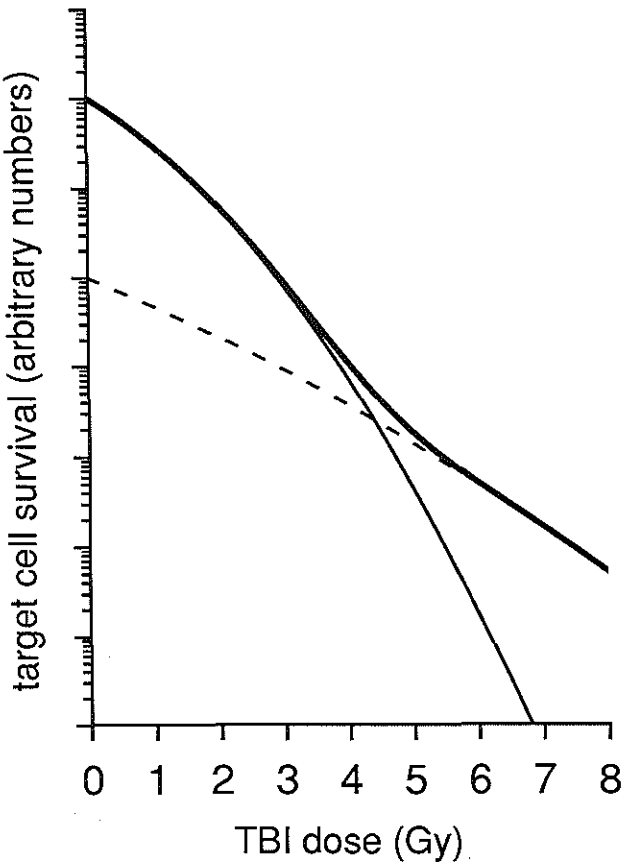


Figure 8.4:
Hypothetical survival of LTR-HSC subsets after TBI. The LTR-HSC with high radiosensitivity (D_0 = 0.64, i.e., weighted mean of the D_0 values for α-thalassemic and normal LTR-HSC), are represented by the thin line and arbitrarily estimated to be 100 x as numerous as the LTR-HSC with low radiosensitivity represented by the broken line (D_0 = 1.3 from reference 387). The thick line represents the sum of both populations. It is obvious that TBI doses above approximately 5 Gy are necessary to be able to evaluate the D_0 of the radioresistant subpopulation.

Table 8.1: Radiosensitivity paramaters of murine hemopoietic stem cell subsets according to the single-hit model and the linear-quadratic (LQ) model.

mouse strain	stem cell subset	single-hit model	LQ-model		
		D_0-value (Gy)	α (Gy^{-1})	β (Gy^{-2})	α/β (Gy)
BALB/c-Hba$^{th/+}$	LTR-HSC	0.68 ± 0.08	1.14 ± 0.53	0.04 ± 0.08*	
BALB/c-Hba$^{+/+}$	LTR-HSC	0.59 ± 0.10	4.70	-0.39	
BALB/c-Hba$^{+/+}$	CFU-S-12	0.67 ± 0.15	2.44 ± 0.54	-0.14 ± 0.77*	
BCBA	CFU-S-12	0.98 ± 0.14	0.77 ±0.01	0.037 ± 0.002	20.73 ± 1.17

* = not significantly different from 0

This is reflected by the relative positions of the dose-effects curves of Figure 8.1. It is not known whether the magnitude of the selective advantage is dependent on the degree of LTR-HSC or peripheral blood red cell chimerism. It is, however, unlikely that such a dependence would influence the results, since firstly, $D_{50/Nt}$ values were calculated in clearly linear parts of the dose-effect relationships (Figure 8.1), and secondly, the selective advantage of the normal erythroid lineage would be similar in the $D_{50/Nt}$ estimates for different BM cell doses. In a recent report, erythroid chimerism assessed by glucose-phophate isomerase isoenzyme analysis, revealed approximately 50% donor type engraftment after 2 Gy TBI and BMT with 1 x 10^7 cells.[514] The degree of erythroid engraftment found is more compatible with the D_0 estimate obtained in this study, than with the estimate obtained with the *in vitro* assays.[387,514]

From murine studies, a model has emerged that the most primitive HSC, i.e., those with the greatest ability to produce progeny for prolonged periods, are the least radiosensitive. Similar observations were done in primate studies. A D_0 for rhesus monkey HSC of 0.6 Gy was estimated by comparing endogenous reconstitution with regeneration after autologous BMT, using survival as an end-point.[530] Using recovery of peripheral blood reticulocytes as an end-point in a study that included extensive supportive care to ensure survival of the animals, a higher D_0 of 1.3 Gy was estimated.[565,566] Short-term survival was proposed to be dependent on more radiosensitive, and perhaps more mature HSC, whereas in the latter study the radiosensitivity of a more immature HSC subset might have been measured.[504]

The data presented here indicate that subsets of HSC with long-term repopulating ability may differ in radiosensitivity.

CHAPTER IX

STABLE MULTILINEAGE HEMOPOIETIC CHIMERISM IN α-THALASSEMIC MICE INDUCED BY A BONE MARROW SUBPOPULATION THAT EXCLUDES THE MAJORITY OF CFU-S DAY-12

We have investigated the contribution of highly purified day-12 spleen colony-forming units (CFU-S-12) as well as more immature hemopoietic stem cells to sustained blood cell production using *in vivo* and *in vitro* assays that allow for frequency analysis. Normal or day-6 post-5-fluorouracil low density bone marrow was sorted on the basis of differences in rhodamine-123 (Rh-123) retention or wheat germ agglutinin (WGA) affinity and tested *in vivo* using a recently developed α-thalassemic chimeric mouse model. In addition, short and long-term clonal activity was assessed *in vitro* using a limiting dilution type long-term bone marrow culture, the CAFC-assay. When sublethally irradiated α-thalassemic mice were transplanted with as many as 281 purified WGA^{bright} CFU-S-12, derived from a fraction containing 95% of all CFU-S-12 from day-6 post-5-fluorouracil low density bone marrow of wild-type mice, detectable chimerism was not observed at 6 months post-transplantation. In contrast, only 3 CFU-S-12 were included in the $Rh\text{-}123^{dull}$ and WGA^{dim} subpopulations that induced 29-58% and 21-31% stable multilineage donor-type chimerism of erythrocytes and leukocytes, respectively. The $Rh\text{-}123^{dull}$ and WGA^{dim} cells were up to 240-fold enriched for long-term repopulating ability (LTRA) as compared to unseparated bone marrow. A comparable level of chimerism was found in the different hemopoietic organs and at the level of bone marrow CFU-S-12. The frequency of the LTRA-unit capable of inducing a 10% sustained level of donor-type erythrocytes in 5 Gy irradiated recipients, was calculated to be 1-2 per 10^5 bone marrow cells.

Several studies have suggested that LTRA and spleen colony formation could be capacities of the same stem cell subset. The present results, however, demonstrate that the majority of CFU-S-12 have only short-term repopulating ability and are physically separable from more immature stem cells with long-term multilineage reconstituting capacities.

adapted from:

Johannes C.M. van der Loo, Cor van den Bos, Miranda R.M. Baert, Gerard Wagemaker and Rob E. Ploemacher: Blood (in press)

9.1 INTRODUCTION

Data from a variety of physical sorting experiments have initiated a debate as to whether cells with *in vivo* long-term repopulating ability (LTRA) and day-12 spleen colony-forming units (CFU-S-12) belong to the same stem cell population or represent distinct and separable subsets.[219,220,281,455,556,557] LTRA and spleen colony-forming ability have been reported to co-purify at high enrichment levels in the population bearing the lineage (Lin) negative (CD4, CD8, Gr-1, Mac-1, TER-119)$^-$ Sca-1$^+$(Ly-6A/E$^+$) Thy-1.1^{lo} phenotype, suggesting that these functions may be attributed to the same cellular subset.[449,455,496] In contrast, bone marrow populations purified by counterflow elutriation have been demonstrated to induce long-term repopulation *in vivo* while being largely depleted of CFU-S activity.[231] Also, flow sorting on the basis of wheat germ agglutinin (WGA) affinity or rhodamine-123 (Rh-123) retention enabled separation of pre-CFU-S from CFU-S-12 quality.[380,383] Cells with marrow repopulating ability (MRA-(CFU-S-13)) or LTRA have been found to have a low affinity for WGA, while CFU-S-12 bind high levels of lectin.[383] Using the supravital dye Rh-123, CFU-S-8, CFU-S-12 and cells with MRA-(CFU-S-13) could subsequently be classified on the basis of their decreasing mitochondrial activity.[48,341,377] It has been demonstrated that almost all CFU-S-12 stain brightly with Rh-123, whereas 90% of the pre-CFU-S activity (determined as MRA-(CFU-S-13)) is contained in the 25% most dull population.[377,381] These studies all demonstrated heterogeneity within the stem cell compartment and indicated that CFU-S activity and long-term repopulating ability are separable.

The present study was designed to separate and highly purify CFU-S-12 and immature stem cells, and to study the contribution of these cells to short and long-term repopulation using *in vivo* and *in vitro* assays. Low density bone marrow was sorted using the differences in either Rh-123 retention or WGA affinity. Also, bone marrow from mice treated with 5-fluorouracil was used, as such a pretreatment has been shown to result in very high enrichments for stem cells if combined with sorting on the basis of WGA.[383] Using this marrow, however, Rh-123 was not able to discriminate the stem cell populations as well as it did in untreated bone marrow and was therefore not included.

