Differentiation of Hemopoietic Stem Cells in the Thymus

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DIFFERENTIATION OF HEMOPOIETIC STEM CELLS

IN THE THYMUS

(Differentiatie van hemopoietische stamcellen in de thymus)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
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ABBREVIATIONS

BC3	C57Bl/RijxC3H/Law F1 mouse strain
BM	bone marrow
BMT	bone marrow transplantation
BSA	bovine serum albumin
C3AK	C3H/LawxAKR F1 mouse strain
C3H	C3H/Law mouse strain
CFC	colony forming cell
CFU-C1	colony forming unit in culture, growth factors: post-endotoxin serum and PMUE
CFU-C2	colony forming unit in culture, growth factor: PMUE
CFU-GEMM'	in vitro colony forming unit producing granulocyte, erythrocytes megakaryocytes and macrophages
CFU-GEM	CFU producing granulocytes, erythrocytes and macrophages
CFU-GMM'	CFU producing granulocytes, macrophages and megakaryocytes
CFU-stem	CFU producing undifferentiated blast cells
day-n CFU-S	colony forming unit-spleen, colonies were counted n days after BMT
CSF-1	colony stimulating factor-1
d	day
Do	radiation dose which kills 63 % of the measured cells
ED50	effective dose 50 %, dose required to protect 50 % of the mice from the development of BM failure
FACS	fluorescence activated cell sorter
FITC	fluorescein isothiocyanate
5-FU	5-fluorouracil
day-n FUBM	BM obtained from mice n days after injection of 150 mg/kg 5-FU
Gy	gray
h	hour
HPP-CFC	high proliferative potential-colony forming cell
HPCM	human placenta conditioned medium
HSC	hemopoietic stem cell
IL-3	interleukin-3
irr.	irradiated
leth. irr.	lethally irradiated
MCA	monoclonal antibody
NBM	normal BM
PHSC	pluripotent hemopoietic stem cell
PMUE	pregnant mouse uterus extract
RBM	regenerating BM
Rh123	rhodamine 123
SAF	stem cell activating factor
SAFBM	BM cell cultured in the presence of SAF
T cell	thymus dependent lymphocyte, usually Thy-1 positive
TBI	total body irradiation
TNC	thymic nurse cell
WGA	wheat germ agglutinin

Chapter 1

GENERAL INTRODUCTION

Encased within the bones of the body, there exists an organ system, the bone marrow, in which a large number of different cell types develops. Most bone marrow (BM) cells belong to the hemopoietic and lymphatic lineages (i.e. erythrocytes, platelets, granulocytes, macrophages and lymphocytes). Other cell types have also been shown to be derived from precursor cells that are present in the BM: tissue mast cells (Kitamura et al., 1977 and 1981), dendritic cells (Steinman et al., 1974; Pugh et al, 1983), Kupffer cells (van Furth, 1980; Bouwens and Wisse, 1980) and osteoclasts (Ash et al, 1980). The bone marrow is thus a source of cells which have a wide variety of functions and which can be found in many tissues of the body. Most BM cells are mature cells. Although the mature cells are important for the homeostasis of the body, they will not be considered in this thesis.

Many experiments have been and still are performed with the intention to characterize and isolate the cell(s) that give rise to the above mentioned mature cell types. The concept of a pluripotent hemopoietic stem cell (PHSC) has arisen from these experiments. It has been hypothesized that the PHSC, after the proper stimulation, can give rise to all BM derived cell types. Another important characteristic of the PHSC is its capacity for self renewal. This will be discussed below.

Other experiments have pointed to the existence of cells in the BM that have only a limited capacity for differentiating and self renewal potential when compared to the PHSC. These "restricted" stem cells can only give rise to descendants in one or two closely related differentiation pathways. These stem cells are often referred to as progenitor cells or committed precursor cells and as uni(or bi)potent hemopoietic stem cells. This stem cell type receives little attention in this thesis.

In between the two compartments of hemopoietic stem cells (HSC)(i.e. PHSC and unipotent hemopoietic stem cells), other stem cells can be detected. They have a certain capacity to remake themselves (self renewal) and to generate a,

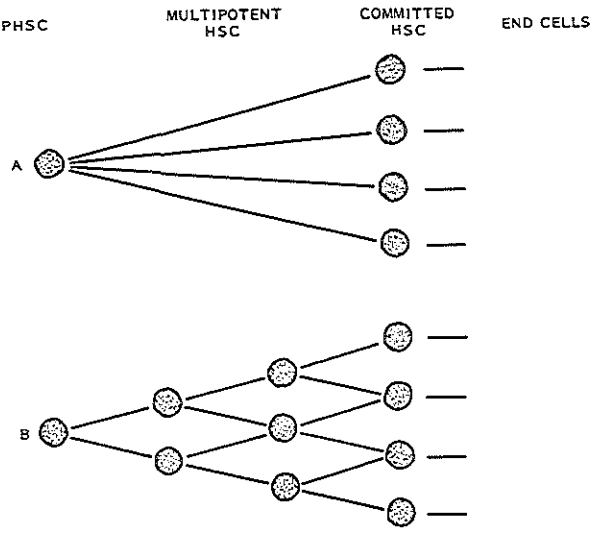


Fig. 1.1 Models for differentiation of hemopoietic stem cells. A. Committment of stem cells. Differentiation (e.g. induced by hemopoietic factors) causes stem cells to produce end cells that belong to only one lineage. Relatively few different stem cells are necessary in such a model. B. Restriction of stem cells. Differentiation causes the exclusion of one (or more) lineages in the differentiation repertoire of the stem cells. If the restriction of the various lineages can occur independently many different types of stem cells are formed. Neither differentiation mechanism can, presently, be excluded.

sometimes, large number of diverse cell types. These cells are called multipotent hemopoietic stem cells (MHSC)(see below). It is highly probable that there exists an overlap between the three compartments. Some investigators favor the idea that there exists a continuum of cells, changing gradually in self renewal capacity and in differentiation status (Fig. 1.1) (Hellman et al, 1978; Botnick et al, 1979; Ogawa et al, 1983). Others favor the existence of a few distinct cell types with different properties (Rosendaal et al, 1979, Hodgson and Bradley, 1979; Micklem, 1983). It is not known whether hemopoietic stem cells differentiate as a result of committment to a certain lineage or whether stem cells lose the potential to differentiate into certain lineages (restriction). Most multipotent and committed stem cells are defined by their ability to produce a particular type of colony or cluster in vitro. This occurs under the influence of factors. Each cell lineage is under the regulation of specific growth factors. However, it is very difficult to ascertain whether the

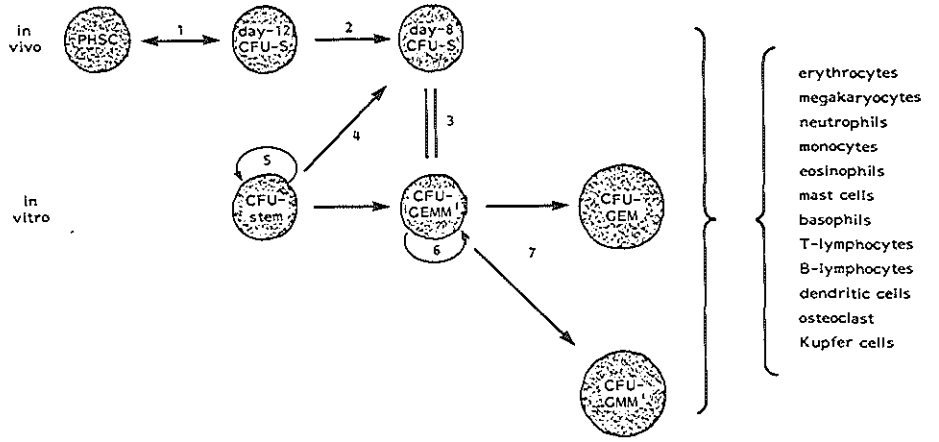


Fig. 1.2 A differentiation scheme of in vivo stem cells and early in vitro progenitor cells. The scheme is constructed from literature data. 1: Visser et al, 1984; 2: Roosendaal et al, 1976, 1979, and Hodgson and Bradley, 1979; 3: Johnson, 1980 and Hara and Ogawa, 1978; 4: Keller et al, 1982; 5: Nakahata et al, 1982; 6: Humphries et al, 1981; 7: Nakahata et al, 1982.

absence of a cell lineage in a colony is due to the lack in differentiation potential of the colony forming cell or due to culture conditions and random events. A definite differentiation scheme of hemopoietic stem cells into mature cells is therefore not yet possible. Fig. 1.2 shows a differentiation model of the most primitive hemopoietic stem cells which is constructed from literature data. It compares stem cells that can be detected in vivo with those that grow in vitro. A recently published scheme of stem cells that grow and differentiate in vitro is presented in Fig. 1.3.

Among the diverse array of BM cells there are cells that have the capacity to travel to the thymus and to give rise to thymocytes. These cells are defined as prothymocytes. There is evidence that the prothymocyte is distinct from other progenitor cells (Abramson et al, 1977; Boersma et al, 1981b; Mulder et al, 1984). The prothymocyte and its predecessors have not yet properly been placed among the above mentioned HSC. For instance, it is not known at which level, beyond that of the PHSC, the HSC lose the potential to generate thymocytes (Fig. 1.4). Very few murine hemopoietic stem cells produce (T) lymphocytes in vitro (e.g.: Lim et al, 1984). This resulted in the notion that the potential for lymphoid differentiation separates very early from that for the other lineages. In most published schemes differentiation into lymphocytes

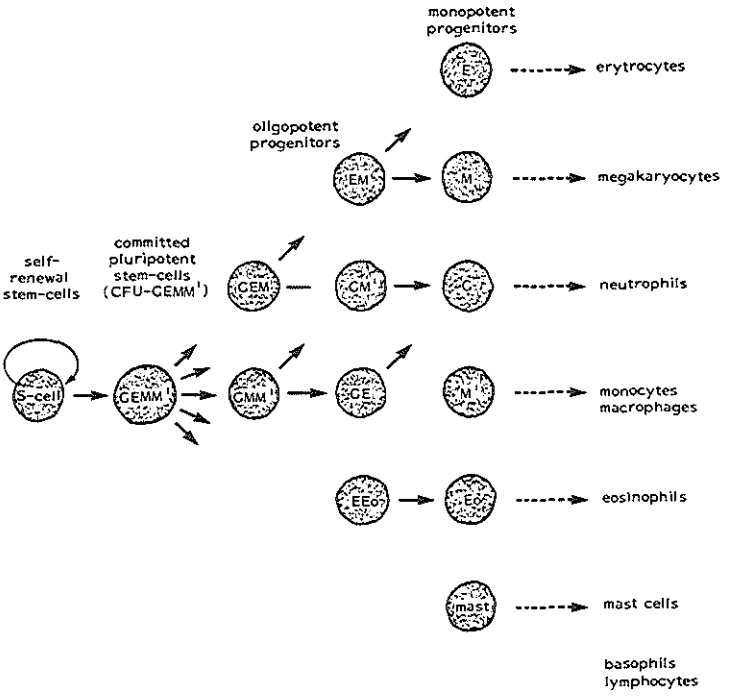


Fig. 1.3 A differentiation scheme of in vitro progenitor cells. Derived from Ogawa et al, 1983. Abbreviations indicate: G: neutrophil; M': macrophage (monocyte); Eo: eosinophil; M: megakaryocyte; E: erythrocyte.

deviates from that of the other lineages at the level of the pluripotent stem cell (e.g.: Moore and Metcalf, 1971; Ogawa, 1983; Fig. 1.3). One of the purposes of this thesis is to delineate more clearly which of the most primitive HSC do or do not have the potential to generate T lymphocytes. This may help to clarify the interrelationships of various HSC more clearly.

Prothymocytes travel to the thymus. Few cells do so under steady state conditions in the adult mouse (Harris et al, 1964). Large numbers enter the avian thymus at three specific times during ontogeny (Le Douarin and Jotereau, 1975; Jotereau and Le Douarin, 1982). This has not (yet) been observed in the murine and human thymus. After total body irradiation (TBI) with a lethal dose and transplantation with a relatively high number (10^7) of BM cells, donor cells can be detected in the thymus as early as the third to fourth day after transplantation (Boersma et al, 1982). Other investigators reported on a later detection (8-12 days) of donor type cells (Kadish and Basch, 1976; Ceredig and

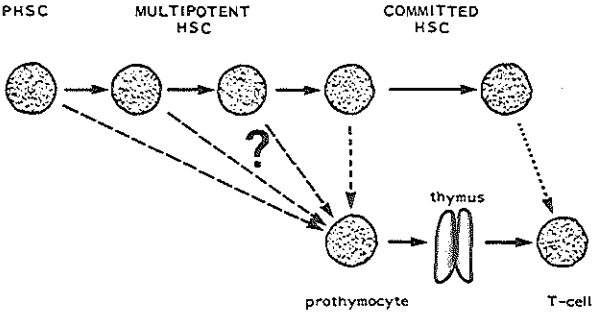


Fig. 1.4 Precursor cells of prothymocytes. It is not known at which level of hemopoietic differentiation the prothymocyte is derived.

MacDonald, 1982; Sharrow et al, 1983). Virtually nothing is known about what happens in the thymus between transplantation and the detection of the first donor thymocytes. For example, it is not known when and at which site the prothymocytes enter the irradiated thymus. We also lack the knowledge about the first differentiation events of prothymocytes. The study of the events that occur to recognizable progenitor cells that have recently entered the thymus may help to clarify the first differentiation steps in the thymus. It is one of the purposes of this thesis to solve the problem when thymocyte precursor cells enter the thymus after lethal irradiation and BMT.

In the following sections a detailed description is presented of the murine (pluripotent) hemopoietic stem cell and their assays: the spleen colony assay (CFU-S assay) and several in vitro assays. The prothymocyte is reviewed and several assays are discussed with emphasis to the so-called thymus repopulation assay. Next, the thymus, its possible functions, the thymocyte subpopulations and their known and unknown relationships are discussed.

Pluripotent hemopoietic stem cells

After irradiation of mice with a high dose of TBI, followed by BMT, it was found that the bone marrow contains precursor cells that can multiply and differentiate in the host (Ford et al, 1956; Vos et al, 1956; Nowell et al, 1956). The take of a BM graft depends on the dose of irradiation: with higher irradiation doses one finds a higher frequency of permanent takes and less transient ones (Gengozian and Makinodan, 1957; Santos et al, 1958). This is due to a decrease in the number of surviving lymphoid host cells that can reject the graft. Lethal irradiation of mice damages the hemopoietic cells severely. This leads to a rapid depletion of the hemopoietic tissues and most animals die in the second week after irradiation. This is caused by anemia, hemorrhages and infections (van Bekkum and de Vries, 1967). The hemopoietic tissues can be restored by bone marrow transplantation and the survival of lethally irradiated mice depends only on the dose of BM cells transplanted (van Bekkum and Vos, 1957). Visser showed that an almost pure stem cell population enriched from normal bone marrow cells possessed the ability to protect lethally irradiated mice (Visser et al, 1984). This indicates that survival after lethal irradiation depends on a small fraction obtained from normal BM which consists most probably of pluripotent hemopoietic stem cells (van Bekkum et al, 1983).

By using a chromosome marker technique, it was established that there are cells in the bone marrow that could give rise to descendents in all hemopoietic lineages (Ford et al. 1956; Micklem et al, 1968; Wu et al 1968a; Abramson et al, 1977). These experiments showed that some of the cells in the bone marrow are pluripotent.

The study of hemopoietic stem cells was greatly aided by the discovery that spleen colonies appear on the spleen surface after transplantation of bone marrow cells into lethally irradiated mice (Till and McCulloch, 1961). These spleen colonies were shown to be clones and to contain cells of the erythroid, granulocytic and megakaryocytic lineages, but no lymphocytes (Becker et al, 1963; Curry and Trentin, 1967; Wu et al, 1967). The cells responsible for the colony formation (colony forming cells-spleen; CFC-S) were found to relate closely with survival after lethal irradiation. This indicates that CFC-S are, if not identical, closely related to the pluripotent hemopoietic stem cell. A proportion of injected CFC-S give rise to a spleen colony in lethally irradiated mice. The number of spleen colonies is called the number of CFU-S (colony forming unit-spleen). Each CFU-S represents a number of CFC-S.

The CFU-S assay

The spleen colony assay estimates the number of hemopoietic stem cells in a graft. Discrete nodules appear on the surface of the spleen of irradiated and bone marrow transplanted mice (Till and McCulloch, 1961). They become macroscopically visible from the sixth day after BMT onwards (Curry and Trentin, 1967; Hodgson and Bradley, 1979, Magli et al, 1982). Only 50 % of total nine day spleen colonies are macroscopically visible (Lewis et al, 1968). The number of spleen colonies is linear with the number of transplanted cells (Till and McCulloch, 1961). The colonies were shown to be clones by Wu et al, (1967) using irradiated bone marrow cells containing CFC-S with unique karyotypes. Using the same technique it was also shown that more than one spleen colony may contain the unique karyotype (Barnes et al, 1968). This suggests that a stem cell may produce more than one spleen colony, probably by (re)circulation of the CFU-S. When normal bone marrow cells are transplanted the number of spleen colonies is constant until 14 days after transplantation (Lewis and Trobaugh, 1964; Curry and Trentin, 1967; Hodgson and Bradley, 1979). However, after lethal irradiation combined with tail shielding or after sublethal TBI, spleen colonies are present at day 5. They dissappear rapidly in the subsequent days (Robinson, 1967; Gregory et al, 1975; Boggs et al, 1973). Direct inspection of the spleen after irradiation and BMT showed that many 7-8 day colonies disappear within 72 hours (Magli et al, 1982; Wolf, 1984). Other spleen colonies can persist for a longer period and still other colonies appear as late as the 12-14th day after transplantation (Hodgson and Bradley, 1979; Magli et al, 1982; Wolf, 1984). The question that arises from these experiments is whether the CFC that give rise to the colonies at different times (i.e. day-8 CFU-S, day-12 CFU-s etc.) are different cells or whether they belong to a single cell type, but start to proliferate at different times or home differently after BMT. Recently, an antigenic difference was found between day 8 and day-12 CFU-S: day-12 CFU-S were Qa-m2 positive, while day-8 CFU-S lacked this antigen (Harris et al, 1984). Subsequent analysis of other Qa determinants on the CFU-S, revealed that there were similar differences in the expression of Qa-m7 and Qa-m8 between CFU-S that give rise to early and late colonies (Harris et al, 1985). Two other antigenic differences between these cell types (H-2K and Thy-1) are presented in Chapter 4. Day-8 CFU-S were further shown to stain more intensely with the supravital DNA stain Hoechst 33342 than day-12 CFU-S (Baines and Visser, 1983). From this it can be concluded that spleen colonies appearing

Table 1.1

Effects of various treatments on the seeding efficiency of CFU-S

Treatment or source of stem cells	Effect of the treatment	Effect of f-factor compared to NBM 3h	24h	Explanation of change by the authors	Reference
BM, Endotoxin, -24h	?	↓		none	Fred and Smith, 1968
BM, Vinblastin, -24h	kill + ?	↓			
BM, Vinbastin	,,	↓		qualitative difference in the CFU-S population	Smith et al, 1968
BM, Vincristin	,,	↓			
Regenerating spleen	cycling CFU-S	↓		trauma to cycling cells causes lower f-factor	Kretchmar and Conover, 1969
Regenerating spleen	cycling CFU-S	↓	-	loss of CFU-S by early differentiation	Lahiri et al, 1970
Fetal liver cells		↓		functional difference between the CFU-S	Moore et al, 1970
Peripheral blood		↑		subpopulation of the heterogenous BM CFU-S	Gidali et al, 1974
BM, hydroxyurea, -12h	cycling CFU-S	↓	↓	cycling CFU-S have a lower f-factor	Monette and Oemello, 1979
Regenerating BM	cycling CFU-S	↓			
BM separated by velocity sedimentation	size difference and cycle difference	↓			
BM, Ara-C, -16h	cycling CFU-S	-		cycling CFU-S do not differ in f-factor	Dumenil et al, 1984

at different days after BMT are the products of different cells. Experiments with mice that were pretreated with ^{89}Sr or with mice whose spleens were reirradiated three days after irradiation and BMT showed that part of the day-12 CFU-S can arrive in the spleen with a delay of at least three days (van Zant, 1984; Wolf, personal communication). This suggests that day-8 and day-12 CFU-S have a different seeding fraction to the spleen and the bone marrow.

Only a fraction (3-25%) of the HSC present in a bone marrow transplant arrives in the spleen three hours after transplantation (reviewed in Quesenberry and Levitt, 1979; Siminovitch et al, 1963; Schooley, 1966; Lahiri and van Putten, 1969, and many others). This fraction, the f-factor, is regarded as a measure for the seeding efficiency to the spleen and is determined by retransplantation of spleen cells shortly after BMT. The f-factor has been shown to depend both on the donor BM and the recipient mice. For instance, the f-factor decreases when the interval between irradiation and transplantation is lengthened (Lahiri et al, 1970). This is probably not caused by the shrinkage of the spleen following irradiation (Till and McCulloch, 1972), but may be related to an increased removal of transplanted cells during the first days after transplantation (Orbach-Arbouys et al, 1982). Alternatively, the CFC-S may differentiate with loss of the ability to produce a colony. Manipulation of the BM also may change the f-factor (Table 1.1). Most treatments cause a decrease in the seeding fraction of the CFU-S to the femur. Many of the treatments also cause an increase in the fraction of cycling CFU-S (Table 1.1). This led Monette and Demello (1979) to postulate that cycling cells have a lower f-factor when compared to non-cycling cells. However, peripheral CFU-S, which are actively cycling, have an increased f-factor (Gidali et al, 1974). Furthermore, sixteen hours after i.v. injection of cytosine arabinoside (Ara C) when approximately half of the CFU-S are in S-phase (Frindel et al, 1980), the f-factor is similar to that of NBM (Dumenil et al, 1984). Mitotic activity of the CFU-S population, therefore, does not sufficiently explain differences in the f-factor. When day-8 CFU-S arrive in the spleen early after transplantation and day-12 CFU-S do so a few days later, it can be expected that the seeding fraction of CFU-S in cell suspensions that are depleted of early appearing CFU-S is low. Preliminary results from our laboratory indicate that there is no clear difference in the f-factor of day-8 and day-12 CFU-S obtained from normal bone marrow at 2h after BMT (Visser, personal communication; Mulder, unpublished results). This is in contradiction with the results by van Zant and by Wolf, which were both mentioned above. It is clear that for a good understanding of

the CFU-S assay, the homing of CFU-S to spleen or bone marrow should be resolved.

The use and the interpretation of the spleen colony assay, regarding the recent developments, has become complicated. Spleen colony counts should be performed at different days after transplantation. If differences in the f-factor exist between the different types of CFU-S, comparison of CFU-S counts from two different sources of BM cells are quite useless when the f-factors are not known.

In vitro detection of (pluripotent) hemopoietic stem cells

An in vitro assay of the PHSC may be the one described by Keller and Phillips (1982) and by Nakahata and Ogawa (1982). They described a colony type, the stem cell colony that consists exclusively of undifferentiated cells. The colonies grow in methyl-cellulose cultures that are supplemented with conditioned medium obtained from pokeweed mitogen-stimulated spleen cells. The colony is present between 8 and 16 days after the initiation of the culture. The progenitor cells that give rise to this colony ("CFU-stem") has the capacity to renew itself and the colony contains CFU-S (Nakahata and Ogawa, 1982; Keller et al, 1984). The spleen colonies produced by these CFU-S are present at eight days after transplantation, but not at twelve days (Keller at al, 1984). Stem cell colonies also contain large numbers of multipotent stem cells (e.g. the CFU-GEMM, a CFU that gives rise to colonies containing granulocytes, erythrocytes, macrophages and megakaryocytes (Nakahata and Ogawa, 1982). It has previously been suggested that the CFU-GEMM and day-8 CFU-S represent similar cell types (Hara and Ogawa, 1978; Johnson, 1980). This indicates that the cell that gives rise to the stem cell colony (CFU-stem) may be a more primitive cell than the day-8 CFU-S.

Self renewal of (pluripotent) hemopoietic stem cells

Self renewal or the ability to produce a daughter cell that has the same properties as the parent cell is one of the presumed characteristics of the PHSC. Differentiated hemopoietic stem cells also have this ability, but probably to a lower extent. Self renewal has been detected in several assays:

After lethal irradiation and BMT a recovery of the bone marrow occurs. All the quantitative assays of hemopoietic stem cells show normal numbers of stem cells three to four weeks after BMT. After serial BMT a decline in the bone marrow repopulating ability occurs (e.g. Barnes et al, 1959; van Bekkum and Weyzen, 1961). This is not caused by ageing of the stem cell, because young and old BM were shown to have equal repopulating ability (Ogden and Micklem, 1976; Harrison, 1982, 1983; Boggs et al, 1984). This indicates that PHSC are lost during retransplantation. When the interval between transplantations is lengthened, the repopulating capacity improves (van Bekkum and Weyzen, 1961). Up to 13 passages could be passaged without evidence of diminishing repopulating ability (van Bekkum and Weyzen, 1961). This must be due to renewal of the PHSC. It has been observed that the loss of repopulating capacity can occur with, initially, little change in CFU-S numbers (Ogden and Micklem, 1976; Ross et al, 1982). This suggests that at least some CFU-S are not pluripotent stem cells.

Self renewal of the CFU-S is measured by the presence of CFU-S in spleen colonies (Siminovitch et al, 1963; Lewis and Trobaugh, 1964). The number of CFU-S per spleen colony is higher in later appearing nodules when compared to those present early after BMT (Vogel et al, 1968, Magli et al, 1982; Johnson and Nicola, 1984). These CFU-S could be derived from stem cells migrating into the spleen colony or could be produced in it. Although, direct evidence for either of the two possibilities has not been presented, it is assumed that the CFU-S are produced in the spleen colony. The distribution of the number of CFU-S per spleen colony was shown to follow a gamma distribution, but not that of a Poisson distribution (Till et al, 1964). A gamma distribution is a continous distribution for which the variance may exceed the mean. This indicates that the variation from colony to colony is greater than might be expected from sampling errors alone (Till et al, 1964). From such a distribution of CFU-S among spleen colonies, it can be expected that self renewal and differentiation of stem cells is governed by random processes (Till et al, 1964). In these studies, it was assumed that the CFU-S belong to a homogenous population of stem cells. However, this seems not to be the case: CFU-S with a relatively low sedimentation rate (i.e. small cells) have an increased capacity to produce secondary CFU-S (Worton et al, 1969a). The CFU-S surviving treatment of the BM donors with isopropyl methane sulphonate produce spleen colonies that contain very few secondary CFU-S (Schofield and Lajtha, 1973). In contrast, the CFU-S that survive treatment with hydroxyurea (HU) and 5-fluoruracil (5-FU) and CFU-S from fetal liver produce spleen colonies that contain very large numbers of

secondary CFU-S (Rosendaal et al, 1976,1979; Hodgson and Bradley, 1979). In addition it has been observed that circulating CFU-S have a low capacity for self renewal (Micklem et al, 1975). The population of cells that gives rise to spleen colonies is thus heterogeneous with respect to self renewal. The distribution of CFU-S in the spleen colonies can then also be explained by differences between the CFU-S instead of stochastic processes which govern self renewal.

In in vitro assays of hemopoietic stem cells, self renewal has also been shown (Dicke et al, 1971). Thirty-five out of 68 stem cell colonies were able to produce new stem cell colonies upon replating (Nakahata et al, 1982). Thirteen out of 49 GEMM colonies were found to produce new GEMM colonies upon replating (Humphries et al, 1981). In contrast, every stem cell colony contains, sometimes large numbers of CFU-GEMM (Nakahata et al, 1982). Very few colonies containing erythroid and megakaryocytic cells produced the same colonies after replating (Humphries et al, 1979; Metcalf et al, 1979). And the colonies containing cells of a single lineage do not contain precursor cells for secondary colonies (Suda et al, 1983).

From the above studies it can be concluded that there are several different stem cells that have self renewing capacity although to a different extent (PHSC, as measured by survival, CFU-S, CFU-stem, CFU-GEMM). From this the notion appears that hemopoietic stem cells are arranged such that more primitive stem cells have a higher self renewal capacity than do more differentiated precursors (Figs. 1.1 and 1.2; Hellman et al, 1978; Rosendaal et al, 1979, Ogawa et al, 1983). This model favours the continued presence of PHSC and predicts, eventually, the disapearance of clones produced by more differentiated cells.

Our knowledge of the mechanisms that regulate the process of self renewal (proliferation) is far from complete: in normal cells the control of growth and differentiation is coupled. Uncoupling of pathways of gene expression that control growth and differentiation can occur in malignant cells (Sachs, 1980). Culture of bone marrow cells infected with the erythroleukemic Friend virus (FV) complex, which consists of the lymphoid leukemia helper virus and the defective spleen focus forming virus causes prolonged production of the CFU-S (Dexter et al, 1977; Greenberger et al, 1983). However, the CFU-S in cultures that are 10-35 weeks old lose the ability to protect lethally irradiated mice (Dexter et al, 1977; Greenberger et al, 1983). The spleen colonies of these CFU-S consist predominantly of small granulocytic colonies (Dexter et al, 1977; Greenberger et

al, 1983). In contrast, serial transplantation of FV infected BM into leukemia resistant C57Bl/6 mice could be continued as long as the FV complex was present (i.e. up to 18 passages)(Eckner et al, 1982). The molecular events that lead to the production of large numbers of CFU-S is not yet known. Infection of long term bone marrow cultures (LTBMC) with a recombinant Rous sarcoma virus has also been shown to extend the self-renewal of the CFU-S (Boettiger et al., 1984). In contrast with the CFU-S in cultures infected with the FV complex, the CFU-S in src infected LTBMC protected lethally irradiated mice as good as those in NBM. Serial transplantation of src infected BM could also be performed much longer than that of NBM (Boettiger et al, 1984). Thus, although the exact regulation of self renewal of hemopoietic stem cells is still unclear, manipulation of gene expression with viral genes and or gene products can cause prolonged proliferation and maintenance of hemopoietic stem cells in vitro.

The prothymocyte

It was mentioned above that the prothymocyte is a precursor cell which is committed to the T cell lineage with the capacity to home to and to have progeny in the thymus. Extrathymic thymocyte precursors (e.g. PHSC, prothymocyte) have the potential to give rise to thymocytes. Thymocyte precursors have been identified in mouse bone marrow (Gengozian et al., 1957; Popp, 1962; Micklem et al., 1968), spleen (Muramatsu et al., 1976; Shisa et al., 1977) and fetal liver (Vos et al., 1960; Tyan et al., 1964; Löwenberg, 1974). In the chicken, the yolk sac has also been shown to contain thymocyte precursor cells (Tyan, 1968). Although the exact mechanism that induces the migration of prothymocytes to the thymus is not known, it has been demonstrated in vitro that the thymus can generate a (chemotactic) stimulus that causes migration of BM or fetal liver cells into the thymus (Fontaine-Perus et al, 1981; Pyke and Bach, 1981; Pyke et al, 1983). The prothymocytes enter the thymus which is a lobulated organ located in the upper mediastinum in the thoracic cavity. The thymus can microscopically be devided in a cortex and a medulla. The cortex is very densily packed with thymocytes, while the medulla has a lower density of lymphoid cells with many epithelial cells in between. Functional thymocytes are mostly found in the medulla.

Assays of prothymocytes

Thymus repopulation after lethal irradiation and BMT can be used to determine the presence of and quantify the number of thymocyte precursors (Kadish and Basch, 1976; Boersma et al, 1981a). The repopulation kinetics has been used as a measure of the number of prothymocytes. A linear relationship between the number of donor cells transplanted and the number of donor thymocytes was described during the repopulation phase (Boersma et al, 1981a, Boersma, 1982). The assay has the disadvantage that it is not clonal and that all cells that can produce thymocytes (i.e. the PHSC, the prothymocyte and unknown intermediate cell types) are measured in the same assay. It was shown that thymocytes and other hemopoietic cells can be derived from one hemopoietic stem cell (Abramson et al, 1977) and that thymocyte precursor cells and hemopoietic stem cells share more characteristics with each other than with most thymocytes or T cells (Basch and Kadish, et al, 1977; Boersma, et al, 1981b: Boersma, 1982; Mulder et al, 1985). This indicates that thymus repopulating ability is one of the characteristics of hemopoietic stem cells.

The expression of T cell antigens after in vivo presentation of a stimulus has been used as a measure for the number of prothymocytes in cell suspensions (Komuro and Boyse, 1973; Jones-Villeneuve et al., 1980; Greiner et al., 1982). However, many T cell markers are present only on a subpopulation of T cells, or if present on all T cells, they are not confined to the T cell lineage (e.g. the Thy-1 antigen; Prystowsky et al.,1978; Schrader et al., 1982; Basch and Berman, 1982). A T cell marker can in this case not be used for quantification of prothymocytes. With mouse thymus extract and thymopoietin, spleen and BM cells can be induced to express the Thy-1 antigen (Komuro and Boyse, 1973). Thymus repopulating ability of the induced cells resided among the Thy-1 positive cells, while thymocyte precursors from untreated, normal BM were Thy-1 negative (Komuro et al, 1975). Thymocyte precursor cells can, apparently, express the Thy-1 antigen without loss of repopulation function. It is very unfortunate that the sensitivity of the CFU-S to anti-Thy-1 treatment was not also tested, because recent results indicate that the CFU-S may also express low levels of the Thy-1 antigen on the cell membrane (Basch and Berman, 1982; see also Chapter 4). This indicates that the expression of Thy-1 on the cell surface of bone marrow cells by thymus hormones cannot be used for the quantification of prothymocytes.

In vitro T cell colonies are another way of enumerating T cell precursors.

The murine T cell colony was reported to be derived from two (perhaps three) cells (Ching and Miller, 1980, 1981, 1982). One of the cells is Thy-1 + and the second is Thy-1 - (Ching and Miller, 1981). The frequency of precursors of these T cell colonies in various organs was found to correlate with the frequency of mature T cells : low in BM and thymic cortex, higher in peripheral lymph nodes, and highest in the blood and thoracic duct lymphocytes (Röpke 1984). Human T cell colony precursor cells were shown to have a similar organ distribution and to be derived from mature T cells. (Klein et al, 1981, 1982; Claeson et al, 1977a and b, 1981; Goube de Laforest and Rozenszajn, 1984). Few (±1 %) human mixed colonies contain T cells (Lim et al, 1984). Multilineage colonies containing T cells that develop from glucose-6-phosphate-dehydrogenase heterozygous human peripheral blood cells were shown to be clones, because of the presence of only one isoenzyme in the cells (Lim et al, 1984). These results indicate that pluri - or multipotent stem cells can produce T cells. However it remains to be determined whether the lineages in the colony reflects the total differentiation potential of the stem cells or whether there is a variable expression of the differentiation potential by the stem cells (e.g. stochastic processes) at the initiation of the culture. Due to this and the low frequency of T cell containing multilineage colonies, it is unlikely that multilineage colonies can be used as a quantitative measure of T cell precursors. These experiments do show, however, that T cells can develop in the absence of a thymus (Ching and Miller, 1981; Triebel et al, 1981; Lim et al, 1984). This agrees with the presence of T cells in athymic, nude mice (Raff, 1973; MacDonald et al, 1981; Reimann and Miller, 1983).

Because of the lack of specificity for mouse pan-T cell markers, the formation of T cell colonies by mature T cells and the very low incidence of multilineage colonies with T cells it may be concluded that, at present, a thymus repopulation assay is the most reliable and quantitative assay for thymocyte precursors. Since this assay may also measure stem cells that are developmentally younger than prothymocytes, experiments were performed, with the object of finding differences between the prothymocyte and other hemopoietic precursors (most often the CFU-S). The thymocyte precursors as defined in the thymus repopulation assay and the CFU-S were shown to have the same (low) density, sedimentation velocity, electrophoretic mobility and not to differ in the presence of several membrane markers (Basch and Kadish, 1977; Boersma et al, 1981b; Boersma, 1982). On the other hand, cell suspensions from NBM, regenerating BM, BM cultures, spleen and fetal liver show large differences in

the ratio of the number of CFU-S and the potential for thymus repopulation. For instance, regenerating BM and long term BM cultures have few thymus repopulating capacity as compared to the number of CFU-S (van Bekkum et al, 1984; Boersma, 1983). Secondly, with the use of BM with unique radiation-induced chromosomal markers, it was shown that the BM contains precursor cells that gave exclusively descendants in the thymus (Abramson et al, 1977). And thirdly, the sensitivity to in vitro gamma irradiation of day-8 CFU-S (Do=1.22Gy) was significantly lower than that of thymocyte precursors (Do=0.92Gy)(Boersma et al, 1983). The sensitivity to gamma irradiation of day-12 CFU-S and thymocyte precursors has not been compared directly, but it has been observed that the day-12 CFU-S are less sensitive than the day-8 CFU-S (Visser and Platenburg, personal communication). This indicates that with regard to the sensitivity to in vitro gamma irradiation, the thymocyte precursor differs from both the day-8 and the day-12 CFU-S. Progenitor cells, responsible for early thymus repopulation (prothymocytes), were found to express less class I H-2 antigens than do day-12 CFU-S (Mulder et al, 1984). These results show that the prothymocyte and the CFU-S are two different cell types.