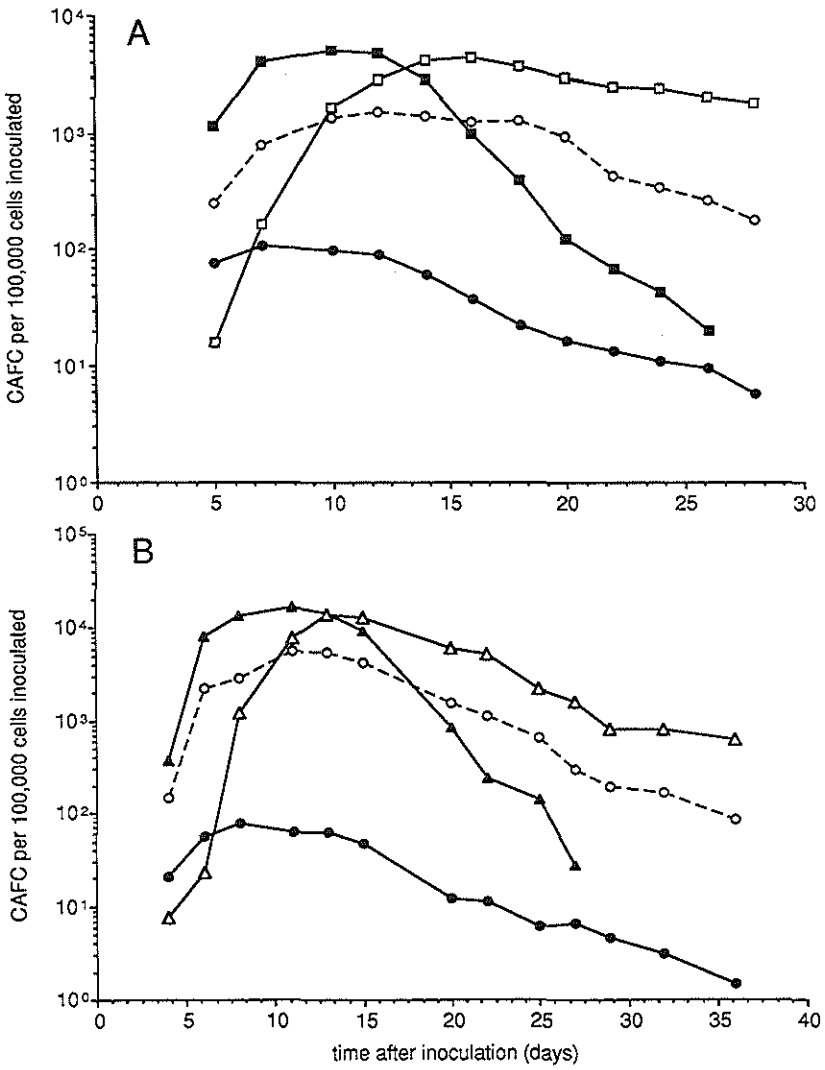


Figure 9.1:
CAFC-frequencies of (A): unseparated BMC (closed circles), low density ER-MP20$^-$ cells (open circles), and the 10% most Rh123^{bright} (closed squares) and Rh123^{dull} cells (open squares) sorted from the LD/ER-MP20$^-$ population; and (B): unseparated BMC (closed circles), LD/FU$_{6d}$BM (open circles), and its 6% most WGA^{bright} (closed triangles) and 6% WGA^{dim} (open triangles) subpopulations. Frequencies were calculated by limiting dilution analysis using Poisson statistics. Data represent 2 of 4 individual experiments.

The repopulating ability of purified CFU-S-12 and of a population highly enriched for immature stem cells were analyzed *in vivo* using an α-thalassemic sex mismatched chimeric mouse model[540,541] (Chapters III[508] and V[505]), and *in vitro* using a limiting dilution type long-term bone marrow culture, the CAFC-assay.[384-386] The transplantation model was evaluated by comparison of the enrichment for donor-type erythrocytes determined by FACScan analysis and nucleated blood cells determined by fluorescent *in situ* hybridization (FISH) using a murine Y-chromosome specific probe. In addition, lineage expression of the grafted LTRA cells was studied at 12 months after transplantation. Chimerism at the level of bone marrow CFU-S-12 was further assessed by secondary transplantation.

9.2 RESULTS

9.2.1 Transient and persisting cobblestone area formation in the CAFC-assay

Low density bone marrow from untreated and 5-fluorouracil treated mice was separated on the FACS using Rh-123 or WGA, and tested for its short and long-term repopulating ability (STRA and LTRA) *in vitro* using the CAFC-assay. Normal low density bone marrow was depleted of contaminating monocytes and granulocytes by MACS or FACS using antibody ER-MP20. From this LD/ER-MP20$^-$ population, the 10% most Rh-123^{bright}, the 10% most Rh-123^{dull} cells, and the 4% most WGA^{bright} cells were sorted from within a light scatter window as previously described (Figure 9.1-A).[377,379,521] Low density day-6 post-5-fluorouracil bone marrow (LD/FU$_{6d}$BM) was not further depleted of monocytes and granulocytes, as the percentages of these cells were already low. The WGA^{bright} and WGA^{dim} cells, sorted from LD/FU$_{6d}$BM, each comprised 6% of the nucleated cells (Figure 9.1-B).[383] The results showed that both the Rh-123^{bright} and WGA^{bright} subpopulations were 50 to 200-fold enriched for cells which gave rise to an early but transient cobblestone area formation at day 10 after inoculation (CAFC-10) (the *in vitro* equivalent to CFU-S-12[385,386]). In contrast, the Rh-123^{dull} and WGA^{dim} subpopulations showed persisting cobblestone area formation and were 250- to 300-fold enriched for late CAFC (CAFC-28/35). This indicates that the *in vitro* short and long-term repopulating ability can largely be separated using Rh-123 retention or WGA affinity.

To examine as to whether subpopulations of short- and long-term repopulating stem cells had been selected from the low density bone marrow, the respective recoveries of the sorted populations were calculated from the CAFC data. The ER-MP20$^-$ population contained 95 to 100% of all early and late CAFC that were originally present in low density bone marrow (Figure 9.2). The Rh-123^{bright} and WGA^{bright} subpopulations represented 65% and 95%, respectively, of the early appearing CAFC from LD/ER-MP20$^-$ bone marrow. The Rh-123^{dull} and WGA^{dim} cells, sorted from LD/ER-MP20$^-$ and LD/FU$_{6d}$BM, respectively, comprised 40 to 50% of the late appearing immature CAFC and only 5 to 10% of the original CAFC-10. These results together indicated that the sorted populations were representative for almost all STRA (including the CFU-S-12) and about half of the LTRA that was contained in the low density populations.

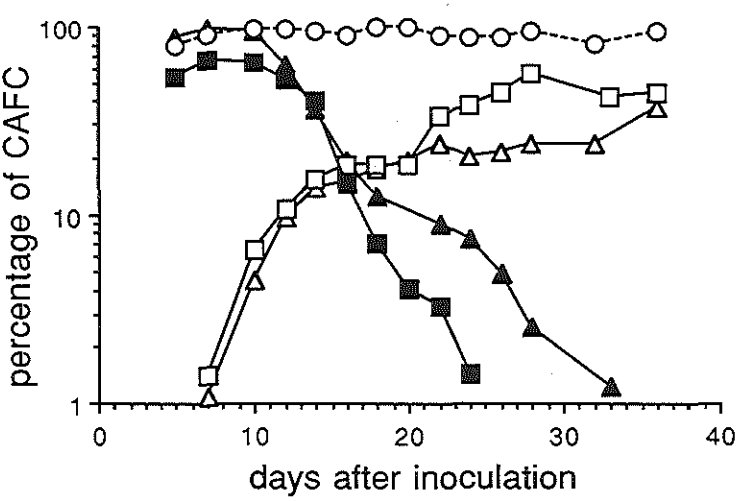


Figure 9.2: Percentages of the total number of CAFC in the ER-MP20$^-$ population as compared to low density bone marrow (open circles); the number of CAFC in the sorted Rh123^{bright} (closed squares), Rh123^{dull} (open squares) and WGA^{bright} (closed triangles) subpopulations as compared to LD/ER-MP20$^-$ cells; and the number of CAFC in the sorted WGA^{dim} subpopulation as compared to LD/FU$_{6d}$BM (open triangles).

Apparently, about 50% of the LTRA was contained in the Rh-123 and WGA intermediately staining cell populations which had not been sorted in our procedure.

9.2.2 Transient and persisting hemopoiesis *in vivo*

Bone marrow fractions tested in the CAFC assay were in parallel analyzed for STRA and LTRA *in vivo*, using the α-thalassemic chimeric mouse model. Sorted and unseparated BM cells, derived from normal male mice, were transplanted into groups of 3, 4 or 5 Gy irradiated female α-thalassemic mice (4 to 8 animals per group). In the subsequent 12 months, the percentage of peripheral blood donor-type erythrocytes was determined by FACScan analysis.

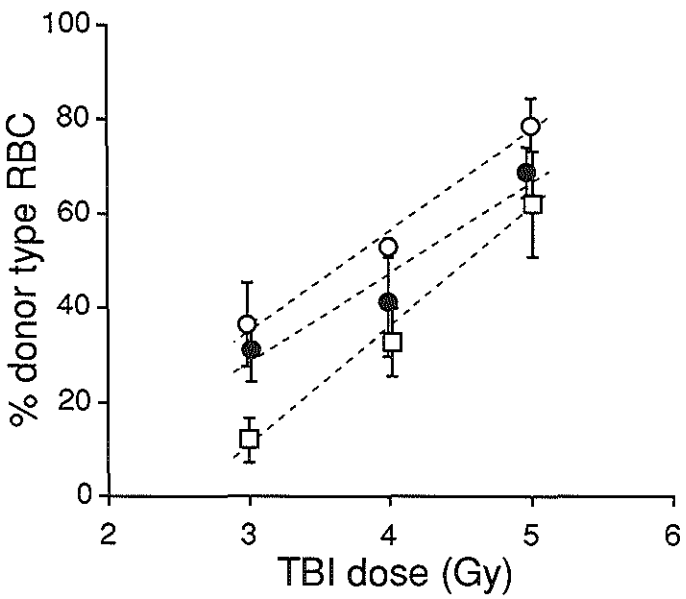


Figure 9.3: Percentage of donor red blood cells at 6 weeks after transplantation of $3x10^5$ unseparated BMC (open circles), $15x10^3$ LD/ER-MP20$^-$ cells (closed circles), or 670 Rh123^{dull} cells (open squares), in groups of 3, 4 or 5 Gy irradiated female α-thalassemic mice (4-8 animals per group). The lines are fitted using linear regression analysis. Bars denote 1 s.d.