Entry of precursor cells in the thymus

The entry of thymocyte precursors in the thymus has been the subject of various investigations. Four weeks after joining mice into parabiosis, only ten percent of the dividing thymocytes were shown to be derived from the partner (Harris et al, 1964). After lethal irradiation and transplantation of mixtures of syngeneic but chromosomally distinguishable BM cells a high variation in the ratio of descendants from the two BM sources was found between the two lobes (Wallis et al, 1975). This was later also found with the use of Thy-1 congenic mouse strains as BM donors (Ezine et al, 1984). These results suggest that low numbers of precursor cells enter the adult thymus with (Wallis et al, 1975; Ezine et al, 1984) or without (Harris et al, 1964) previous irradiation. Precursor cells present in the yolk sac were found to arrive in the thymus, only after passage through the bone marrow (Stutman, 1976, 1977). This indicates that at least some thymocyte precursor cells are not attracted directly to the irradiated thymus after transplantation and suggests that these cells receive a differentiation stimulus before their travel to the thymus.

An interesting experiment presented by Kadish and Basch (1976) deals with

the time of entry of thymocyte precursors. The experiment was designed as follows. 48 hours after lethal irradiation and transplantation of spleen cells from donor A, variable numbers of BM cells from donor B were transplanted. The percentage of donor A derived thymocytes was determined 14 days after the first transplant. It was considered that thymocyte precursor cells from BM B should compete with those in the first graft. The results showed that there was a decrease in the number of A derived thymocytes when higher numbers of B BM cells were injected and it was concluded that "the entry into the irradiated thymus must occur soon after injection of the precursor cells" (Kadish and Basch, 1976). However, because the number of A derived thymocytes can be influenced by BM B as late as 48 h after the first grafting, these results rather suggest that the precursor cells enter the thymus at 48 h after BMT.

If it is assumed that thymocyte progenitors arrive in the thymus soon after BMT and that thymus repopulation occurs exponentially, then extrapolation of the thymus repopulation curve to the day of BMT results in 300 precursor cells per 10^6 BM cells (Boersma et al, 1981a). In a direct assay, the group of Weissman showed that less than 0.1 % of normal, labeled BM cells home within three hours into an irradiated thymus (Lepault and Weissman, 1981). Analysis of the immigrants indicated that 1/4-1/3 of the cells were macrophages or mature lymphocytes (Lepault et al, 1983). These experiments did not show whether the immigrated cells could give rise to thymocytes. The entry of injected cells decreased when the interval between irradiation and BMT was increased (Varlet et al, 1982). In addition, the number of immigrants at 16 h post-transplantation was 6-fold higher than that at 3 h. (Varlet et al, 1982). These result show that the number of thymus-seeking cells depends not only on the size of the BMT, but also on the conditioning of the recipient. From these studies it can be concluded that within a few hours after BMT there are donor cells present in the thymus, but it remains to be determined whether these cells are prothymocytes.

It is uncertain where thymocyte progenitor cells lodge after their arrival in the thymus. Early experiments show that most immigrants (at 3 days after BMT) are present in the medulla and the corticomedullary area (Brumby and Metcalf, 1967). The few migrants in the cortex are in close proximity of blood vessels (Brumby and Metcalf, 1967). The presence of lymphocytes in the wall of the postcapillary venule, which is situated at the corticomedullary junction indicates that here lymphocytes passage to and from the medulla or corticomedullarry junction (Hwang, et al, 1974). Other studies suggest that there is a traffic of cells in the thymus from the subcapsular area to the

cortex and then to the medulla (Weissman, 1967, 1973; Fathman et al, 1975). This would indicate that intrathymic thymocyte precursors are present in the subcapsular area. Recent data have shown that there exists a small population in the thymus that has the capacity to give rise to thymocytes after isolation and transplantation (Mathieson and Fowlkes, 1984; Fowlkes et al, 1985). The phenotype of this subpopulation (see below) has been described to be present in the subcapsular area (van Ewijk et al, 1981). The above experiments point to two places where thymocyte precursor cells can be found: the subcapsular area and the corticomedullary junction. Other contributions to the literature suggest that the thymus contains more than one, parallel or independent T cell lineages (Shortman and Jackson, 1974; Boersma et al, 1983; Ezine et al, 1984). One for the cortex and one for the medulla. Although definite proof of this has not been presented, these studies suggest that the cells that enter the thymus can be expected to immigrate at different sites.

The thymus

The thymus is a complex organ in which epithelial cells, macrophages, dendritic cells and lymphocytes are present. The non-lymphoid cells comprise only a small fraction of the number of thymus cells (<5%). However, a close interaction occurs between all the cell types (Wekerle et al, 1980; Kyewski et al, 1982, 1984). The thymus produces several hormones (Bach and Dardenne, 1973; Goldstein, 1975; Goldschneider et al, 1981) which have important immunological functions. Neonatal thymectomy of mice severely impairs immunological functions (e.g. Miller et al, 1962). The immunological defect could be restored with thymus transplants (Miller, 1962) or with a thymoma graft (Stutman et al, 1969). The restored immune function in these recipients was mediated by host derived lymphocytes (Stutman et al, 1969). This indicates that the nonlymphocytic elements in the thymus play an important role in the development of T cell function. Several of the various non-lymphocytic cell types that produce the thymic hormones will be described next.

Three, phenotypically different epithelial cells have been described in the thymus. One type can be recognized in the cortex and two types (either I-A positive or negative) are present in the medulla (van Vliet et al, 1984, van Vliet, 1985). Thymic epithelial cells together with thymocytes can be isolated in complexes that are called thymic nurse cells (TNC; Wekerle et al, 1980). The

TNC consists of a large epithelial cell enclosing many thymocytes. They can also be found in situ (van de Wijngaert et al, 1983). TNC are predominantly located in the outer cortical area (Kyewski and Kaplan, 1982). The TNC are not the port of entry of the prothymocyte (Kyewski et al, 1982; Mulder and van Bekkum, 1983; Houben deFresne et al, 1984). However, relatively early after lethal irradiation and BMT the TNC enclose relatively higher proportions of donor type thymocytes (Kyewski et al, 1984). The thymocytes in the TNC resemble, phenotypically, the thymocytes that are present in the cortex, which are predominantly non-functional cells (Kyewski and Kaplan, 1982). The thymocytes in the TNC, however, were shown to display several functions that are usually ascribed to more mature thymocytes, like helper activity (Vakharia and Mitchison, 1984), a high spontaneous proliferation rate and the presence of cytotoxic lymphocyte precursor cells (Fink et al, 1984). These results supported the notion that TNC are involved in one of the maturation steps of thymocytes (Wekerle, et al, 1980).

Thymic epithelial cells can also be grown succesfully in vitro (Jason and Janeway, 1984; Small, 1984). Cultured epithelial cells can ingest large numbers of syngeneic thymocytes, which remain viable for at least 24 h. (Jason and Janeway, 1984). Haptenated cultured thymic epithelial cells, injected i.v. can induce a contact hypersensitivity which can be transferred to a second recipient by transplantation of spleen cells (Britz, 1984). This indicates that some of the epithelial cells can function as an antigen presenting cell, like the Langerhans cell of the skin (Ptak et al, 1980) and the spleen dendritic cell (Steinman et al, 1974). Thymic epithelial cells and/or culture supernatants were shown to increase the proliferative response of lectin stimulated lymphocytes (Waksal et al, 1975; Stimson and Crilly, 1981; Boniver et al, 1981) and the mixed lymphocyte reaction (MLR), to augment the production of cytotoxic lymphocytes (Kruisbeek et al, 1979; Glimcher et al, 1983) and to induce Graft versus Host reactivity to cocultured spleen cells (Waksal, et al, 1975). These results support the notion that thymic epithelial cells stimulate thymocytes, during their maturation process.

Thymic macrophages, also a heterogeneous population, were shown to increase the expression of H-2 antigens of cortical thymocytes (Beller and Unanue, 1977). I-A positive macrophages augment and I-A negative macrophages suppress mitogen induced proliferation of thymocytes (Zepp et al, 1984a and b). These findings suggest that macrophages also have a regulatory role in the differentiation and proliferation of thymocytes. Closely related to macrophages are the Antigen

Presenting Cells (APC) which include the dendritic cells (Van Voorhis et al, 1983). Although differences between APC and macrophages have been described (Van Voorhis et al, 1983), it is possible that in the studies with thymic macrophages, APC were also included (Beller and Unanue, 1977; Zepp et al, 1984). APC, defined by their ability to stimulate lymphocytes in a mixed lymphocyte reaction appear in the thymus around the fourteenth gestational day (GD) (Robinson, 1983). Thymic lymphopoiesis initiates at this time. Experiments with F1 to parent BM chimeras indicate that APC of donor type appear after the second month after BMT (Longo and Schwartz, 1980). At eight months after BMT many peripheral T cells show proliferative responses against soluble antigens that are characteristic for the host genotype (i.e. they are under control of class II H-2 genes). After administration of anti-thymocytes serum and cortisone and subsequent repopulation of the lymphoid tissues the response pattern to the soluble antigens changes to that of the donor F1 strain (Longo and Schwartz, 1980). In a subsequent study it was shown that with a higher dose of irradiation (i.e. 12 Gy), the APC are present in the thymus as soon as three weeks after BMT. The responses to soluble antigens of the peripheral T cells in these recipients correlated with that of the BM donor (Longo and Davis, 1983). These studies suggest that thymic APC may be responsible for learning the maturing thymocytes to recognize class II I-A antigens. Recognition of class I H-2 antigens, however, appears not to be influenced by the APC (Bradley et al, 1982; Zinkernagel, 1982). The APC can also stimulate syngeneic thymocytes in vitro which start to proliferate (Born and Wekerle, 1982; Papiernik et al, 1983).

From the above it appears that most if not all the non-lymphocytic cells in the thymus are somehow involved with the differentiative and proliferative processes of the thymocytes. Why divers cell types such as the epithelial and dendritic cells display similar immunological activities is unanswered, but may be related to the difficulty in obtaining good separation between these relatively infrequent cells.

Thymocytes

The vast majority of the cells in the thymus are lymphocytes. Despite intensive investigation, the differentiation pathways of the lymphocytes in the thymus is not known. T cells which develop from thymocytes have been shown to

be present in athymic (nude) mice (Raff, 1973; MacDonald et al, 1981; Reimann and Miller, 1983). One of the most prominent features of the thymus is that there is much proliferative activity. However, few (1%) cells leave the thymus. This indicates that many (cortical) thymocytes die (30% per day) (Metcalf, 1966; McPhee et al, 1979; Shortman and Jackson; 1974, Scollay et al, 1980). From this it was speculated that cortical thymocytes are subject to a selection and that most of them are destined to die (Shortman and Jackson, 1974). Because the events that take place in the thymus are not understood much effort has been put into the description of subpopulations of thymocytes and the investigation of their interrelationships (Shortman and Jackson, 1974; Fathman et al, 1975; Hopper and Shortman, 1976; Cantor and Weissman, 1976; Stutman, 1977; Mathieson et al, 1981; Chen et al, 1983a and b; Leiserson et al, 1983; Scollay and Shortman, 1983; Immunol. Rev. 82). Before reviewing this subject, several thymocyte cell surface markers, their functions, and their appearance during ontogeny will be discussed.

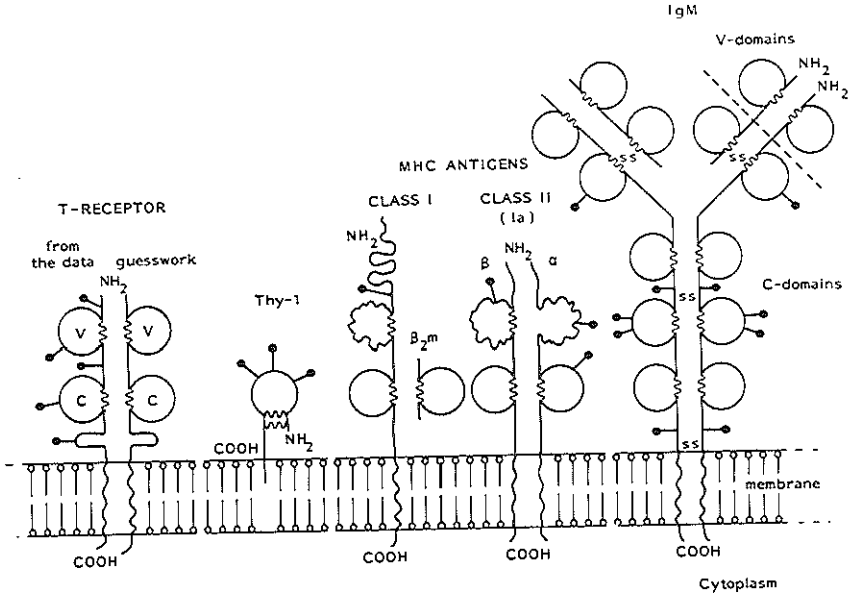


Fig. 1.5 Structure of membrane molecules that are present on thymocytes and T cells. Redrawn from Jensenius and Williams, 1982 and Williams, 1984. For comparison the structure of immunoglobulins is also shown. Circles marked V are like an immunoglobulin V domain, those marked C are more like an immunoglobulin C domain. Also shown are intrachain disulphide bonds (∿∿), interchain disulphide bonds (S S) and N-linked carbohydrate structures (—●). It is highly probable that the class I structure applies also to the Qa and Tl antigens.

Major Histocompatibility Complex antigens on thymocytes and T cells

Class I Major Histocompatibility Complex-2 (MHC; H-2) (Fig. 1.5) antigens are present on all thymocyte precursor cells, but on only 15-25% of thymocytes (Winn et al, 1969 - Mulder et al, 1984). The H-2K positive thymocytes are present in de medulla (Scollay et al, 1980; van Ewijk et al, 1981; Goldschneider et al, 1982; Scollay and Shortman, 1983). All the precursors of cytotoxic and IL-2 producing cells in the thymus are H-2K positive (Ceredig et al, 1982a). The majority of thymocytes that have no or few H-2K antigens on their cell surface belong to a short-lived cell population from which few functional activity can be recovered (Hopper and Shortman, 1976; Hopper et al, 1978). The peripheral T (and B) lymphocytes express large amounts of class I antigens (e.g. Winn et al, 1969). Class II MHC antigens can be detected on 50 % of the thymocytes (Sharrow et al, 1980). Their presence is not associated with thymocyte function (Sharrow et al, 1980).

The function of H-2 antigens in the thymus

The class I and II MHC antigens have been shown to direct the immune response at several levels: antigen recognition and cellular interactions. Recognition of MHC antigens by lymphocytes is often necessary for immune reactions to initiate or to have an effect. A lack of recognition may lead to unresponsiveness of lymphocytes: peripheral T cells may cooperate only with class II histocompatible macrophages (Rosenthal and Shevach, 1973). This has led to the notion that MHC antigens are involved in the restriction of immune responses, the MHC restriction (Zinkernagel et al, 1978, 1983; Klein et al, 1982b; reviewed in Immunol.Rev. 42 and 57). Helper T cells can recognize and are restricted by class II antigens (Katz et al, 1973; Kappler and Marrack, 1977; Sprent, 1978; Hodes et al, 1980; von Boehmer and Haas, 1980). The development of these helper T cells has been suggested to depend on the expression of class II antigens in the thymus (Singer et al, 1981, 1982; Kruisbeek et al, 1984; Kast et al, 1984; Stockinger et al, 1980; van Vliet, 1985). For instance, T cells restricted to class II region determinants are absent from nude, but present in the BM of euthymic littermates. They develop in nude BM when transplanted into parental euthymic mice and they recognize and react only in association with class II antigens expressed by the host thymus

(Kruisbeek et al, 1984). T cells have been shown to recognize and respond to class II antigen on accesory cells. For instance, normal thymocytes (predominantly the 'mature' medullary type) respond in vitro only to Thy-1 negative stimulator cells from the thymus with syngeneic I-A antigens (Born and Wekerle, 1982; Rock and Benacerraf, 1984). From this it may be suggested that class II antigens in the thymus stimulate and learn subpopulations of thymocytes to react only in association with that class II molecule.

Cytotoxic T cells (CTL) are, generally, restricted in their effector functions by class I MHC molecules that are present on the target cell. For instance, CTL originally stimulated against virus infected cells only lyse virus infected target cells with the class I H-2 haplotype of the stimulator cell (Zinkernagel et al, 1978). The acquisition of MHC restriction by CTL is a matter of considerable controversy: the genotype of the responding CTL, the thymus in which the T cells develop and unknown extrathymic influences have all been shown to modify the MHC restriction patterns of CTL (Zinkernagel et al, 1980, Wagner et al, 1980; von Boehmer et al, 1975; Bevan and Fink, 1978; Zinkernagel et al, 1978; von Boehmer and Haas, 1980; Fink and Bevan, 1981; Kruisbeek et al, 1981, 1983a). However, with the finding that the T cell receptor (see below) may have a binding site for both the antigen and the MHC molecule one may hope that the question how class I MHC restriction is generated will be solved (Patten et al, 1984).

The T cell receptor

A receptor on the T cell (Fig. 1.5), specific for antigen and major histocompatibility antigens has been postulated (Doherty et al, 1976; Hunig and Bevan, 1981). Until recently there was no direct evidence for its existence (Jensenius and Williams, 1982). Recently, class I and II specific T cell hybridomas were shown to have heterodimeric receptors of approximately the same size (75-80,000 MW), but differing slightly in charge (Allison et al, 1982; Haskins et al, 1983; Kappler et al, 1983a; Meuer et al, 1983). Tryptic peptide mapping of the different T cell clones showed that these cell surface molecules have variable and constant regions (Kappler et al, 1983b; McIntyre and Allison, 1983; Acuto and Reinherz, 1985). Cloned and sequenced T cell specific mRNA's also showed extensivily similarities to immunoglobulin genes (Hedrick et al, 1984a and b; Yanagi et al, 1983). An interesting difference with

immunoglobulins, the presence of hypervariable regions outside the classical immunoglobulin binding site, suggests that here the interactions may be occurring with MHC determinants (Patten et al, 1984). It was further shown that, similar to that with the immunoglobulin genes in B cells, rearrangements of the β chain of the T cell receptor gene are present in thymocytes, T cells and some lymphoproliferative disorders (Hedrick et al, 1984; Minden et al, 1985, O'Connor et al, 1985). Rearrangements of the β chain was found to be different between functional T cell subsets: helper and cytotoxic T cells show rearrangements of the β gene, while suppressor T cells do not and appear to have the β locus deleted (Hedrick et al, 1985). In a recent publication a small subpopulation of thymocytes was investigated for the expression of the a and β chain (Samelson et al, 1985). This subpopulation, consisting of Ly-1 dull, Ly-2 negative, L3T4 negative thymocytes (see also below), has been suggested to represent intrathymic precursor cells (Fowlkes, 1984, Mathieson and Fowlkes, 1984; Fowlkes et al, 1985). Part of these cells showed no rearrangement of the β chain and no expression of the α chain and others did show rearrangements of the β genes, but still no expression of the α gene (Samelson et al, 1985). The presence of the T cell receptor on cortical type thymocytes suggests that in these cells both the gene loci are rearranged (Roehm et al, 1984). Rearrangements in the T cell receptor gene and the expression of the receptor can thus be used as differerentiation markers for developing thymocytes.

Certain monoclonal antibodies raised against the T cell receptor can predict the antigen and MHC specificity of T cells. This was shown by binding of the MCA to T cell hybridomas and inhibition of their reactivity to an antigen (Marrack, et al, 1983). About 10 % of the thymocytes expressed the cell surface molecule, especially among cortisone resistant and PNA negative thymocytes (Roehm et al, 1984). However, lower amounts were also present on PNA positive thymocytes, suggesting the presence of this receptor on the 'immature' cortical thymocytes (Roehm et al, 1984; Snodgrass et al, 1985).

From these studies it can be concluded that the T cell receptor can explain the recognition of both antigen and MHC products, the generation of diversity for antigen recognition (similar to that of the immunoglobulins) and that the arrangement of the T receptor gene already gives a clearer insight in the first differentiation steps of the thymocyte.

The Thy-1 antigen

The Thy-1 antigen (Fig. 1.5), originally discovered by Reif and Allen (1963) is a cell surface glycoprotein with unknown function and is abundantly present on mouse thymocytes and T cells. Homology of Thy-1 and immunoglobulin at both the protein (Williams and Gagnon, 1982) and DNA level (Moriuchi et al, 1983) suggests that the genes have evolved from a common ancestral gene. In the rat the Thy-1 antigen can be found on all lymphohemopoietic cells and the density of Thy-1 is inversely related to their differentiation status (Goldschneider et al, 1980; Castagnola et al, 1983). In the mouse the Thy-1 antigen was also found on cells lining the otocyst (Prystowsky et al, 1978), brain tissue (Reif and Allen, 1964), fibroblasts (Stern, 1973), epidermal cells (Scheid et al, 1972), mammary cells (Lennon et al, 1978), and on hemopoietic progenitor cells (Schrader et al, 1982; Basch and Berman, 1982; Miller et al, 1985). In the thymus, the Thy-1 antigen is present in a high density on the cortical type thymocytes and in a lower density on the cortisone resistant, medullary thymocytes and the peripheral T cells (Fathman et al, 1975; Shortman et al, 1979). Thy-1 molecules on T cell clones show structural heterogeneity of the Thy-1 molecule. This was found to be due to differences in the N-linked olichosaccharide residues (Pont et al, 1985). In agreement with this it was found that one anti-Thy-1 MCA fails to label cortical thymocytes, while cortisone resistant thymocytes and peripheral T cells are normally labeled (Auchincloss et al, 1982). This could also explain the inconcistencies detected by immunofluorescence staining with different anti-Thy-1 MCA (Scollay, 1982). Together, these data suggests that that the Thy-1 antigen may represent a clonospecific cell surface molecule. In several publications the Thy-1 molecule has been postulated to play a role in cell-cell interactions, but direct evidence is still lacking (Dulbecco et al, 1979; Levy et al, 1985).

Other antigens present on thymocytes

The Ly-1, 2 and 3 antigens were originally described by Boyse et al, 1968. All thymocytes and T cells express some Ly-1 antigen (Ledbetter et al, 1981). A high density of Ly-1 on thymocytes and peripheral T cells (and the absence of Ly-2) is correlated with helper T cell function (Cantor and Boyse, 1975). Addition of anti-Ly-1 MCA to mixed lymphocyte reactions (MLR) enhances DNA

synthesis (Hollander et al, 1981). This is probably caused by the production of IL-2, because the culture supernatants, collected after 24 h, were also able to augment a mixed lymphocyte reaction (Hollander et al, 1981).

A new monoclonal antibody, L3T4, delineates more clearly the helper lymphocytes. The L3T4 cell surface molecule, recognized by MCA GK1.5 resembled the human T4 molecule in tissue distribution and in biochemical properties (reviewed in Dialynas et al, 1983). 87-91, 33 and 19 percent of the nucleated cells in thymus, bone marrow and spleen, respectively, can be labeled with GK1.5 (Ceredig et al, 1983c; Dialynas et al, 1983). Ly-2 (see also below) and L3T4 are expressed on separate cell populations of cortisone resistant thymocytes (Ceredig et al, 1983c; Dialynas et al, 1983). An association of the murine L3T4 with reactivity to class II H-2 antigens has been demonstrated (Dialynas et al, 1983; Wilde et al, 1982, Greenstein et al, 1983, Swain et al, 1984). One possible function of the L3T4 molecule was studied with T cell hybridomas that are reactive to ovalbumine in association with I-A. These hybridomas showed a difference in sensitivity to ova/I-A: the clones with a low sensitivity to ova/I-A contained only small amounts of L3T4 on the cell surface and were difficult to inhibit with the MCA (Marrack et al, 1983b). These data suggest that L3T4 may stabilize the binding of the T cell to its target.

Ly-2 antigens are glycoproteins that are present on subpopulations of thymocytes and T cells (Boyse et al, 1968). Cytotoxic T cells and their precursors have been reported to be Ly-2 positive (=Ly-2 +) (Kisielow et al, 1975; Cantor and Boyse, 1975; Ceredig et al, 1982a). Loss of Ly-2 expression was accompanied by the loss of cytotoxic activity Dialynas et al, 1981). Most Ly-1 negative (=Ly-1 -), Ly-2 + cytotoxic T cells or clones recognize class I H-2 antigens (Swain, 1981; Rao et al, 1983). This and the finding that CTL directed against class II H-2 antigens are Ly-1 + Ly-2 -, has led to the notion that the Ly-2 gene product is associated with class I H-2 antigens instead of a particular (killer) T cell function (Swain, 1981; Vidovic et al, 1984; Rao et al, 1983). In contradiction to this hypothesis it was shown that primary (anti-class II) CTL (Miller and Stutman, 1982) and secondary (anti-class I) CTL (Vidovic et al, 1984) can be Ly-2 +. Antisera or MCA directed against Ly-2 were shown to block cytolytic activity (Nakayama et al, 1979; Shinohara and Sachs, 1979; Sarmiento et al, 1980). However not all cytolytic T cell clones are equally inhibited by anti-Ly-2 (MacDonald et al, 1982) and anti-class II CTL are relatively resistant to blocking by anti-Ly-2 (Shinohara and Kojima, 1984). Thus, although the function of the Ly-2 molecule is not exactly known at the

present time, it is evident that the molecule is necessary for the killing of target cells (Dialynas et al, 1981) and primarily associated with class I H-2 molecules (Swain, 1981).

Ontogeny of some cell surface markers in the thymus

Around the eleventh gestational day (GD) large basophilic stem cells arrive in the thymus (Owen and Ritter, 1969). Until the 14th GD the thymocytes are a homogenous population with the expression of the following membrane antigens: H-2K+, Thy-1+, Ly-1-, Ly-2-, L3T4- (Kamarck and Gottlieb, 1977; van Ewijk et al, 1982; Ceredig et al, 1983b; Kisielow et al, 1984). At the 13-14th GD the first Ly-1+ cells appear (Mathieson et al, 1981; Ceredig et al, 1983b; Habu and Okumura, 1985) and one day later Ly-2 and L3T4 can be detected on small numbers of thymocytes (Kamarck and Gottlieb, 1977; Mathieson et al, 1981; Kisielow et al, 1984; Ceredig et al, 1983b). Two thymocyte subpopulation, the Ly-1-, Ly-2+ and Ly-1+, Ly-2- have previously been assumed to be derived from a Ly-1+, Ly-2+ precursor population (Cantor and Boyse, 1975). The appearance of Ly-1 before that of Ly-2 in the fetal thymus suggests that this may not be the case and that the two populations may be derived from other intrathymic precursor cells (Mathieson et al, 1981). In the subsequent days the number of thymocytes expressing T cell markers increases to that present in the neonatal thymus. The ontogeny of T cell markers has also been studied by culturing fetal thymus in vitro. These cultures were shown to result in the development of viable and functionally active thymocytes (Mandel and Russel, 1971, Robinson and Owen, 1976, 1977; Ceredig et al, 1982b; Kisielow et al, 1984). The expression of cell membrane markers of an 13-14 GD fetal thymus in vitro follows the intra-uterine development (Ceredig et al, 1982b; Kisielow et al, 1984). Thereafter there is, in general, a selection for the medullary type thymocyte, which may vary widely in the expression of Ly-2 and L3T4 (Kisielow et al, 1984).

Thymocyte subpopulations

Ly-1, Ly-2 and L3T4 delineate at least four major subpopulations that differ in phenotype (Mathieson and Fowlkes, 1984). These are listed in Table II. The first expresses low levels of Ly-1 and is negative for both Ly-2 and L3T4.

Isolation and transplantation of these cells results in the production of thymocytes belonging to the other subpopulations (Fowlkes, 1984; Leiserson et al, 1984; Fowlkes et al, 1985), but not that of spleen colonies (Mathieson and Fowlkes, 1984). Further study of this interesting subpopulation (<3% of total thymocytes) has shown that many of the cells express IL-2 receptors, while most thymocytes do not (Ceredig et al, 1985). The genes for the T cell receptor are not rearranged in most cells (see above, Samelson et al, 1985). This is good evidence that these subcortical cells may function as intrathymic progenitors for other thymocytes.

Table 1.2

Thymocyte subpopulations

Group	Percentage of thymocytes	Phenotype	Localization
I	<3	dull Ly-1, Ly-2-, L3T4-	subscapsular
II	80-85	Ly-1+, Ly-2+, L3T4+	cortex
III	8	Ly-1++, Ly-2-, L3T4+	medulla
IV	5	dull Ly-1, Ly-2+, L3T4-	medulla

The other thymocyte subpopulations can, broadly, be devided in cortical (high expression of Thy-1) and medullary thymocytes (low Thy-1). The phenotypic characterization of thymocytes which is usually performed by flow cytometry, does not give information about the localization of the cell in the thymus. But, comparison of flow cytometric data and immunohistochemical staining of frozen thymus sections indicates that a good correlation exists between the phenotype and the localization of the major subsets in the thymus (van Ewijk, et al, 1981). The majority of the thymocytes expresses Ly-1, Ly-2 and L3T4 (II, Table 1.2). This subpopulation comprises the cortical thymocytes. The medulla contains at least two subsets of thymocytes: with the 'helper' Ly-2-, L3T4+ and 'suppressor' Ly-2+, L3T4- phenotype (III and IV, Table 1.2). All these subsets contain proliferating cells (Goldschneider et al, 1982; Scollay and Shortman, 1983; Mathieson and Fowlkes, 1984). In addition to these major subsets, several

minor subsets can be discriminated when other T cell markers are used (Scollay et al, 1984; Mathieson and Fowlkes, 1984; Huiskamp and van Ewijk, 1985; see also van Vliet, 1985).

The study of thymocytes subpopulations has given little insight in the interrelationships of the various subpopulations (see below). One exception is the delineation of the subcapsular weakly Ly-1 postive blast cell which was shown to be an intrathymic precursor cell (Fowlkes, 1984; Mathieson and Fowlkes, 1984). Another exception may be the finding that the two major medullary subsets (dull Ly-1, Ly-2+, L3T4-; Ly-1+, Ly-2-, L3T4-) do not necessarily have to be derived from Ly-1+, Ly-2+ precursor cells that are very abundant in the thymus (Mathieson et al, 1981; Ceredig et al, 1983a). In these instances the static delineation of subpopulation was aided by sequential analysis of the development of other thymocyte subpopulations in vivo (Mathieson et al, 1981) or in vitro (Ceredig et al, 1983a).

Interrelationship of thymocyte subpopulations

Very few facts can be reviewed concerning the relation between the various thymocyte subpopulations. This was amply reviewed by Scollay and Shortman (1983). As stated above, there are precursor cells (probably present in the subcortical area) that can give rise to the other subpopulations of thymocytes (Mathieson and Fowlkes, 1984). A major dilemma concerns the relation of cortical cells to those in the medulla. Initially, a single pathway from cortex to the medulla was proposed (Weissman, 1973; Cantor and Boyse, 1975). The cortex containing the maturing and proliferating cells which finally arrived in the medulla. More recent experiments indicate that cortex and medulla may have separate precursor cells. Boersma showed different repopulation kinetics of thymocytes expressing a low or a high amount of Thy-1: the Thy-1 dull thymocytes were the first cells that appeared after TBI and BMT (Boersma et al, 1982). Similarly, the first donor derived thymocytes are present in the medulla (Ceredig and Schreyer, 1984). In another study, regenerating thymuses from bone marrow chimeras injected with low doses of BM cells were sectioned and analyzed for the presence of donor derived thymocytes. It was demonstrated that regeneration of either the cortex or the medulla could occur (Ezine et al, 1984). These results suggest that two independent differentiation pathways may be present in the thymus. The two lineage model was first proposed by

Schlesinger (1972) and Shortman and Jackson (1974). The latter proposed that, because of the large number of dying cortical thymocytes (30 % per day) cortical thymocytes represent a sterile pathway with no end cell (Shortman and Jackson, 1974, Hopper and Shortman, 1976, Scollay et al, 1984). Another two lineage model proposes that the two functional subpopulations (helper and cytotoxic/suppressor) develop independently (Mathieson et al, 1981; Mathieson and Fowlkes, 1984; Scollay, 1983). Where the split between these two populations occurs is, however, 'totally unclear' (Mathieson and Fowlkes, 1984).

Aim and outline of this thesis

From the literature reviewed in this Chapter it is concluded that, the differentiation of hemopoietic stem cells into the T lymphoid lineage can best be studied in a thymus repopulation assay in vivo. The characterization of progenitor cells that have thymus repopulating ability can give insight in the early differentiation events of the T cell lineage. Since the potential for differentiation into T cells appears to be confined to primitive hemopoietic stem cells and to committed precursors cells, the characterization of stem cells with and without thymus repopulating ability can also give information about the organization and interrelationships of hemopoietic stem cells. These experiments are described in Chapter 3, 4 and 5.

BM cells that arrive shortly after BMT into the thymus can be regarded as a cell population enriched for stem cells committed for the T cell lineage. Variables that influence the direct homing of BM cells into the thymus may help characterize these immigrant cells. These experiments are described in Chapter 6. The functional assay of thymus repopulation was also used for the determination of the time of entry of thymocyte precursors after lethal irradiation and BMT. This was measured by reirradiation of the thymus only or by reirradiation of the mice with shielding of the thymus at several times after BMT. The presence of donor derived thymocytes was measured from 14-30 days after BMT. These experiments are described in Chapter 7 of this thesis. One may expect that more data concerning the entry of thymocyte precursor cells, may help elucidating the unknown relationships of thymocyte subpopulations and the maturation events in the thymus.

Chapter 2

MATERIALS AND METHODS

Mice

The mice that were used in this study were bred at the Radiobiological Institute TNO. They were maintained under specific pathogen free (SPF) conditions until the initiation of the experiment. Thereafter, the mice were housed in a conventional animal room. Bone marrow was obtained from mice which were 8 to 9 weeks old. The recipient mice in the survival studies, in the thymus regeneration -, the competitive repopulation - and the homing assays (see below) were 8 weeks old. For the CFU-S assay the oldest mice were used, usually 12-14 weeks old. The age of the recipient mice did not influence the number of spleen colonies in the CFU-S assay. The mouse strains used in this thesis are shown in Table 2.1. Several cell surface markers present on BM cells or thymocytes are also shown.

Table 2.1

Mouse strains used in this study

mouse strain	abbrevation	H-2-haplotype	Thy-1-haplotype
(C57Bl/RijxC3H/Law)F1	BC3	b/k	Thy-1.2
(C3H/LawxAKR)F1	C3AK	k/k	Thy-1.1 and Thy-1.2
C3H/Law	C3H	k	Thy-1.2

In vivo pretreatment of BM donor mice with 5-fluorouracil (5-FU)

In some experiments, donor mice were injected i.v. with 150 mg 5-FU per kg body weight. At the times indicated BM was obtained from these mice. BM cells from mice four days after 5-FU injection is referred to as day-4 FUBM, etc.

Irradiations

Lethal irradiations were performed with a ^{137}Cs Gamma-Cell-20 small animal irradiator (Atomic Energy of Canada, Ltd). A maximum of 25 mice was irradiated in an animal container flushed with air. The radiation unit contains twin ^{137}Cs gamma-ray sources, one located above and one below the irradiation compartment. The dose rate at the position of the mice was 0.79 Gy/min. Irradiated mice received a total dose of 9.0 Gy.

Reirradiation of lethally irradiated and BM transplanted mice was performed with a Philips-M.E.L. linear accelerator, type SL 75. It produces 6 MV X Rays at a dose rate of 2.3 Gy/min. The total dose delivered refers to the absorbed dose in muscle tissue. The mice were irradiated either with thymus shielding or at the thymic area only. For thymus shielding a leaden, cylindrical bar (Ø=12 mm) or block (30 mm x 35 mm), both with a height of 10 cm were used. For irradiation of the thymus only a 10 cm high leaden block with an aperture of 23 mm diameter was used. Dosimetry was performed with a calibrated ionization chamber. The dose under the shielded area was about 10 % of the total delivered dose (Zoetelief).

Cell suspensions

Organs were removed after asphyxiation of mice with CO_2. These included the spleen, thymus, mesenteric lymph nodes, femur and tibia. The cell suspensions were prepared with Hanks' balanced salt solution (300 mosmol; pH 6.9), containing 5 % fetal calf serum (FCS, Seromed) and when indicated 0.02 % sodium azide. This medium is abbreviated as HSA (Hanks', Serum, Azide). The BM cells were prepared by flushing the femur and tibia with medium from a needle inside the bone. The BM cells were subsequently pipetted through a nylon gauze. Spleen, thymus and lymph node suspensions were prepared by gently pressing the organ through a nylon gauze premoistened with medium. The total number of nucleated cells was counted in a Bürker hemocytometer.

Labeling of cell suspensions with monoclonal antibodies

Nucleated cells were labeled with monoclonal antibodies (MCA) as follows: 10^6 nucleated cells were incubated in 50 ul of the appropriate dilution of the MCA in HSA for 45 minutes on ice. The cells were washed with HSA and

centrifuged once. The cells were subsequently labeled for 30 minutes on ice with either goat-anti-mouse-FITC (GAM/FITC, Nordic Immunological Laboratories B.V., Tilburg, the Netherlands), diluted 50 times in HSA or avidin/FITC, diluted 50 times in HSA. The MCA's used in this study were: anti-H-$2K^k$, clone 11-41 (Oi et al, 1979) which reacts with class I H-2 antigens from the k, p, q and r haplotype. The antibody was diluted 100 times in HSA for incubations. Anti-Thy-1.1 MCA (New England Nuclear, NEI014) and anti-Thy-1.2. MCA (Becton Dickinson, Sunnyvale CA) were diluted 100 times in HSA.

Labeling of cell suspensions with rhodamine 123 (Rh123)

BM cells were labeled with Rh123 as described by Bertoncello et al, (1985). Rh123 (Eastman Kodak, Rochester, N.Y.) was dissolved in distilled water (1 µg/ml). 10^6 BM cells per ml were incubated in Hanks' salt solution containing 5 % FCS and 0.1 µg/ml Rh123 for 20 minutes at 37°C. The cells were centrifuged twice, resuspended and incubated for 15 minutes in Hanks' with 5 % FCS. The cell suspension was centrifuged again and resuspended (3-4x10^6/ml).

Labeling of cell suspensions with Hoechst 33342

BM cells were labeled with Hoechst 33342 as described by Baines and Visser (1983). 10^7 cells per ml were incubated for 90 minutes in HSA containing 20 µg/ml bisbenzimide Hoechst 33342 (Riedel- de Haan AG, Seelze-Hannover, W.Germany) at 37°C. Hoechst 33342 was added to the medium shortly before use from a stock solution, containing 200 µg/ml Hoechst 33342 in saline. After the incubation the cells were washed once, centrifuged and resuspended in HSA.

Flow microfluorometric analysis of (labeled) cells

Cell suspensions were analyzed on a modified fluorescence activated cell sorter (FACS II, Becton Dickinson, Sunnyvale, CA). The machine was adapted to measure two light signals perpendicular (70-110 degrees) to the laser beam by introducing an achromatic beam splitter and a second photomultiplier. Forward light scatter (FLS) was collected over the angles 0.5-13 degrees with respect to the axis of the laser beam. The horizontal laser light blocking bar was set as narrowly as possibly (1 mm). Green fluorescence was measured by an S20 type photomultiplier after passing through a broad band multicavity interference

filter (520-550 nm, Pomfrett, Stamford, Conn.) and a colored glass filter transmitting light above 530 nm (Ditric, series D). In most studies the laser was tuned at 488 nm with an output of 0.4-0.5 W. In sorting experiments the sheath fluid consisted of Hanks' balanced salt solution. In experiments were cells were analyzed for scatter of laser light or fluorescence intensity the sheath fluid was saline.

The light scatter characteristics of BM cells discrimate between several subpopulations. Since this was used in most cell sorting experiments, it will be reviewed briefly. FLS was shown to be proportional to the square of the radius of Sephadex spheres (Visser et al, 1978a). Therefore, FLS is regarded as a parameter for cell size. PLS is a reflection of light illuminated by a particle. It was suggested by van den Engh that the PLS signal reflects cell structure in the sense that cells with many intracellular particles or cells with an oddly shaped nucleus give relatively high scatter signals in the perpendicular direction (van den Engh et al, 1979). Visser et al, (1978b) and van den Engh et al (1979) described that with normal BM cells four clusters can readily be recognized by light scatter alone. This is shown in Fig. 2.1. Erythocytes have a low FLS (because they are small cells) and a wide variety in PLS (because of the variable positioning of the concave cells with respect to the laser beam). In sorting experiments the erythrocytes were usually neglected by setting a trigger threshold for the FLS signal. A narrow cluster of cells with higher FLS and low PLS consists of lymphocytes and maturing erythroid cells (normoblasts). At still higher FLS, two cell populations are present which can be discriminated by the PLS signal: the granulocytes which have a high PLS (because of their lobulated nuclei) and undifferentiated cells with a relatively low PLS. This so-called blast-cell population was comprised of large, undifferentiated cells. It was found to contain 80 % of the CFU-S (van den Engh et al, 1979).

Preparation of purified stem cells

Purified stem cells were prepared as described by Visser et al. (1984). This procedure consists of three separation steps. In the first step, low density BM cells were obtained with the use of a discontinuous metrizamide density gradient (Nyegaard, Oslo). Discontinuous gradients were prepared by pipetting 1 ml of a 1.100 g/cm^3 solution (in Hanks' with 1 g BSA per 100 ml) onto the bottom of a 15 ml tube (Falcon, Oxnard, CA, 2057). 3 ml of a 1.081

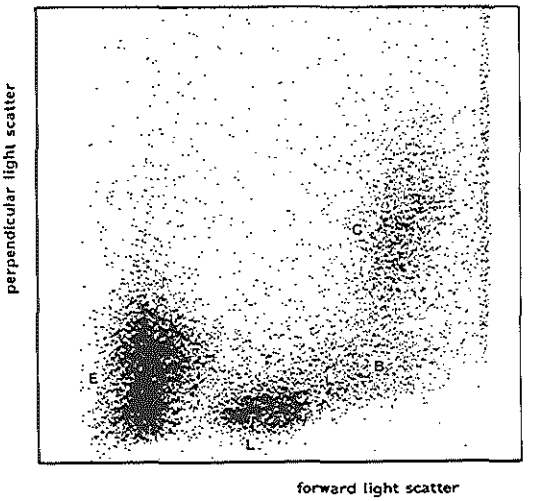


Fig. 2.1 Forward versus perpendicular light scatter intensity of normal mouse bone marrow cells. Each point represents one cell. E=Erythrocytes; L=Lymphocytes; G=Granulocytes; B=Blast cells. The last population contains 80 % of the CFU-S.

g/cm^3 solution was put on top of the bottom layer and 1 ml of a 1.055 g/cm^3 solution, containing 5x10^7 cells was used as the top layer. The cells were centrifuged for 10 min at 1.000 g, the cells at the interfaces were collected, washed, centrifuged and counted. During centrifugation of the gradient, the bone marrow cells were exposed to wheat germ agglutinin-FITC (WGA-FITC; final concentration 0.03-0.1 µg/ml; Polysciences, Inc., Warrington, PA). The low density cells were subsequently analyzed on a fluorescent activated cell sorter (FACS II). The WGA-FITC positive cells with relatively high FLS and low PLS (the so-called blast window) were sorted. The collected cells were then incubated with an isotonic solution containing 0.2 M N-acetyl-D-glucosamine (Polysciences) for 15 minutes at 37°C. This removes the WGA-FITC. The BM cells were subsequently incubated with biotinylated anti-H-2K MCA and avidin/FITC as described above. In a second run on the cell sorter, cells with a high fluorescence intensity, a relatively high FLS and a low PLS were sorted. In a typical experiment, this fraction contained 30 % of the nucleated cells that were collected during the first sort, 3 % of the low density cells and 0.3 % of the original BM cell suspension.

Chromium labeling of cell suspensions

Cell suspensions were centrifuged (500 g, 5 min.) and resuspended in 300 µl medium, consisting of 200 µl HSA and 100 µl saline, containing 20 µCi $Na^{51}CrO_4$ (Amersham, Buckinghamshire, England; specific activity 200 µCi/µg chromium). The cells were incubated at 37°C for 30 minutes. The cells were washed three times with HSA, centrifuged and resuspended. The last supernatant was checked for the absence of radioactivity. The suspensions were adjusted to the given concentrations and injected into lethally irradiated animals. At the times indicated the spleen, liver, femoral BM, thymus, kidneys, heart, lungs and injections site at the tail were removed and counted for the presence of radioactivity by a Packard 5360 gamma counter.

Velocity sedimentation

Velocity sedimentation was performed as described by Miller and Phillips (1969). A siliconized glass sedimentation chamber with a diameter of 14.5 cm and a glass baffle mounted above the inlet was used. A buffered step gradient from 0.2-2 % BSA (Sigma, St.Louis, Mo.) in phosphate buffered saline (PBS) was used. This prevents the occurrence of streaming. After loading the sedimentation chamber with 30 ml PBS, $2x10^8$ nucleated BM cells in 30 ml 0.2 % BSA in PBS were entered. Subsequently, a 1-2 % BSA in PBS gradient of 800 ml was generated underneath the cells. After sedimentation of the cells for 3 to 3.5 h in the cold room, the chamber was unloaded. The first 150 ml was discarded and the remainder was collected in fractions of 30 ml. The cells were recovered by centrifugation (500 g, 10 min), resuspended in 1 or 2 ml Hanks' solution containing 5 % FCS and counted in a hemocytometer. 80 % of the nucleated cells were recovered with this procedure. The cells were subsequently diluted and injected for the assay of day-8 and day-12 CFU-S.

Bone marrow cultures

Bone marrow cells were incubated in preparations containing stem cell activating factor (SAF, which is identical to interleukin-3, IL-3), in serum free suspension cultures at 37°C for a period of four days. SAF was a gift from

G. Wagemaker. In some experiments the culture was terminated at other times (as indicated). One unit of SAF is defined as the quantity for 50 % proliferation of the IL-3 dependent cell line DA-1. In exp. I listed in Table 3.1, SAF was prepared from mouse spleen conditioned medium by affinity chromatography using ConA sepharose and gel filtration as described by Wagemaker et al (1980). The specific activity as measured by the CFU-S assay increased approximately 50,000-fold by this procedure. For experiments II-IV listed in Table 3.1, and Figs. 3.2-3.8 a preparation from WEHI-3 conditioned medium was used that was partially purified by concentration using hollow fibers, adsorption to ConA-Sepharose and reverse phase chromatography as described by Wagemaker (manuscript in preparation). The active material shares many properties with IL-3 (Dorssers et al, 1984; Ihle et al, 1981). The specific activity of this preparation was approximately 200,000 units per mg protein. The cells were incubated at a concentration of $1\text{-}2\text{x}10^6$ cells per ml in the presence of approximately 60 units SAF. The medium consisted of an enriched Dulbecco's medium supplemented with bovine serum albumine, human transferrin, selenite, cholesterol, linoleic acid and nucleosides as described before (Merchav et al, 1984). The medium was further supplemented with 1 µM hydrocortisone (Sigma, St.Louis) and 1 µM isoproterenol (Sigma). The cultures were terminated with Hanks' balanced salt solution prior to intravenous injection into recipient mice. Bone marrow cells cultured for four days as described here will be abbreviated as SAFBM throughout this thesis. Unless noted otherwise, the number of cells or cell types in SAFBM refer to the number of nucleated BM cells at the onset of the culture.

CFU-S assay

The spleen colony assay of Till and McCulloch (1961) was used. Lethally irradiated mice received the indicated number of cells. The spleens were removed at several times after transplantation, fixed in Tellyesniczky's solution and counted for the presence of macroscopically visible spleen colonies. Hemopoietic stem cells that gave rise to spleen colonies at day-8 are referred to as day-8 CFU-S, at day-12 as day-12 CFU-S, etc. The standard deviation of the presented data fell within that described for a poisson distribution (i.e. the square root of the mean value; see also van den Engh, 1976 and Bol, 1982).

Microscopy of spleen colonies

The morphology of spleen colonies was determined on sections from spleens fixed in 4% formaldehyde. The sections were 2-3 μm thick and were stained with hematoxilin, phloxin and saffrane (HPS).

Survival assay

NBM, SAFBM, BM cells incubated with Rh123 or fractions of the latter cell suspension were transplanted at several dilutions in groups of 10 mice. A dilution of the same suspensions was used for determination of the CFU-S content of the graft. The fraction of mice surviving 30 days after irradiation was determined. Most of the nonsurviving animals died in the second week after irradiation. The number of BM cells or the number of day-8 or day-12 CFU-S needed for survival of 50 % of lethally irradiated mice (ED50 values; effective dose) was calculated from these data by probit analysis.

Thymus regeneration assay

The prothymocyte assay (Fig. 2.2) was based on that described by Kadish and Basch (1976) and modified by Boersma et al. (1981). C3AK donor bone marrow was transplanted into C3H recipients. C3AK derived thymocytes are Thy-1.1 and Thy-1.2 positive, while C3H thymocytes have only the Thy-1.2 determinant on their cell surface. Donor-derived thymocytes could thus be recognized because of this alloantigenic difference. At various times after transplantation of 10^6 nucleated BM cells, the animals were killed and their thymuses removed. The total number of thymocytes per organ was determined. Thymocytes were incubated with anti-Thy 1.1 and anti-Thy-1 as described above. The labeled cells were analyzed in a fluorescence activated cell sorter and the fraction of Thy 1.1-positive was determined. The results of thymus regeneration experiments are expressed either as the total number of donor cells per thymus or as the total number of donor thymocytes per CFU-S transplanted. The sensitivity of the thymus regeneration assay was determined by Boersma (1982). He showed that a twofold difference in the content of thymocyte progenitor causes a significant delay (i.e. 1 day) in thymus regeneration (Boersma, 1982).

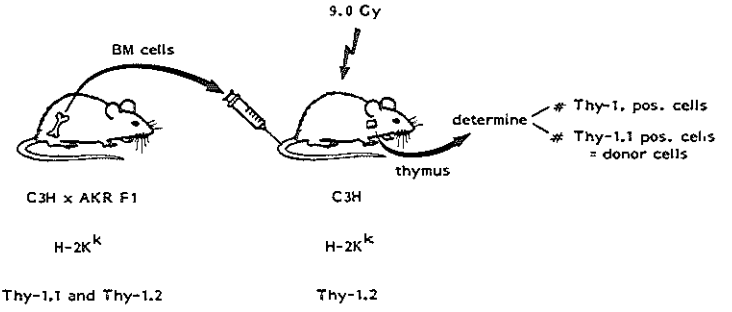


Fig. 2.2 The prothymocyte assay. See text for explanation.

Competitive repopulation assay

In experiments, described in Chapter 3, mixtures of BM cells were transplanted into lethally irradiated mice. These mixtures consisted of C3H and C3AK BM cells and were treated or isolated as indicated. After a relatively long period of regeneration (i.e. 1, 2 and 4 months) the proportion of C3H or C3AK derived thymocytes was determined with methods similar to that of the thymus regeneration assay.

Direct homing assay

The direct homing assay was similar to that described by Lepault and Weissman (1981). Variable numbers of BM cells labeled with Hoechst 33342 (see above) were injected into preirradiated mice. The interval between irradiation and BMT was varied in several experiments and is indicated. At several times after BMT suspensions were prepared from thymus, spleen and BM. The number of cells per organ was counted and the number of Hoechst 33342 labeled cells was analyzed on a fluorescence activated cell sorter. The laser was set to produce UV light (351-362 nm). For the detection of Hoechst 33342 fluorescence the S-20 photomultiplier was protected from scattered light by two Schott (Mainz, W.Germany) KV 408, UV absorbing filters of 3 mm thickness each.

The frequency of labeled cells in a cell suspensions was determined as follows: with the use of labeled cells that were not injected, a scatter and fluorescence window was set that contained all the fluorescent nucleated cells.

Next, the frequency of cells, fullfilling the criteria of the electronic window in cell suspensions from irradiated, but not transplanted mice was determined. Finally, the cell suspensions from the recipients of the labeled BM cells were analyzed (10^5-10^6 cells were analyzed, depending on the frequency of labeled cells) and the number of cells falling in the electronic window was counted. The results are expressed as the frequency of labeled cells per 10^5 cells or as the frequency of labeled cells per organ per 10^6 transplanted cells. The results were corrected for background fluorescence by subtraction of the number of fluoresceing cells in the cell suspension of not transplanted control mice. In initial experiments FITC was used as a tracer of the injected cells. A high background fluorescence was measured with this dye. Hoechst 33342, which is excited at a different wave lenghth did not present such problems and was used in all subsequent experiments.

Chapter 3

SPLEEN COLONY FORMATION, RADIOPROTECTION AND THYMUS REPOPULATION BY BONE MARROW CELLS CULTURED WITH STEM CELL ACTIVATING FACTOR

Introduction

After culture of normal bone marrow cells in the presence of fibroblast-conditioned medium, spleen-conditioned medium or WEHI-conditioned medium a production of CFU-S occurs (Löwenberg and Dicke, 1974; Löwenberg and Dicke, 1975; Wagemaker et al, 1982, Garland and Crompton, 1983; Prystowsky et al, 1984; Mulder et al, 1985). The active material in these media, called stem cell activating factor (SAF) in our laboratory (Löwenberg and Dicke, 1974), is identical to interleukin-3 (IL-3)(Dorssers et al, 1984)(Ihle et al, 1981). Recently it was found that NBM cultured in the presence of stem cell activating factor has a different ratio of day-8 and day-12 CFU-S. (Mulder et al, 1985; Migliaccio et al, 1986, in press). Differences between day-8 and day-12 CFU-S were reviewed in Chapter 1 (antigenic differences, differences in homing to spleen and bone marrow, copurification of day-12 CFU-S and PHSC). Since BM cells cultured with SAF (SAFBM) are one of the few sources in which day-8 CFU-S are enriched, it was of interest to study the functional capacity of the CFU-S in these cultures.

The cultured BM cells were tested for the presence of day-8 and day-12 CFU-S after stimulation with a relatively high dose of SAF. The morphology of the spleen colonies was studied. The capacity of cultured BM cells to protect lethally irradiated mice was tested and the ability of SAFBM to produce offspring in the thymus was determined. Finally SAFBM was tested for its capacity to compete with other BM cells (NBM and BM from mice pretreated with 5-FU) after injection in lethally irradiated mice. This was measured by the ability of SAFBM to produce progeny among thymocytes in comparison with that by NBM and FUBM.

Table 3.1

CFU-S content of bone marrow cells cultured with SAF

Exp.		CFU-S/10^5 nucleated bone marrow cells				
		day-6	day-8	day-9	day-10	day-12
I	NBM		19.1		16.6	21.8
	SAFBM		35.4		20	0
II	NBM	20.0		24.4		26.0
	SAFBM	118.0		40.8		24.8
III	NBM	14.0		14.8		15.6
	SAFBM	71.0		62.4		37.2
IV	NBM	18.4		19.6		19.6
	SAFBM	65.6		48.0		29.6

$5x10^5$ bone marrow cells were cultured under serum free conditions with SAF as described in Chapter 2. The number of CFU-S from SAFBM is expressed per 10^5 initially cultured cells. After four days the recovery of nucleated cells was determined (25% in exp. I, 60-65% in exp. II-IV).

Day-8 and day-12 CFU-S content of NBM and of SAFBM

(C3HxAKR)F1 (C3AK) BM cells were cultured for four days in the presence of SAF, transplanted into lethally irradiated syngeneic recipients and tested for the ability to produce spleen colonies at various days after transplantation (Table 3.1). Normal bone marrow cells produced similar numbers of spleen colonies at various days after transplantation. This is in agreement with results obtained by others (Curry and Trentin, 1967; Hodgson and Bradley, 1979). Bone marrow cells cultured for four days in the presence of SAF (SAFBM) showed an increase in the number of early spleen colonies. The number of spleen colonies at day-12 was maintained or somewhat increased in SAF cultures. The average day-12 CFU-S/day-6 CFU-S ratio was 0.33. In normal BM the day-12/day-6 CFU-S ratio is 1.10. This indicates that SAF predominantly induces the production of cells which form early spleen colonies.

Table 3.2

Gross morphological appearance of spleen colonies

Source of the CFU-S	Total number of spleen colonies	Desintegrating colony		Partly desint. colony		Dense colony	
		n	(%)	n	(%)	n	(%)
NBM d 8	66	4	(7)	6	(9)	56	(85)
d 12	70	3	(4)	7	(10)	60	(86)
SAFBM d 8	99	32	(32)	15	(15)	52	(52)
d 12	51	13	(25)	5	(10)	33	(65)

Spleens from lethally irradiated mice injected with NBM or SAFBM were removed at 8 and 12 days after BMT for spleen colony counts and histology of the colonies. The colonies were categorized in desintegrating colonies (i.e. a colony that consists of many areas of maturing (erythroid) cells intermingled with large numbers of erythocytes), dense colonies (i.e. a colony consisting entirely of nucleated and mostly immature cells with few erythrocytes) and partly desintegrating spleen colonies (a mixture of the two colony types). n=number.

Gross morphology of the spleen colonies from NBM and SAFBM

Spleens from mice that were injected with NBM or SAFBM were sectioned and stained as described in Chapter 2. Two sections, one at one third and one at two thirds of the thickness of the spleen were examined. Double observations of the same spleen colony were avoided by drawing the position of the colony in relation to the spleen contour. The gross morphology of spleen colonies revealed a large difference between NBM and SAFBM: one third of day-8 and one quarter of day-12 spleen colonies in SAFBM were of a desintegrating type whereas less than 10 % of the spleen colonies from NBM showed desintegration (Table 3.2). Typical examples of these and other colonies are shown in Fig. 3.1. Desintegrating colonies consisted of many small areas of maturing nucleated cells (mostly erythroid) with many erythrocytes in between.

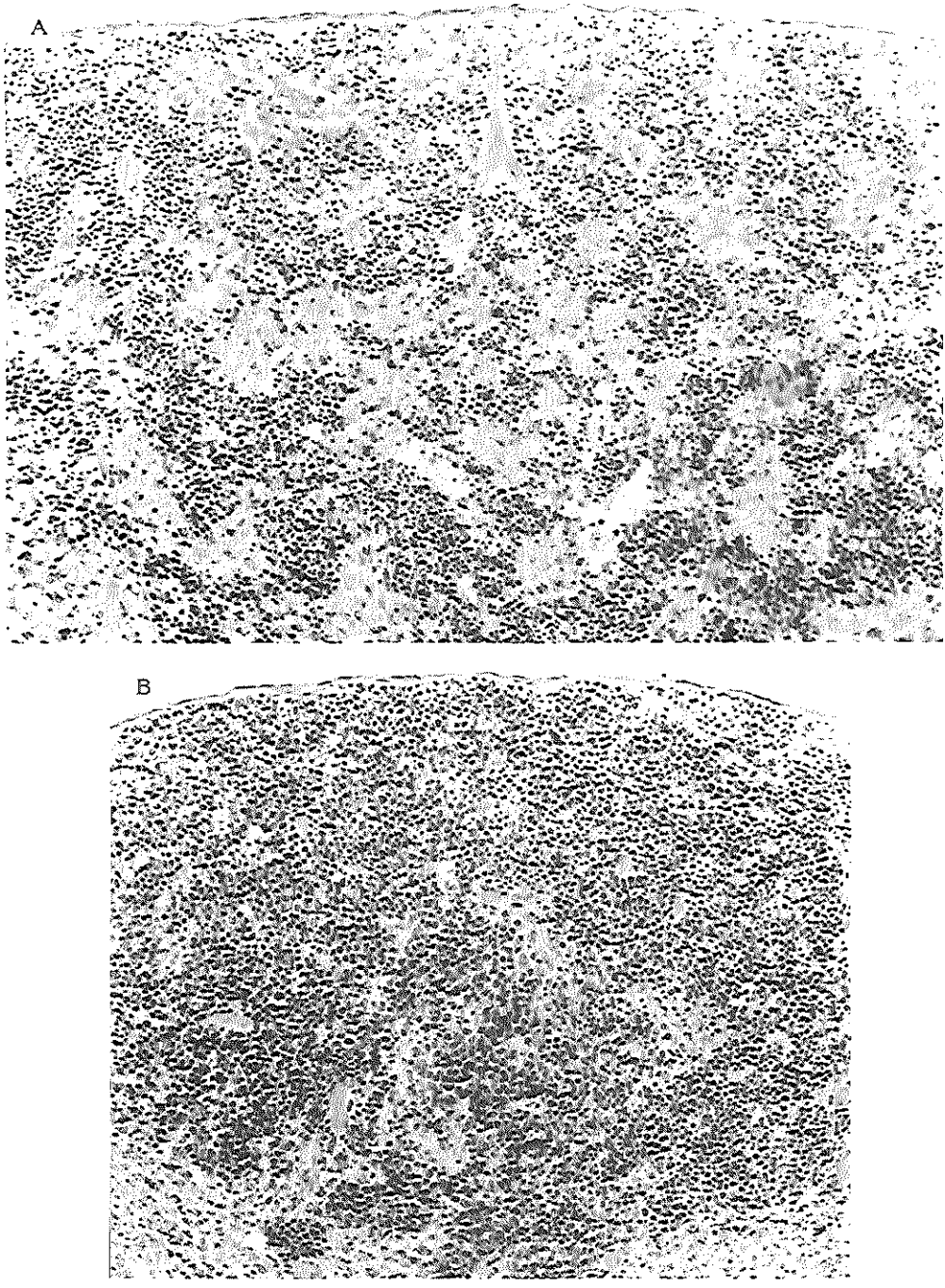
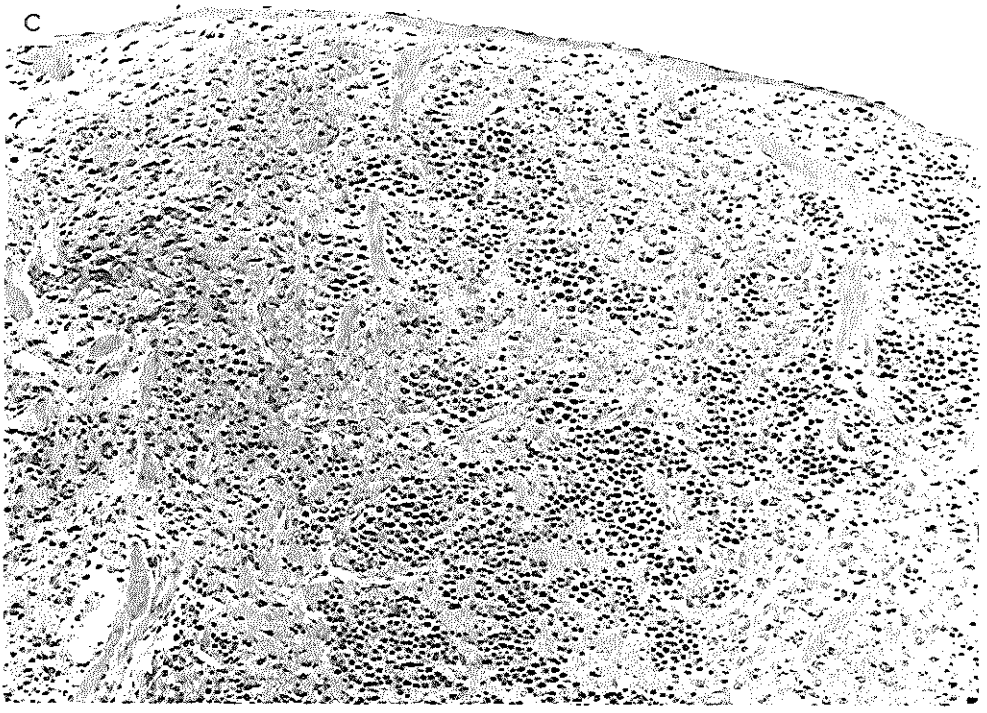


Fig. 3.1 Morphology of spleen colonies. Day 8 (A-B) and day 12 (C-D) spleens were prepared as described in Chapter 2. A and C: Desintegrating spleen colonies B and C: dense colonies.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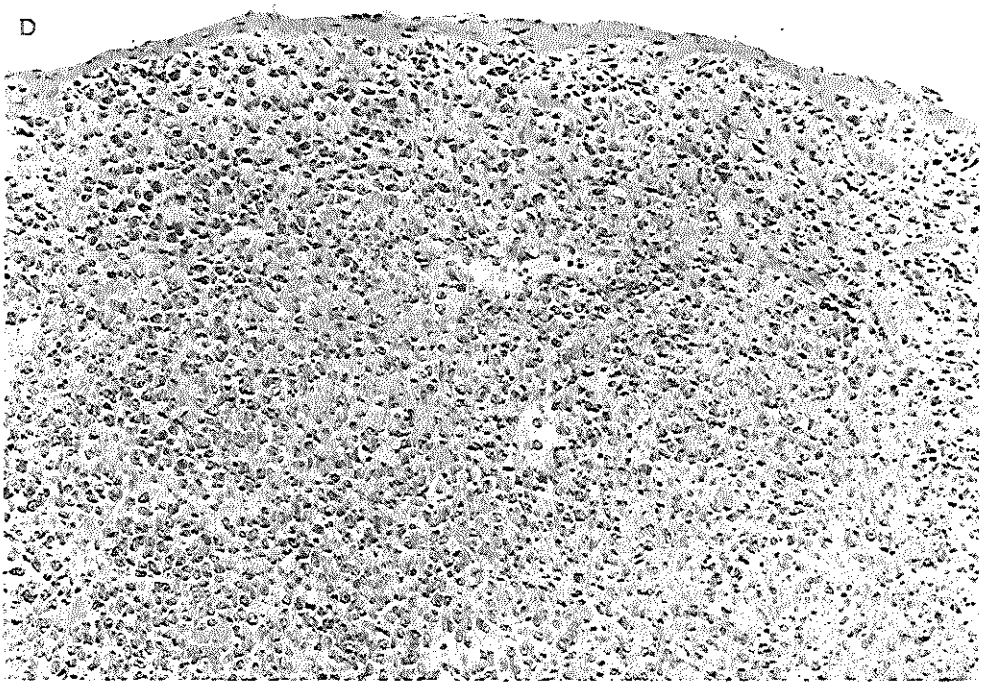
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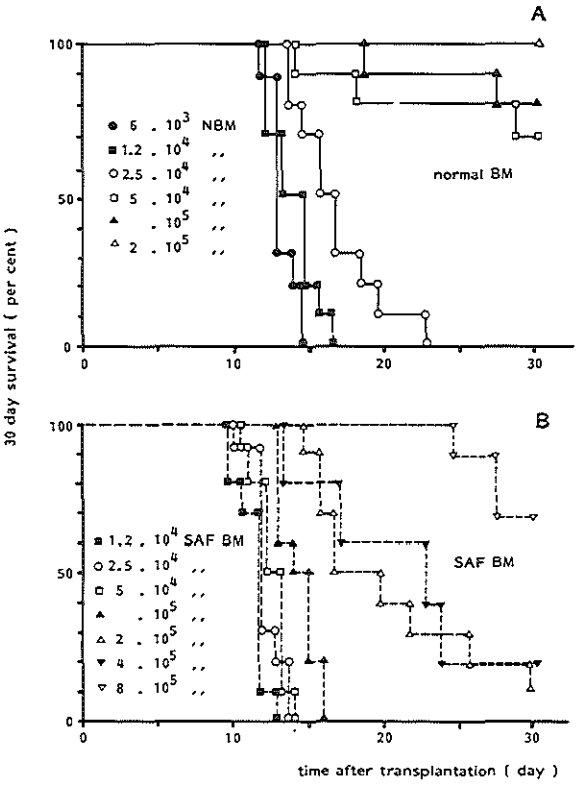


Fig. 3.2 Survival of lethally irradiated mice after transplantation of NBM (A) or SAFBM (B). Time versus survival. A. Groups of ten lethally irradiated C3H mice were transplanted with the indicated number of bone marrow cells and checked for survival. The BM contained 28 day-8 CFU-S and 23.5 day-12 CFU-S per 10^5 cells. B. Similar to A., but transplanted with SAFBM. The number of cells transplanted represents the number of cells that were initially cultured. SAFBM contained 40.8 day-8 CFU-S and 15 day-12 CFU-S per 10^5 initially cultured cells.

Survival of lethally irradiated mice with SAFBM

Pluripotent hemopoietic stem cells (PHSC) can be measured by their ability to protect lethally irradiated mice from bone marrow failure. Bone marrow cells enriched in day-12 CFU-S were shown to have a similar radioprotective capacity as did unseparated bone marrow cells on a per day-12 CFU-S basis (Visser et al, 1984). Bone marrow cells from donor mice pretreated with 5-FU or hydroxyurea (HU) (devoid of day-8 CFU-S) were shown to have a greater capacity for self

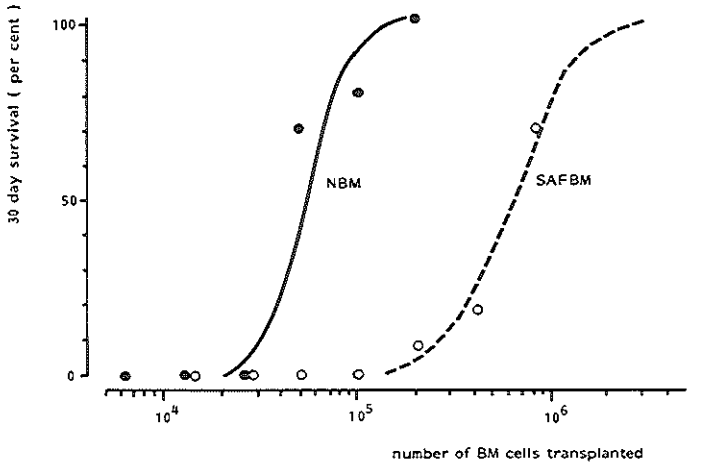


Fig. 3.3 Survival of lethally irradiated mice after transplantation of NBM (●) or SAFBM (o). Number of cells transplanted versus 30 day survival (see also Fig. 2). The lines result from probit analysis (see also Chapter 2).

renewal and to produce more megakaryocytic precursors than did those from normal bone marrow (Rosendaal et al, 1976; Hodgson and Bradley, 1979). Such treated BM cells when coinjected with untreated BM cells was found to gradually replace the progeny that was produced by the NBM (Ross et al, 1982). Although it has been established by now that day-8 - and day-12 CFU-S are different cells (Harris et al, 1984; Baines and Visser, 1983; Table 4.1 and Figs. 4.1, 4.2 and 4.4), it is not known to what extent these cells are functionally different. Therefore, we determined the radioprotective capacity of bone marrow cells cultured for four days with SAF. These cells, as shown in Table 3.1, are enriched in day-8 CFU-S. In the survival experiment presented, fresh bone marrow cells contained 28 day-8 CFU-S and 23.5 day-12 CFU-S per 10^5 cells. After culture for four days in the presence of SAF, there were 40.8 day-8 CFU-S and 15 day-12 CFU-S. In Fig. 3.2A and B survival of lethally irradiated mice transplanted with varying dosages of fresh (Fig. 3.2A) or cultured (Fig. 3.2B) bone marrow cells is shown. Fig. 3.3 presents the 30-day survival versus the number of bone marrow cells transplanted (in the case of fresh bone marrow cells) or versus the number of bone marrow cells that were cultured and transplanted four days later. Fig. 3.3 shows that culture of bone marrow with SAF is associated with a loss of survival capacity. This occurs despite a production of CFU-S. The ED50 (effective dose that protects 50 % of lethally irradiated mice) of normal and

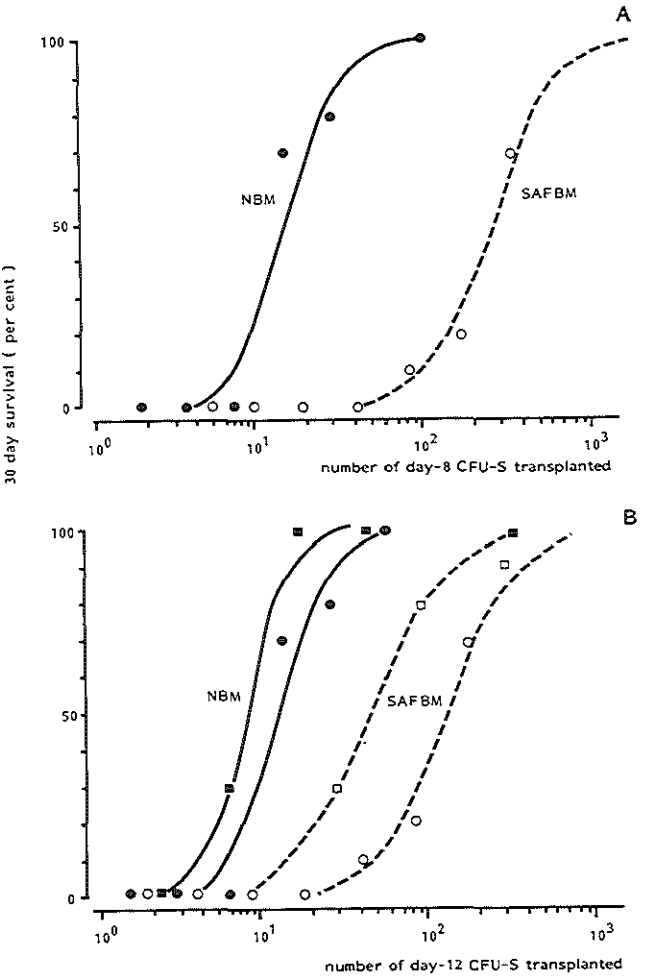


Fig. 3.4 Survival of lethally irradiated mice after transplantation of NBM (●;■) or SAFBM (○;□). Number of CFU-S transplanted versus 30 day survival (see also Figs. 2 and 3). A. Survival per day-8 CFU-S. B. Survival per day-12 CFU-S. Squares and circles represent different experiments. The lines result from probit analysis.

cultured bone marrow cells is $5.1 \times 10^4 \pm 0.77$ and $58.5 \times 10^4 \pm 16$ nucleated cells, respectively (see Chapter 2 for the calculations). Fig. 3.4 A and B presents the 30-day survival versus the different types of CFU-S in fresh and cultured bone marrow cells. The ED50 level of normal day-8 and day-12 CFU-S is approximately the same: 14.4 ±2.17 and 12.1 ±1.8, respectively. These numbers are in agreement with previously obtained ED50 values in this laboratory (e.g.

Visser et al, 1984). These figures are considerably higher for SAFBM: SAFBM: 234.9 ±56.8 day-8 CFU-S or 86.4 ±20.8 day-12 CFU-S are needed for a 50% survival of lethally irradiated mice. In another experiment we found similar high ED50 values for SAFBM: 194 ±52 day-6 CFU-S; 66.9 ±18.3 day-9 CFU-S and 40.8 ±11.1 day-12 CFU-S. This experiment is also shown in Fig. 3.4. These data indicate that the CFU-S produced in SAFBM (Table 3.1) have a decreased capacity to protect recipients from radiation induced bone marrow failure (Figs. 3.2-4).