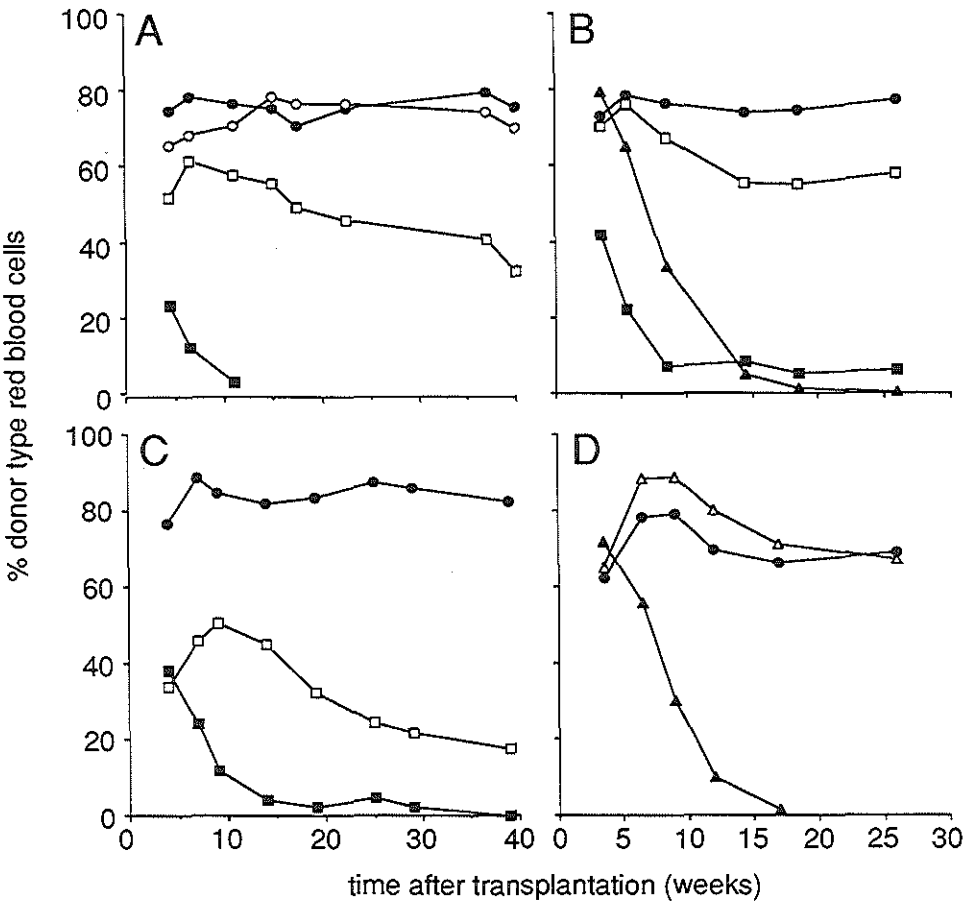


Figure 9.4:
Mean percentage of donor type red blood cells in 5 Gy irradiated α-thalassemic mice (4-8 animals per group) after transplantation of unseparated BMC or BMC sorted on the basis of Rh123 or WGA. A-D indicate 4 individual experiments. (A-D) $3x10^5$ unseparated BMC infused per recipient (closed circles); (A) $15x10^3$ LD/ER-MP20$^-$ cells (open circles); (A-C) 1930, 5950, 6770 Rh123^{bright} cells in A-C, respectively (closed squares); (A-C) 670, 1700, 2000 Rh123^{dull} cells in A-C, respectively (open squares); (B and D) 4700 and 3700 WGA^{bright} cells in B and D, respectively (closed triangles); and (D) 1700 WGA^{dim} cells (open triangles). Cells in experiment A-C were sorted from normal bone marrow, cells in D were sorted from 5-fluorouracil treated animals.

At each time point, the lines fitted through the mean percentages donor-type erythrocytes at 3, 4 and 5 Gy ran parallel for all bone marrow fractions tested. This is illustrated by Figure 9.3 at 6 weeks after transplantation. The mean repopulation data of the animals which received a dose of 5 Gy in each of the 4 individual experiments are shown in Figure 9.4. The enrichment for the *in vivo* erythroid repopulating ability of the sorted cells was calculated for each of the three irradiation groups. This was done by dividing the mean percentage of donor-type erythrocytes of animals which received sorted cells by that of recipients of unseparated bone marrow, after correction for the number of cells injected. Figures from the three individual radiation dose groups were then combined to calculate the mean enrichment, shown in Figure 9.5. Transplantation of 6770 Rh-123^{bright} or 4700 WGA^{bright} cells into α-thalassemic recipients led to a transient donor-type repopulation lasting only 4 to 5 months. In contrast, infusion of 670 or 1700 Rh-123^{dull}, or 1700 WGA^{dim} cells resulted in sustained chimerism of approximately 40 to 70% donor-type RBC (Figures 9.4 and 9.5). These populations were 50- to 240-fold enriched for *in vivo* long-term repopulating ability. In support of the *in vitro* data, these results showed that also the *in vivo* STRA and LTRA have largely been separated. In addition, our observations indicated that the erythroid progeny of the transiently repopulating cell fractions can be measured up to 5 months after transplantation.

9.2.3 Analysis of the number of CAFC-10, CAFC-28 and CFU-S-12 injected per mouse.

The contribution of CFU-S-12 and immature hemopoietic stem cells in the various fractions to long-term repopulation was analyzed by comparing the number of CAFC-10, CFU-S-12 and CAFC-28 with peripheral blood cell chimerism at 6 months after transplantation (Table 9.1). Injection of 1930 to 6770 Rh-123^{bright}, or 3700 to 4700 WGA^{bright} cells per mouse, containing low numbers of CAFC-28 but as many as 281 CFU-S-12 and high numbers of CAFC-10 injected per mouse, resulted in only short-term repopulation (Figure 9.4). Injection of 670 to 2000 Rh-123^{dull} cells, on the other hand, which contained low numbers of CAFC-10 (in one case even 0.7 CAFC-10, equivalent to about 0.1 CFU-S-12 injected per mouse[385]) led to stable long-term

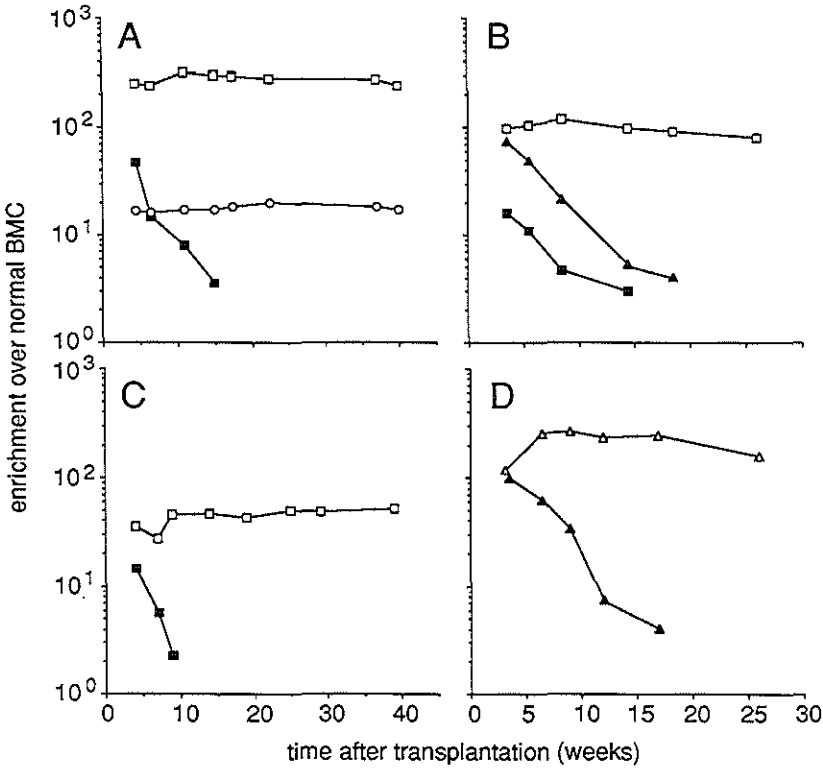


Figure 9.5: Mean enrichments for erythroid repopulating ability of sorted cells over unseparated BMC (see legend Fig 4 for the different populations) in groups of 3, 4 or 5 Gy irradiated α-thalassemic mice. Enrichments were separately calculated for each irradiation group, corrected for the number of cells injected, and then combined (see Results section). A-D indicate separate experiments. The control BMC level is at 1. Mean Coefficient of Variation of groups with stable chimerism was 55% (± 28%)

repopulation ranging from 24 to 58% at 6 months after transplantation (Table 9.1). In addition, injection of 189 WGA^{dim} cells from $LD/FU_{6d}BM$, which contained 0.9 CFU-S-12, led to more than 10% donor-type repopulation in 50% of the mice (Table 9.2), and more than 40% repopulation in 17% of the mice injected (not shown). Therefore, the data clearly demonstrated that long-term repopulation is independent of the number of CFU-S-12 injected and may be attributed to more immature $Rh\text{-}123^{dull}$ and WGA^{dim} cells.