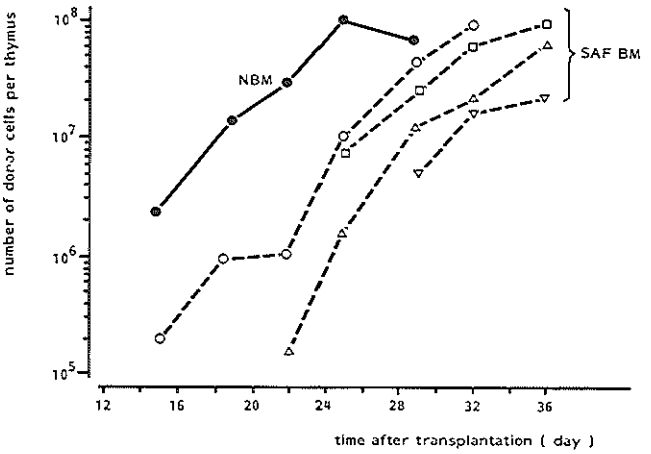


Fig. 3.5 Thymus repopulation by bone marrow cells incubated in the presence of SAF. Lethally irradiated mice were transplanted with either 10^6 NBM (●) or graded numbers of SAFBM. ($3x10^6$: ○ ; 10^6 : □ ; $0.3x10^6$: △ ; $0.1x10^6$: ▽). CFU-S content of NBM (per 10^6 cells): day-6: 200; day-9: 244; day-12: 260. CFU-S content of SAFBM (per 10^6 cells): day-6: 1180; day-9: 408; day-12: 248. Each point represents the mean value of donor thymocytes per 3-4 mice.

Thymus repopulation by SAF cultured bone marrow cells

The previous results showed that the capacity of stem cells in SAFBM to produce spleen colonies early after transplantation is not correlated with radioprotection. Another function of hemopoietic stem cells, the capacity to repopulate the thymus is limited to few progenitor cells: pluripotent hemopoietic stem cells and prothymocytes. This is illustrated in several differentiation schemes in which the capacity for lymphoid differentiation is the first lineage to separate from all the others (Moore and Metcalf, 1971;

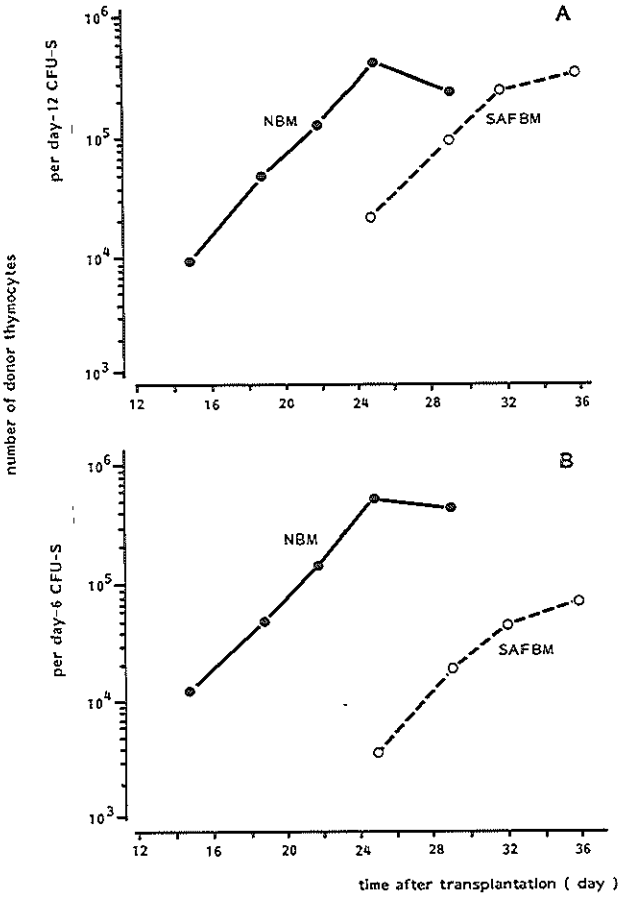


Fig. 3.6 Thymus regeneration by bone marrow cells incubated in the presence of SAF. Lethally irradiated mice were transplanted with either 10^6 NBM (●) or 10^6 SAFBM (○). For CFU-S content of BM transplants see Fig. 3.5. A. Thymus regeneration per day-6 CFU-S. B. Thymus regeneration per day-12 CFU-S.

Ogawa et al, 1983; Fig. 1.4). In order to test the functional capacity of CFU-S in SAFBM, the thymus regeneration ability of SAFBM was tested. The CFU-S data of these experiments were shown in Table 3.1 (exp. II). In Fig. 3.5 thymus repopulation following transplantation of SAFBM is presented and compared to that following grafting of fresh bone marrow cells which were obtained from littermates of the donors of the SAF cultures. Normal bone marrow cells (10^6 nucleated cells containing 244 day-9 CFU-S) show a rapid increase in the number

of donor thymocytes in the third week after transplantation. A plateau is reached around day 23. This is in agreement with results obtained by others (Kadish and Basch, 1976; Boersma et al, 1981a). SAFBM on the other hand did not give rise to donor thymocytes in the first three weeks in four out of five experiments (data not shown). As shown in Fig. 3.5 transplantation of 10^6 cultured BM cells, containing 1180 day-6 CFU-S, 408 day-9 CFU-S and 248 day-12 CFU-S, results in a 7-8 day delay in thymus repopulation despite the presence of a large number of day-6-9 CFU-S. Thereafter there is an increase until a plateau is reached at 35-40 days after transplantation. In one experiment we observed low numbers of donor thymocytes as early as 18 days after transplantation of 10^6 initially cultured BM. The titration experiment shown in Fig. 3.5 also indicates the extent to which SAFBM is depleted in thymus repopulating ability: after extrapolation of the data with SAFBM, it can be calculated that one needs 100 times more cells cultured with SAF to obtain an equivalent thymus regeneration as that obtained with fresh bone marrow cells. Expression of thymus repopulation per transplanted CFU-S relates the presence of thymocyte precursor cells in the various cell suspensions to that of CFU-S. This is shown for SAFBM in Fig. 3.6 A and B. Thymus regeneration by SAFBM is expressed per transplanted day-12 and day-6 CFU-S, respectively. Thymus regeneration per day-6 CFU-S is delayed twelve days. Thymus regeneration per day-12 CFU-S from SAFBM is delayed eight days when compared to that by day-12 CFU-S from NBM.

Competitive repopulation of the thymus by normal bone marrow or bone marrow cells enriched in either day-8 or day-12 CFU-S

Experiments have shown that under steady state conditions there is a continuous influx of cells from the bone marrow into the thymus (Ford et al, 1968). This indicates that ultimately the thymus of a stable mixed chimera reflects the mixed composition of pluripotent stem cells in the bone marrow. C3H recipient mice were lethally irradiated and transplanted with a mixture of C3H and C3AK BM cells. Either NBM, SAFBM or BM cells from mice pretreated with 5-FU four days earlier (day-4 FUBM) was used. The CFU-S content of the experimental groups is presented in Table 3.3. At one, two and four months after BMT, the thymuses were removed and the percentage of Thy-1.1 (i.e. C3AK derived) positive thymocytes was determined as described in Chapter 2. Fig.

Table 3.3

CFU-S content of mixed bone marrow transplants into C3H mice

Group	Strain	Source	Number of CFU-S transplanted		
			day-6	day-9	day-12
I	C3AK	NBM	18.4	19.6	19.6
	C3H	NBM	14.0	14.8	15.6
II	C3AK	SAFBM	65.6	48.0	29.5
	C3H	NBM	14.0	14.8	15.6
III	C3AK	FUBM	2.3	2.8	16.0
	C3H	NBM	14.0	14.8	15.6
IV	C3AK	FUBM	2.3	2.8	16.0
	C3H	SAFBM	71.2	62.4	37.2
V	C3AK	SAFBM	65.6	48.0	29.5
	C3H	FUBM	0	0	2.7

The CFU-S content of the mixed BM grafts was determined by i.v. injection of the separate components into lethally irradiated C3H mice. The results are expressed as the total number of CFU-S that were transplanted into the mixed chimeras (NBM and SAFBM 10^5 cells and day-4 FUBM $4x10^4$ cells). See also Fig. 3.7.

3.7A shows the percentage of C3AK derived thymocytes in mice that received 10^5 normal C3H BM together with variously treated C3AK bone marrow cells: normal C3AK BM, SAFBM, or day-4 FUBM. C3AK SAFBM produced less offspring in the thymus when coinjected with C3H NBM than C3AK NBM and C3AK FUBM (Fig. 3.7A). This occured despite the presence of 3-4 fold higher numbers of CFU-S that give rise to early spleen colonies and also higher numbers of day-12 CFU-S in SAFBM than in NBM and FUBM (Table 3.3). Fig. 3.7B shows the percentage of donor thymocytes in thymuses of mice that had received a mixture of SAFBM and FUBM: (IV) C3AK SAFBM and C3H FUBM or (V) C3AK FUBM and C3H SAFBM. SAFBM of either C3AK or C3H origin produced very few offspring when coinjected with FUBM of the other strain (Fig. 3.7B). The SAFBM in these transplants contained much higher numbers of day-6-9 CFU-S, and also larger

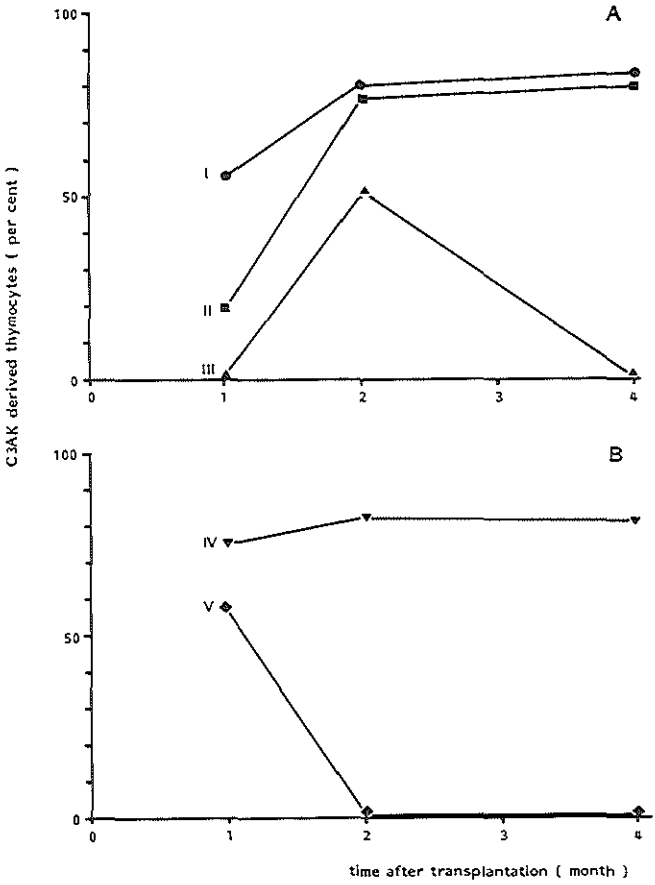


Fig. 3.7 A. Competitive thymus repopulation after injection of mixtures of variously treated BM cells. All lethally irradiated C3H mice received 10^5 C3H NBM and either I. 10^5 NBM (●) or II. $4x10^4$ day-4 FUBM (■) or III. $5x10^5$ SAFBM (▲) from C3AK mice. B. Lethally irradiated C3H mice received either IV. $4x10^4$ C3AK day-4 FUBM and 10^5 C3H SAFBM (▼) or V. 10^5 C3AK SAFBM and $4X10^4$ C3H day-4 FUBM (◆). See Table 3.3 for the CFU-S content of the various BM grafts.

numbers of day-12 CFU-S than the FUBM (Table 3.3). The recipient mice showed no obvious signs of Graft versus Host disease, there was no mortality, and the thymuses had a normal cellularity.

Discussion

The results can be summarized as follows: SAFBM is enriched in CFU-S producing early spleen colonies (Table 3.1). One third of day-8 and one quarter of day-12 CFU-S from SAFBM are desintegrating spleen colonies (Fig. 3.1 and Table 3.2). Radioprotection by SAFBM is, per day-8 and day-12 CFU-S lower than by NBM (Figs. 3.2-3.4). SAFBM causes delayed thymus regeneration despite a production of CFU-S (Figs. 3.5 and 3.6). SAFBM injected with other BM sources has relatively very little offspring among thymocytes (Figs. 3.8).

Bone marrow cells cultured in the presence of SAF is a rich source of CFU-S that give rise to early spleen colonies 6-8 days after transplantion (Table 3.1). Late appearing spleen colonies are, however, still present. The possibility exists that, after transplantation of day-8 colony forming cells a number of early appearing colonies are still visible around twelve days after BMT. Such persistent spleen colonies have been described (Magli et al, 1982; Wolf and Priestly, 1984). The production of progenitor cells giving rise to "early" spleen colonies from NBM after in vitro stimulation by SAF may be the result of self renewal of stem cells or of differentiation from more primitive precursor cells. The experiments described in this Chapter cannot discriminate between these possibilities. In experiments, described in the two following Chapters, indirect evidence was obtained suggesting that the day-12 CFU-S can give rise to day-8 CFU-S. The origin of day-8 CFU-S in SAFBM will therefore be discussed later.

The appearance of up to one third, desintegrating spleen colonies was a surprising finding. The almost exclusive presence of maturing nucleated erythroid cells and erythocytes at both day-8 and day-12 in these colonies, suggests that the CFU resembles the in vitro growing burst-forming unit (BFU-E). The difference in the number of desintegrating colonies between NBM and SAFBM shows that a CFU-S in SAFBM, no matter at what day it is scored, cannot be compared to a CFU-S in NBM. This is also apparent from the survival data presented in Figs. 3.2-3.4. Of any type of CFU-S present in SAFBM 4-20 times more are necessary for survival after lethally irradiation then after transplantation of NBM. The difference in ED50 expressed in CFU-S numbers from NBM and from SAFBM becomes less with spleen colonies counted later. This indicates that the day-12 CFU-S from SAFBM is more closely associated with the pluripotent hemopoietic stem cell than the day-8 CFU-S. Survival after lethally irradiation should occur after transplantation of the same number of day-12

CFU-S from NBM or SAFBM if the day-12 CFU-S and the PHSC were two totally overlapping cell populations. This is clearly not the case and this suggests that either the day-12 CFU-S in SAFBM and NBM are not similar or that NBM contains an even more immature stem cell, the pre-CFU-S, which is depleted in SAFBM.

It was shown that SAFBM contains relatively large numbers of day-8 CFU-S (Table 3.1). Despite this, there is a loss of thymus repopulating ability (Figs. 3.5 and 3.6). From titration studies of NBM and SAFBM it follows that the thymus repopulating ability is decreased 100-200 fold when compared to that of fresh bone marrow cells (see also below). The most likely explanation for this is that part of the CFU-S have lost the capacity for lymphoid differentiation. These restricted CFU-S are probably the day-8 CFU-S. Thymus regeneration, although delayed, does occur eventually after transplantation of SAFBM. Therefore a source of thymocyte progenitors must be present in this material. It is possible that these precursors are present among the remaining day-12 CFU-S. Thymus regeneration per day-12 CFU-S, as was the case in the survival experiments, remains delayed when compared to that by NBM. This indicates that either the day-12 CFU-S in SAFBM and NBM are different or that SAFBM lacks another, as yet not identified precursor cell, the pre-CFU-S.

From the competition experiments in which two types of BM cells (two from NBM, SAFBM or day-4 FUBM) were grafted to proliferate in a host for several months, it also appeared that SAFBM gave rise to relatively low and transient numbers of thymocytes. This indicates that the progenitor cells present in SAFBM, apart from causing a delayed thymus regeneration, are at a disadvantage in settling in a host in the presence of progenitor cells from NBM and day-4 FUBM. From this it is concluded that the day-8 CFU-S in SAFBM are not pluripotent progenitor cells and that they have lost the potential to differentiate into thymocytes. It explains the loss of the capacity to protect lethally irradiated mice and the markedly delayed thymus regeneration and the inability to compete with NBM and FUBM or SAFBM. It has, however, been reported that some 7-day old spleen colonies contain day-12 CFU-S (Johnson and Nicola, 1984). This suggests that the day-7 CFU-S can give rise to the day-12 CFU-S. After observing the appearance and disapearance of spleen colonies by a spleen window technique, it was found that about half of the spleen colonies that are present at twelve days are already visible at eight days after BMT (Wolf and Priestley, 1984). The day-12 CFU-S present in an early spleen colonies may therefore be derived from persistent and not from a transient spleen colony that

is enriched in the SAFBM. Spleen colonies were shown to contain more CFU-S when the colony was excised at a later time after transplantation (Vogel et al, 1968; Magli et al, 1982; Johnson and Nicola, 1984). It has been assumed that this was due to the proliferation of CFU-S in the spleen colony (Vogel et al, 1968). However, when early and late spleen colonies are derived from different cells (see the next Chapter), it is also possible that secondary CFU-S are absent in the transient and present in the persistent and late appearing spleen colonies. In summarry, the data described in this Chapter, support the existence of CFU-S that differ in differentiation potential (Rosendaal et al, 1979; Hellman et al, 1978; Hodgson and Bradley, 1979; Micklem, 1983; Ogawa et al, 1983).

Conclusions

1. Bone marrow cells cultured in the presence of SAF are enriched in day-8 CFU-S.

2. Part of the spleen colonies produced by SAFBM are desintegrating.

3. SAFBM, despite its net increased number of CFU-S, has fewer capacity to protect lethally irradiated mice.

4. SAFBM causes a delayed thymus regeneration.

5. SAFBM, coinjected with NBM or FUBM, has few or no progeny in the thymus late after transplantation. This is probably caused by the gradual replacement of the stem cells in SAFBM by those present in NBM and FUBM.

6. The day-8 CFU-S in SAFBM and possibly also in NBM is a restricted hemopoietic stem cell that has lost the potential for T cell differentiation.

7. The stem cell population in SAFBM differs from that in NBM. The data indicate that SAFBM contains relatively many differentiated progenitor cells that can still produce spleen colonies. Expression of the functional tests (thymus regeneration and radioprotection) per day-6-8 or day-12 CFU-S indicates that more of these cells from SAFBM have to be injected for a similar effect when compared to NBM. This indicates that either

   - day-12 CFU-S in SAFBM are different from those in NBM which is supported by the relatively high frequency of desintegrating spleen colonies by day-12 CFU-S from SAF or that
   - SAFBM contains less precursor cells that are responsible for radioprotection and thymus repopulation. This cell precedes the day-12 CFU-S in the differentiation sequence.

Chapter 4

CELLS FORMING LATE SPLEEN COLONIES (DAY-12 CFU-S) IN RELATION TO CELLS FORMING EARLY SPLEEN COLONIES (DAY-8 CFU-S).

Introduction

The literature about day-8 - and day-12 spleen colony forming units has been reviewed in Chapter 1. It can be summarized as follows: Some of the spleen colonies present at 8 days after transplantation of normal bone marrow cells (NBM) have disappeared at day 12 (Magli et al, 1982; Wolf and Priestley, 1984), other spleen colonies appear late (i.e. after 8-9 days) after transplantation (Hodgson and Bradley, 1979, Magli et al, 1982, Wolf and Priestley, 1984). In two instances, day-8 and day-12 CFU-S from NBM were separated: Firstly, a difference in binding of an anti-Qa-2 monoclonal antibody was found between day-8 and day-12 CFU-S (Hogarth et al, 1982; Harris et al, 1984). The two types of CFU-S could be separated with the use of a light-activated cell sorter (Harris et al, 1984). Secondly, after incubation of NBM with Hoechst 33342, the day-12 CFU-S contains less dye than the day-8 CFU-S (Baines and Visser, 1983). These data allow the conclusion that different cells can produce spleen colonies that appear at different times after transplantation.

It has been argued that a difference in the cell cycle status of CFU-S causes the difference in the time of appearance of spleen colonies (i.e. cycling CFU-S give rise to early spleen colonies and quiescent CFU-S produce late colonies; Baines and Visser, 1983). However, removal of cycling cells in fetal liver, regenerating BM, regenerating spleen (i.e BM or spleen seven days after lethal irradiation and BMT) or long-term bone marrow cultures by short-term exposure to hydroxyurea did not affect the ratio of day-7-8 - to day-11-12 CFU-S (Johnson and Nicola, 1984; van Zant, 1984; Chertkov and Drize, 1984). Most CFU-S in these cell suspensions are rapidly cycling (Boersma, 1982; Duplan, 1970; Chertkov and Drize, 1984). Also no difference in the fraction of cycling day-7 and day-11 CFU-S in regenerating spleen could be detected with the

use of other procedures ([^{3}H]TdR, Ara-C)(Chertkov and Drize, 1984). Furthermore, day-4 FUBM contains almost exclusively day-12 CFU-S, most of which are cycling. However, in bone marrow from mice that received 2.0 Gy TBI three days previously a larger fraction of the day-7 CFU-S was cycling when compared to that of the day-11 CFU-S (Chertkov and Drize, 1984). Others did not find this difference (Wright et al, 1985). All day-8 CFU-S have finished at least one cell cycle in a 7-17 day period, while only 20-40% of day-12 CFU-S went through S-phase in such a time period (Hodgson and Bradley, 1984; N.S. Wolf, personal communication). From these data it cannot be concluded that day-8 CFU-S in NBM are cycling cells, and that day-12 CFU-S are quiescent. The shorter turnover time of day-8 CFU-S may then be explained by a difference in the length of G1 or the presence of a Go phase among the day-12 CFU-S.

The relationship of day-8 and day-12 CFU-S is not yet clear. The most plausible relationship is that the day-12 CFU-S is the predecessor of the day-8 CFU-S. It has been postulated that there exists a pre-CFU-S that can give rise to the day-12 CFU-S (Hodgson and Bradley, 1979). The pre-CFU-S has been suggested to survive treatment with 5 fluorouracil (5-FU) (Hodgson and Bradley, 1979). We tried to separate the day-8 and day-12 CFU-S and to deduce their relationship by studying the appearance of day-8 CFU-S in vivo and in vitro from cell populations devoid of day-8 CFU-S. BM cells were separated or isolated by various methods and analyzed for the content of day-8 and day-12 CFU-S. These methods included flow cytometry, velocity sedimentation and differential killing of CFU-S with 5-FU. Separation of day-8 and day-12 CFU-S by flow cytometry turned out to be possible with monoclonal antibodies directed against class I H-2 and Thy-1.1 antigens and with rhodamine 123, a supravital fluorescent dye which is specific for mitochondria. Velocity sedimentation of BM cells was carried out because it was reported that slowly sedimenting cells have a high rate of self renewal (Worton et al, 1969a). This might have been due to differences in the proportions of day-8 and day-12 CFU-S in the various fractions. This hypothesis turned out to be incorrect. 5-FU was used to enrich for day-12 CFU-S (Hodgson and Bradley, 1979). The recovery of the CFU-S was studied in vivo and BM cells were also cultured in vitro in the presence of stem cell activating factor, because this substance was found to result in the production of large numbers of early appearing spleen colonies from NBM (Chapter 3).

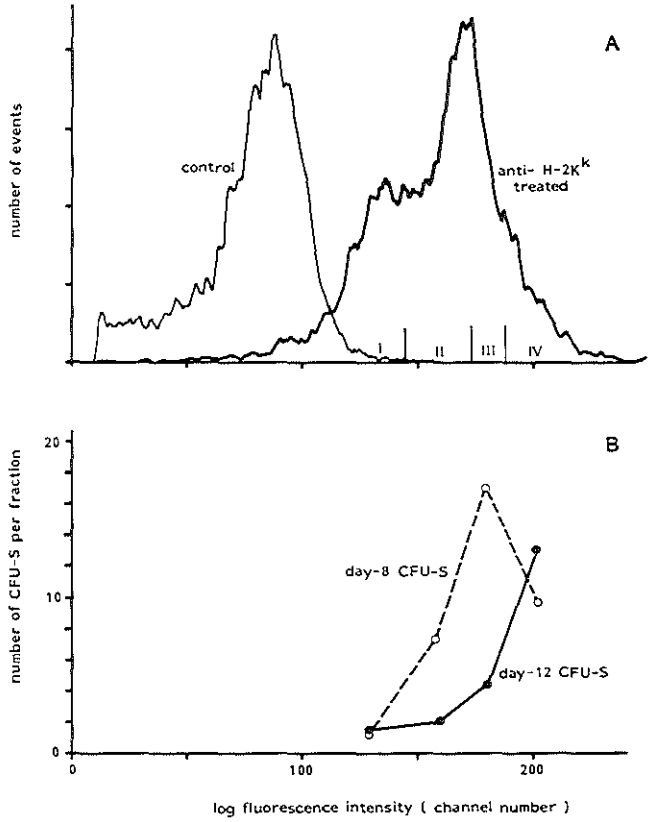


Fig. 4.1 A. Fluorescence of anti-H-2K treated bone marrow cells. C3H BM cells were incubated with biotinylated anti-H-2K and avidin/FITC as described in Chapter 2. The cells were sorted in the fractions indicated by roman figures. The thin line shows the fluorescence of BM cells incubated with avidin/FITC only. B. CFU-S content of BM fractions differing in H-2K density. The CFU-S content is expressed as the number of spleen colonies per fraction sorted from 10^5 incubated cells. Day-8 CFU-S (o); day-12 CFU-S (•). Unseparated BM cells contained 29 and 17.7 day-8 and day-12 CFU-S.

Density of H-2K molecules on day-8 and day-12 CFU-S

H-2K antigenic determinants are located on class I molecules which are encoded for by the H-2 complex on chromosome 17 (Klein, 1975; Klein et al, 1983). This class consists of molecules controled by at least three different loci in the H-2 complex: H-2K, H-2D, and H-2L. The monomeric gene product consists of three domains outside the plasma membrane, a transmembrane and a

Table 4.1

Distribution of day-8 and day-12 CFU-S in bone marrow fractions differing in density of class I H-2 antigens

Treatment	% of all nucl. cells sorted	number of day-8 CFU-S /10^5	number of day-12 CFU-S /10^5	Ratio of day-12/day-8
Incubation with medium	93	29	17.7	0.61
Incub. with MCA	95	28	20.8	0.74
Incub. with MCA Fr.I	39	1.3	1.4	-
Incub. with MCA Fr.II	30	7.4	2	0.27
Incub. with MCA Fr.III	19	17.2	4.4	0.25
Incub. with MCA Fr. IV	8	4.4	9.8	2.23

See legend of Fig. 4.1 for details

cytoplasmic sequence (Maloy and Coligan, 1983). The molecule has a size of 40.000-45.000 daltons (Coligan et al, 1982). It was reported that CFU-S have such class I H-2 antigens on their cell surface (Russel and van den Engh, 1979). With the use of a monoclonal antibody (MCA) directed against H-2K molecules a high enrichment of CFU-S can be obtained (Mulder et al, 1984; Visser et al, 1984). The MCA used in this study reacts with cells from strains carrying the k, p, q, and r haplotype (Oi et al, 1979). This strain distribution has not been observed with alloantisera before (Oi et al, 1979), but it corresponds with the distribution of H-2K.m3 which is detected by a monoclonal antibody (Klein et al, 1979).

A difference in the density of Qa antigens was found on day-8 and day-12 CFU-S (Harris et al, 1984). Since both the Qa and H-2K molecules belong to the

Table 4.2

Histology of spleen colonies from bone marrow fractions differing in density of class I H-2 antigens.

Treatment	Colony age (days)	Undiff.	E	G	Meg	E/Meg	E/G	G/Meg	E/G/Meg
Fr. I	8	1	0	0	0	0	0	0	0
	12	6	3	0	0	0	2	0	0
Fr.II	8	8	11	0	0	0	1	0	0
	12	3	5	0	0	0	1	0	1
Fr.III	8	15	37	0	0	0	1	0	1
	12	1	10	1	0	0	2	1	5
Fr. IV	8	12	20	1	1	0	0	1	2
	12	6	22	0	1	3	10	0	2

The spleens from lethally irradiated mice that received BM fractions, separated on the amount of H-2K antigens, were sectioned and the colony type was scored microscopically. Undiff=undifferentiated, E=erythroid; G=granulocytic; Meg=megakaryocytic. See also Fig.1 and Table 4.1.

class I antigens of the Major Histocompatibility Complex (MHC) (Klein et al, 1983), it seemed worthwhile to investigate whether day-8 and day-12 CFU-S also differ in the density of H-2K molecules.

Normal C3H bone marrow cells were incubated with biotinylated anti-H-2K MCA and avidin/FITC as described in Chapter 2. The fluorescence distribution of the nucleated cells is presented in Fig. 4.1A. Virtually all nucleated cells are labeled with the MCA when compared to cells that were incubated with avidin/FITC only. Anti-H-2K treated cells were separated on fluorescence intensity with a fluorescence activated cells sorter (see Chapter 2 for details). The fractions that were sorted are shown in Fig. 4.1A The distribution of the CFU-S among the various fractions is shown in Fig. 4.1B and Table 4.1. 63 % of day-12 CFU-S were recovered from the brightest fluoresceing fraction. This fraction contained only 27.4 % of the day-8 CFU-S. In contrast, fraction III contained most day-8 CFU-S, while only 20 % of the day-12 CFU-S were present (3 exp.). This shows that day-12 CFU-S have a higher density of H-2K molecules than have day-8 CFU-S.

The H-2K separation was added to two other purification methods (separation on boyant density and on the density of membrane receptors for wheat germ agglutinin) that were shown to enrich for CFU-S by Visser (Visser et al, 1984). The purified cells were enriched for day-12 CFU-S (ratio day-12/day-8 CFU-S=4.2). In NBM this ratio is 1.2 (Visser et al, 1984; See also Chapter 5). Culture of these cells with SAF resulted in the production of large numbers of day-8 CFU-S (2.5 to 3.2 fold increase), CFU-C2 (7.9 to 14 fold increase) and BFU-E (9.4 to 13 fold increase), but loss of day-12 CFU-S (0.9 to 0.4 fold increase) (Visser, personal communication).

Spleen colonies have been shown to change in morphology with time: Early spleen colonies were shown to be mainly erythroid, while later spleen colonies were shown to contain large numbers of other cell types (Curry and Trentin, 1967, Moore and Metcalf, 1971; Johnson and Nicola, 1984). If the morphology of the spleen colonies is dependent on the type of CFU-S transplanted instead of the age of the colony one can expect to find a relation between the morphology of the spleen colonies and the fluorescence intensity with anti-H-2K. The spleens were sectioned, stained and their morphology was determined as described in Chapter 2. The results are presented in Table 4.2. Spleen colonies consisted predominantly of erythroid and undifferentiated cells at 8 days after transplantation. Four days later the spleen colonies showed a more heterogeneous cell distribution. This pattern was found in all groups. However, half of the day-12 erythroid spleen colonies from Fraction III are desintegrating. In unseparated BM less than 10 % of the day-12 spleen colonies are falling apart. Example of these colonies were presented in Fig. 3.1. These results indicate that some spleen colonies produced by a fraction enriched in day-8 CFU-S, are still present at day-12, but in a desintegrating state.

Density of Thy-1 molecules on day-8 and day-12 CFU-S

The Thy-1 antigen was described as an antigen with specificity for T cells (Reif and Allen, 1963). The antigen was, however, also found on several other cell types as brain cells, cells lining the otocyst, fibroblasts and epidermal cells (Reif and Allen, 1963; Prystowsky et al, 1978; Stern, 1973; Scheid et al, 1972). Hemopoietic stem cells were reported to be Thy-1 negative (Thierfelder, 1977, Russel and van den Engh, 1979). Prothymocytes were also shown to be Thy-1 negative (Kadish and Basch, 1976; Boersma, 1982). More recent experiments,

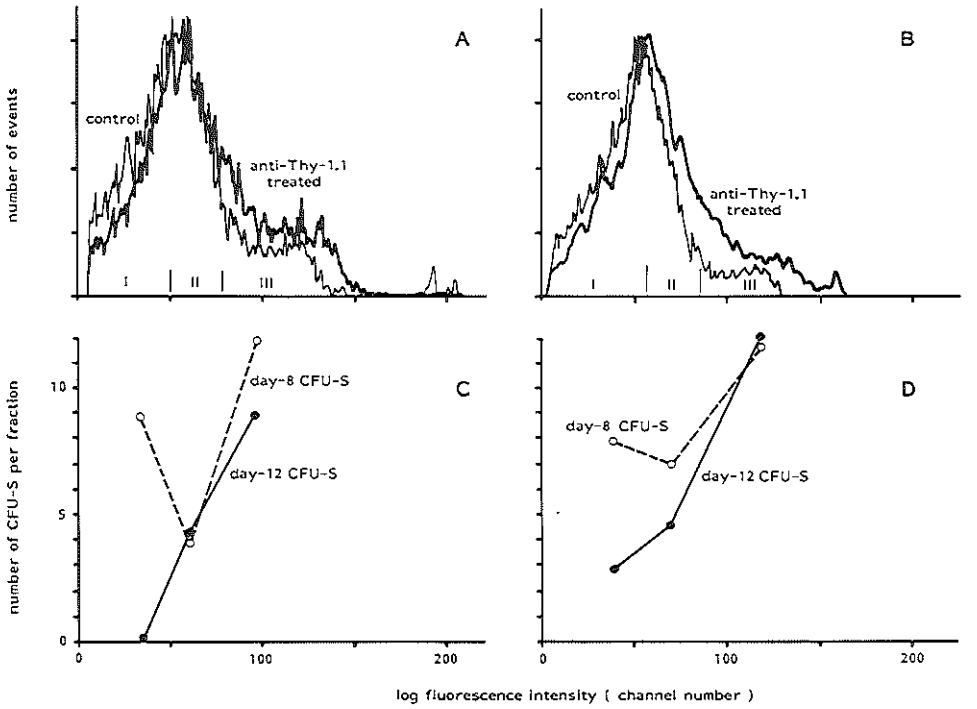


Fig. 4.2 A/B. Fluorescence of anti-Thy-1.1 treated bone marrow cells. C3AK F1 BM cells were incubated with anti-Thy-1.1 and GAM/FITC as described in Chapter 2. The cells were sorted in the fractions indicated by the roman figures. The thin lines shows the fluorescence distribution of BM cells incubated with GAM/FITC only. C/D. CFU-S content of BM fractions differing in Thy-1.1 density. The CFU-S content is expressed as the number of spleen colonies per fraction sorted from 10^5 incubated cells. Day-8 CFU-S (o); day-12 CFU-S (●). Unseparated BM cells contained 16.5 and 19.5 day-8 and day-12 CFU-S (A/C) and 18.6 and 17 day-8 and day-12 CFU-S (B/D), respectively per 10^5 cells.

employing monoclonal antibodies indicate that the CFU-S and the thymocyte progenitor do have low amounts of Thy-1 antigens on their cell surfaces (Basch and Berman, 1982).

In initial experiments in which C3AK F1 BM cells were incubated with anti-Thy-1.1 and complement an insignificant reduction of day-10 CFU-S was observed (data not shown). In sorting experiments in which BM cells were labeled with anti-Thy-1.1 and goat-anti-mouse/FITC and separated on fluorescence intensity, day-10 CFU-S were predominantly present in the fluoresceing fractions (data not shown). These experiments were repeated and spleen colonies were

Table 4.3

Effect of anti-Thy-1.1 treatment of C3AK BM on day-8 and day-12 CFU-S

Treatment	day-8 CFU-S/10^5 NBM	day-12 CFU-S/10^5 NBM
medium control	19.1±4.6	17.2±3.7
Thy-1.1 incub.	16.0±7.2	17.3±3.4
C' incub.	19.4±4.2	16.2±4.0
Thy-1.1 and C'	19.1±5.1	10.0±1.5

C3AK F1 BM cells were incubated with anti-Thy-1.1 and/or complement as described in Chapter 2. Appropriate numbers of cells were subsequently injected into lethally irradiated recipients and assayed for day-8 and day-12 spleen colonies.

counted at both day-8 and day-12. Table 4.3 shows the cytotoxic effect of incubation of C3AK BM cells with anti-Thy1.1 and complement. Neither of the incubation procedures affected the number of day-8 CFU-S, but antibody labeling and complement treatment reduced the number of day-12 CFU-S to 55 % (Table 4.3).

Fig. 4.2A and C shows the fluorescence distribution of C3AK BM cells labeled with anti-Thy-1.1 and GAM/FITC or with GAM/FITC only. When BM cells are labeled with GAM/FITC, B cells and small numbers of large cells fluoresce. After incubation with anti-Thy-1.1 and GAM/FITC a larger number of cells is labeled. Most of the additional fluoresceing cells are T cells, as indicated by there presence in the lymphocyte population (see Chapter 2). The distribution of day-8 and day-12 CFU-S in the sorted fractions is presented in Fig. 4.2B and D. Two third of the day-12 CFU-S are found in a fraction labeled with anti-Thy-1.1. In the negatively stained fraction there are few or no day-12 CFU-S. Part of the day 8-CFU-S are Thy-1.1 positive, but there is also a fraction Thy-1.1 negative day-8 CFU-S.

The presence of CFU-S in the fluorescing fraction might be due to binding of GAM/FITC to the CFU-S. In order to exclude this (C57Bl/RijxC3H/Law)F1 (BC3) mice (Thy-1.1 negative) were incubated with anti-Thy-1.1 and GAM/FITC and sorted on fluorescence intensity. The fractions were assayed for the content of day-8 and day-12 CFU-S. This is presented in Fig. 4.3A and B. The Thy-1.1 antigen

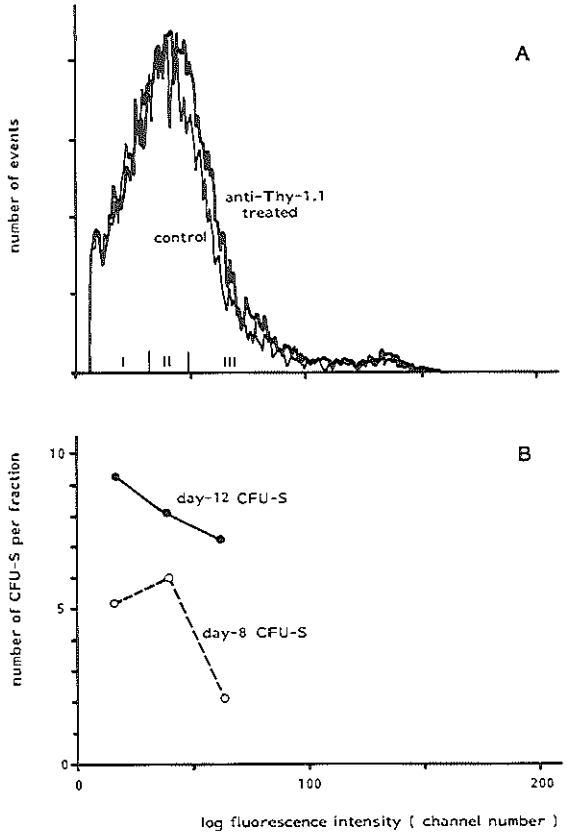


Fig. 4.3 A. Fluorescence of anti-Thy-1.1 treated bone marrow cells. BC3 F1 BM cells were incubated with anti-Thy-1.1 and GAM/FITC as described in Chapter 2. See also legend of Fig. 4.2A. B. CFU-S content of BM fractions differing in Thy-1.1 density. Unseparated BM cells contained 18.8 and 19.6 day-8 and day-12 CFU-S per 10^5 cells.

is absent from BC3 BM cells (Fig. 4.3A) and day-8 or day-12 CFU-S are not enriched in the fluorescing fraction. This indicates that GAM/FITC does not bind to day-8 or day-12 CFU-S.

These results indicate that the day-12 CFU-S from C3AK mice are Thy-1.1 positive and that day 8-CFU-S can be Thy-1.1 positive or negative.

Table 4.4

Distribution of day-8 and day-12 CFU-S in bone marrow fractions differing in the uptake of Rhodamine 123

Fraction	% of all nucl. cells sorted	number of day-8 CFU-S /10^5	number of day-12 CFU-S /10^5	Ratio of day-12/day-8
Weakly Rh123 positive	40	2.0±2.1	7.5±3.9	3.8
Brightly Rh123 positive	40-50	17.6±5.9	12.2±2.1	0.7

Normal bone marrow cells were labeled with Rhodamine 123 as described in Chapter 2. The BM cells were subsequently separated in two fractions with a light activated cell sorter and assayed for the content of day-8 and day-12 CFU-S. The results are expressed as the total number of CFU-S sorted from 10^5 cells.

Uptake of Rhodamine 123 by day-8 and day-12 CFU-S

Rhodamine 123 (Rh123) is a supravital dye which accumulates specifically in mitochondria (Johnson et al, 1980). Mitochondria are synthesized throughout the cell cycle (James and Bohman, 1981). Lymphocytes stimulated with phytohemagglutinin show maximal Rh123 uptake when maximal numbers of cells are in S phase and in mitosis (Darzynkiewicz et al, 1981). Other studies also showed that there is a relationship between active cell cycle and Rh123 uptake (Cohen et al, 1981; James and Bohman, 1981). This indicates that cycling CFU-S may be discriminated from resting ones by their uptake of Rh123.

C3AK BM cells were labeled with Rh123 as described in Chapter 2. They were subsequently separated in fractions that differed in Rh123 fluorescence and assayed for CFU-S content. Fig. 4.4A shows the fluorescence distribution of BM cells incubated with or without Rh123. Fig. 4.4B shows the distribution of day-8 and day-12 CFU-S in four fractions differing in Rh123 uptake. Most day-8 CFU-S contained a relatively high amount of Rh123, while the day-12 CFU-S were present in all the fractions. Table 4.4 shows the mean results of six

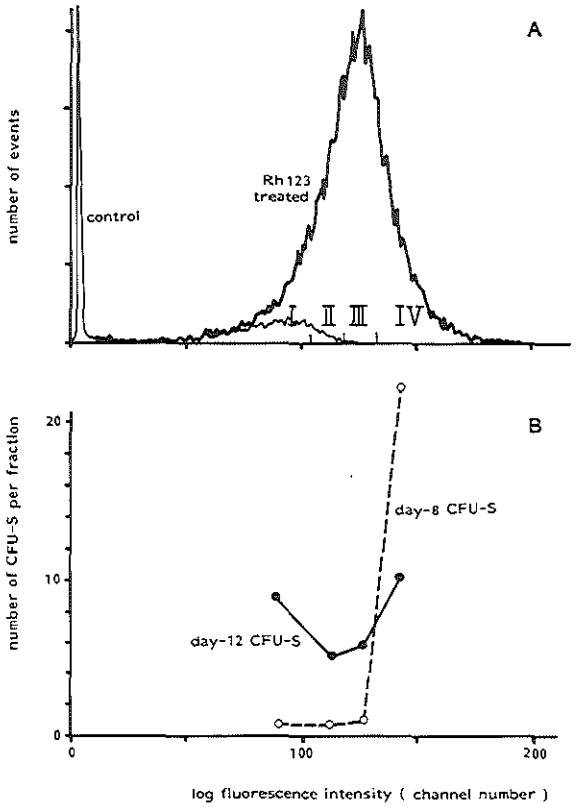


Fig. 4.4 A. Fluorescence of bone marrow cells incubated with Rh123. C3AK F1 BM cells were incubated with Rh123 as described in Chapter 2. The cells were sorted in the fraction indicated by the roman figures. The thin line shows the fluorescence distribution of control BM cells. B. CFU-S content of fractions differing in Rh123 content. The number of CFU-S is expressed as the number of spleen colonies per fraction sorted from 10^5 BM cells.

experiments in which Rh123 labeled cells were devided in two fractions that differed in Rh123 fluorescence. Most day-8 CFU-S were brightly labeled, while the day-12 CFU-S are present in both fractions. This indicates that day-8 CFU-S have more or more active mitochondria than most of the day-12 CFU-S.

In two experiments the radioprotective capacity of the weakly and brightly labeled BM cells was tested in a 30-day survival assay (See chapter 2). The two experiments are shown in Fig. 4.5. Thirty day survival is expressed per day-12 CFU-S injected. In both experiments the survival of lethally irradiated mice was better with BM cells that were weakly labeled with rhodamine 123. The

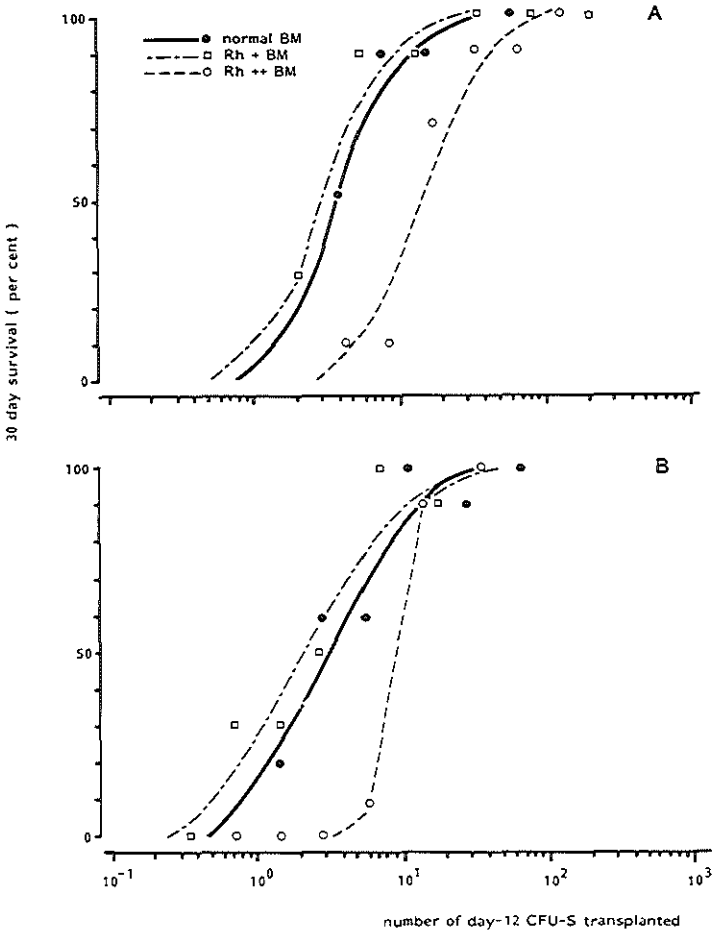


Fig. 4.5 Survival of lethally irradiated mice after transplantation of NBM (●), Rh+ BM (□) and Rh++ BM (o). Groups of ten irradiated mice were transplanted with varying numbers of cells. The survival is expressed per transplanted day-12 CFU-S. Two experiments are shown (A and B). The lines result from probit analysis.

numbers of day-12 CFU-S for 50 % survival by the Rh123+ fraction were 1.22±0.28 and 2.1±0.54,. respectively. For the Rh123++ fractions these values were 14.2±2.88 and 9.6±1.4 respectively. This indicates that the CFU-S with low Rh123 uptake have better repopulating ability when compared to those that incorporate a larger amount of Rh123.

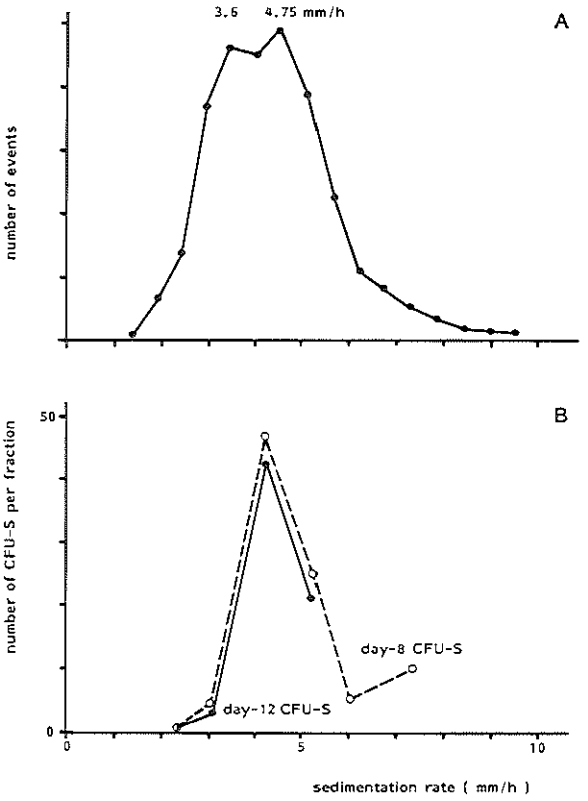


Fig. 4.6 A. Velocity sedimentation distribution of BC3 F1 BM cells. BM cells sedimented for 3 hours at 1 g as described in Chapter 2. B. CFU-S content of BM fractions differing in velocity sedimentation. The CFU-S are expressed as the number of CFU-S per 10^5 fractionated cells. Unseparated BM cells contained 22 and 23.4 day-8 and day-12 CFU-S per 10^5 cells.

Velocity sedimentation of day-8 and day-12 CFU-S

Fig. 4.6A shows the velocity sedimentation profile of nucleated NBM after three hours of sedimentation. The technique is described in Chapter 2. The two peaks that can be recognized sediment at 3.60 and 4.75 mm/h. This is in agreement with results that were obtained before (van den Engh, 1976). Fig. 4.6B shows the number of day-8 and day-12 CFU-S in the various fractions. From this it is concluded that there is no difference in sedimentation velocity of the day-8 and the day-12 CFU-S, suggesting that the two cell types have a similar size.

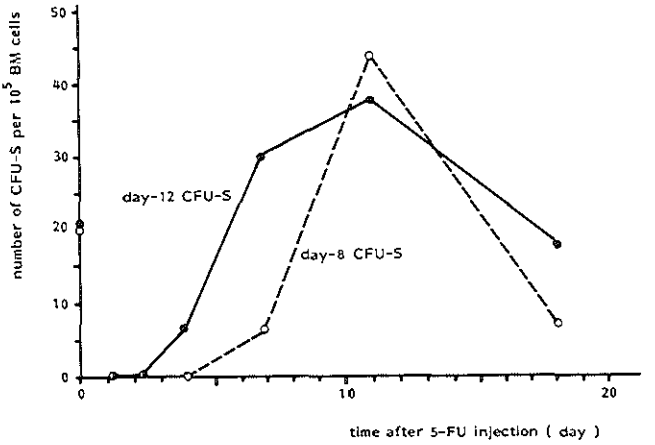


Fig. 4.7 Recovery of day-8 (o) and day-12 CFU-S (•) after injection of 150 mg/kg 5-FU, i.v.. Control values of untreated BM are shown on the Y-axis.

Regeneration kinetics of CFU-S in vivo and in vitro after treatment of mice with 5-flurouracil

5-Fluorouracil (5-FU) has been shown to be toxic for the CFU-S (Bruce et al, 1966). During the first days after i.v administration of 150 mg/kg 5-FU very few day-8 and day-12 CFU-S are detectable. The day-12 CFU-S recover before the day-8 CFU-S. Between 7 and 11 days after treatment high numbers of day-8 and day-12 CFU-S can be recovered from the bone marrow which return to normal values a few days later. This pattern of regeneration is presented in Fig. 4.7.

Culture of bone marrow cells in the presence of SAF results in the production of CFU-S (Tables 3.1 and 3.3). However, NBM is a mixture of early and late spleen colony forming cells. 5-FUBM contains virtually no day-8 CFU-S during the first days after administration. The development of these precursors may therefore be studied by culturing 5-FUBM with SAF. Fig. 4.8A shows the results of culture of FUBM, collected four days after 5-FU treatment (day-4 FUBM) in the presence of SAF. At the beginning of the culture there were very low numbers of day-8 CFU-S. The day-12/day-8 CFU-S ratio at the initiation of the cultures was 20.8. During a one week culture the number of day-12 CFU-S increases slowly. Parallel to this, with a delay of three-four days, followed a production of day-8 CFU-S.

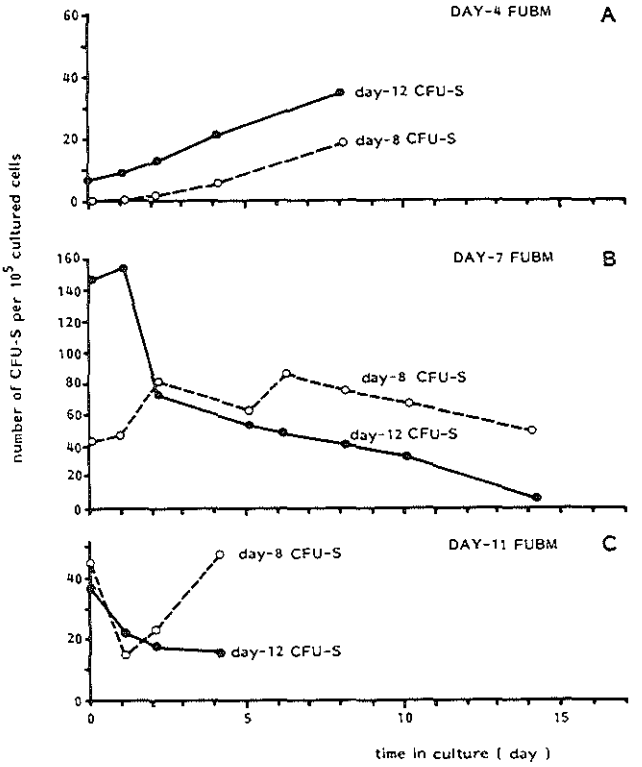


Fig. 4.8 CFU-S production in vitro from FUBM. A. BM, four days after 5-FU administration, was cultured in the presence of SAF. See Chapter 2 for culture conditions. At the days indicated, the cultured cells were collected, counted and assayed for day-8 and day-12 CFU-S content. The CFU-S are expressed as the number of CFU-S per 10^5 initially cultured cells. B. BM, seven days after 5-FU administration was cultured in the presence of SAF. C. BM, eleven days after 5-FU administration was cultured in the presence of SAF.

In day-7 FUBM high numbers of day-12 and some day-8 CFU-S were present (Fig 4.7). These cells were also cultured in the presence of SAF (Fig. 4.8B). The number of day-12 CFU-S increased until the first (2 exp.) or second (1 exp.) day after initiation of the culture, after which a rapid decrease could be observed. A second phase with a slower decline rate occured until 14 days of culture, after which the day-12 CFU-S was no longer present. Day-8 CFU-S from day-7 FUBM showed a different growth pattern in vitro (Fig. 4.8B): Maximal numbers of day-8 CFU-S were found later and the decline also starts at a later time. The decline in day-8 CFU-S paralleled that of the Day-12 CFU-S with a

delay of about one week.

Culture of day-11 FUBM resulted in the immediate onset of the decline of the day-12 CFU-S. The frequency of day-8 CFU-S was also initially decreased, but a production occured at a later time (Fig. 4.8C). This suggests that the day-8 CFU-S do not renew themselves but are produced from other stem cells, like the day-12 CFU-S or a pre-CFU-S.

Discussion

CFU-S can be separated into two subsets that produce spleen colonies at different days after transplantation (Harris et al, 1984; Baines and Visser, 1983). Day-12 CFU-S differ from day-8 CFU-S in the cell surface density of class I MHC antigens (Harris et al, 1984; Fig. 4.1). Part of the day-8 CFU-S are Thy-1 negative, while most day-12 CFU-S and the remaining day-8 CFU-S are (weakly) Thy-1 positive. Day-12 CFU-S can be subdivided in the ability to take up the mitochondrial dye Rh123 (Fig. 4.4). Both day-8 CFU-S and day-12 CFU-S show the same sedimentation velocity (Fig. 4.6), indicating these cells have the same diameter. From this it can be speculated that the day-12 CFU-S are heterogenous (Rh123-+ and Rh123++) and that there are two day 8-CFU-S (Thy-1 + and Thy-1 -).

The function of a large number of class I H-2 antigens on the surface of day-12 CFU-S is unknown. The only cell types that have a comparable antigen density are B and T lymphocytes (see also Chapter 4). Immature BM cells and Kupffer cells also have many membrane bound class I H-2 antigens, but at a lower density (Fig. 4.1, Basch and Stetson, 1962). Class I H-2 antigens are further present on other tissues like the epithelial cells of the uterus, colon, salivary ducts, small intestine, breast and skin (Ponder et al, 1983). All these tissues contain stem cells. However, other tissues with stem cells like sperm and ova have very few class I H-2 antigens (Palm et al, 1971, Johnson and Edidin, 1972; Klein, 1975). Class I H-2 antigens are recognized by cytotoxic T lymphocytes (CTL) in association with an antigenic determinant. Absence of the class I H-2 antigen on a cell causes nonresponsiveness of the CTL, despite the presence of the antigen. This indicates that cells with a high level of class I antigens are potential target cells for a CTL. From this it could be speculated that stem cells have a high density of class I antigens to facilitate the recognition of abnormalities, like malignancies, by CTL (Klein, 1975).

The labeling of CFU-S with anti-Thy-1.1 MCA is in agreement with the results obtained by Basch and Berman (1982). However, the heterogenous labeling of day-8 CFU-S with anti-Thy1.1 is as yet unexplained. The most likely possibility appears to be that there are two day-8 CFU-S, the Thy-1.1 positive cell being an intermediate CFU-S type. Experiments are in progress to determine whether in a cell population consisting of relatively mature CFU-S (SAFBM) the day-8 CFU-S are predominantly Thy-1.1 negative. The relatively high uptake of Rh123 by all day-8 CFU-S (Fig. 4.4) and the notion that Rh123 uptake and state of cell cycle are correlated (Cohen et al, 1981; Darzynkiewicz et al, 1981; James and Bohman, 1981), suggests that all day-8 CFU-S are in active cell cycle. The variable uptake of Rh123 by the day-12 CFU-S suggests that this population consists of cycling and resting cells. This difference in cell cycle status between the day-8 and day-12 CFU-S is in contrast with studies showing that few day-8 and day-12 CFU-S are cycling (<10 %; See introduction; Johnson and Nicola, 1984; van Zant, 1984; Chertkov and Drize, 1984; Wright, 1985). It could be that the S-phase specific treatments used in these studies (cytosine arabinoside, (^{3}H)TdR and hydroxyurea) are less sensitive to detect differences in the cell cycle status of day-8 and day-12 CFU-S than studies with Rh123, because mitochondria are synthesized throughout the cell cycle (James and Bohman, 1981). However, when all day-8 CFU-S are cycling, as indicated by the high Rh123 uptake, about 50 % of these cells should be killed by the S-phase specific agents. This is clearly not the case and suggests that a high uptake of Rh123 depends not only on cell cycle status. It is suggested that the differentiation processes in which the day-8 CFU-S appears to be involved also cause the presence of many or very active mitochondria.

The difference in radioprotective capacity per day-12 CFU-S between Rh123+ and Rh123++ cell populations shows, similar to the results with SAFBM (Chapter 3), that the number of spleen colonies at day-12 does not accurately predict the presence of pluripotent hemopoietic stem cells. On the other hand it has been demonstrated that in a bone marrow fraction in which day-12 CFU-S have been enriched 130-fold, the radioprotective capacity was enriched 180-fold. This indicates that day-12 CFU-S and PHSC are at least coenriched in the purified stem cell suspension. The present results indicate that the purified stem cell population is either a selection of the day-12 CFU-S from NBM (i.e. Rh+ day-12 CFU-S) or that another more immature stem cell, the pre-CFU-S is also present. Experiments are in progress to determine the ability to incorporate Rh123 by the purified stem cells. The presence of most of the day-8 CFU-S in the Rh++ cell

population and the loss of radioprotective capacity when compared the the Rh+ cells suggests that they have no major role in the protection against bone marrow failure after lethal irradiation.

In a recent publication it was shown that low density BM cells, weakly labeled with Rh123, contained 92 % of the precursor cells that can give rise to CFU-C2 at 13 days after BMT (marrow repopulating ability), 77 % of the platelet precursors (platelet repopulating ability) and 38 % of the day-13 CFU-S (see also Table 1) when compared to unseparated low density BM cells (Bertoncello et al, 1985). These results are in agreement with ours and indicate that the hemopoietic stem cells in the weakly and brightly Rh123 labeled fraction are qualitatively different.

The recovery of the day-12 CFU-S before that of the day-8 CFU-S after treatment of mice with 5-FU in vivo suggests that day-8 CFU-S are derived from the day-12 CFU-S (Fig. 4.7). This is also indicated by the appearance of day-8 CFU-S after culture of day 4 FUBM, which is virtually devoid of these cells, in the presence of SAF as well as by the disappearance of the day-8 CFU-S one week after the disappearance of the day-12 CFU-S in such cultures (Fig. 4.8A). The growth patterns of day-8 and day-12 CFU-S from BM collected at different days after 5-FU treatment in vitro are markedly different. During the early regeneration phase a production of day-12 CFU-S occured and a delayed appearance of day-8 CFU-S was apparent (Fig. 4.8A). Culture of BM cells 7 days after 5-FU caused a transient (1-2 days) production of day-12 CFU-S, an immediate production of day-8 CFU-S followed by a decline that paralleled that of the day-12 CFU-S with a delay of four-five days (Fig. 4.6B). Culture of FUBM after the in vivo regeneration had reached its maximum, was associated with a drop in the numbers of day-12 CFU-S and a diminished production of day-8 CFU-S (Fig. 4.8C). These results suggest that the day-8 CFU-S are derived from a precursor cell, like the day-12 CFU-S or the pre-CFU-S (Hodgson and Bradley, 1979).

From the results described in this Chapter it is concluded that day-8 and day-12 CFU-S are phenotypically different cells. Indirect evidence suggests that day-12 CFU-S give rise to day-8 CFU-S. This is in agreement with results obtained after culture of purified hemopoietic stem cells from NBM which, after in vitro culture in the presence of SAF, also gave rise to large numbers of day-8 CFU-S, CFU-C2 and BFU-E (Visser et al, manuscript in preparation). These results are further in agreement with data alluding to the heterogeneity in self renewal and differentiation potential among the most primitive hemopoietic stem cells (i.e. CFU-S; Worton et al, 1969a; Schofield and Lajtha, 1973; Micklem et

al, 1975; Rosendaal et al 1976, 1979; Hodgson and Bradley, 1979; see also Chapter 1). The organization of hemopoietic stem cells is further discussed in Chapter 8.

Conclusions

1. Day-8 and day-12 CFU-S from normal bone marrow differ in the density of H-2K antigens.

2. Day-12 CFU-S are Thy-1 positive and day-8 CFU-S are Thy-1 positive or Thy-1 negative

3. Day-8 CFU-S incorporate relatively much rhodamine 123, while day-12 CFU-S show a variable uptake of rhodamine 123

4. Day-8 and day-12 CFU-S have the same sedimentation velocity.

5. Day-12 CFU-S regenerate in vivo earlier than the day-8 CFU-S after treatment with 5-FU.

6. The day-12 CFU-S or its precursor, the pre-CFU-S, in FUBM is one of the targets of SAF as measured by the production of day-12 CFU-S after stimulation with SAF.

7. The appearance and disappearance of day-8 CFU-S in in vitro cultures of FUBM in relation to that of the day-12 CFU-S suggests that the day-12 CFU-S is the precursor of the day-8 CFU-S.

8. The presence of day-12 CFU-S in fractions of NBM does not accurately predict the radioprotective capacity. This indicates that day-12 CFU-S is a functionally heterogenous stem cell population or that radioprotection is mediated via another stem cell, the pre-CFU-S.

Chapter 5

THE LATE APPEARING SPLEEN COLONY FORMING UNIT (DAY-12 CFU-S) IN RELATION TO THYMOCYTE PRECURSORS

Introduction

Repopulation of a thymus after lethal irradiation and bone marrow transplantation has been studied for more than twenty years. It is well known that precursors of thymocytes exist in the bone marrow (Harris, et al., 1964; Kadish and Basch, 1976 and Boersma et al., 1981a, 1982). Boersma reported a linear relationship between the number of bone marrow cells transplanted and the number of donor cells present in the thymus at 14 days after transplantation (Boersma, 1982). He showed that the number of donor type thymocytes present in a regenerating thymus is a quantitative measure of the number of thymus precursors in normal bone marrow suspensions.

The thymocyte precursor cells have been characterized as follows: They have a low density, an intermediate sedimentation velocity and are relatively cortisone resistant (Basch and Kadish, 1977 and Boersma et al., 1982). Prothymocytes have further been reported to be Thy-1 negative and to share antigens present on brain tissue (Basch and Kadish 1977; Boersma et al., 1981b). The sensitivity to in vitro gamma irradiation of prothymocytes (Do=0.92 Gy) was significantly higher than that of the CFU-S (Do=1.22 Gy)(Boersma etal., 1981b). From these data it appears that the prothymocyte shares several characteristics with the CFU-S which was usually measured at 9-10 days after transplantation, but that there are also differences between the two cell types. Separation of the CFU-S and the prothymocyte has however not yet been accomplished.

This Chapter describes experiments that determine the number of thymocyte precursors and CFU-S in: normal, unseparated bone marrow cells and in fractions from NBM differing in the density of cell membrane bound class I H-2 antigens. In other experiments the thymus repopulating capacity of BM fractions differing in the uptake of rhodamine 123 and of BM pretreated in vivo with 5-fluorouracil (FUBM) is investigated.

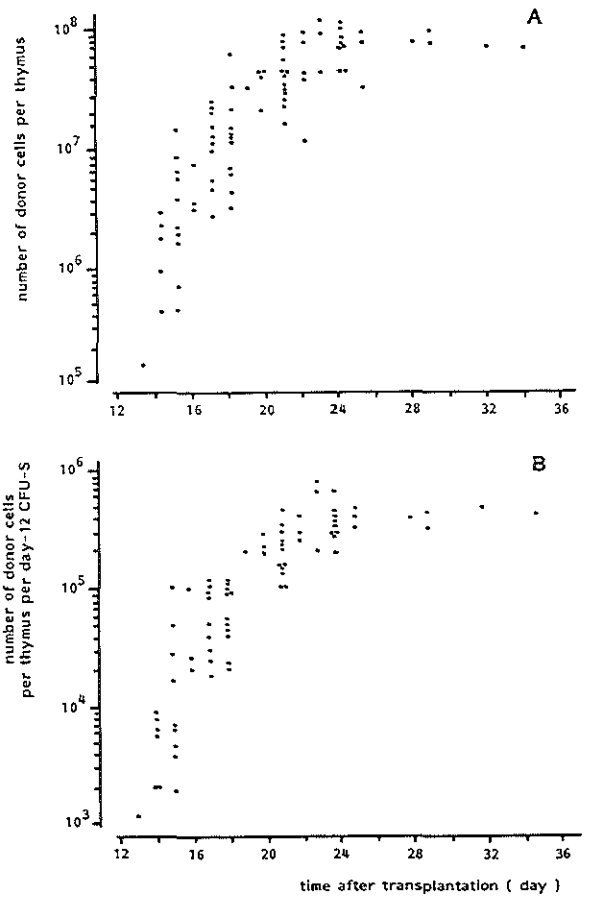


Fig. 5.1 Thymus repopulation after transplantation of 10^6 normal C3AK F1 BM cells into lethally irradiated C3H recipients. Each dot represents the mean value of groups of three to four mice. A. Number of donor thymocytes versus the time after BMT. B. Number of donor thymocytes per CFU-S transplanted versus the time after BMT.

Thymus repopulation by normal bone marrow cells

Most of the thymus repopulation data by normal bone marrow cells that were performed in 1981-1984 were pooled and analyzed (see Chapter 2 for details about the CFU-S- and prothymocyte assay). Fig. 5.1A shows the appearance of donor thymocytes after irradiation and bone marrow transplantation. Each point represents the mean number of donor thymocytes from groups of three C3H mice that were transplanted with 10^6 C3HxAKR F1 (C3AK) nucleated bone marrow cells.

The average day-10 CFU-S content of these bone marrow cells was 204±40 CFU-S per 10^6 nucleated cells. The average ratio of day-12 CFU-S/day-8 CFU-S in C3AK F1 bone marrow cells was 1.10±0.15. Donor thymocytes were detectable by day 14 after bone marrow transplantation after which a swift increase was apparent. At day 22-24 the thymus was fully repopulated. Fig: 5.1B shows thymus repopulation by normal bone marrow cells expressed as the number of donor type thymocytes per day-10 CFU-S transplanted.

Thymus repopulation by fractionated bone marrow cells

Sorting of H-2K labeled hemopoietic progenitors from mouse bone marrow

Bone marrow cell were incubated with biotinylated anti-H-2K MCA and avidin/FITC and separated on a fluorescence activated cell sorter. The incubation and sorting procedure are described in Chapter 2. The density of H-2K molecules on day-8 and day-12 CFU-S from C3H mice was described in Chapter 4. In the thymus regenerating experiments we used C3AK donor mice. C3AK mice have a higher mean density of H-2K molecules (Bauman et al, 1985). The CFU-S were initially assayed at day 10 only. The recovery of such CFU-S in the brightly fluorescent fraction ranged from 89 to 104 % (see also Chapter 2). This indicates that virtually all day-10 CFU-S from C3AK F1 mice are present among the cells with the highest density of H-2K antigens. Later experiments in which the day-8 and day-12 CFU-S were determined showed that two third of day-8 CFU-S were recovered from a fraction staining less intense with anti-H-2K, while the most densily stained cells were relatively enriched in day-12 CFU-S. The mean day-12/day-8 CFU-S ratio of this fraction was 2.02±0.39. These results are in agreement with those described in the previous Chapter in which C3H mice were used.

Determination of the density of H-2K antigens on thymocyte precursors

Concomitantly with the CFU-S assay, the thymus repopulating capacity of the sorted cell suspensions was assayed. Fig. 5.2 gives a summary of the results with untreated BM cells, with cells incubated with anti-H-2K and with brightly H-2K-positive bone marrow cells (4% of total cells). The results are expressed

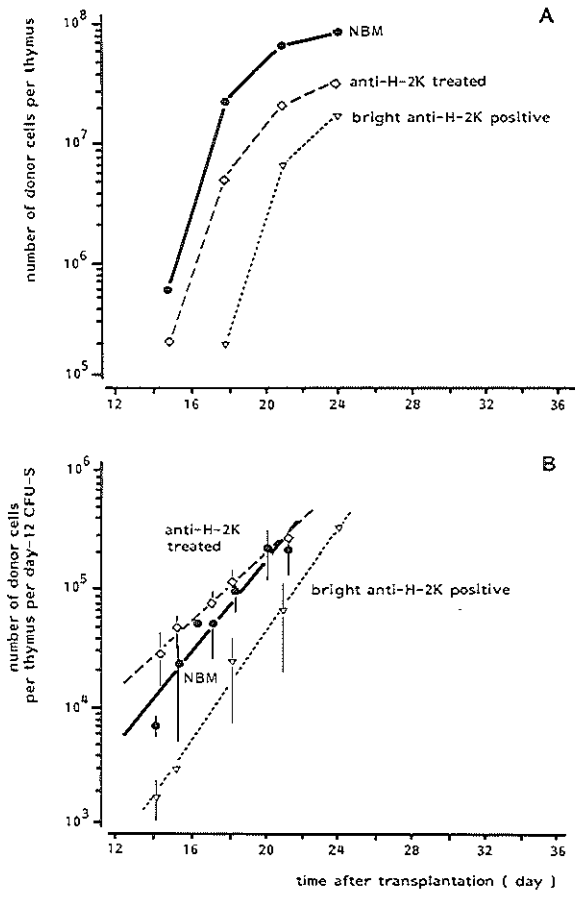


Fig. 5.2 A. Number of donor cells in the thymus versus the time after BMT per 10^6 NBM cells (●; 236 day-10 CFU-S), per 10^6 BM cells treated with anti-H-2K (◇; 112 day-10 CFU-S) and by a brightly fluoresceing fraction sorted from 10^6 anti-H-2K labeled BM cells (▽; 99 day-10 CFU-S). All BM cells were run through a light activated cell sorter. B. Number of donor cells in the thymus per day-10 CFU-S transplanted versus the time after BMT. NBM, H-2K-treated and H-2K-positive BM contained 19.9±6.3, 10.2±5.0 and 8.4±1.1 day-10 CFU-S, resp. The regression lines were calculated from the means of the obtained data. Each bar represents one standard deviation. The correlation coefficients were 0.95 with 5 degrees of freedom for normal bone marrow cells, 0.99 with 3 degrees of freedom for anti-H-2K-treated bone marrow cells, and 0.99 with 3 degrees of freedom for brightly anti-H-2K-positive bone marrow cells in the FLS-PLS window (see also Chapter 2). Comparison of the regression lines of NBM and anti-H-2K treated BM showed that they were parallel (intercept Y-axis p=0.027;slope p=0.13). The regression line of brightly anti-H-2K-positive BM was parallel with that of NBM (intercept Y-axis p=0.027; slope p=0.40) and different from that of H-2K-treated BM (intercept Y-axis p<0.001; slope p<0.01).

as donor cells per day-10 CFU-S injected. This makes a comparison of the three groups possible, since different numbers of CFU-S were injected in each experiment. The treatment of BM cells with anti-H-2K caused a reduction to 40 % of the number of CFU-S. Thymus regeneration by anti-H-2K treated BM cells expressed per number of cells injected was delayed when compared to that by untreated BM cells (Fig. 5.2A). Accordingly, thymus regeneration when expressed per day-12 CFU-S transplanted by anti-H-2K treated BM was similar when compared to that by untreated BM cells (Fig. 5.2B). This indicates that the treatment with anti-H-2K MCA affects CFU-S and the thymocyte precursor cells to the same extent. The brightly labeled H-2K-positive bone marrow cells showed a delay in thymus repopulation when compared with unsorted anti-H-2K-labeled bone marrow cells (Fig. 5.2B). This highly H-2K-positive bone marrow graft contained the same absolute number of CFU-S as that of unsorted H-2K-labeled bone marrow cells. Comparison of the regression lines of H-2K positive bone marrow cells with sorted, but unseparated, anti-H-2K labeled bone marrow cells shows that they are not parallel ($p<0.01$) and that the intercepts with the Y-axis are different ($p<0.001$). We conclude from these results that the H-2K-bright bone marrow cells, which are enriched in CFU-S (Chapter 3), are depleted in prothymocytes (Fig. 5.2).

Animals receiving the CFU-S poor-fraction (about 11 % of total nucleated cells) in the cell sorting experiments described above died from radiation exposure before thymus repopulation could be determined. Therefore, the prothymocyte assay was altered slightly by coinjection of a small dose of syngeneic bone marrow cells into the recipient ($5x10^4$ cells per animal). Fig. 5.3 shows the results of a representative experiment (out of 3). The dim H-2K positive cells gave rise to a significant progeny of donor-derived cells in the thymus. These cells appeared relatively early during repopulation (day 15) and disappeared after 24 days (Fig. 5.3). From this pattern it is concluded that prothymocytes have fewer H-2K antigens on their cell surface than do day-10 CFU-S. The committed progenitor cells or prothymocytes, apparently, are present in the bone marrow. They migrate to the thymus and give rise to relatively early and transient thymus repopulation. After transplantation of NBM this transient repopulation then must be overtaken by the swift increase due to prothymocytes produced by the grafted stem cells. The presence of fewer H-2K antigens on the prothymocyte also explains the somewhat higher early thymus repopulation after anti-H-2K treatment in comparison with NBM as well as the absence of this early thymus repopulation by sorted H-2K brightly positive BM.