Table 9.1: In vitro and in vivo repopulating ability of sorted cells

		infused per recipient			% donor-type at 6 months	
population	# cells	# CAFC-10	# CFU-S-12	# CAFC-28	RBC	WBC
NBMC	$3x10^5$	297	nd	18	76	31
	$3x10^5$	504	nd	24	87	32
	$3x10^5$	495	53	12	77	43
	$3x10^5$	213	73	12	69	41
LD/ER-MP20$^-$	$1.5x10^4$	106	nd	27	78	20
Rh-123^{bright}	6770	879	nd	< 0.9	5	0.5
	5950	532	63	< 16.1	6	nd
	1930	96	nd	< 0.5	5	1.0
WGA^{bright}	4700	1592	171	< 7.1	0	nd
	3700 *	555	281	< 9.9	< 2	nd
Rh-123^{dull}	2000	0.7	nd	4.3	24	11
	1700	18.7	3.1	76.8	58	31
	670	11.2	nd	12.2	47	12
WGA^{dim}	1700 *	78.4	8.3	140.4	67	43
	567 *	26.1	2.8	46.8	29	21
	189 *	8.7	0.9	15.6	14	7
	63 *	2.9	0.3	5.2	7	nd
	21 *	1.0	0.1	1.7	2	nd

Low density ER-MP20-negative cells (LD/ER-MP20$^-$), or day-6 post-5-fluorouracil BMC, were sorted on the basis of their rhodamine-123 (Rh-123) retention or wheat germ agglutinin (WGA) affinity. Long-term repopulation was determined after transplantation into 5 Gy irradiated female α-thalassemic mice. Donor-type red blood cells (RBC) and white blood cells (WBC) represent mean percentages of groups of 4-8 animals. RBC chimerism was determined by FACScan

analysis, WBC chimerism by FISH. * indicates the populations sorted from 5-fluorouracil treated animals. nd = not determined.

In the present study, the ratio between CAFC-10 and CFU-S-12 varied from 2.0 to 9.7. This could be due to intrinsic differences between individual stromal layers, or differences in the spleen seeding efficiency of the sorted cells. Previously, a more extensive regression analysis has shown that 4.7 to 6.7 CAFC-10 were detected for each CFU-S-12.[385]

Using this particular transplantation model, the LTRA unit was defined as the number of male cells that needs to be transplanted to reach a sustained level of at least 10% donor-type RBC in 5 Gy irradiated female α-thalassemic mice. Because of the limitations of the RBC and WBC analyses, a signal of less than 5% was not considered as indicative for donor-type engraftment. Using Poisson statistics and limiting dilution analysis, the frequency of the LTRA unit in the 160-fold enriched LD/FU_{6d} WGA^{dim} cells (Figure 9.5-D) was calculated to be 1 in 412 (Table 9.2).

9.2.4 Fluorescent in situ hybridization (FISH)

To test the concordance of the FISH technique, 120 peripheral blood slides were prepared from the same sex mismatched chimeric mouse and stored at -20°C. Groups of slides were subsequently processed in 3 different FISH sessions. The Y-chromosome-specific probe was derived from 6 different nick-translation batches that we had used in our experiments in the past two years. Series of 100 as well as 200 cells were counted, some by two independent observers. Analysis of variance and t-statistics (data not shown) showed no significant differences between: a) the different Y-chromosome-specific probe, b) the different FISH sessions, c) the counting of 100 or 200 cells, or d) between observers. The standard deviation of the 120 samples, at a level of 63.3% male cells, was 10.2%.

9.2.5 Comparison of the enrichment for LTRA *in vitro* and *in vivo*

In order to compare the enrichment of the sorted populations, as determined *in vitro* as well as *in vivo*, data from different experiments were combined. The percentage of donor-type nucleated cells was determined using FISH on blood

Table 9.2: Frequency analysis of LTRA units, 6 months after transplantation of WGA^{dim} cells sorted from $LD/FU_{6d}BM$

Number of cells injected	Fraction of mice with less than 10% donor-type erythrocytes
1700	0/4
567	2/7
189	3/6
63	7/8
21	8/8
LTRA-frequency*	1/412
95% conf. limits	207-817

The frequency of the LTRA-unit* responsible for 10% donor-type erythroid repopulation was calculated from the proportion of mice with less than 10% donor-type RBC using limiting dilution analysis according to Poisson statistics. Recipient mice were irradiated with 5 Gy and transplanted with WGA^{dim} cells from a low density fraction of day-6 post-5-fluorouracil bone marrow ($LD/FU_{6d}BM$).

smears at 2, 4, and 6 months, and in one experiment also at 9 months after transplantation. Linear regression analyses showed a high and significant correlation between the enrichment for CAFC-28/35 *in vitro*, and donor-type RBC and nucleated blood cells *in vivo* (Figure 9.6), supporting our earlier findings.[383] Data at 4 months after transplantation also revealed that the enrichment for RBC and nucleated blood cell chimerism correlated strongly. The correlations at 2, 6 and 9 months gave similar results (not shown). Therefore, the results indicate that RBC chimerism can be used to determine the LTRA of a graft *in vivo* as of 2 months after transplantation. The use of α-thalassemic mice reduced the time required for analyzing 100 samples from almost 4 days, which was required for FISH, to less than 1 hour for FACScan analysis of the RBC. The method also provides a direct way to determine chimerism without the need for prior immunochemical or biochemical staining.[508] Therefore, the model is suitable for a longitudinal study of long-term repopulation using large groups of mice.

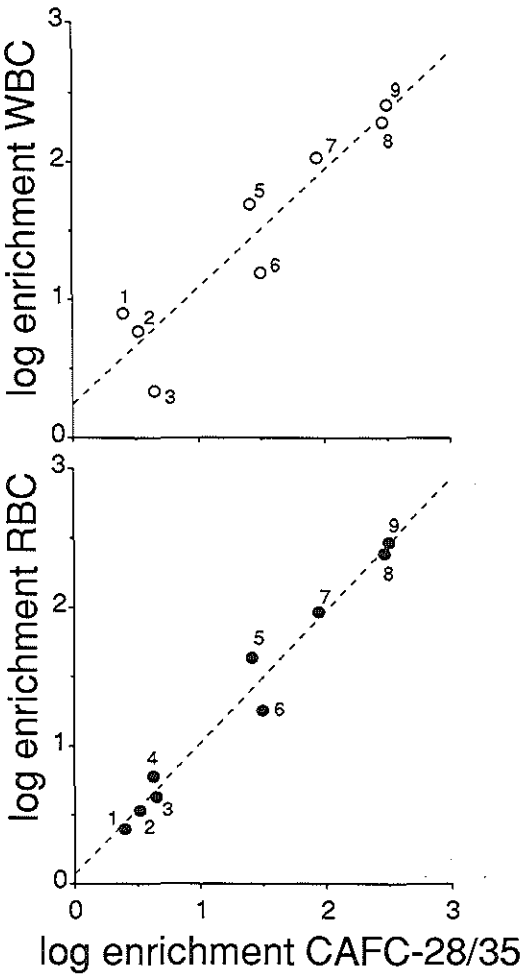


Figure 9.6:
Correlation between the enrichment for CAFC-28/35 *in vitro* and (top panel): donor-type nucleated blood cells (WBC) (r=0.94; n=8; p<0.001), or (bottom panel): donor-type red blood cells (RBC) *in vivo* (r=0.99; n=9; p<0.001), at 4 months after transplantation. Symbols represent enrichments calculated on groups of 12-24 recipients of sorted cells compared to 12-24 recipients of control BMC. All three irradiation groups are included, as indicated in the Results section. Numbers indicate different grafts: 1-2 Rh^{bright}; 3-4 WGA^{bright}; 5,7,9 Rh^{dull}; 6 LD/ER-$MP20^{-}$ and 8 WGA^{dim}. RBC chimerism was determined by FACScan, nucleated blood cell chimerism by FISH using a murine Y-chromosome specific probe.

9.2.6 Multilineage donor-type reconstitution

In addition to the repopulation in the RBC compartment, chimerism was also determined in the major hemopoietic organs and differentiation lineages of individual mice. This was done at 9 and 12 months after transplantation of 3 x 10^5 unseparated BM cells, 670 or 1700 LD/ER-MP20$^-$ Rh-123^{dull} cells (Tables 9.3 and 9.4).

Table 9.3: Percentage donor type red blood cells and nucleated cells in 8 individual chimeric mice, 9 months after transplantation

TBI (Gy)	grafted cells*	peripheral blood red cells	peripheral blood white cells	bone marrow	spleen	thymus	BM CFU-S-12
5	NBMC	72	46	31	46	77	24 ± 7.5 (8/33)
5	Rh123^{dull}	50	24	24	25	23	13 ± 11.7 (1/8)
5	Rh123^{dull}	25	4	2	10	4	<7 (0/14)
5	Rh123^{dull}	72	39	36	32	20	36 ± 10.3 (8/22)
4	NBMC	52	47	43	43	90	87 ± 5.0 (40/46)
4	Rh123^{dull}	43	27	31	22	10	35 ± 8.6 (11/31)
4	Rh123^{dull}	29	12	4	12	7	6 (2/12)
4	Rh123^{dull}	9	10	5	14	26	<6 ± 6.0 (0/16)

*3x10^5 unseparated BMC (NBMC) and 670 Rh123^{dull} cells sorted from low density ER-MP20$^-$ bone marrow were transplanted per mouse after 4 or 5 Gy total body irradiation (TBI) as indicated. CFU-S-12 chimerism is depicted as the percentage donor-type CFU-S-12 colonies (± standard error) after secondary transplantation. Between brackets the number of male colonies per total number of spleen colonies analyzed. The standard error of the proportion was approximated on the assumption of normally distributed data. RBC chimerism was determined by FACScan analysis of peripheral blood. Other data were generated using FISH.