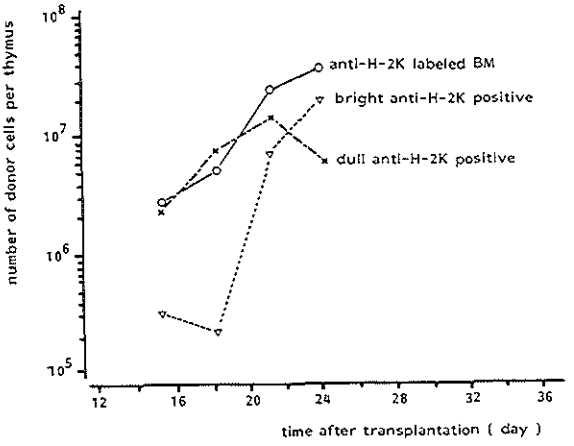


Fig. 5.3 Thymus repopulation by BM cells treated with anti-H2K-biotin, and avidin-FITC (o), by sorted cells brightly positive for H-2K (▽) and by cells intermediately positive for H-2K (x). Thymus repopulation is expressed as the number of donor thymocytes produced by 10^6 BM cells or by a fraction sorted from 10^6 BM cells. The numbers of day-10 CFU-S in these grafts were 100, 89, and 20, respectively.

Thymus repopulation by purified stem cells

CFU-S were shown to have the following characteristics: They have a low density (Worton et al., 1969b; Bol et al., 1977; Visser and Bol, 1981) and they contain a large number of receptors for wheat germ agglutinin (Visser and Bol, 1981). CFU-S scatter laser light relatively strong when measured at low angles, but relatively weakly when measured perpendicular from the direction of the laser beam (van den Engh et al. 1979). CFU-S were also shown to stain with antibodies directed against class I H-2 antigens (Russell and van den Engh, 1979; Chapters 2 and 4). Visser et al. (1984) combined these methodologies which resulted in an enrichment of day-12 CFU-S with a factor of about 135 (range, 90-230). The purity of these cell suspensions ranged from 35-110% when an f-factor of 10 % is taken into consideration. Morphological analysis by electron microscopy indicated that the purified cell suspension contains two cell types: 43-59 % of the cells resemble the "candidate stem cell" which was described by van Bekkum et al (1971), while 24-43 % have somewhat more cytoplasm and deeper nuclear indentations (Visser et al, 1984). The spleen colony data indicated that the average ratio of day-12 CFU-S/day-8 CFU-S is 4.2 in these

Table 5.1

CFU-S content of bone marrow cells after density separation and fluorescence activated cell sorting.

Experiment	CFU-S/10^5 purified cells	
	Day 8	Day 12
I	4250	8070
II	1500	2380
III	1360	2480
IV	2750	2660
V	1110	2320
VI	1580	4390
VII	2560	5640
VIII	1160	1440
	2000±1000	3680±2100

The isolation procedure is described in Chapter 2. 150-250 purified cells derived from C3AK mice were transplanted for the CFU-S assay. Unseparated bone marrow cells contained 20.4±4.0 day-10 CFU-S per 10^5 transplanted cells.

sorted suspensions (in NBM this ratio was 1.2; Visser et al., 1984). The two cell types, discerned by electron microscopy, do not to correlate with the day-12 and day-8 spleen colony data.

The method described above appeared to give the highest enrichment of CFU-S from normal bone marrow cells and to result in cell suspensions that resembles pure pluripotent hemopoietic stem cells best with the presently available techniques. Purified stem cells derived from C3AK mice were prepared as described by Visser et al (1984) (see Chapter 2 for more technical details) and they were tested for thymus repopulating ability. Table 5.1 gives the spleen colony data that were obtained in the thymus repopulation experiments with enriched hemopoietic stem cells. An average of 2000±1000 day-8 CFU-S and 3680±2100 day-12 CFU-S per 10^5 nucleated cells were obtained. Compared to normal bone marrow a 100 fold enrichment of day-8 CFU-S and a 180 fold enrichment of day-12 CFU-S was observed. These results, similar to those of

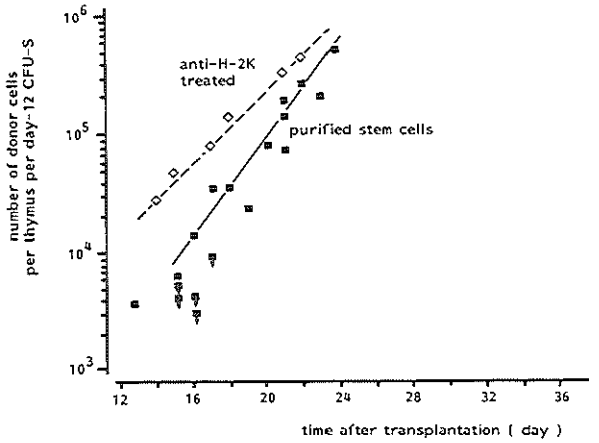


Fig. 5.4 Thymus repopulation by purified C3AK stem cells. See Chapter 2 for the isolation procedure. anti-H-2K treated, unseparated BM cells (◇), purified stem cells (■). The recipients of both groups received similar numbers of day-12 CFU-S. The dots with arrows represent values calculated from the number of thymocytes and the detection limit (1%) of the flow cytometric assay. In these cases no donor thymocytes were observed.

Visser et al (1984) show that both day-8 and day-12 CFU-S were enriched in the purified cell suspension, but that the recovery of day-8 CFU-S was lower. Similar data were obtained with C3H mice (Chapter 4).

Thymus repopulation by purified stem cells was tested by injection of low density, WGA+ and H-2K+ C3AK bone marrow cells into lethally irradiated recipients. At various days after transplantation animals were sacrificed and the thymuses were removed. The number of donor cells per thymus was determined as described in Chapter 2. The results are expressed as the number of donor cells per day-12 CFU-S (Fig. 5.4). Fig. 5.4 shows that purified stem cells gave a thymus repopulation that is delayed when compared to that by unseparated, anti-H-2K treated bone marrow cells. This delay was similar to that observed between brightly H-2K-positive, sorted cells and unseparated, anti-H-2K treated cells as was shown in Fig. 5.2) and indicates that the cell, responsible for early thymus regeneration, the prothymocyte, is depleted from the brightly H-2K positive BM cell fraction.

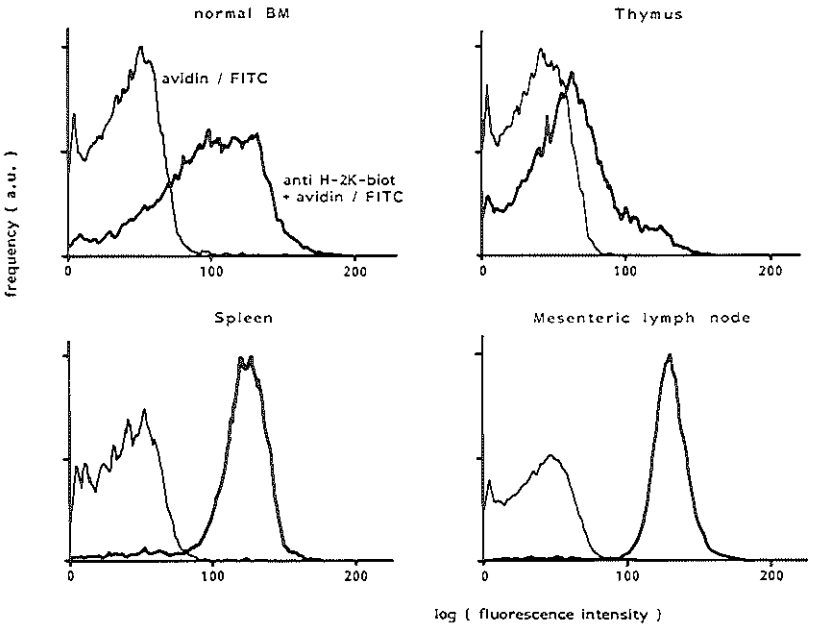


Fig. 5.5 Fluorescence intensity distributions of various cell types incubated with biotinylated anti-H-2K and avidin/FITC. The thin lines are fluorescence distributions of cells incubated with avidin/FITC only.

Staining of hemopoietic and lymphatic tissues with anti-H-2K

The expression of H-2K among thymocytes and T cells varies widely (e.g. Winn, 1969). In order to determine the changes in the density of H-2K antigens from pluripotent hemopoietic stem cells to mature T cells the fluorescence intensity after incubation with anti-H-2K MCA was measured. C3AK F1 cells from four tissues were incubated with biotinylated anti-H-2K monoclonal antibody (MCA), washed and incubated with avidin/FITC as described in Chapter 2. Fluorescence distributions of anti-H-2K treated cells are shown in Fig. 5.5. Almost all nucleated bone marrow cells had the H-2K determinant on their cell membranes, although in variable density. The fluorescence distribution of C3AK F1 bone marrow (Fig. 5.5A) had the same shape as that of C3H BM (Fig. 4.1A). Most thymocytes are weakly labeled, with the exception of about 20 percent, which had a medium to high fluorescence intensity (Fig. 5.5B). Spleen cells and mesenteric lymph node cells stained very brightly with this monoclonal antibody. There was clearly no evidence of an H-2K negative subpopulation in

these tissues. These results are in agreement with results reported in the literature (e.g. Winn, 1969).

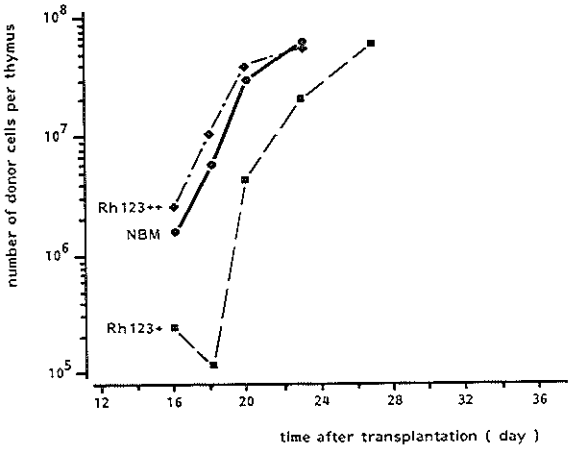


Fig. 5.6 Thymus repopulation by BM sorted on the uptake of rhodamine 123. C3AK F1 BM cells were labeled with rhodamine 123 as described in Chapter 2. Lethally irradiated C3H mice received BM cells sorted from 10^6 BM cells. Rh+ BM contained 0 day-8 CFU-S and 10 day-12 CFU-S per 10^5 cells. Rh++ BM contained 15.6 day-8 CFU-S and 11.2 day-12 CFU-S. Each point represents the mean value of four mice.

Thymus repopulation by BM fractions differing in the uptake of rhodamine 123

In the previous Chapter it was shown that day-8 CFU-S take up a high amount of Rh123, while day-12 CFU-S are heterogenously labeled. It was further shown that the capacity for radioprotection after lethally irradiation is predominantly present in the weakly labeled fraction which contained only day-12 CFU-S. In other experiments the thymus repopulating ability of weakly (Rh+) and brightly (Rh++) labeled fractions was assessed. This is shown in Fig. 5.6. The Rh++ cells repopulated the thymus similar to NBM, but the Rh+ fraction repopulated the thymus with a delay of about four days. This delay was similar to that observed with the H-2K bright BM cells and the purified stem cells. These results indicate that the cell responsible for early thymus regeneration (the prothymocyte) differs from the Rh+ day-12 CFU-S, but that prothymocytes and day-8 CFU-S incorporate a similar amount of rhodamine 123.

Table 5.2

CFU-S content of bone marrow cells after 5-FU treatment

Treatment	day-8		day-12	
	CFU-S/10^5 BM	CFU-S/femur	CFU-S/10^5 BM	CFU-S/femur
normal bone marrow	16.7 ± 4.0	3106 ± 55	14.8 ± 7.2	3124 ± 177
day-4 FUBM	15.5 ± 6.7	434 ± 33	89.1 ± 11.1	2495 ± 112

FUBM was obtained as described in Chapter 2. The total number of nucleated cells per femur was decreased from 18.6×10^6 in normal femora to 2.8×10^6 in 5-FU treated mice. The data represent the average of 4 experiments.

Bone marrow from mice pretreated with 5-FU (FUBM)

As a source of day-12 CFU-S we used murine bone marrow cells four days after injection of 5-FU (Hodgson and Bradley, 1979). The delayed appearance of spleen colonies from these cells has been suggested to result from transient toxicity of the drug to CFU-S (Rosendaal et al., 1981). Rosendaal distinguished however between the toxicity to early and late colony formers, the latter being less affected (Rosendaal et al., 1981). The remaining day-12 CFU-S have been shown to repopulate the hemopoietic system at a higher rate than do CFU-S from normal BM (Hodgson and Bradley, 1979; Ross et al, 1982). The day-12 CFU-S present in bone marrow after treatment with 5-FU or HU have also been shown to have a higher rate of self renewal than those in normal bone marrow (Hodgson and Bradley, 1979; Roosendaal et al., 1979). These results indicate that the surviving CFU-S do not suffer from any detectable toxicity. We have therefore considered the 5-FU treated cells as representative for day-12 CFU-S which are present in normal bone marrow. Table 5.2 shows the effect of 5-FU on the presence of CFU-S. The frequency of day-8 CFU-S is normal at 4 days after 5-FU. Due to the decrease in the total number of nucleated cells per femur, the total number of day-8 CFU-S was 14 % of normal. The total number of day-12 CFU-S per

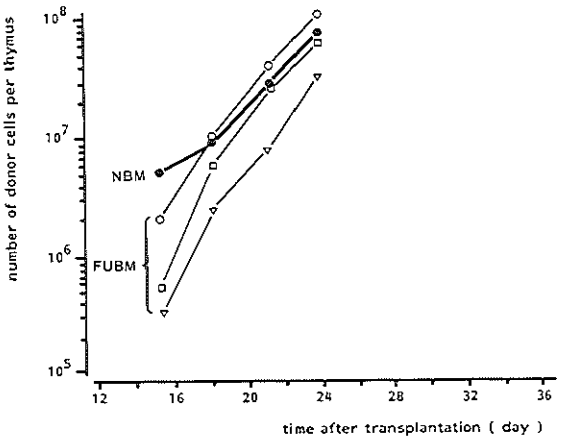


Fig. 5.7 Thymus repopulation by FUBM. Lethally irradiated mice received 10^6 NBM (●), or graded numbers of 5-FUBM ($5x10^5$ (○), $2.5x10^5$ (□), $1.25x10^5$ (▽). NBM contained 166, 172 and 232 day-8, -10 and -12 CFU-S, resp. FUBM contained 230, 760, and 1000 day-8,-10 and -12 CFU-S per 10^6 cells, respectively. Each point represents the mean value of three to four mice.

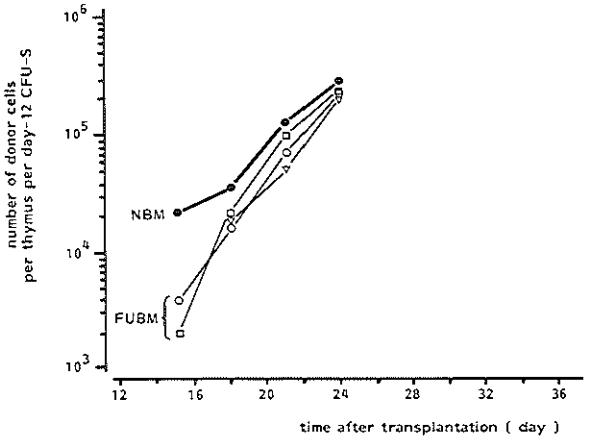


Fig. 5.8 Thymus repopulation by FUBM. Thymus repopulation per day-12 CFU-S transplanted. See Fig. 5.7 for details.

femur was 80 % of normal after treatment with 5-FU and the concentration of day-12 CFU-S was 6 times that in normal bone marrow cells.

Thymus repopulation by FUBM

The bone marrow cells remaining after in vivo treatment with 5-FU (day-12 CFU-S) were tested for thymus repopulating ability. Fig. 5.7 shows the relationship between the number of cells transplanted and the number of thymocytes at different days after transplantation. Mice injected with FUBM showed diminished thymus repopulation at 15 days after transplantation. After injection of high numbers of nucleated cells, containing large numbers of CFU-S, thymus repopulation is still less than that of normal bone marrow (Fig. 5.7). In other experiments even larger numbers of 5-FUBM were transplanted, but at the saturating dose thymus repopulation was still less than that of NBM (data not shown). Since we wanted to determine the thymus repopulation in relation to the day-12 CFU-S, the thymus repopulation was expressed per day-12 CFU-S transplanted. This is shown in Fig. 5.8. A delay of three to five days is apparent in the early repopulation period. This difference disappears rapidly due to a higher growth rate in the thymuses injected with FUBM. These results indicate that the prothymocyte is sensitive to 5-FU.

Comparison of thymus repopulation by NBM, purified stem cells, FUBM and SAFBM

In order to compare thymus regeneration by different cell suspensions, the dose-response relationship (i.e. the number of CFU-S transplanted versus the number of donor derived thymocytes) was determined at several days after BMT. The curves are shown in Fig. 5.9. The lines indicate that the number of donor derived thymocytes depends log linear on the number of day-12 CFU-S (and also the total number of cells) transplanted. The regression lines from these data were determined and the values at a dose of 200 and 500 transplanted CFU-S were calculated. These doses were within the range of most experiments (except for those of 500 CFU-S with the purified stem cells which is omitted in Fig. 10B). The values are presented versus the time after BMT in Fig. 5.10A and B. Differences in thymus repopulating ability of the various cell suspensions is reflected by a different onset of thymus repopulation.

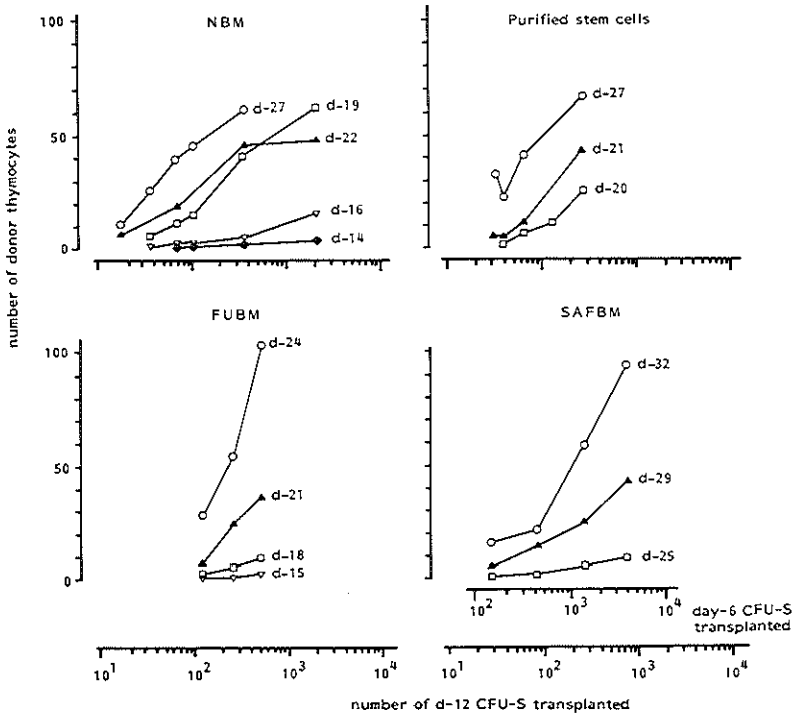


Fig. 5.9 Thymus repopulation by NBM, purified stem cells, FUBM and SAFBM. Dose-response relationship between the number of CFU-S transplanted and the number of donor thymocytes present at the indicated days.

The delay in thymus repopulation between NBM and purified stem cells and FUBM disappears during the early repopulation phase. The delay in thymus repopulation between NBM and SAFBM (either per day-6 CFU-S or per day-12 CFU-S) is much more pronounced. This is about ten days.

Discussion

In the experiments that are described in this Chapter two biological assays are applied to the same cell supensions: The spleen colony assay and the thymus repopulation assay (Till and McCulloch, 1961; Kadish and Basch, 1976). The CFU-S assay measures a heterogenous population of cells that produces spleen colonies at different times after transplantation (Hodgson and Bradley, 1979; Magli et al., 1982; Harris et al., 1984; Visser et al., 1984). In Chapter 3 it

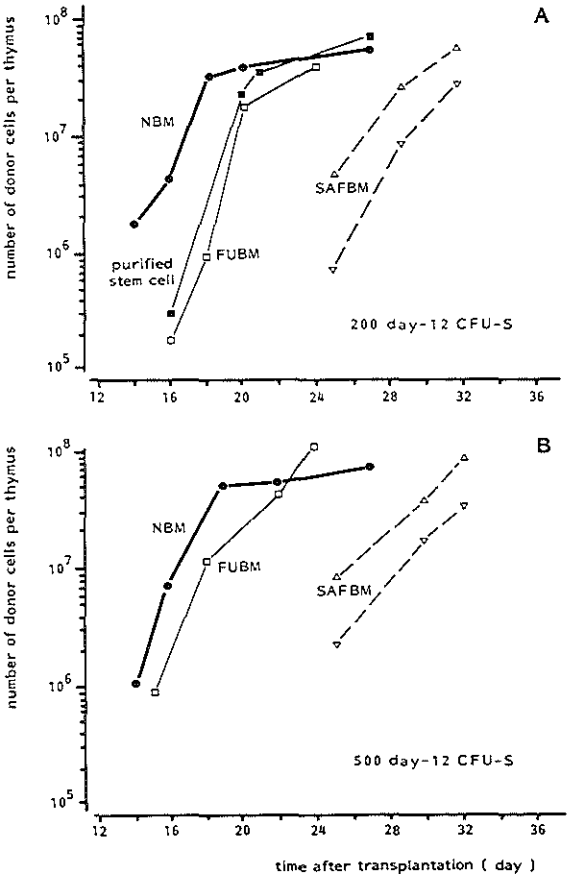


Fig. 5.10 Comparison of thymus repopulation by the various BM sources. The curves were constructed from the dose response curves presented in Fig. 5.9. A. At a dose of 200 day-12 CFU-S in NBM (●), purified stem cells (■), 5-FUBM (□) and SAFBM (△) or day-6-8 CFU-S in SAFBM (▽). B .At a dose of 500 CFU-S, see A for characters.

was described that BM cell populations enriched in day-8 CFU-S (SAFBM) showed a dimished capacity to repopulate the thymus, indicating that these day-8 CFU-S had no longer thymus repopulating ability. The experiments described in this chapter were performed in order to evaluate the thymus repopulating capacity of day-12 CFU-S in enriched cell suspensions.

The results can be summarized as follows: Thymus repopulation by normal bone marrow cells is, with Thy-1.1 as a marker, detectable at 14-15 days after transplantation after which a rapid growth could be observed (Figs. 5.1A and

Table 5.3

Presence of day-8 CFU-S, day-12 CFU-S and prothymocytes in cell suspensions

Cell suspension	Day-8 CFU-S	Day-12 CFU-S	Delay in thymus regeneration = absence of prothymocytes
normal BM	0.9	1.0	-
bright H-2K pos.	0.5	1.0	3-4 days
purified stem cells	0.5	1.0	3-4 days
dull H-2K pos.	4.0	1.0	none
day-4 FUBM	0.2	1.0	4 days
Rh123+	0.3	1.0	4 days
Rh123++	1.4	1.0	none
SAFBM	3.0	1.0	8 days

Summary of the content of day-8 CFU-S, day-12 CFU-S and prothymocytes in various BM cell suspensions. The presence of prothymocytes is indicated by the absence of a delay in thymus regeneration. The data are derived from experiments described in Figs. 3.6, 4.4, 5.1, 5.2, 5.3, 5.4, 5.6, and 5.8 and Tables 3.1, 4.1, 4.4, 5.1 and 5.2.

B). This is in agreement with results obtained by others (Kadish and Basch, 1976; Boersma et al., 1981a). Bone marrow cells were separated in fractions differing in the content of class I H-2 antigens. All day-12 CFU-S, at least 90 % of the day-10 CFU-S and 35 % of the day-8 CFU-S were present in 4-5 % of bone marrow cells selected for FLS, PLS and high fluorescence intensity after labeling with anti-H-2K and avidin/FITC (see also Chapter 4). Thymus repopulation by this fraction was delayed 3-4 days when compared to labeled, unseparated bone marrow cells (Fig. 5.2B). Relatively large numbers of day-8 CFU-S (65 %) and low numbers of day-10 CFU-S (10 %), but virtually no day-12 CFU-S were present in the weakly H-2K positive fraction. This fraction gave rise to transient progeny between 14 and 24 days after irradiation and transplantation (Fig. 5.3). Purified stem cells were prepared as described in Chapter 2. The last step also separates cells on fluorescence intensity after

labeling with anti-H-2K. The average enrichment of day-8 CFU-S was 100 fold, whereas day-12 CFU-S were enriched 180 times. Thymus repopulation by these highly enriched bone marrow stem cells was, on a per CFU-S basis, similar to that by bone marrow cells that were separated on fluorescence intensity with anti-H-2K only (Fig. 5.4). Both these cell suspensions showed a delay in thymus repopulation of 3-4 days.

Early thymus regeneration is mediated with BM cells that label brightly with Rh123 (Fig. 5.6). This fraction contains most day-8 CFU-S and part of the day-12 CFU-S. These results are consistent with the separation experiments in which anti-H-2K was used. In both cases early thymus repopulation is correlated with day-8 CFU-S, but not with day-12 CFU-S. Three to four days delayed thymus repopulation is correlated with the presence of day-12 CFU-S.

FUBM contains a preponderance of CFU-S that produce spleen colonies late after transplantation (Hodgson and Bradley, 1979; Table 5.2). Several committed progenitors with restricted differentiation capacity have been reported to be sensitive to the cytotoxic action of the drug (day-8 CFU-S, BFU-E, GM-CFU-1 and GM-CFU-2)(Fig. 4.7 and Table 5.2; Hodgson and Bradley, 1979; Suda et al, 1983). Thymus repopulation is delayed several days, indicating that prothymocytes are also killed by 5-FU. The delay in thymus regeneration disappears rapidly with time (Figs. 5.7-5.10). The similarities between FUBM, H-2K bright BM cells, purified stem cells and weakly Rh123 labeled BM cells suggests that delayed thymus repopulation in these preparation is caused by the absence of the prothymocyte. This suggests that the thymus repopulation stems following transplantation of these cell suspensions is effectuated not by prothymocytes present in the graft, but by prothymocytes that derive from grafted pluripotent stem cells.

These results can be interpreted as follows (Fig. 5.11). Pluripotent stem cells, present in BM fractions enriched in day-12 CFU-S, give rise to spleen colonies late after transplantation. Thymus repopulation by PHSC is delayed several days when compared to that by NBM. This indicates that PHSC (part of the day-12 CFU-S or the pre-CFU-S, see Chapter 4) first have to produce prothymocytes in the bone marrow. Directly grafted prothymocytes are responsible for the early thymus repopulation. They are present in NBM, in bone marrow fractions staining intermediately dense with anti-H-2K and bright with Rh123 (Table 5.3). They are absent from FUBM, SAFBM, BM cells weakly labeled with Rh123 and from brightly H-2K-positive bone marrow cells (purified stem cells) (Table 5.3). The thymic progeny of the prothymocytes contained in the BM

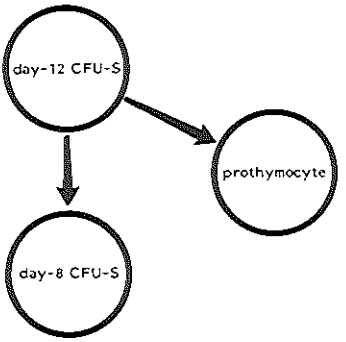


Fig. 5.11 Proposed model of early hemopoietic differentiation. See text for details.

graft is relatively small and is gradually replaced by the progeny of prothymocytes that are produced by PHSC in the bone marrow. In this model it is postulated, that the CFU-S that produces early spleen colonies (day 6-8) have lost the differentiation potential for the T cell lineage and can no longer give rise to prothymocytes (see also Chapter 3, SAFBM).

If this model is correct, then prothymocytes, although identifiable and separable from day-12 CFU-S have limited proliferative and few self renewing capacity. The literature supports that such characteristics belong to the prothymocyte: Transplantation of thymus suspensions from mice irradiated and bone marrow transplanted 2 days previously, resulted in the appearance of low numbers of donor cells with little proliferative activity. Donor cells were absent 18 days after the transplantation (Boersma, 1982). Similar results were obtained after transplantation of normal thymocytes (Takada and Takada, 1973; Boersma, 1982).

The changes in H-2K expression during T-cell development are interesting. The PHSC have a higher density than do prothymocytes (Fig. 5.3) and most thymocytes contain barely detectable H-2K antigens (Fig. 5.5). Those with a "mature" phenotype do express again high levels levels of H-2K antigens (Ceredig et al., 1982a). Phenotypic characterization of thymocytes leaving the thymus showed that all migrating cells are brightly H-2K-positive, compared with 20-50% H-2K positive cells in the thymus (Scollay et al., 1980). Peripheral T cells, that are smaller than CFU-S have a fluorescence intensity comparable to that of the CFU-S (Fig. 5.5C and 5D). The density of H-2K on these cells must therefore be higher as compared with the CFU-S. H-2K expression, therefore,

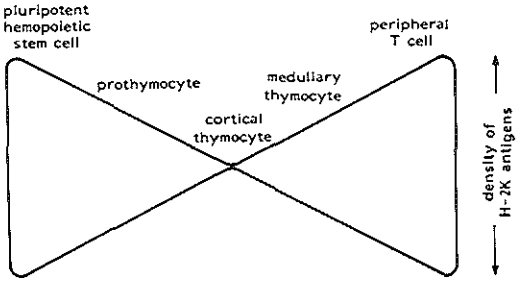


Fig. 5.12 Density of H-2K antigens on increasingly mature T cells. See text for explanation.

decreases early during T cell differentiation (CFU-S → prothymocyte → cortical thymocyte) and is increased again in cells with a 'mature'phenotype (medullary thymocyte → migrating thymocyte → peripheral T cell (Fig. 5.12). Reexpression of H-2K on immature thymocytes also occurs after in vitro stimulation with factors that are probaly derived from macrophages (Beller et al., 1977; Di Sabato et al., 1979; Abbott, et al., 1981). Why only a minority of the thymocytes (re)express H-2K antigens and leave the thymus as mature cells remains to be elucidated.

Conclusions

1. Day-10 to 12 CFU-S and prothymocytes differ in the density of H-2K proteins

2. Although day-8 CFU-S and prothymocytes are different cells, they have a similar density of H-2K proteins and a similar uptake of rhodamine 123.

3. FUBM is depleted of prothymocytes.

4. Thymus repopulation stems from prothymocytes contained in the graft and from prothymocytes produced by grafted PHSC. Prothymocytes are present in a fraction staining intermediately with anti-H-2K and brightly with rhodamine 123. They cause early thymus repopulation. Pluripotent stem cells are present in a fraction densely labeled with anti-H-2K, in a fraction weakly labeled with rhodamine 123 and in FUBM. PHSC cause a delayed thymus repopulation, probably via in vivo generation of prothymocytes in the BM.

5. Prothymocytes have limited proliferative and no self renewing properties

6. The expression of H-2K molecules is high in PHSC, is less in prothymocytes, is very low in cortical thymocytes and is again high in mature T cells.

Chapter 6

HOMING OF BONE MARROW CELLS INTO THE THYMUS

Introduction

Several methods, determining the presence of thymocyte precursors in cell suspensions were reviewed in Chapter 1. Most are indirect measurements because they determine the progeny of the precursor cell. Boersma (1982) extrapolated thymus repopulation data to the day of transplantation. Assuming that prothymocytes arrive in the thymus shortly after irradiation, it was calculated that there are about 300 prothymocytes per 10^6 BM cells. One method, which counts the number of cells that arrive in the thymus after transplantation of supravitally stained cell suspensions into lethally irradiated mice, has been used as an assay of prothymocytes (Lepault and Weissman, 1981; Lepault et al, 1983). This assay, if specific for thymocyte precursor cells, should be a valuable tool for the study of the very first differentiation step from thymocyte precursor cell to thymocyte. These studies suggested that the BM contains more thymus seeking cells than do spleen, mesenteric lymph nodes and thymus. The frequency of these cells was found to be about 1 in 1000 nucleated BM cells transplanted (Lepault and Weissman, 1981). Determination of the phenotype of the immigrated cells revealed that 1/4-1/3 of these cells express markers of mature B and T lymphocytes and macrophages. It was, however, not determined whether the thymic immigrants are also thymocyte precursors. Therefore, experiments were performed with the intention to relate the number of thymic immigrants to thymus regeneration. The entry of BM cells into the thymus three hours after BMT of mice which were irradiated 3 hours, 1 day or 4 days before BMT was determined by using fluorescent labeling techniques and chromium 51 labeled cells. The light scatter characteristics of the thymus immigrants was compared to that of the thymocyte precursor cells. The entry of cells into the thymus from BM cells depleted of thymocyte precursors (FUBM or purified stem cells; see Chapter 5) was compared to that by NBM. The first two BM suspensions showed a delay in thymus regeneration of 3-4 days which is indicative for a

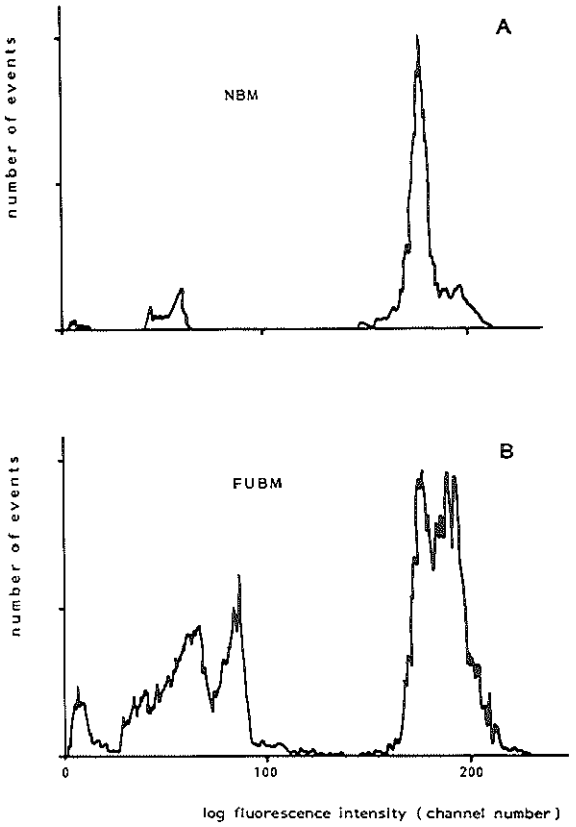


Fig. 6.1 A. Fluorescence of NBM labeled with Hoechst 33342 NBM was labeled with Hoechst 33342 as described in Chapter 2. The cells were analyzed on a light activated cell sorter with the laser in UV mode (351-364nm). B. Fluorescence of BM cells four days after injection of 150 mg per kg 5-FU (i.v.) and labeled with Hoechst 33342.

depletion of prothymocytes (Chapter 5). One would expect very low numbers of thymus seeking cells from these cell suspensions.

Homing of BM cells labeled with Hoechst 33342

Homing experiments with fluorescein-isothiocyanate (FITC) labeled BM cells and analysis of cell suspensions by flow cytometry showed that there was a high background fluorescence in thymus cell suspensions. Other supravital dyes were tested and Hoechst 33342 was selected. Normal BM cells were labeled with

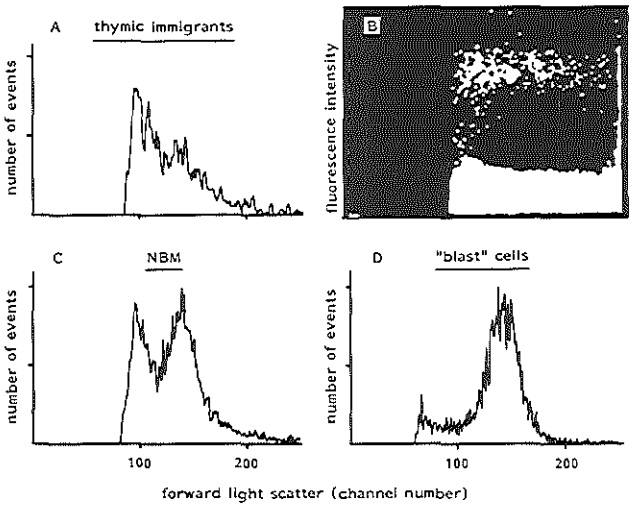


Fig. 6.2 A. Forward light scatter of the fluoresceing cells. B. Dotplot of thymocytes from mice irradiated at -6h and transplanted with $30x10^6$ Hoechst 33342 labeled NBM at -3h. 10^6 events are shown. For comparison the FLS of NBM (C) and the 'blast' (D) population containing the prothymocytes are shown.

Hoechst 33342 as described by Baines and Visser (1983). This supravital dye binds to DNA. The fluorescence distribution of these cells is shown in Fig. 6.1A. Due to the bright fluorescence of the labeled cells, transplanted cells could easily be recognized among unlabeled cells of the recipient as shown in Fig. 6.2B. After transplantation of BM cells incubated with Hoechst 33342 into preirradiated mice (-3h) a linear relationship was observed between the number of cells transplanted and the frequency of donor cells in the spleen, bone marrow and thymus (Fig. 6.3A). The small deviation from linearity after transplantation of relatively low numbers of BM cells, with respect to thymus homing, may be due to the selective homing of BM lymphocytes, which comprise most thymic immigrants, to other organs (Yoshida and Osmond, 1978). At three hours after BMT 10-15 % of the injected cells could be recovered from the both the spleen and the bone marrow. However, very few, i.e. less then 0.1 % of the injected cells, could be recovered from the thymus.

From these experiments it was concluded that the number of donor cells recovered from the spleen, BM or thymus, is linear with the number of BM cells transplanted.

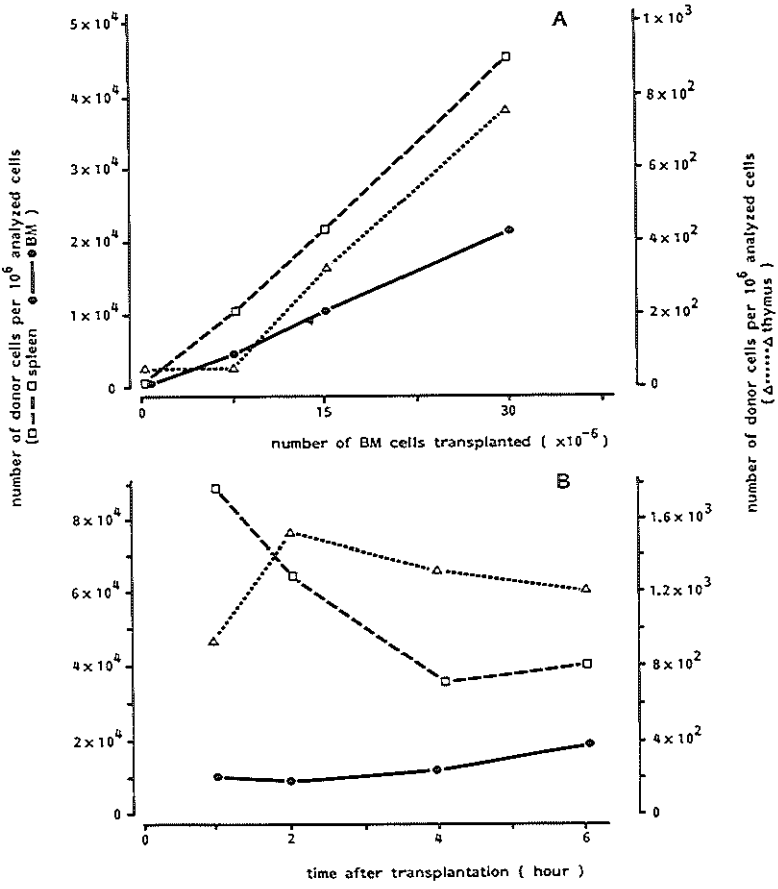


Fig. 6.3 A. Relationship between the number of Hoechst 33342 labeled BM cells transplanted and the frequency of labeled cells in the BM (●), spleen (□) and thymus (Δ) at 3h after BMT. Note that the scales for the different organs are different. B. Relationship between the time after transplantation of 21×10^6 Hoechst 33342 labeled NBM and the frequency of labeled cells in the BM (●), spleen (□) and thymus (Δ).

The appearance of BM cells in the three organs in relation to the time after transplantation is shown in Fig. 6.3B. Transplanted cells appeared very soon in the spleen and the thymus after BMT and there was a tendency to a decrease in frequency in the subsequent hours. A somewhat later appearance of transplanted cells was evident in the bone marrow.

Table 6.1

Number of thymus seeking BM cells

Time between irradiation and BMT	Total nucleated cells per thymus 6 x 10	Number of donor cells per organ per 10^6 cells transplanted from NBM	FUBM
2-3h	85 ±7	1200±400	ND
1d	14.4±1.1	795±140	314/480
4d	1.2±0.2	19±5	ND

BM cells were labeled with Hoechst 33342 as described in Chapter 2. 15-30x10^6 nucleated BM cells were injected into mice that were preirradiated at the times indicated. The frequency of transplanted cells and the total number of nucleated cells was determined at three hours after BMT. From this, the recovery per organ per 10^6 injected cells was calculated. The data are derived from 5 experiments. In each experiment five mice were used per point. Two experiments with FUBM are shown. The data are corrected for background fluorescence, which ranged from 100-400 events per 10^6 analyzed cells. Values ± 1 standard deviation are shown. ND=not done.

Number of NBM cells homing to the thymus

Bone marrow cells (15-30x10^6) were transplanted into mice that were lethally irradiated at 2-3 hours, 1 day or 4 days before transplantation. The frequency of transplanted cells in the thymus at three hours after transplantation was determined by counting the number of fluoresceing cells among 2x10^5-10^6 thymocytes in a flow cytometer. The total recovery per thymus was calculated from the frequency of labeled cells and from the total number of nucleated cells per thymus. Table 6.1 shows that early after irradiation relatively large numbers of injected cells could be recovered from the thymus. When recipient mice are irradiated 1 day before BMT lower numbers of injected cells are recovered and with a four day interval a very small part of the inoculum could be detected. Concominant with this decreased seeding fraction of labeled BM cells there was a 70-fold decline in the number of nucleated cells in the thymus during the first four days post-irradiation (Table 6.1).

Analysis of the forward light scatter intensity of the fluoresceing cells (a measure of cell size, see also Chapter 2) showed that the majority of the labeled cells are small (Fig. 6.2). They have the size of lymphocytes. Sorting experiments have shown that virtually all thymocyte precursor cells can be recovered from the large 'blast' population (e.g. the exps. described in Fig. 5.2-5.4, see also Chapter 2).

These experiments show that, apart from the number of BM cells injected, the recovery of donor cells from the thymus is also dependent on the interval between lethal irradiation and BMT.

Homing of FUBM labeled with Hoechst 33342

In two experiments BM cells from mice treated four days previously with 5-FU were labeled with Hoechst 33342 and injected into mice that were irradiated one day before transplantation. Fig. 6.1B shows the fluorescence distribution of FUBM prior to injection. The unstained cells (channel number 100) were erythroid cells which are very abundant in FUBM. The nucleated cells had a bimodal fluorescence distribution, which was due to the large fraction of cells in S - and G-2/M phase: the right peak (channel number 180) represents cells with about twice the fluorescence intensity (=2nDNA) when compared to that of the left peak (Go/G1, channel number 195). The number of FUBM derived cells that appeared in the thymus is shown in Table 6.1. Per 10^6 cells transplanted 314-480 cells could be recovered from the thymus (irradiated one day before BMT). This is half of that observed after injection of normal BM cells. Extrapolation of the thymus repopulation data by FUBM (Fig. 5.7) to the day of BMT, showed that 0.4 per 10^6 injected BM cells seeded into the thymus (95 % range: 0.06-2.5). Extrapolation of thymus repopulation data by NBM (Fig. 5.1A), resulted in 460 BM cells per 10^6 injected BM cells at the day of BMT. The extrapolation data are compatible with the notion that FUBM is devoid of committed thymocyte precursor cells which was indicated by the delayed thymus regeneration by FUBM (Chapter 5). However, the large discrepancy between the direct homing assay and the functional, but indirect repopulation assay, strongly suggests that the FUBM derived cells present in the thymus early after BMT are not thymocyte precursor cells.

Table 6.2

Homing of ^{51}Chromium and Hoechst 33342 labeled purified stem cells

Organ	Recovered specific radioactivity per organ (% of injected)		Recovery of Hoechst 33342 labeled cells per thymus (% of injected cells)
	Exp. I	Exp. II	
Femur	1.0±0.9	1.8±1.2	
Spleen	5.5±2.6	12.2±2.6	10
Liver	27.7±10	16.9±3.4	
Kidney	3.0±1.8	10.4±2.2	
Thymus	-1.4±1.9	0.07±0.8	0.13 / 0.10

Purified stem cells were isolated and labeled with ^{51}Cr or Hoechst 33342 as described in Chapter 2. In exp I there were 0 day-8 CFU-S and 4 day-12 CFU-S per 100 cells and in exp II there were 4.4 day-8 CFU-S and 7.7 day-12 CFU-S per 100 cells injected. The recipients received 5100, 1700 or 570 cells in exp I and 6400, 2100 or 710 cells in exp II at one day after lethal irradiation. At three hours after BMT the recipients were killed, the organs were removed and the radioactivity per organ was determined. Each datum represents the mean value obtained from 9 mice ± 1 standard deviation and is corrected for background. The total recovery was 42±6 %. One femur comprises about 6 % of the total BM compartment (Smith and Clayton, 1970). The calculated total recovery is then 52±6 %. The radioactivity of faeces and urine at 3 h after grafting was negligible, but after 24 h 20- 44 % of total injected radioactivity could be recovered from filter paper on which the urine was disposed. Lethally irradiated mice also received 4000 or 5000 Hoechst 33342 purified stem cells. After three hours the recipients were killed, the thymus was removed, frozen and cut in 16µm thick sections. The slides were examined by fluorescence microscopy and the number of Hoechst labeled cells in the thymus was counted (less than 5 labeled cells were present). In both experiments the thymuses from three mice were counted.

Homing of purified stem cells labeled with Hoechst 33342 and ^{51}Cr

Hemopoietic stem cells purified by density separation and light-activated cell sorting for both a high density of WGA receptors and H-2K antigens were shown to be enriched in day-12 CFU-S (Visser et al, 1984; Chapter 5). In the previous chapter it was described that the onset of thymus regeneration by such purified stem cells as well as FUBM are both similarly delayed by 3 to 4 days, despite the presence of large numbers of CFU-S. Comparison of the seeding of FUBM and purified stem cells into the thymus at three hours after

transplantation may help answering the question whether these seeded cells are involved in thymus repopulation.

Purified stem cells were isolated and labeled with Hoechst 33342 or ^{51}Cr as described in Chapter 2. When Hoechst 33342 labeled cells were used, thymuses of recipient mice were carefully removed, frozen and the total organ was cut in 16 µm thick sections. The slides were examined by fluorescence microscopy and the number of fluoresceing cells per organ was scored. Very few (i.e. less than 5) fluoresceing cells were present per thymus after injection of 4000-5000 purified stem cells (Table 6.2). In other organs with a larger seeding fraction (e.g. the spleen) fluoresceing cells could easily be scored (data not shown). 570-6400 purified stem cells, labeled with ^{51}Cr were also injected into preirradiated (-3h) mice. Three hours later several organs were removed and the radioactivity per organ was determined (see also Chapter 2). Table 6.2 shows the results of two experiments. The recovery of radioactivity in the spleen and BM is in the same range as that of CFU-S that are recovered in a retransplantation assay (Visser and Eliason, 1983). In the thymus no radioactivity above that of untransplanted, irradiated controls was observed. Titration experiments showed that the detection limit of this assay is about 50 cells. These measurements indicate that purified stem cells do not home to the thymus in significant numbers. FUBM, in contrast, which is also depleted of prothymocytes, is a much richer source of thymus immigrants.

Discussion

The experiments described in this Chapter were performed in order to relate the presence of transplanted cells in the thymus to that of thymocyte progenitors. The extrapolated curve of NBM in Fig. 5.1 leads to a seeding of 400±200 thymocyte precursor cells in the thymus early after BMT. Instead, 1200±400 donor cells were observed in the thymus at 3 h after BMT (Table 7.1). It has been shown that thymus repopulation by donor cells is not related to the time between irradiation and BMT (Boersma, 1982). Therefore, from the extrapolated curve in Fig. 5.1 400±200 prothymocytes would be expected to be present at 3 h after BMT in the thymus of mice that were irradiated 4 days previously. However, only 19±12 donor cells were observed per thymus per 10^6 transplanted cells if the irradiation was given 4 days earlier. This has also been reported by Varlet et al, (1982). These two inconsistencies indicate that

most thymic immigrants consist of other cells than prothymocytes. The low number of thymic immigrants observed in mice that were irradiated 4 days earlier suggests that the prothymocyte enter the thymus later than 3 h after BMT. In addition, this indicates that extrapolation of the thymus repopulation data to a time early after BMT may lead to an erronous outcome.

Extrapolation of the thymus regeneration data suggests that there exists a 1000 fold difference in the number of donor cell in the thymus early after BMT between NBM and FUBM. In the direct homing assay there is only a two fold difference in the number of donor cells in the thymus from NBM and FUBM. This indicates that the migration of donor cells to the thymus early after BMT is not related to the repopulation of the thymus by the donor stem cells and progenitor cells.

Light scatter measurements indicated that the size of most of the thymic immigrants is less than that of thymocyte precursor cells in the BM (Fig. 6.2). The FLS distribution is in agreement with the observation that a significant fraction of the seeded cells are mature lymphocytes and macrophages (Lepault and Weissman, 1981).

Comparison of the results with FUBM and purified stem cells also showed inconsistencies with regard to thymus repopulation and thymus homing. These cell suspensions showed a similar, delayed thymus repopulation kinetics which is characteristic for a depletion of prothymocytes (Chapter 5). Thymus repopulation by these cell suspensions is closely related to the number of grafted day-12 CFU-S. Less than 0.13 % of labeled (Hoechst 33342 or ^{51}Cr) purified stem cells could be detected in the thymus at 3 h after BMT (in the direct homing assay: 2 to 4 cells per 4000 to 5000 injected purified stem cells). This is in agreement with earlier observations that CFU-S do not home to the thymus (Boersma, 1982). 310 to 480 per 10^6 (i.e. 0.03 to 0.05 %) FUBM cells transplanted into lethally irradiated recipients seeded into the thymus. However, the concentration of day-12 CFU-S (day-12 CFU-S) in FUBM was less than that of the purified stem cells (Chapter 5). In order to obtain similar thymus repopulation kinetics as by purified stem cells more cells had to be transplanted. This indicates that the number of cells that have entered the thymus 3 hours after BMT is not correlated with the number of transplanted day-12 CFU-S and thus with thymus repopulation, but with the total number of cells transplanted. These results confirm the previous conclusion that early immigrants are not prothymocytes.

Because the direct homing assay measures cells that appear to be unrelated

to thymocyte precursors, experiments were designed to determine functionally the time of entry of thymocyte precursors. These are described in the next Chapter.

Conclusion

The majority of BM cells that are present in the thymus early after irradiation are not thymocyte precursors.

Chapter 7

THE ARRIVAL OF THYMOCYTE PRECURSORS IN THE THYMUS AFTER BONE MARROW TRANSPLANTATION

Introduction

In the previous Chapter it was described that the number of transplanted BM cells that were present in the thymus at 3 h after irradiation is not related to thymus repopulation kinetics. The earliest donor BM derived thymocytes can be detected around 8-12 days by various methods applied to thymus cell suspensions (Kadish and Basch, 1976; Stutman, 1977; Boersma et al, 1981; Ceredig and MacDonald, 1982; Sharrow et al, 1983; Hirokawa et al, 1985). In one report donor thymocytes were detected as early as 6 days after BMT (Boersma et al, 1982). Thus, thymocyte precursor cells must enter the thymus before the sixth day after BMT.

So far all investigators determined the appearance of the progeny of the thymocyte precursor. These results do not provide sufficient information on the time of entrance of thymocyte precursors. Determination of the time after BMT that precursor cells, committed to the T cell lineage (prothymocytes) enter the thymus is important for the study of the earliest differentiation events in the T cell lineage. If the time at which prothymocytes enter the thymus were known, it would become possible to characterize these cells more closely, to define their port of entry and to study the first differentiation steps.

In this Chapter attempts to determine the time of entry of thymocyte precursors are described. They employ procedures involving two irradiations (Fig. 7.1). C3H mice were lethally irradiated and transplanted with C3AK BM cells. At various times after the BMT, mice were reirradiated on the thymus. Thymus repopulation was determined subsequently between 15 and 35 days after the BMT. If prothymocytes entered the thymus before reirradiation of the thymus, thymus repopulation should be delayed when compared to that of mice that were not reirradiated. By varying the interval between BMT and the second irradiation it should be possible to determine when precursor cells begin to

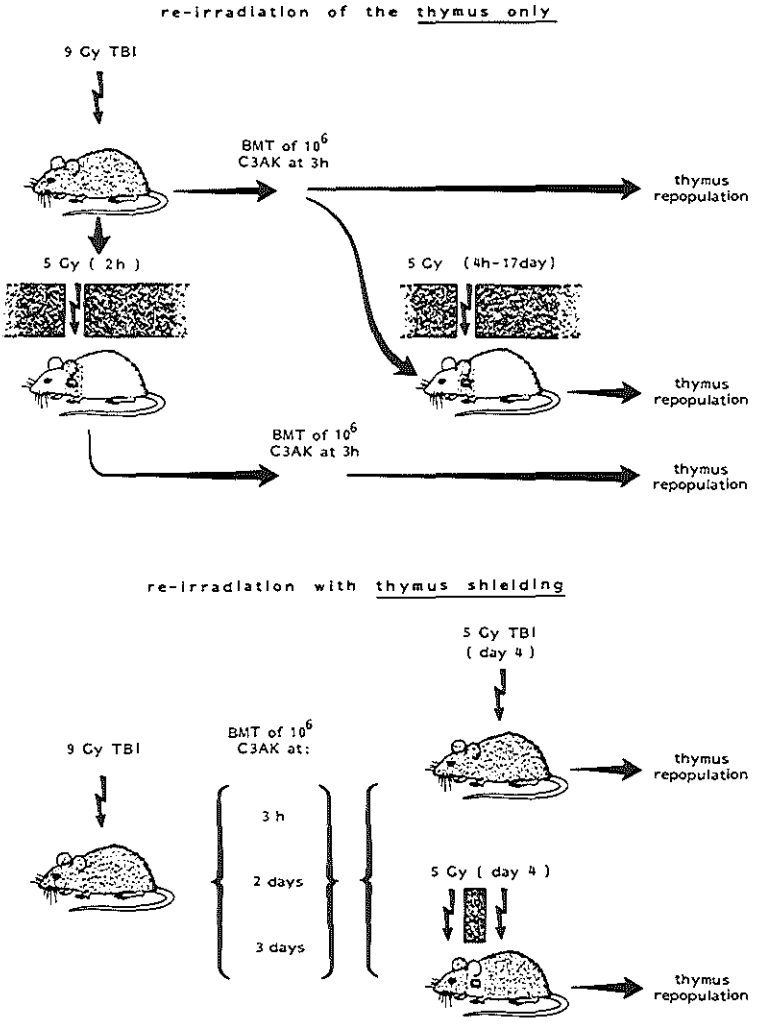


Fig. 7.1 Outline of the reirradiation experiments. See text for explanation

enter the thymus. In a second set of experiments lethally irradiated and BM transplanted mice were reirradiated with or without thymus shielding at various times after BMT. When prothymocytes are present in the thymus at the time of reirradiation, thymus repopulation can be expected to start earlier in mice which were shielded on the thymus than in those which were not shielded.

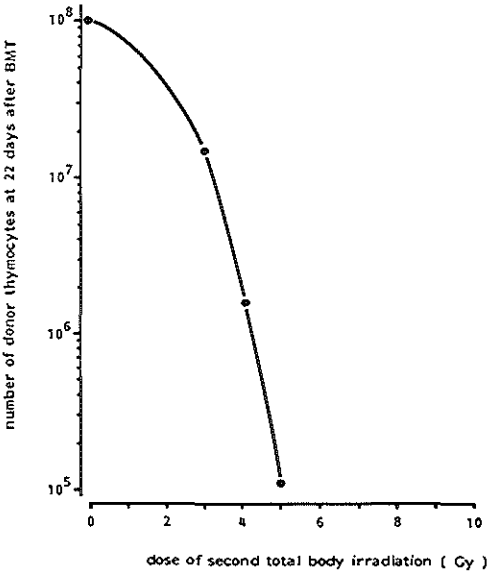


Fig. 7.2 Relationship between the dose of the second irradiation and thymus repopulation at 22 days after BMT. Lethally irradiated and BM transplanted mice were reirradiated on day 4 with various doses of X rays (see Chapter 2). At 22 days after BMT the number of donor thymocytes was determined.

Preliminary experiments

In order to determine the appropiate dose for the second irradiation, mice were lethally irradiated, transplanted with 10^6 C3AK BM cells and whole body reirradiated (TBI) at day 4 with increasing doses of 6 MV X rays. The number of donor type thymocytes was determined 22 days later. Without reirradiation, the thymus is regenerated at this time (Fig. 5.1). Fig. 7.2 shows the relationship between the dose of the second irradiation and the number of donor type thymocytes. The maximum dose of irradiation that is tolerated was 5 Gy, which caused a reduction of the number of donor type thymocytes at day 22 by a factor of 1000. Reirradiation with 6 Gy caused some mortality and all mice reirradiated with 7 Gy died in the second week after the first irradiation. Therefore, a radiation dose of 5 Gy was selected for reirradiation.

The effectiveness of thymus shielding with a leaden pole (Ø=12 mm; 10 cm high) was tested by counting the total number of thymocytes at various times after 5 Gy whole body irradiation of normal mice with or without thymus

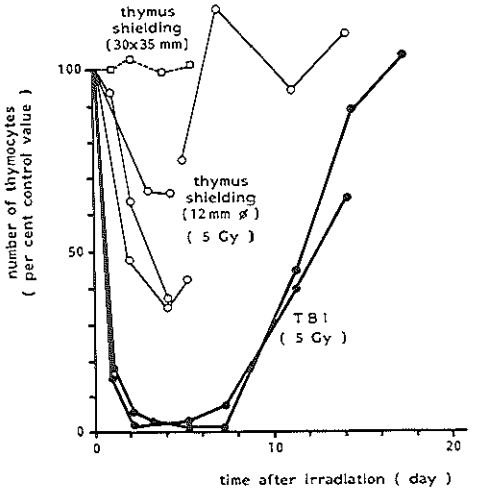


Fig. 7.3 Effectivenes of thymus shielding. C3H mice were irradiated with 5 Gy whole body irradiation (●) or irradiated and shielded on the thymus with a bar of 12 mm diameter (○) or with a block of 3x3.5 cm (□).

shielding. Two days after whole body irradiation virtually all thymocytes were eliminated (Fig. 7.3). Thymus repopulation started 7 days after the irradiation and the thymus was repopulated at about 16 days after TBI (Fig. 7.3). Thymus shielding resulted in much less reduction in the number of thymocytes than that following whole body irradiation (Fig. 7.3). At four days after irradiation with thymus shielding a 35-65 % decrease in the number of thymocytes was observed and the thymus had a normal cellularity as early as 7 days after irradiation. The shielded thymuses showed focal necrosis that was present throughout the thymus (data not shown). Flow cytometric analysis of thymocytes from mice that were irradiated with thymus shielding showed no difference in cell size, and in the density distribution of Thy-1, H-2K and Ly-2 antigens when compared to normal thymocytes (data not shown). These results indicated that thymus shielding with a circular field of 12 mm in diameter was partially protective. It caused a 35 to 65 % reduction in the number of thymocytes but there appeared to be no selective advantage for any of the thymocyte subpopulations. With the use of a larger shield (3.5 cm x 3.0 cm, 10 cm high) and irradiation of normal mice with 5 Gy, no decrease in the number of thymocytes was observed. (Fig. 7.3). This indicate that some radiation was delivered under the circular shield (Ø=12 mm).

The fraction of the delivered dose under the shielded area (Ø=12mm) was measured and found to be about 10 % (see Chapter 2). Thus, 0.5 Gy was delivered under the shielded area. The sensitivity of prothymocytes to in vivo gamma irradiation has been determined previously (Do=0.85 Gy; Boersma, 1982). From his data it can be calculated that after an exposure to 0.5 Gy, 50 % of the precursor cells should be viable according to in the thymus repopulation assay. The sensitivity of the thymus repopulation assay was determined by Boersma (1982). He calculated that a two fold difference in the number of thymocyte precursors in a BM transplant caused a significant delay of 1 day in thymus repopulation kinetics. Therefore, the results of the thymus shielding experiments have to be corrected for the death of 50 % of the prothymocytes or in terms of thymus repopulation corrected for a 1 day delay. Although the correction could have been circumvented with the use of a larger shield, it was considered better to use the smallest possible shielding area, because this allows for more complete elimination of prothymocytes in the extrathymic sites.

Thymus repopulation after lethal irradiation, bone marrow transplantation and a second irradiation on the thymic area only

Reirradiation of the thymus after lethal irradiation and BMT has the advantage that it does not affect the bone marrow and the spleen. It should kill the thymocyte precursors that are present in the thymus which results in a delayed thymus repopulation. Fig. 7.4 shows the means of three experiments in which lethally irradiated and BM transplanted mice were reirradiated on the thymus 1 hour before BMT or 1 h or 1, 2, 3 or 4 days after BMT. The mice that were reirradiated on the thymus at 1 h and 1 d after BMT contained similar numbers of donor thymocytes at all times post BMT as the control mice (not reirradiated mice and mice reirradiated 1 h before BMT). The observation that reirradiation at these times does not affect thymus repopulation indicates that thymus precursor cells are absent from the thymus until 1 day post BMT. The number of donor thymocytes at 14 to 19 days post BMT in the mice reirradiated on the thymus 2 to 4 days after BMT was significantly lower than control values (day 14: $p<0.01$; day 18: $p<0.01$; day 19: $p<0.05$). This indicates that significant numbers of thymocytes start to enter the thymus from the second day after BMT onwards.

The results of one experiment are shown in detail in Table 7.1. The table

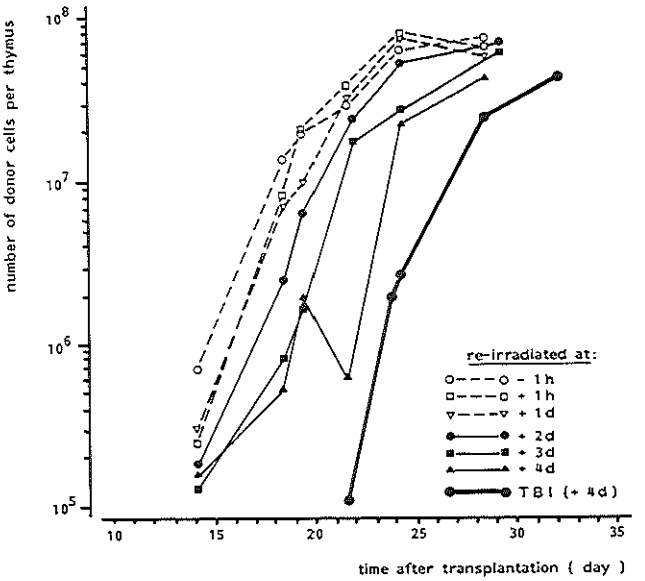


Fig. 7.4 Thymus repopulation by mice reirradiated on the thymus. Lethally irradiated and BM transplanted mice were reirradiated on the thymus 1 h before BMT (o), or 1 h (Δ), 1 d (□), 2 d (●), 3 d (▲) and 4 d (■) after BMT. ●—● represents thymus repopulation after total body irradiation at day 4. Thymus repopulation was determined as described in Chapter 2. The mean results of 3 experiments are shown. The Wilcoxon-Mann-Whitney test was used to disprove the zero-hypothesis that the data at day 14 to 19 of the groups reirradiated at 2, 3 and 4 days after BMT have the same frequency distribution as the control values: day 14 p 0.01; day 18: p 0.01; day 19: p 0.05. The test showed that at these time points the groups reirradiated 2, 3, and 4 days after BMT differed significantly from non-reirradiated controls. The W-M-W test could not be applied to the results at day 21 for reirradiation at 4 days after BMT, because this value represented the mean of only two determinations.

Thymus repopulation by mice that were lethally irradiated (Leth. irr.), transplanted with 10^6 BM cells and reirradiated on the thymus (Thymus irr.) 1 h before and 1 h, 1 d, 2 d, 3 d, 4 d and 7 d after BMT. At the times indicated the recipients were killed and the number of donor derived thymocytes was determined as described in Chapter 2. The Wilcoxon-Mann-Whitney test was used for the analysis of a significant difference between the data at day 15 to 19 of the groups of mice reirradiated at 2, 4 and 7 days after BMT and the ones that were reirradiated earlier or those that received no reirradiation. *: p 0.05; £:p 0.01.

Table 7.1

Thymus repopulation in lethally irradiated, bone marrow transplanted mice that were reirradiated on the thymus only

Treatment	Day of assay after BMT	Number of donor and host cells per thymus $x10^6$	% Donor cells	Number of donor cell per thymus $x10^6$
Leth. irr. (0h)	15	32	1	0.32
BMT (3h)	17	57.2	11	7.4
	19	43	65	28.0
	21	49	88	43.1
	24	71	96	68.2
Leth. irr. (0h)	15	1.54	10	0.15
Thymus irr.(2h)	17	11.6	65	7.5
BMT (3h)	19	21.9	91	20.0
	21	41.5	90	37.4
	24	63	97	61.1
Leth. irr. (0h)	15	5.6	5	0.28
BMT (3h)	17	9.6	40	3.8
Thymus irr.(4h)	19	22.2	91	20.2
	21	45	95	42.8
	24	48	98	47
Leth. irr. (0h)	15	1.9	30	0.57
BMT (3h)	17	10.9	52	5.7
Thymus irr.(1d)	19	18.3	84	15.4
	21	34	92	31.3
	24	62	95	58.9
Leth. irr. (0h)	15	0.25	10	0.025 £
BMT (3h)	17	8.8	18	1.6 *
Thymus irr.(2d)	19	9.9	81	8.0 *
	21	15.6	91	14.2 *
	24	33	96	31.7
Leth. irr. (0h)	15	0.36	4	0.014 £
BMT (3h)	17	3.6	1	0.036 *
Thymus irr.(4d)	19	2.3	18	0.41 *
	21	0.8	81	0.64 *
	24	3.0	93	2.8
Leth. irr. (0h)	15	0.44	5	0.022 £
BMT (3h)	17	5.5	1	0.055 *
Thymus irr.(7d)	19	17.6	73	12.8 *
	21	26.2	85	22.3
	24	54.6	97	53

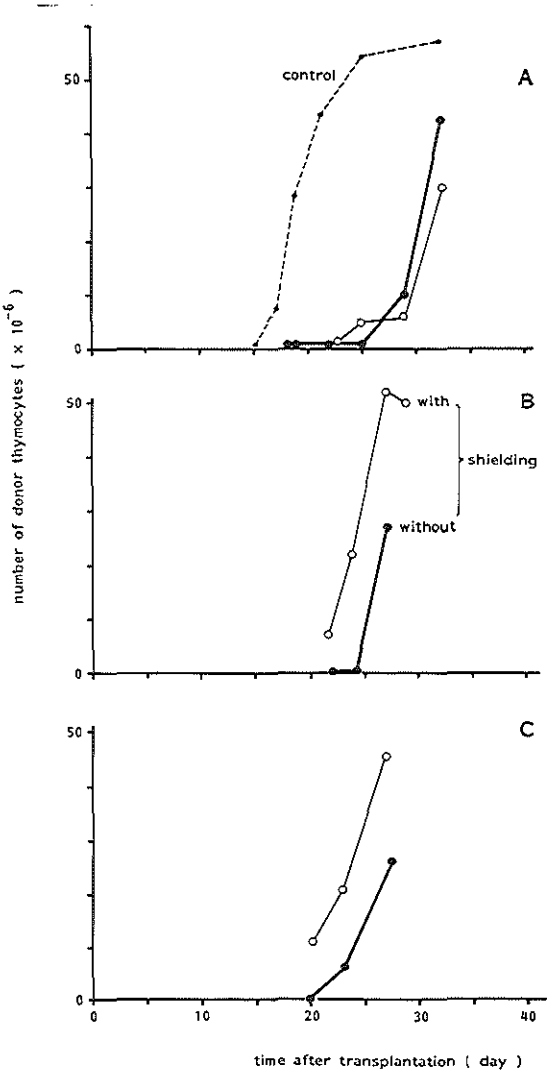


Fig. 7.5 Thymus repopulation in mice transplanted 3 days (A), 2 days (B) or immediately after lethal irradiation (C) with 10^6 C3AK BM cells. The recipients were reirradiated on the fourth day with 5.0 Gy with (o) or without (•) thymus shielding or were not reirradiated (•—•).

lists part of the data incorporated in Fig. 7.4: a delay in thymus repopulation occured in the groups that were reirradiated on the thymus at days 2 and 4. However, reirradiation of the thymus at 7 days after BMT caused less delay in thymus generation as compared to that at 4 days after BMT. This may be related to the presence of larger numbers of intrathymic precursors cells at day 7 after BMT (see section on intrathymic precursor cells).

Thymus repopulation after lethal irradiation, bone marrow transplantation and a second irradiation with or without thymus shielding

The kinetics of thymus repopulation after lethal irradiation, grafting of 10^6 BM cells on day 1 and 5 Gy total body reirradiation on day 4 is shown in Fig. 7.5A. The first donor thymocytes can be detected between 25 and 30 days after BMT. This is more than 10 days later than in mice that were not reirradiated. Boersma et al (1981a) showed that a 10 day delay in thymus repopulation corresponds to an, at least 200 fold difference in the number of BM cells transplanted. This is in accordance with the results in Fig. 7.2 and indicates that very few thymocyte precursor cells are left in the body after the second irradiation.

Lethally irradiated mice were transplanted after 1 h, 2 and 3 days with 10^6 BM cells and reirradiated with or without thymus shielding on the fourth day. The mice that were reirradiated with the thymus shielded at one day after BMT showed no difference in thymus repopulation when compared to mice that were reirradiated without thymus shielding (Fig. 7.5A). The mice that were reirradiated with thymus shielding 2 and 4 days after BMT showed an enhanced thymus repopulation as compared to mice that were reirradiated without thymus shielding (Fig. 7.5B and C). These results are in accordance with those reported in the previous paragraph in showing that one day after BMT thymocyte precursor are absent from the thymus and that they are present at two and four days after BMT.

Presence of thymocyte precursors in the bone marrow shortly after bone marrow transplantation

The absence of migration of prothymocytes into the thymus during the first

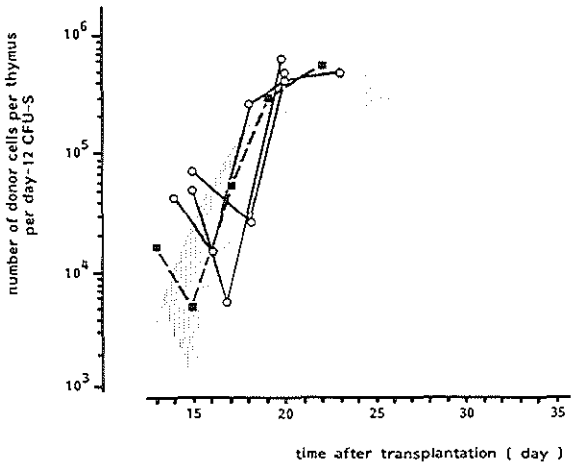


Fig. 7.6 Thymus repopulation by BM cells which were obtained from lethally irradiated C3H mice transplanted with $30x10^6$ C3AK BM cells. At 24 (o) and 36 h (■) after BMT BM cells were collected from the femura and tibiae and injected into lethally irradiated C3H recipients. Thymus repopulation is expressed per day-12 CFU-S transplanted and compared to that by NBM which is represented by the shaded area (see Fig. 5.1B).

day post BMT would suggest that prothymocytes, delivered with the BM graft, home elsewhere. The most obvious site is the bone marrow. In order to test this hypothesis BM was obtained from lethally irradiated C3H mice at 24 and 36 hours after grafting of $3x10^7$ normal C3AK BM cells and tested for thymus repopulating ability. It was found (Fig. 7.6) that between 13 and 16 days post BMT there was a higher number of donor derived cells than after injection of NBM (compare with Fig. 5.1B). These data are in agreement with the hypothesis that at 24 to 36 hours after transplantation, the BM contains a relative abundance of prothymocytes. The decline in donor cells observed thereafter probably reflects the time required for the production of prothymocytes by pluripotent stem cells.

Intrathymic thymocyte precursors

It is well known from thymus regeneration studies that after lethal irradiation, the thymus first involutes, but that between 7 and 21 days many host derived thymocytes appear, reaching maximum values 13 to 15 days after irradiation. (Kadish and Basch, 1975; Boersma et al, 1981a; Ceredig et al,

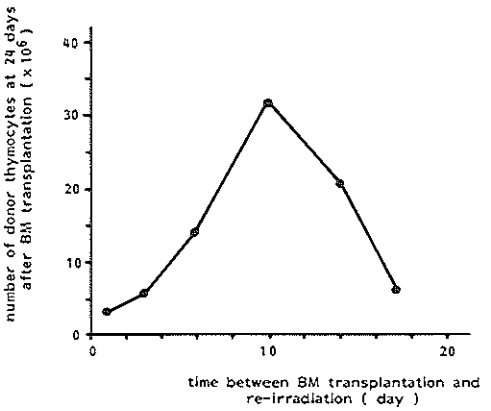


Fig. 7.7 Thymus repopulation in regenerating mice that received 5 Gy total body irradiation at various times after BMT. At 24 days after BMT the number of donor thymocytes was determined as described in Chapter 2.

1982). The experiments in which the thymus was reirradiated showed that host thymus repopulation can be abolished by reirradiation of the thymus with a dose of 5 Gy (Table 7.1: the difference between the first and third columns). From this observation it can be concluded that the host derived thymocytes stem from intrathymic precursor cells and that the precursor cells, which survived 9.0 Gy, were killed when an additional dose of 5 Gy was given to the thymus.

In the experiments in which mice were reirradiated with thymus shielding, control groups received a total body reirradiation. Thymus repopulation was determined in these groups also. It was found that prolonging the interval between BMT and reirradiation to 10 days led to an increase in the number of donor cells at 24 days after BMT. A further increase of the interval between BMT and reirradiation led to a progressive decline in the thymus repopulation at day 24. This decline is due to the shorter interval between reirradiation and determination of thymus cellularity, which is insufficient for full repopulation (10 and 7 days respectively).