Microscopic inspection of the May-Grünwald-Giemsa stained cytospin preparations of the sorted monocytes and granulocytes proved these cells to be more than 95% pure (not shown). Peripheral blood cell chimerism, induced by unseparated BM cells or Rh-123^{dull} cells, was present in all lineages, whereas its level was comparable to that in most hemopoietic organs. The selective advantage of the erythroid repopulation has been described previously (Chapter V[505]). CFU-S-12 chimerism and nucleated blood cell chimerism were of the same magnitude (Table 9.3). The results indicated that the LTRA cells, acquired by buoyant density centrifugation and sorting on the basis of their low Rh-123 retention, are qualitatively comparable to the LTRA cells in unseparated bone marrow and give rise to stable long-term multilineage donor-type reconstitution.

9.3 DISCUSSION

The present results clearly demonstrate a lack of LTRA activity *in vivo* and *in vitro* in the populations 50 to 200-fold enriched for CFU-S-12 using Rh-123 or WGA. The sorted Rh-123^{bright} and WGA^{bright} cells represented 65% and 95%, respectively, of all CFU-S-12 (CAFC-10) originally present in pre-enriched low density bone marrow. It has been previously reported that CFU-S are unable to maintain hemopoiesis in long-term bone marrow culture.[88,461,512] In the CAFC-assay, Rh-123^{bright} and WGA^{bright} sorted cells gave rise to an initial but short-lived hemopoietic burst.[383,385,386,512] Injection of as many as 281 WGA^{bright} CFU-S-12 in sublethally irradiated mice, equivalent to 2800 day-12 spleen colony-forming cells (corrected for a 10% seeding efficiency), did not result in any significant repopulation beyond 4 to 5 months after transplantation. In contrast, the populations which were 160-240 fold enriched for LTRA (Figure 9.5) were severely depleted of CFU-S-12 activity. Injection of 670-2000 Rh-123^{dull} or 200-500 WGA^{dim} cells with extremely low CFU-S-12 numbers induced sustained chimerism (Table 9.1). In one experiment, CFU-S-12 numbers injected per mouse approximated 0.1. Chimerism involved all hemopoietic organs and lineages tested, including the bone marrow CFU-S-12 as determined in secondary recipients, indicating that our sorting procedure enriched for LTRA subsets with similar multilineage expression ability as have LTRA cells in unseparated bone marrow. The results therefore imply that

Table 9.4: Percentage donor-type cells in the different hemopoietic lineages of 8 individual chimeric mice, 12 months after transplantation

grafted cells*	peripheral blood red cells	peripheral blood white cells	mono-cytes	granulo-cytes	B-cells	T cells
NBMC	75	53	51	34	38	51
NBMC	86	56	62	45	30	58
NBMC	91	63	54	62	15	46
NBMC	56	36	44	30	10	60
$Rh123^{dull}$	93	42	22	52	18	nd
$Rh123^{dull}$	62	33	40	10	9	32
$Rh123^{dull}$	78	56	23	58	23	32
$Rh123^{dull}$	48	20	10	25	18	nd

*$3x10^5$ unseparated BMC (NBMC) or 1700 $Rh123^{dull}$ cells sorted from low density ER-MP20$^-$ bone marrow cells were transplanted in 5 Gy irradiated mice. Monocytes and granulocytes were sorted from bone marrow using ER-MP20, B cells using RA3-6B2 (anti-B220). T cells were harvested from the thymus without sorting. RBC chimerism was determined by FACScan analysis. Other data were generated by FISH. nd = not determined.

hematopoietic stem cells contain a distinct stem cell subset with LTRA that can be separated physically from the vast majority of CFU-S-12. Although the data make it likely that LTRA stem cells have pre-CFU-S properties, we do not exclude that some LTRA cells may form a spleen colony.

Partial or total separation of LTRA from CFU-S has been reported earlier using plastic-adherence or counterflow elutriation.[231,242] However, final enrichment levels for the respective hemopoietic stem cell populations were lower than presently reported. Frequency analysis of the plastic-adherent BM cells, which contained 30% of the long-term repopulating stem cells as detected by competitive repopulation in lethally irradiated animals, showed about equal

numbers of CFU-S-12 and LTRA.[242] The graft that induced long-term engraftment still contained 15 CFU-S-12, whereas we observed long-term repopulation with only 0.1 to 3.1 CFU-S-12 injected per animal.

Previously, the population of Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells has been thought to comprise stem cells purified to almost unit efficiency.[449,455,456,496] These cells have been attributed the capacity to protect lethally irradiated animals from radiation induced death, as well as to provide short- and long-term repopulation. However, it was recognized that most of the Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells possessed STRA, with only a minority being true LTRA cells.[220] The number of CFU-S-12/13 in the Lin$^-$ Sca-1$^+$ population, calculated from their frequency of 1 in 21 cells, comprised almost 50% of the population.[276,457] Further analysis of the phenotype of the Lin$^-$ Sca-1$^+$ cells, using several lectins and antibodies, revealed a heterogeneity for Thy-1 expression, WGA affinity and Rh-123 retention.[277,456,457] Although Lin$^-$ Sca-1$^+$ cells contained functionally different Rh-123^{lo} and Rh-123$^{med/hi}$ subpopulations, responding differently to hemopoietic growth factors,[277] the use of neither Rh-123 nor Thy-1 allowed for a separation of CFU-S activity from LTRA.[277,456,496] Analysis of the fluorescence profile of Lin$^-$ Sca-1$^+$ cells labeled with WGA, however, revealed a clearly distinguishable large peak of WGA^{bright} cells,[457] comprising about 50% of the population at a fluorescence level characteristic for CFU-S-12 in our laboratory. The remaining 50% WGA^{dim} cells appeared highly similar to our definition of LTRA cells, a population depleted of CFU-S-12.[383] Therefore, this observation suggests that the Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ population could be divided into 2 subpopulations containing either WGA^{bright} CFU-S or the more immature WGA^{dim} non-CFU-S LTRA cells, using the criteria applied in the present study.

The enrichment for LTRA *in vivo* correlated strongly with the enrichment for the CAFC-28/35 *in vitro*, as did the enrichments for CFU-S-12 and CAFC-10 (data not shown). Therefore, these data support the earlier contention that the CAFC-assay can be used as a tool for the simultaneous and quantitative assessment of populations with STRA as well as LTRA.[383,385] Using either competitive repopulation or the CAFC-assay, the frequency of the LTRA cell in marrow has been estimated to range from 0.5 to 2.5 per 10^5 normal BM cells.[191,331,383,449] In the present study, using sublethally irradiated α-

thalassemic recipients in a sex mismatched transplantation setting, the frequency of the *in vivo* LTRA cell could not be determined directly, but was estimated by defining the number of cells capable of inducing a sustained level of 10% donor-type RBC in 5 Gy irradiated recipients as an LTRA unit. For the 160-fold enriched LD/FU_{6d} WGA^{dim} population (Figure 9.5-D), the LTRA unit was calculated to contain 412 cells. The frequency of the LTRA unit in unseparated bone marrow therefore approximates 1-2 per 10^5 cells, which is in agreement with previous estimates of *in vivo* LTRA cells in different mouse models.[191,331,383,449]

At 12 months after transplantation of 1700 Rh^{dull} cells, containing 2-3 LTRA units, clear differences can be seen in their contribution to the monocytic and granulocytic lineages in individual mice (Table 9.4). Such a segregation of monocytic and granulocytic lineage expression has been observed earlier using retrovirally marked stem cell clones.[232] It has been suggested that in addition to the stochastic mechanisms operating at the level of stem cell commitment, lineage specific demands might influence the clonal expansion of particular lineages by microenvironmental and humoral factors.[232,268,475] Also, the percentage of donor-type cells in the thymus, which varied considerable when compared to the other hemopoietic tissues (Table 9.3), might be influenced by these mechanisms. The presence of intra-thymic long-lived T cell clones and/or a shift in the relative proportion of donor-type cells during involution of the thymus may play an additional role. The contribution to the T-cell lineage may therefore temporary differ from the expression in other lineages.

In summary, we achieved a stringent separation of CFU-S activity from stem cells that are capable of inducing a stable long-term multilineage reconstitution of sublethally irradiated mice. As determined *in vitro* as well as *in vivo*, the LTRA cells were 160 to 240-fold enriched as compared to unseparated bone marrow. A highly enriched population of CFU-S-12, on the other hand, did only contribute to repopulation in the first months after transplantation. This transient role of CFU-S in repopulation has also been suggested by other investigators.[58,242] The spleen colony-forming cells could be held responsible for the strong short-term clonal fluctuations observed in retroviral transfection experiments in the first 4 to 6 months after transplantation.[80,232,269,450] To study the early clonal engraftment of the LTRA cells, separation from CFU-S

populations is therefore desirable. Such a study on the *in vivo* behavior of the LTRA cells could eventually provide clues to design more effective protocols for therapeutic gene transfer studies.

CHAPTER X
GENERAL DISCUSSION

10.1 INTRODUCTION

The thalassemias are the most common group of hereditary disorders in humans.[12,13,553] Thalassemia major has a high morbidity and mortality,[62,63,155] for the greater part due to transfusion and thalassemia related[83] iron overload which results primarily in cardiac disease.[62,155] Conventional treatment, which is expensive and often characterized by poor patient compliance,[533,551] consists of hypertransfusion and iron chelation therapy to mitigate the effects of iron overload.[271] Bone marrow transplantation (BMT) offers the possibility of permanent correction of the disorder,[484] but has not found widespread application in the management of thalassemic patients due to transplantation related risks which include transient pancytopenia and immunosuppression, as well as graft rejection and graft-versus-host disease. BMT patients thus require high level medical care which is mostly not available at the scale required in countries were thalassemia has a high prevalence. BM graft rejection remains a problem in thalassemia patients with an extensive history of blood transfusions, who may have become sensitized to histocompatibility antigens and do not tolerate intensive conditioning regimens due to complications related to iron accumulation in various tissues.[299] Also precluding widespread application of BMT is the lack of an HLA-identical family donor in approximately 60% of the thalassemic children.[299] The expected growth of the world population will occur in countries where the thalassemias are common.[551] Therefore, the development of more generally applicable treatment modalities in addition to genetic counseling programs would be necessary to cope with the expected worldwide increase in thalassemia patients.[551] The development of a transplantation regimen that combines relatively non-toxic conditioning with the use of HLA-mismatched donors could lead to a substantial increase in the number of thalassemia patients being considered for BMT. In the experiments presented in this thesis, conditions that allow for induction of stable allogeneic partial chimerism were studied, using murine α- and β-thalassemia models.

10.2 RELEVANCE OF THE MURINE THALASSEMIA MODELS

Murine models with characteristic microcytic anemia are available for α-thalassemia as well as β-thalassemia.[416,443] The properties of murine β-thalassemic red blood cells closely resemble human β-thalassemic red cells.[411]

The sustained anemia results in compensatory hemopoiesis. Similar to α-thalassemic mice,[540] compensatory hemopoiesis in β-thalassemic mice was located primarily in the spleen and was not restricted to the erythroid lineage (Chapter IV[506]). A quantitative difference between the two murine models was observed, with more severe anemia[396,443] and a correspondingly higher level of compensatory hemopoiesis in β-thalassemic mice.[506] In contrast to α-thalassemia, the β-thalassemic mice had excess lymphocytes in addition to a granulocytosis. Also human patients develop a compensatory hemopoiesis which is not restricted to the erythroid lineage.[89,154,229] As the pathophysiological changes of hemopoiesis are similar in both thalassemia models, generalization of the results obtained by experiments with either one may be justified.
The thalassemic mice are presently the best available preclinical animal models to study new treatment modalities for thalassemia. Extrapolation of the results obtained in a laboratory setting with the murine thalassemia models to the treatment of thalassemia patients would preferably require additional preclinical studies in large, outbred animal models, which are, however, not available.

10.3 SELECTIVE ADVANTAGE OF NORMAL RED CELL PRODUCTION

Initial studies demonstrated that correction of murine α-thalassemia can be obtained without complete eradication of the endogenous thalassemic hemopoietic stem cells.[27,541] This was achieved by use of relatively non-toxic, low dose total body irradiation (TBI), followed by transplantation of a clinically feasible number of normal BM cells (approximately $3x10^8$ cells per kg body weight). It was hypothesized that this phenomenon had been the result of partial hemopoietic chimerism and a selective advantage of donor type normal red blood cell production.[541]
In the present study, the selective advantage of normal red cell production was examined in more detail. For this purpose, quantitative methods were developed to assess chimerism in thalassemic recipients of normal bone marrow in red blood cells, white blood cells and CFU-S. Peripheral blood red cell chimerism was determined by a flow cytometric method, using a specific calibration curve in α-thalassemic mice and a semi-quantitative system in β-thalassemic mice. Fluorescent *in situ* hybridization, using a murine Y-chromosome specific probe, was used to distinguish donor and recipient nucleated cells in recipients of sex

mismatched bone marrow. Evidence was found for a selective advantage of normal red blood cell production in partial chimeras for both types of thalassemic recipients (Chapters V and VI), in that the percentages of donor type peripheral blood red cells were consistently higher than the percentages of donor type immature hemopoietic cells (CFU-S) or peripheral blood white cells. Comparison of peripheral blood red cell chimerism and CFU-S chimerism demonstrated that the selective advantage was smaller than originally assumed[27,541] as higher levels of CFU-S-12 chimerism were found than initially calculated on the basis of the known radiosensitivity of hemopoietic stem cells. However, if conditioning TBI and transplanted cell numbers were optimally chosen, still as few as 20 - 30% donor type immature bone marrow cells appeared to produce over 50% of normal peripheral blood red cells.

Recently, reports have been published of partial chimerism in human thalassemic recipients of allogeneic BM cells.[31,288,347,361,422] In these patients, mixed chimerism was partly transient,[31,288] and patients with high levels of mixed chimerism at 2 months post-BMT uniformly rejected the BM graft.[347] However, long-term stable allogeneic partial chimerism was reported in a small number of patients that remained transfusion free at three or more years after BMT, although at relatively low hemoglobin levels (4.4 and 5.0 mmol/l).[422] Immature hemopoietic cell chimerism has as yet not been evaluated. The magnitude of the selective advantage of normal RBC production in allogeneic partially chimeric human thalassemia patients is therefore not known. The reports may suggest that stable allogeneic partial hemopoietic chimerism is feasible in human thalassemia patients as well.

The potential benefit of partial bone marrow chimerism following transplantation of normal bone marrow in human thalassemia patients may be inferred from a comparison of the degree of anemia in the murine models and various forms of the disease in humans. In murine α-thalassemia, the globin mRNA ratio is approximately equal to that reported for humans with α-thalassemia trait.[8,310,550] In murine β-thalassemia, the β-minor/α-globin chain synthesis ratio of approximately 0.75 is somewhat higher than the ratio observed in thalassemia minor.[102,396,424,443,550] Both α-thalassemia trait and thalassemia minor result, at the most, in a slight anemia, which does not require transfusion.[363] Those thalassemic patients with severe transfusion dependent

forms of the disease may therefore benefit even more from a selective advantage of normal RBC production in case of partial hemopoietic stem cell chimerism, since thalassemic erythropoiesis in these patients is probably more deficient than that of thalassemic mice. This aspect needs evaluation in preclinical as well as clinical studies, as species differences in proteolysis of excess globin chains[564] may influence the red cell membrane characteristics and thereby the degree of phagocytosis of red blood cells and their precursors in the bone marrow.

10.4 ALLOGENEIC PARTIAL HEMOPOIETIC CHIMERISM

Relatively non-toxic immunosuppressive regimens to prepare for bone marrow transplantation may be of benefit to all recipients of a bone marrow graft, in particular to those patients which have a reduced tolerance to the usual cytoreductive agents due to tissue damage, as is the case in thalassemia.[299] Sustained allogeneic partial chimerism, in addition, requires the immunosuppressive regimen to induce mutual tolerance of donor and recipient derived immunocompetent cells to ensure disease free survival. In Chapter VII of this thesis, it is shown that sustained allogeneic BM chimerism can be induced by a conditioning regimen that combines low-dose TBI with additional immunosuppression by monoclonal antibodies directed at immunocompetent cells. The monoclonal antibodies selected (anti-CD4, anti-CD8 and anti-LFA-1α) had previously been shown to induce tolerance to donor type skin grafts if combined with bone marrow transplantation.[55,94,95,142,214,371,436] The regimen described in Chapter VII was specifically designed to induce stable allogeneic partial hemopoietic chimerism in α-thalassemic mice. Although not tested in this study, it is conceivable that donor type skin-graft tolerance is also induced in partially allogeneic chimeric mice. Partial chimerism in the α-thalassemic mice was maintained without the need for continuous post-transplant immunosuppression.

Preclinical subhuman primate studies using combinations of anti-human CD4, CD8 and CD11a and other anti-T cell MCAs need to be done to evaluate the feasibility of a similar conditioning regimen for human patients. The development of such a conditioning regimen would not only benefit thalassemia patients, but also patients suffering from another hereditary disease which may

in principal be cured by bone marrow transplantation, such as Sickle cell anemia, certain lysosomal storage diseases, phagocytic disorders and osteopetrosis.
Besides the advantages of relatively non-toxic conditioning regimens for the treatment of thalassemia patients with allogeneic BMT, it is expected that such regimens will also reduce the duration of post-transplant pancytopenia and its complications.

10.5 RADIOSENSITIVITY OF HEMOPOIETIC STEM CELLS

Comparison of peripheral blood red cell chimerism and CFU-S chimerism in α-thalassemic mice showed that the selective advantage of the normal erythroid lineage in the BALB/c thalassemic recipients, although clearly demonstrable, was less than originally assumed.[27,541] Therefore, the assumption of a radiosensitivity of stem cells characterized by a D_0 of 1.0 Gy, on which the original estimate of the selective advantage of the normal RBC production in partially chimeric mice was based, needed reappraisal. The discrepancy was explained by demonstrating that immature bone marrow cells of BALB/c mice are more radiosensitive than those of other mouse strains. For this purpose, the radiosensitivity of the long-term repopulating hemopoietic stem cells was specifically evaluated by a competition assay in the TBI dose range used in the conditioning regimen which resulted in sustained partial chimerism (Chapter VIII). The calculated D_0 values of α-thalassemic and normal BM LTR-HSC of BALB/c mice were 0.68 ± 0.08 Gy and 0.59 ± 0.10 Gy, respectively. Although only repopulation in the erythroid lineage was measured in these experiments, the results obtained with transplantation of HSC populations which are highly enriched for LTR-HSC (Chapter IX) make it very unlikely that at more than 20 weeks after BMT the progeny of other than multilineage reconstituting LTR-HSC was measured. As the D_0 values calculated for LTR-HSC corresponded closely to those found in the same mouse strain for CFU-S-12, these results are at variance with a series of experiments that demonstrated higher D_0 values for more immature cells, such as LTR-HSC, than for cells measured by the spleen colony test.[317,318,319,387]. It was hypothesized that LTR-HSC are heterogeneous with respect to radiation sensitivity. Unfortunately, the competition assay used here cannot be used in the high TBI dose range, as the required levels of

chimerism will not be reached. Direct proof, that BALB/c mice also have an LTR-HSC population characterized by a higher D_0 value can, therefore, not be obtained with the same method, but should be sought by use of the CAFC-assay.[384-386] Alternatively, the radiosensitivity of LTR-HSC of other mouse strains in the low TBI dose range may be analyzed. Back-crossing of the gene deletions leading to murine α- or β-thalassemia into other mouse strains, e.g., (BALB/c x C57BL) F1 mice, will allow for estimation of D_0-values of LTR-HSC of mice with other genetic backgrounds, as well as further evaluation of the differences obtained so far between BALB/c α-thalassemic and C57BL β-thalassemic mice. Other markers than the size difference between normal and thalassemic red cells, such as glucose-phosphate isomerase may also be used. Further evaluation of the radiosensitivity of (sub)populations of HSC of mice and other species may provide clues for the development of treatment modalities for victims of radiation accidents and cancer patients requiring TBI.

10.6 HEMOPOIETIC STEM CELL PURIFICATION

The experiments using BM cell fractions which were highly enriched for LTR-HSC (Chapter IX) demonstrated that these cells are responsible for the correction of thalassemia and that purified hemopoietic stem cells can be used to correct thalassemia. The latter observation is important because accessory cells may (partly) determine the immunogenicity of BM allografts[125] and the future development of partial allogeneic chimerism for human patients may be facilitated by stem cell purification and graft engineering.[16] The development of clinically applicable stem cell purification methods is partly hampered by lack of reliable assays for human LTR-HSC. The development of a primate version of the CAFC-assay[384-386] would therefore be of great value.

On the basis of the results presented in this thesis, attempts to correct thalassemia by insertion of normal globin genes and regulatory elements in thalassemic HSC will depend on highly effective transfection protocols.[16,17,49,235] The results obtained in α-thalassemic (Chapter V) as well as β-thalassemic mice (Chapter VI) indicate that total ablation of endogenous BM is still necessary if only one out of five LTR-HSC can be corrected. Further studies on LTR-HSC physiology and *in vivo* and *in vitro* behavior of these cells

may provide clues for more effective transfection protocols than are presently available.

SUMMARY EN SAMENVATTING

SUMMARY

The thalassemias are a heterogeneous group of hereditary disorders of hemoglobin synthesis, characterized by a microcytic, hypochromic anemia. Thalassemia patients not only suffer from anemia, but also from the effects of iron overload, such as cardiac disease, growth impairment, delayed or absent sexual maturation and other endocrinological disturbances. Conventional treatment with hypertransfusion and iron chelation therapy is partly effective, whereas bone marrow transplantation (BMT) offers a possibility of cure. However, BMT has found only limited application in the management of thalassemic patients due to the risks of the procedure, including allograft rejection and GvHD. Since the long-term effects on the outcome of thalassemia related problems after BMT or with conventional therapy are still largely unknown and not all transplantation related risks have been reduced sufficiently, the choice between BMT using currently available conditioning and transplantation regimens or conventional therapy remains difficult (Chapter I).

Previous experiments in a murine model for α-thalassemia (Chapter I) had shown that the anemia associated with thalassemia could be corrected without eradication of endogenous stem cells. It was hypothesized that partial hemopoietic chimerism could lead to correction due to a selective advantage of normal red cell production. This hypothesis was verified and the magnitude of the selective advantage was further evaluated in murine models for α- as well as β-thalassemia. A flow cytometric method to determine peripheral blood red cell chimerism rapidly for a large number of samples was developed. The method takes advantage of the differences in forward light scatter (FLS) characteristics between the microcytic thalassemic and the normal red blood cells (Chapter III). Chimerism at a developmentally immature hemopoietic stage (CFU-S-12) as well as in differentiated hemopoietic lineages was determined by evaluation of donor and recipient origin of nucleated cells in recipients of sex mismatched donor cells by use of a murine Y-chromosome specific probe, detected by fluorescent in situ hybridization.

In the α-thalassemic model, it was demonstrated that as little as 20 - 30% donor type immature hemopoietic cells may produce over 50% normal peripheral blood erythrocytes (Chapter V). By comparing repopulation characteristics in normal recipients of normal or thalassemic BM cells as well as in thalassemic

recipients of normal cells, it was shown that the selective advantage did not result from either an intrinsic defect of thalassemic stem cells or from microenvironmental differences between normal and α-thalassemic mice. Transplantation of a mixture of α-thalassemic and normal cells in lethally irradiated mice excluded the possibility that the selective advantage could be attributed to differences between exogenous and endogenous repopulation. It was concluded that the preponderance of normal red cells resulted from a selective advantage in the hemoglobin synthesizing stages of erythropoiesis, presumably on the basis of increased cell death of thalassemic cells (Chapter V). A selective advantage of normal erythropoiesis was also observed after the development of partial hemopoietic chimerism in β-thalassemic mice following BMT with syngeneic normal BM cells (Chapter VI). A small quantitative difference between the two thalassemia models was observed in that often greater selective advantage of normal red cell production was observed in β-thalassemic mice. This is most likely due to the more severe anemia in β-thalassemic mice resulting in a greater compensatory hemopoiesis than observed in α-thalassemic mice (Chapter IV). However, it is not excluded that mouse strain characteristics were partly responsible for the observed differences between α- and β-thalassemic mice.

The selective advantage of the normal stem cell derived erythropoiesis in partially chimeric α-thalassemic mice was less pronounced than thought on the basis of the original observations and the assumption of a radiosensitivity of hemopoietic stem cells characterized by a D_0 of 1 Gy. In the experiments described in Chapter VIII, the radiosensitivity of LTR-HSC was determined *in vivo* by analysis of endogenous repopulation in relation to repopulation from graded numbers of transplanted bone marrow (BM) cells. The experimental design enabled estimation of the D_0 of LTR-HSC independent of the actual number of bone marrow cells per mouse or the homing fraction of transplanted BM cells. Assessment of the radiosensitivity of the LTR-HSC in the dose range used to induce partial chimerism revealed that these cells were more radiosensitive than originally assumed (Chapter VIII), which was in accordance with the actual selective advantage of normal erythropoiesis found in α-thalassemic recipients.

In thalassemia patients, tolerance to the usual cytoreductive agents used in clinical BMT is limited by tissue damage due to iron overload. The murine thalassemias offer suitable models in the search for conditioning regimens that are relatively non-toxic and sufficiently immunosuppressive to allow for engraftment of transplanted allogeneic BM cells. To this end, α-thalassemic mice were conditioned with graded doses of TBI in combination with immunosuppressive monoclonal antibodies (MCAs) before BMT with clinically relevant numbers of allogeneic BM cells. It was shown that sustained allogeneic partial hemopoietic chimerism can be induced by a combination of low dose TBI and anti-CD4, anti-CD8 and anti-CD11a MCAs (Chapter VII). Immunosuppressive capacity of anti-CD18 MCAs could not be demonstrated. Antigenic differences between donor and recipient influenced the immunosuppressive capacity of combinations of MCAs. The efficacy of combinations of MCAs directed against the human counterparts of these antigens needs to be evaluated in studies with primates, e.g. rhesus monkeys, as well as in clinical trials.

The development of protocols allowing for effective insertion of normal globin genes and regulatory elements in HSC will strongly depend on insight into the normal regulation of hemopoietic stem cell replication and identification of the stem cell subset with the capacity of sustained hemopoietic reconstitution. In Chapter IX, experiments are described to identify this stem cell subset, using transplantation into α-thalassemic mice as a direct assay for sustained hemopoietic regeneration as well as competitive capacity. These experiments showed that murine LTR-HSC are Rh-123^{dull} and have a low affinity for WGA, and can be separated from short term repopulating cells on the basis of these characteristics. In addition they demonstrated that correction of the anemia in thalassemic mice by partial hemopoietic chimerism results from engraftment of multilineage reconstituting LTR-HSC. A frequency of 1-2 / 10^5 was estimated for the LTR-HSC in bone marrow of normal mice, indicating that these cells are a very rare subset. The responsiveness to hemopoietic growth factors of these cells remains to be determined.

Currently, thalassemic mice are the best available preclinical animal models to study new treatment modalities for thalassemia. Large outbred animal models for thalassemia are lacking. Still, studies in such animal models aimed at the

development of stable allogeneic hemopoietic chimerism may provide clues for the (future) development of conditioning regimens that combine the requirements of relatively low toxicity and sufficient immunosuppression to allow for stable allogeneic hemopoietic chimerism.

SAMENVATTING

De thalassemieën vormen een heterogene groep van erfelijke aandoeningen van de hemoglobinesynthese, gekenmerkt door een microcytaire en hypochrome anemie. Thalassemie patienten lijden niet alleen aan anemie, maar ook aan de effecten van ijzerstapeling, welke zich uiten in de vorm van hartaandoeningen, vertraagde groei, vertraagde of afwezige sexuele ontwikkeling en andere endocrinologische stoornissen. Beenmergtransplantatie (BMT) biedt, naast de conventionele behandeling met bloedtransfusies en ijzerchelatie therapie, de mogelijkheid tot permanente genezing van deze aandoeningen. Beenmergtransplantatie wordt echter, tengevolge van de frequent optredende complicaties zoals "Graft-versus-Host Disease" en beenmergafstoting, niet op grote schaal toegepast bij de behandeling van thalassemie. Aangezien de lange termijn effecten van zowel de conventionele behandeling als de behandeling met BMT op de bij thalassemie behorende problemen onbekend zijn en de aan beenmergtransplantatie verbonden risico's nog onvoldoende zijn beperkt, blijft de keuze tussen conventionele behandeling en beenmergtransplantatie moeilijk (Hoofdstuk I).

Eerdere experimenten in een muizemodel voor α-thalassemie (Hoofdstuk I) hadden aangetoond dat de bij de thalassemie behorende anemie gecorrigeerd kon worden zonder volledige uitschakeling van de endogene thalassemische stamcellen. De hypothese werd opgesteld, dat partieel hemopoëtisch chimerisme kon leiden tot deze correctie op basis van een selectief voordeel van de vorming van normale rode bloedcellen. Toetsing van deze hypothese én bepaling van de omvang van het selectieve voordeel werd verricht in de muizemodellen voor α-thalassemie en β-thalassemie. Hiervoor werd een flowcytometrische methode ontwikkeld om snel het perifere rode bloedcel chimerisme te kunnen bepalen in een groot aantal monsters. Deze methode is gebaseerd op de verschillen in voorwaartse lichtverstrooiing tussen de microcytaire thalassemische en de normale erythrocyten (Hoofdstuk III). Chimerisme op het niveau van zowel onrijpe hemopoëtische cellen (milt-kolonie-vormende cellen) als gedifferentieerde hemopoëtische cellen werd geanalyseerd door het bepalen van donor of ontvanger origine van cellen in ontvangers van "sex mismatched" beenmerg met een probe, die specifiek is voor het Y-chromosoom van de muis.

In het α-thalassemie model werd aangetoond dat 20 - 30% donor type chimerisme op het niveau van de milt-kolonie-vormende cellen leidde tot meer dan 50% normale erythrocyten in het perifere bloed (Hoofdstuk V). Door het vergelijken van de repopulatie in enerzijds normale ontvangers van zowel normale als thalassemische BM cellen en anderzijds thalassemische ontvangers van normale BM cellen, kon worden aangetoond dat het selectieve voordeel niet een gevolg is van intrinsieke defecten van de thalassemische stamcellen, noch van verschillen tussen de hemopoëtische micro-omgeving in normale en thalassemische muizen. Door het transplanteren van een mengsel van normale en thalassemische cellen in letaal bestraalde muizen werd tevens de mogelijkheid uitgesloten, dat een verschil tussen exogene en endogene repopulatie voor het selectieve voordeel verantwoordelijk is. De conclusie werd getrokken dat een toegenomen sterfte van de thalassemische cellen in de hemoglobine synthetiserende fase van de erythropoëse leidt tot het selectieve voordeel van de normale rode bloedcelvorming (Hoofdstuk V).

Een selectief voordeel van normale rode bloedcel vorming werd ook aangetoond bij partieel chimerisme in β-thalassemische muizen na transplantatie van normale syngene BM cellen (Hoofdstuk VI). Tussen beide thalassemie modellen werd een klein kwantitatief verschil gevonden, aangezien vaker een groter selectief voordeel van de normale erythropoëse in β-thalassemische muizen werd waargenomen. Dit verschil wordt waarschijnlijk veroorzaakt door de ernstiger anemie van β-thalassemische muizen, die leidt tot een grotere compensatoire hemopoëse dan in α-thalassemische muizen (Hoofdstuk IV). Dat verschillen tussen de gebruikte muizestammen mede verantwoordelijk waren voor de aangetroffen verschillen tussen de α- en β-thalassemische muizen, kon niet geheel worden uitgesloten

In α-thalassemische muizen met partieel chimerisme was het selectieve voordeel van de vorming van erythrocyten door normale stamcellen minder groot dan verondersteld werd op basis van de oorspronkelijke observaties en de aanname van een stralingsgevoeligheid van stamcellen gekenmerkt door een D_0 van 1 Gy. In de experimenten beschreven in Hoofdstuk VIII is de stralingsgevoeligheid van de lange termijn repopulerende stamcellen *in vivo* bepaald door het vergelijken van de endogene repopulatie met de repopulatie door getransplanteerde BM cellen. De opzet van de experimenten was zodanig

gekozen dat de bepaling van de stralingsgevoeligheid onafhankelijk was van zowel het feitelijke aantal BM cellen per muis, als de fractie getransplanteerde cellen die in het beenmerg uitgroeit. De stralingsgevoeligheid van de lange termijn repopulerende stamcellen bij die stralingsdoses, welke gebruikt werden voor het induceren van partieel chimerisme, bleek inderdaad hoger dan oorspronkelijk werd aangenomen, in overeenstemming met het feitelijke selectieve voordeel van de normale erythropoëse in de α-thalassemische ontvangers.

De weefselschade veroorzaakt door ijzerstapeling leidt bij thalassemische patienten tot een lagere tolerantie voor cytoreductieve agentia, zoals gebruikt worden bij beenmergstransplantatie. De muizemodellen voor thalassemie bieden de mogelijkheid tot onderzoek naar conditioneringsschema's die zowel weinig toxisch, als voldoende immunosuppressief zijn om het aanslaan van allogene beenmergcellen te waarborgen. Hiertoe werden α-thalassemische muizen voorbehandeld met een opklimmende doses totale lichaamsbestraling in combinatie met immunosuppressieve monoclonale antilichamen (MCA). Na deze voorbehandeling werden ze getransplanteerd met een klinisch relevant aantal allogene BM cellen. De resultaten van deze experimenten worden beschreven in Hoofdstuk VII. Aangetoond werd dat blijvend allogeen partieel hemopoëtisch chimerisme geinduceerd kon worden door een combinatie van een lage dosering totale lichaamsbestraling met anti-CD4, anti-CD8 en anti-CD11a MCA. Een anti-CD18 monoclonaal bleek niet effectief. Tevens bleek dat de mate van immuunsuppressie die door de MCA wordt gegeven, afhankelijk is van antigene verschillen tussen donor en ontvanger. De werkzaamheid van combinaties van MCA gericht tegen deze antigenen bij de mens dient niet alleen verder onderzocht te worden in primaten zoals de rhesusaap, maar ook in klinische studies.

Voor het ontwikkelen van nieuwe behandelingsmodaliteiten voor thalassemie is dieper inzicht in de fysiologie van stamcellen, zowel *in vivo* als *in vitro*, een vereiste. Zeker de ontwikkeling van protocollen gericht op het overbrengen van de genetische informatie van zowel de globinen als hun regulerende elementen naar hemopoëtische stamcellen is van dergelijke kennis afhankelijk. In Hoofdstuk IX worden experimenten beschreven waarin stamcellen van de muis worden gesorteerd en verrijkt op basis van verschillen in rhodamine-123

retentie en WGA affiniteit. Deze experimenten toonden aan dat de lange termijn repopulerende stamcellen, die zwak aankleuren met rhodamine-123 en een lage affiniteit hebben voor WGA, gescheiden konden worden van de korte termijn repopulerende cellen. Correctie van thalassemie door middel van partieel hemopoëtisch chimerisme bleek het resultaat van het aanslaan van getransplanteerde lange termijn repopulerende stamcellen, die in staat zijn tot bloedcelvorming langs vele hemopoëtische lijnen. De frequentie van de lange termijn repopulerende stamcellen in normaal beenmerg van de muis werd geschat op 1-2 per 10^5, waarmee ook de zeldzaamheid van deze cellen werd aangetoond. De gevoeligheid van deze lange termijn repopulerende stamcellen voor de verschillende hemopoëtische groeifactoren dient nog nader onderzocht te worden.

Op dit moment vormen de thalassemische muizen het beste pre-klinische model voor het onderzoek naar nieuwe behandelingsmodaliteiten voor thalassemie. Modellen in niet-ingeteelde diersoorten, bijvoorbeeld subhumane primaten, ontbreken. Studies, bijvoorbeeld in rhesusapen, gericht op het tot stand brengen van stabiel allogeen partieel chimerisme, zouden aanwijzingen kunnen geven voor de ontwikkeling van nieuwe conditioneringsschema's met relatief lage toxiciteit en voldoende immunosuppressie om de ontwikkeling van stabiel allogeen partieel hemopoëtisch chimerisme mogelijk te maken.

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CURRICULUM VITAE

Cor van den Bos was born in Rotterdam, The Netherlands, on March 16, 1962. After completion of secondary education (gymnasium β, Gymnasium Erasmianum, Rotterdam) in 1980, he studied medicine at the Erasmus University Rotterdam. In the last months before obtaining his bachelor's degree, he stayed for 4.5 months at the Department of Radiobiology, located in the Radiobiological Institute TNO (Director: Prof. dr D.W. van Bekkum), Rijswijk, The Netherlands, for the completion of his research period ('keuzevak'), under supervision of dr G. Wagemaker.

In 1987 he completed his medical studies and was stationed as a military physician in Seedorf, Germany. Leaving the army in the autumn of 1988, he returned to the Department of Radiobiology of the Erasmus University. From 1988 to 1992, the experiments described and presented in this thesis were for the greater part performed there, in the research group headed by dr G. Wagemaker in collaboration with the Radiobiological Institute TNO and in the research group of dr R.E. Ploemacher in the Institute of Hematology of the same university.

In October 1992, he started his training in pediatrics at the Catharina Hospital, Eindhoven, and The University Hospital for Children and Youth, 'Het Wilhelmina Kinderziekenhuis', Utrecht, The Netherlands.

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C. van den Bos, F.C.J.M. van Gils, D. Kieboom, Y.Westerman and G.Wagemaker: Stable partial chimerism in α-thalassemic recipients of allogeneic normal bone marrow cells conditioned with sublethal TBI and immunosuppressive antibodies. Submitted

C. van den Bos, R.W. Bartstra, D. Kieboom and G. Wagemaker: Murine hemopoietic stem cells with long-term repopulating ability and high radiosensitivity. Submitted

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