The increase in the number of donor cells after reirradiation at 6 or 10 days after BMT can be explained as follows: Reirradiation at any time after BMT kills the same fraction of PHSC in the bone marrow compartment. This cannot cause a difference in the number of thymocytes at 24 days or later. The results, therefore, indicate that the difference in thymus repopulation between the groups that were reirradiated at 2 or 10 days after BMT is caused by the development of another compartment of thymocyte precursors after the second day

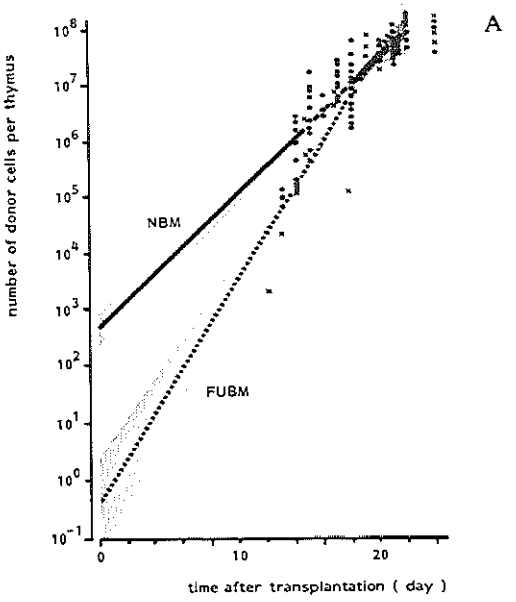
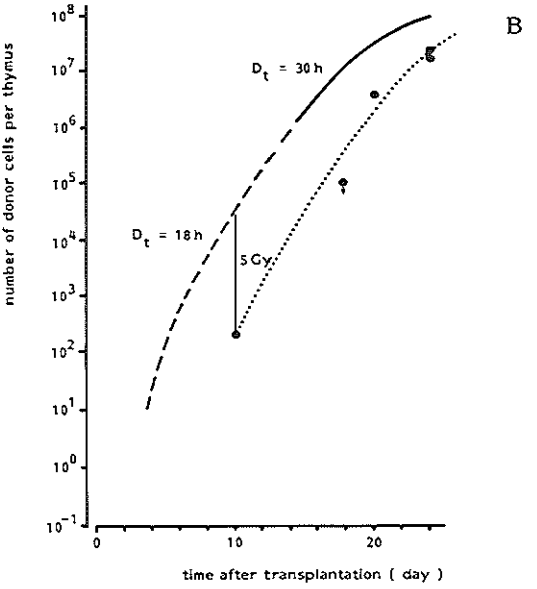


Fig. 7.8 A. Thymus repopulation of lethally irradiated C3H mice injected with 10^6 C3AK F1 BM (•) cells or 10^6 FUBM (x). Regression lines were calculated from the data points during the rapid growth phase. 95 % confidence limits for the regression lines are shown. The doubling times were 30.4 h (normal BM) and 18.4 h (FUBM). B. Thymus repopulation of lethally irradiated mice injected with 10^6 BM cells (————). The doubling time changes from less than 18 h at the initiation of repopulation to 30 h around 16 days after BMT. The dotted line represents thymus repopulation after 5 Gy whole body reirradiation at day 10. * represent the measurements. The symbols with arrows represent values calculated from the number of thymocytes and the detection limit (1 %) of the flow cytometric assay. In these cases no donor thymocytes were observed. The difference between the two curves at day 10 corresponds with a Do=500/(ln(5/1000)/ln0.37)=0.94 Gy.

after BMT. These precursor cells are different from the PHSC. Reirradiation of the thymus only at 7 days after BMT also caused less delay in thymus regeneration when compared to reirradiation of the thymus at day 4. (Table 7.1, last column). This indicates that the other compartment of thymocyte precursor cells is located in the thymus.

There are few data on the development of donor precursor cells in the thymus. In the previous Chapter it was shown that the number of BM cells in the thymus at 3 h after BMT is not related to thymus repopulation kinetics and the



reirradiation experiments, described in this Chapter show that thymus repopulation starts from the second day onwards. Furthermore, repopulation of the thymus by donor cells is usually measured when more than 10^6 donor (Thy-1.1 positive) cells are present in the thymus. At this time it can be expected that the measured growth rate of the total thymocyte population is less than at the initiation of the repopulation. The decreasing growth rate with time is probably due to a progressive increase in cell loss, i.e. death of cortical thymocytes (see also Fig. 5.3). Log-linear regression analysis of data obtained after transplantation of normal BM cells, therefore, describes thymus repopulation erronously during the first two weeks after BMT (Fig. 7.8A). The doubling time of intrathymic thymocyte precursor cells during the first two weeks after BMT must be less than measured during the third week for the total donor thymocyte population (30 hours).

Thymus repopulation after injection of FUBM or purified stem cells is faster than after injection of normal BM cells (Figs. 5.10 and Fig. 7.8A). Thymus repopulation by the first two cell populations stems from pluripotent stem cells but not from prothymocytes. Since there is a difference in the growth rate between thymocytes derived from prothymocytes and those derived from PHSC (Fig.

5.3), as measured between 16 and 21 days, it is assumed that the doubling time of the thymocyte population after injection of FUBM (18 hours) reflects the initial doubling time of the intrathymic precursor population more closely.

In Fig. 7.8B thymus repopulation by donor cells is presented with a doubling time of 30 hours when more than 10^6 donor cells are present in the thymus. In the early repopulation phase, the assumed shorter doubling time of 18 hours is depicted. With this repopulation kinetics the higher number of donor thymocytes after whole body reirradiation at day 10 as compared to that at day 2 (Fig. 7.7) can be explained: At day 2 there are few precursor cells in the thymus. Reirradiation results in the death of virtually all cells. After reirradiation at day 10, however, a substantial number survive which results in a higher number of thymocytes at day 24. This is represented by the dotted line. From the difference between the two curves a Do of the precursor cell population can be calculated: 0.94 Gy (see legend Fig. 7.8B).

The host intrathymic precursor cells have been reported to be radioresistant (Kadish and Basch, 1975; Sato and Sakka, 1969; Sharp and Watkins, 1981). The high Do values calculated by these authors were obtained either by measuring the surviving fraction of nucleated cells, which could have been cells other than thymocytes or their precursors, or by determination of the repopulation of the thymus. When the latter method is used one has to know the doubling time of the precursor cell population during the irradiation. However, this cannot be determined accurately at the present time and therefore the doubling time of the total thymocyte population was used to estimate the Do value of the thymocyte precursor cell. The same argument used above applies to host thymocyte regeneration: The doubling time of the total thymocyte population is probably longer than that of the precursor cell population. When it is assumed that a doubling time of 18 hours corresponds with that of the precursor cells, the following Do can be calculated. After lethal irradiation there are $31(\pm4)\times10^6$ thymocytes at day 15. Lethal irradiation immediately followed by irradiation of the thymus with 5 Gy results in only $8.1(\pm8)\times10^5$ thymocytes at 15 days. With a doubling time of 30 h the Do of the thymocyte precursor is 137 rad and with a doubling time of 18 h the Do is 90 rad. These calculations argue against the presence of radioresistant precursor cells in the thymus.

Discussion

The experiments presented in this Chapter can be summarized as follows:

Thymus shielding with a leaden pole of 12 mm diameter was partially protective (Fig. 7.3). It was calculated that about 50 % of the thymocyte precursor cells located in the thymus survived reirradiation with thymus shielding. Reirradiation of the thymus at 1 hour and 1 day after BMT did not change the kinetics of thymus repopulation, while reirradiation at 2 to 4 days caused a delay in thymus repopulation (Fig. 7.4; Table 7.1). It was concluded that thymocyte precursor cells begin to enter the thymus on the second day post BMT. The experiments in which the mice were reirradiated with thymus shielding confirmed this conclusion: reirradiation with thymus shielding at 1 day after BMT did not show an enhanced thymus repopulation when compared to that after reirradiation without thymus shielding (Fig. 5A). However, reirradiation with thymus shielding at a later time after BMT did show an earlier onset of thymus repopulation, indicating that thymocyte precursor cells were entering the thymus by that time. These results show that all thymocytes are derived from precursor cells that do not home directly to the thymus, in contrast to the direct homing of BM cells to the thymus as suggested by the experiments of Lepault and Weissman (1981).

The period of 8-12 days between BMT and the appearance of the first donor derived thymocytes represents both the time necessary for migration and the time needed for differentiation and proliferation. Since prothymocytes begin to enter the thymus on the second day post grafting (Fig. 7.4), 2 days is probably the minimum time necessary for migration into the thymus. This leaves 6 to 10 days for differentiation and proliferation before significant numbers of donor cell can be detected. In a recent publication it was shown that after intrathymic (i.t.) injection of 10^5 BM cells it took 14 days before donor derived cells could be detected (Goldschneider et al, 1986). Dose-response curves (the number of donor thymocytes at 17 days after grafting versus the number of BM cells injected; Goldschneider et al, 1986) indicated that after i.t. injection of saturating numbers of BM cells, significant numbers of donor thymocytes may be detected as soon as 8 days after i.t. injection. This is within the range estimated from the data obtained after intravenous BMT. It has been suggested that the proliferation of host-derived thymocyte precursors may inhibit the proliferation of the donor cells until the wave of host cells is completed (Greiner et al, 1984). The reirradiation experiments exclude this possibility because reirradiation virtually abolishes host repopulation, while donor thymus repopulation is unaffected (Fig. 7.4 and Table 7.1). These results indicate that the 6 to 10 days between the immigration of prothymocytes

and the detection of thymocytes is only required for differentiation and proliferation of the precursor cells.

In the experiments in which BM cells obtained from mice 24 to 36 hours after lethal irradiation and BMT were retransplanted into lethally irradiated recipients, a biphasic thymus repopulation was observed: Between 13 and 16 days there were more donor thymocytes as compared to injection of normal BM cells. This was followed by a decline after which the rapid growth phase started. These results suggest that the BM soon after BMT contains many prothymocytes as compared to the day-12 CFU-S. The dip in the thymus repopulation curve indicates that during the early repopulation phase there occurs already a considerable loss of thymocytes. This strengthens the notion that the doubling time of the precursor population must be less than that of the total population. The dip also indicates that it takes several days before host type prothymocytes are produced from PHSC in the bone marrow.

Intrathymic thymocyte precursor cells, the progeny of prothymocytes in the thymus, were found to produce very few thymocytes after irradiation with 14 Gy. Since, it is quite likely that the doubling time of these precursor cells, early after irradiation and BMT, is higher than that of the total thymocyte population it could be calculated that they have a Do which is close to or somewhat less than 0.9 Gy. The CFU-S (Do=1.01 Gy), prothymocyte (Do=0.85)(Boersma, 1982) and most peripheral lymphocytes (Do=0.50-1.20 Gy; Sharp and Watkins, 1981) have a similar radiosensitivity. This indicates that the intrathymic precursor cells are not radioresistant precursor cells in contrast to previous reports by others (Sato and Sakka, 1969; Kadish and Basch, 1975; Sharp and Watkins, 1981; Boersma, 1982; Ceredig and MacDonald, 1982; Sharrow et al, 1983).

The donor derived thymocyte precursor cells in the thymus could be detected to be present in relatively large numbers at day 10 after BMT. In the results section it was calculated that these cells have a Do which is at most 0.94 Gy. The repopulation kinetics (the precursor cells give rise to maximal numbers of thymocytes 14-16 days later) and the Do values (host type cells: 0.9 Gy; donor type cells: 0.94 Gy) suggest that the two cell types are similar.

Conclusions

1. The first prothymocytes enter the thymus on the second day after BMT and continue to do so at the third and fourth day.

2. The precursor cells of host thymocytes are present in the thymus, but they are not radioresistant cells. Their Do is calculated as approximately 0.9 Gy.

3. Donor derived intrathymic thymocyte precursor cells are present in relatively large numbers at 10 days after BMT as indicated by the survival of significant numbers after irradiation with 5 Gy. They have a calculated Do of 0.94 Gy.

Chapter 8

GENERAL DISCUSSION

Organization of hemopoietic stem cells

The day-12 CFU-S

The day-12 CFU-S, the hemopoietic stem cell that produces spleen colonies around day 12 after lethal irradiation, was found to coenrich with cells capable of protecting mice from hemopoietic death following lethal irradiation (Visser et al, 1984). This suggests that the day-12 CFU-S in the enriched cell suspension are pluripotent hemopoietic stem cells. However, not all cell populations, containing day-12 CFU-S, protected lethally irradiated mice to the same extent. For instance, strongly Rh123 labeled BM cells protected lethally irradiated mice less than weakly labeled Rh123 BM cells, expressed day-12 CFU-S (Fig. 4.5). This indicates either that a subpopulation of day-12 CFU-S (weakly Rh123+) is the cell responsible for radioprotection or that the radioprotective cells are different from the day-12 CFU-S, but coenrich with these cells in many purification procedures. Strong evidence for the notion of qualitative differences between the two BM fractions obtained after separation on Rh123 fluorescence comes from Bertoncello et al. (1985). They showed that the weakly Rh123 labeled fraction contained 38 % of the day-13 CFU-S, but also 92 % of the precursor cells that can give rise to CFU-C2 at 13 days after BMT (a measure of marrow repopulating ability) and 77 % of the platelet precursors (platelet repopulating ability). In contrast, the brightly Rh123 labeled cells contained 62 % of the day-13 CFU-S, but only 8 % marrow repopulating ability and only 23 % platelet repopulating ability. These results are in agreement with those presented in this thesis, which indicate that the hemopoietic stem cells in the fraction weakly labeled with Rh123 are qualitatively different from those that incorporate more Rh123.

The uptake of Rh123 is higher in cycling than in non-cycling cells (Cohen et al, 1981; Darzynkiewicz et al, 1981; James and Bohman, 1981). Rh123 is a

supravital dye which accumulates exclusively in mitochondria. Therefore it can be concluded that the number of mitochondria per cell type increases during the cell cycle. Day-7-9 CFU-S from NBM are all contained within the fraction brightly labeled with Rh123, but only 10 % of these cells are cycling (Visser et al, 1977; Johnson and Nicola, 1984; Chertkov and Drize, 1984). The high uptake of Rh123 by day-8 CFU-S as compared to the other BM cells must therefore be caused by other factors than an active cell cycle. It has been reported that putative hemopoietic stem cells have a low content of mitochondria (van Bekkum et al, 1971). This suggests that the number of mitochondria as well as the Rh123 uptake increases with the differentiation of hemopoietic stem cells.

Culture of BM cells in the presence of SAF (IL-3), which causes stem cell proliferation, resulted in a diminished capacity, per day-12 CFU-S, to protect mice from the development of bone marrow failure (Fig. 3.4B). Microscopical examination of the day-12 spleen colonies from SAFBM revealed that one third of the spleen colonies were desintegrating colonies which contained many (mature) erythroid cells. NBM produced only few of these desintegrating spleen colonies (Table 3.2). These observations indicate that the day-12 CFU-S of SAFBM produces higher numbers of differentiated stem cells as compared to the day-12 CFU-S of NBM and that therefore the day-12 CFU-S of SAFBM and NBM are different stem cells. As to the nature of the more differentiated stem cells produced in SAFBM, it is likely that they belong to the population of erythroid burst- forming units (BFU-E).

An important conclusion from these experiments is that the number of day-12 CFU-S in a BM graft, which has been subject to separation procedures or in vitro culture, is not representative for the capacity to protect lethally irradiated mice. This indicates that either day-12 CFU-S are a heterogenous population or that there is yet another, more immature cell, the pre-CFU-S that is responsible for survival after lethal irradiation. In other studies a discrepancy between the number of day-12 CFU-S injected and the number of progenitor cells derived from the graft was also observed: day-1 FUBM, per day-12 CFU-S injected, gave rise to at least 5 times more day-12 CFU-S in the spleens and 10 times more day-10 CFU-S in the femur when compared to NBM (Hodgson and Bradley, 1979). A similar difference between the number of injected day-12 CFU-S from FUBM and NBM was observed for the number of megakaryocytes in the spleen (Jones et al, 1980). These results led Hodgson and Bradley (1979) to propose that there exists a pre-CFU-S in the BM whose offspring can produce day-12 and day-8 CFU-S. An in vitro colony was described, the HPP-CFC (high proliferative

Table 8.1

Characteristics of day-12 CFU-S, day-8 CFU-S and prothymocytes

Characteristic	Day-12 CFU-S	Day-8 CFU-S	Prothymocyte
1. Presence in NBM	++	++	++
2. Presence in FUBM	+	-	-
3. Presence in SAFBM	+	+++	-
4. Presence in early RBM	+	+	++
5. Presence in day-7 RBM	+	+	-
6. Presence in long-term BM cultures	N.D.	+	-
7. Presence in spleen	+	+	-
8. Presence in fetal liver	+	+	±
9. Sensitivity to in vitro gamma irradiation (Do)	1.20	1.01	0.85
10. Density of H-2K antigens	++	+	+
11. Density of Thy-1 antigens	+	+/-	+
12. Uptake of Rhodamine 123	+-- ++	++	++

Derived from 1.: Fig. 5.1, Table 3.1; 2: Fig. 5.7 and 5.8, Table 5.2; 3: Fig. 3.5 and 3.6, Table 3.2; 4: Fig. 7.6; 5-8: Boersma, 1982; 9: Boersma, 1982, Visser, personal communication; 10: Figs. 4.1, 5.1-5.4, Tables 4.1 and 5.1; 11: Figs. 4.2 and 4.3, Table 4.3; 12: Figs. 4.4 and 5.6, Table 4.4. Early RBM is regenerating BM from mice injected 1 to 2 days previously with 20 to 30×10^6 BM cells.

potential-colony forming cells), whose presence in a BM graft corresponded more closely with the ability of a BM graft to generate progenitor cells in the femur of a recipient mouse at 13 days post-BMT (marrow repopulating ability) (Bradley et al, 1980) than the number of CFU-S in that graft. The HPP-CFC can be stimulated by growth factors derived from human placenta conditioned medium (HPCM) and pregnant mouse uterus extract (PMUE=M-CSF) and is resistant to the cytotoxic action of 5-FU (Bradley et al, 1980; Baines et al, 1981). Other studies indicate that the growth factor present in HPCM is probably identical to

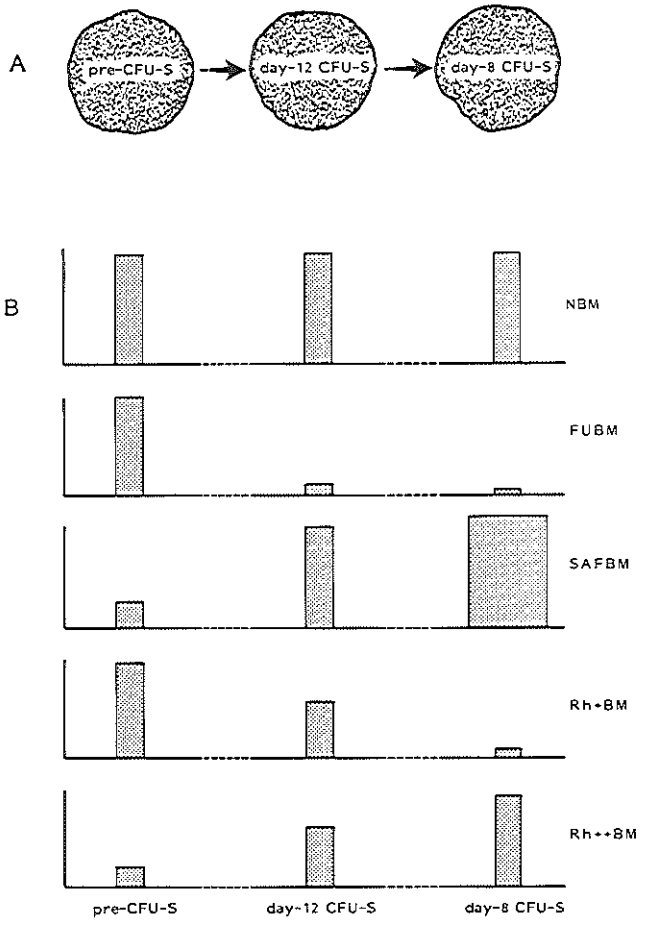


Fig. 8.1 A. Relationship of early hemopoietic stem cells. B. Relative content of hemopoietic stem cells in the BM cell suspensions used in this study.

a stimulator derived from a human bladder carcinoma line, called hemopoietin-1 (Bartelmez and Stanley, 1985). This factor was shown to act on a more primitive cell than the one stimulated by IL-3 (SAF)(Bartelmez and Stanley, 1985; Bartelmez et al, 1985).

Fig. 8.1 depicts one of the likely relationships of the pre-CFU-S, the day-12 CFU-S and the day-8 CFU-S. The relative number of these stem cells in NBM, FUBM and SAFBM and in BM cells fractionated on their Rh123 content is also shown. FUBM is relatively enriched in the pre-CFU-S and contains less mature hemopoietic stem cells as compared to NBM. SAFBM, on the other hand,

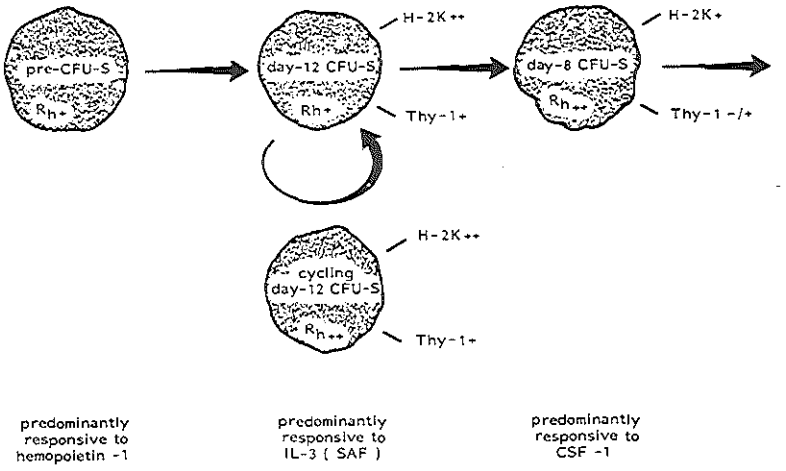


Fig. 8.2 Relationship of early hemopoietic stem cells based on the results of this study. Responsiveness to hemopoietin-1 is derived from Bartelmez et al, 1985.

contains less pre-CFU-S and is enriched in day-8 CFU-S. Rh123+ BM cells resemble FUBM and the composition of Rh123++ BM cells is similar to that of SAFBM in that the cells have a lower proportion of pre-CFU-S. The lower radioprotective capacity of SAFBM and Rh123++ BM must be due to the relative absence of the pre-CFU-S. The higher marrow repopulating capacity of FUBM is ascribed to a relative excess of pre-CFU-S as compared to NBM. This model is also consistent with the observation that after serial transplantation of BM cells, there is a decrease in the radioprotective capacity per CFU-S transplanted (Ross et al, 1982). Preliminary experiments from our laboratory also indicate that larger numbers of day-12 CFU-S obtained from mice after the second retransplantation are required for long-term survival when compared to NBM (Mulder and Platenburg, unpublished results).

As stated above bone marrow cells purified by density separation and light activating cell sorting for a high binding of WGA-FITC and anti-H-2K are enriched for both day-12 CFU-S (130±30 fold) and radioprotective capacity (180±70 fold)(Visser et al, 1984). Electron microscopy identified two, slightly different, cell types in the purified cell population (Visser et al, 1984). The presence of the day-12 CFU-S and a radioprotective cell as two separate morphological entities in the purified cell fraction is therefore possible. Since day-12 CFU-S and the PHSC must be closely related, it is likely that these

cells copurify in many separation procedures. With the use of Rh123 it may be possible to obtain separation between day-12 CFU-S and the PHSC in the purified stem cell suspension and to correlate these cells with one of the two morphological cell types. These experiments are in progress.

Day-8 and day-12 CFU-S

Day-8 and day-12 CFU-S have been reported to differ in the density of membrane bound Qa-2 antigenic determinants (Harris et al, 1984) and in the uptake of the supravital DNA dye Hoechst 33342 (Baines and Visser, 1983). Day-12 CFU-S bind more anti-Qa-2 and incorporate less Hoechst 33342 than do day-8 CFU-S. Other differences that are described in this thesis can be summarized as follows (see also Table 8.1): Day-12 CFU-S contain many class I H-2 molecules (Fig. 4.1) and are weakly positive for the Thy-1 antigen (Fig. 4.2). As mentioned above the Rh123 uptake by day-12 CFU-S is heterogenous (Fig. 4.4). Day-8 CFU-S, in contrast, contain less H-2K molecules on their cell membrane and part of the day-8 CFU-S are Thy-1 negative. Day-8 CFU-S take up relatively high amounts of Rh123 in their mitochondria. These results show that day-8 and day-12 CFU-S belong to distinct cell populations

Functional differences between cell populations differing in the content of day-8 and day-12 CFU-S were described. The experiments in which BM cells were separated on the uptake of Rh123 showed that radioprotection was predominantly present in the weakly labeled fraction. This fraction contained only day-12 CFU-S (Table 4.4 and Fig. 4.5). The day-8 CFU-S are therefore not important for radioprotection after lethal irradiation. Similarly, culturing of BM cells in the presence of SAF(=IL-3) (SAFBM) caused a 3-fold increase in the number of day-8 CFU-S and a maintenance of the number of day-12 CFU-S (Table 3.2). Despite its ability to produce day-8 CFU-S, SAFBM showed a diminished radioprotective capacity when compared to fresh BM (Figs. 3.2-3.4). In another functional assay: thymus repopulation, SAFBM, containing large numbers of day-8 CFU-S, was less effective than day-12 CFU-S from NBM or FUBM (Figs. 3.5, 3.6, 5.4, 5.7 and 5.9). These results show that day-8 CFU-S have less properties ascribed to pluripotent hemopoietic stem cells than the day-12 CFU-S and suggests that the day-8 CFU-S is a more differentiated stem cell.

The most plausible relationship between day-8 and day-12 CFU-S is, that day-12 CFU-S produce day-8 CFU-S. Indirect results in support of this notion

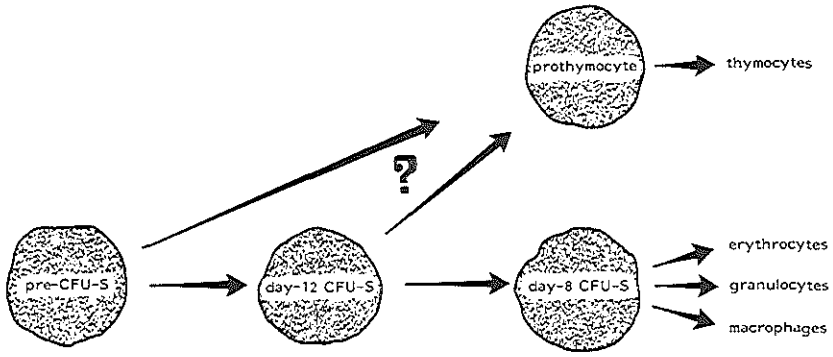


Fig. 8.3 Model describing the relationship between the pre-CFU-S, the day-12 CFU-S, the day-8 CFU-S and the prothymocyte.

was obtained with BM from mice, injected with 5-FU. Day-8 CFU-S are more sensitive to 5-FU than the day-12 CFU-S. The day-12/day-8 CFU-S ratio is 24 one day after 5-FU injection as compared to 1 in NBM (van Zant, 1984). After treatment of mice with 5-FU, day-12 CFU-S recovered before the day-8 CFU-S in vivo (Fig. 4.7). A similar pattern of both proliferation and loss of day-12 and day-8 CFU-S has been obtained in SAF containing cultures of BM cells of 5-FU treated mice. The day-12 CFU-S kinetics was always a few days ahead of that of the day-8 CFU-S.

From these results it is concluded that day-8 CFU-S are not pluripotent hemopoietic stem cells and may suggest that day-12 CFU-S produce day-8 CFU-S (Fig. 8.2). The differentiation from day-12 CFU-S to day-8 CFU-S is associated with a loss of H-2K determinants and possibly with a loss of Thy-1 antigens.

Prothymocytes

Prothymocytes, the committed precursor cells of thymocytes show several similarities with day-8 and day-12 CFU-S, but are clearly different from them. This is summarized in Table 8.1. Day-12 CFU-S and day-8 CFU-S were present in most hemopoietic cell suspensions, although the ratio of the two CFU-S varied widely (see also Table 5.3). Prothymocytes, whose presence was measured by the ability to induce early thymus regeneration were absent from several of these cell suspensions, like day-1-4 FUBM, SAFBM, day-7 RBM, and spleen (Boersma, 1982; Boersma, 1983b; Table 8.1). In regenerating BM early after BMT (1 day),

there is a relative excess of prothymocytes when compared to the day-12 CFU-S (Fig. 7.6). These results indicate that prothymocytes, day-8 and day-12 CFU-S are present in different proportions in the various cell populations.

Day-8 CFU-S and prothymocytes had a similar density of class I H-2 antigens and showed a similar uptake of Rh123 (Figs. 4.1, 4.4, 5.3 and 5.6). Thymus repopulation capacity, however, was confined to Thy-1.1 positive cells (data not shown; Basch and Berman, 1982), while about 40% of the day-8 CFU-S were negative for Thy-1.1 (Fig. 4.2). SAFBM, enriched in day-8 CFU-S (Table 3.1) is severely depleted in thymus repopulating capacity (Fig. 3.5 and 3.6). From these data it is concluded that the day-8 CFU-S and the prothymocyte are different cell types and that day-8 CFU-S do not differentiate into prothymocytes.

Day-12 CFU-S and prothymocytes differed in the density of class I H-2 antigens (Figs. 4.1, 5.2-5.4). Only a fraction of the day-12 CFU-S, but all prothymocytes incorporated relatively much Rh123 (Fig. 5.6). Day-12 CFU-S and prothymocytes are, thus, different cells (see also Table 8.1). The question remains whether the day-12 CFU-S or the pre-CFU-S (or both) are the precursors of prothymocytes. Two possible differentiation pathways are shown in Fig. 8.3. Since a separation between pre-CFU-S and day-12 CFU-S (and an absence of prothymocytes) has not yet been obtained, it is not yet possible to discriminate between these options (Fig. 8.3).

The entry of the prothymocyte into the thymus

Prothymocytes travel from the BM to the thymus by an unknown mechanism. For instance, it is not known whether the prothymocytes receive a stimulus before their exit from the BM. Experiments in which the thymus could receive precursor cells from two different sources of BM cells (e.g. after parabiosis or in mixed BM chimeras) showed that there was a high variability in the ratio of descendents from the two BM sources with time and also between the two thymus lobes (Harris et al, 1964; Wallis et al, 1975; Ezine et al, 1984). From these studies it was concluded that few prothymocytes enter the thymus during steady state conditions. Another, equally likely, explanation for the high variability in the ratio of descendants is that the progeny of the precursor cells is transiently present in the thymus and that a few weeks later the thymus may contain thymocytes that are derived from other prothymocytes. This is

supported by the high turnover of thymocytes (Metcalf, 1966; Shortman and Jackson, 1974; McPhee et al, 1979; Scollay et al, 1980). Furthermore, the thymus (normal or 2 days regenerating) contains thymocyte precursor cells that can give rise to only a limited and transient progeny in the thymus (Boersma, 1982). These experiments show that a continous inflow of precursor cells is necessary for the maintenance of thymus cellularity. In recent experiments the phenotype of the thymocyte population, responsible for the early and transient thymus repopulation was determined: low Ly-1 positive and Ly-2 and L3T4 negative (Mathieson and Fowlkes, 1984; Fowlkes et al, 1985).

When do the prothymocytes enter the thymus after BMT? Homing experiments have shown that BM cells labeled with a supravital fluoresceing dye can be recovered from the thymus as early as three hours after lethal irradiation and BMT (Lepault and Weissman, 1981). The experiments described in Chapter 6 (Figs. 6.2 and 6.3 and Table 6.1) are in agreement with this. However, several observations argue against the notion that these cells have anything to do with repopulation of the thymus. Firstly, most of the immigrant cells were smaller than the prothymocyte (Fig. 6.2). Secondly, a longer interval between lethal irradiation and BMT, which does not influence thymus repopulation kinetics (i.e. thymus repopulation starts at the same time after BMT; Boersma, 1982), caused a decrease in the number of BM cells that could be obtained at 3 h after BMT from the thymus (Table 6.1). Thirdly, extrapolation of the thymus repopulation data after injection of FUBM, indicated that 1000-times less precursor cells entered the thymus at the day of grafting than with NBM (Fig. 7.8), while in our direct homing experiments only 2-times fewer FUBM derived cells entered the thymus at 3 h post-BMT. Therefore the definition of prothymocytes does not apply to the unidentified BM cells that enter the thymus immediately after grafting. At present it has to be assumed that entrance into the thymus is not an exclusive property of prothymocytes.

The experiments in which regenerating mice were reirradiated on the thymus only (Fig. 7.4 and Table 7.1) or reirradiated with thymus shielding (Fig. 7.5) showed that prothymocytes are absent from the thymus until the second day after BMT. Two days after BMT the presence of prothymocytes in the thymus could be demonstrated with both reirradiation procedures. These results show that prothymocytes enter the thymus from the second day onwards and indicate that the prothymocyte first migrates to another site before transfer to the thymus. Evidence was presented that this primary homing site is the bone marrow: It was shown that soon after BMT (24-36 hours) prothymocytes can be recovered from

the BM.

Intrathymic thymocyte precursor cells

After their entry in the thymus, the prothymocyte differentiates and becomes part of the intrathymic pool of proliferating thymocyte precursor cells. Due to the production of host thymocytes after lethal irradiation, it has been argued that the thymus contains radioresistant thymocyte precursors (Kadish and Basch, 1975; Boersma et al, 1981a; Ceredig and MacDonald, 1982; Sharrow et al, 1983). However, reirradiation (5 Gy) of the thymus 2 h after a TBI (9 Gy) Gy, results in a strong reduction in the number of host derived thymocytes (Table 7.1). Assuming that the precursor cells have a shorter doubling time early after irradiation than the total thymocyte population 12 to 15 days later it could be calculated that these precursor cells are not radioresistant and have a Do of approximately 0.9 Gy.

6 to 10 days after BMT relatively large numbers of these intrathymic progenitor cells must already be present in the thymus. Their presence was observed by a dimished delay in thymus regeneration when the thymus is reirradiated at or later than 6 days after BMT (Table 7.1 and Fig 7.7).

SUMMARY

The presence of hemopoietic stem cells in cell suspensions can be determined by a variety of in vivo or in vitro techniques. The spleen colony assay, the 30 day survival and the thymus repopulation assay are in vivo assays. In addition, there are a multitude of in vitro tests, using different hemopoietic growth factors (or combinations of growth factors). The difficulty with these tests is that it is largely unknown to which extent they measure similar cell types. This makes it impossible to determine the differentiation pathways of the various lineages exactly and to define where a lineage deviates from the others. In order to obtain a clearer insight in the organization of hemopoietic stem cells a variety of BM cell suspensions or fractions thereof were applied to the three in vivo assays. From the results it could be estimated to which extent different stem cells were measured in these assays and what the differences between the stem cells were. These experiment are described in Chapter 3, 4 and 5.

The spleen colony assay measures a proportion of the stem cells (colony forming unit-spleen; CFU-S) injected into lethally irradiated mice as discrete nodules which consist of hemopoietic cells from several lineages. They appear on the surface of the spleen between 6 and 14 days after BMT (colonies counted at 8 days after BMT are derived from day-8 CFU-S and those present at twelve days from day-12 CFU-S, etc.). The CFU-S assay has long been regarded as an assay of pluripotent hemopoietic stem cells (PHSC, the least mature hemopoietic stem cell). However, it was found that CFU-S are a heterogenous cell population and that day-8 and day-12 CFU-S are different cells. Cell populations enriched in day-8 CFU-S can be obtained by culture of BM cells in the presence of stem cell activating factor (SAF, which is identical to IL-3) (Chapter 3). The day-8 CFU-S in these cultures could be produced by self renewal of day-8 CFU-S or by differentiation from less mature cells. In order to test the hypothesis that day-12 CFU-S give rise to day-8 CFU-S, experiments were performed with BM cells obtained from mice treated with 5-fluorouracil (5-FU; FUBM). 5-FU kills predominantly cycling cells. Shortly after 5-FU injection very few day-8 CFU-S survived as compared to the day-12 CFU-S. The kinetics of regeneration of day-12 CFU-S was several days ahead of that of the

day-8 CFU-S. This suggests that the day-12 CFU-S is an earlier cell type than the day-8 CFU-S. A similar pattern of proliferation of day-12 and day-8 CFU-S has been obtained in SAF containing cultures of FUBM. The generation of day-12 CFU-S was always a few days ahead of that of the day-8 CFU-S. These results suggest that the day-12 CFU-S is one of the targets of SAF and that stimulation with SAF leads to differentation of day-12 CFU-S into day-8 CFU-S (Chapter 4).

Several BM suspensions or fractions thereof where tested for the ability to protect lethally irradiated mice for 30 days. It was found that after culture of BM cells with SAF there is a loss of protective capacity, despite the production of day-8 CFU-S. Day-8 CFU-S, therefore, do not represent pluripotent hemopoietic stem cells (Chapter 3). A completely different technique allowed the separation of day-12 CFU-S from normal BM cells containing day-8 CFU-S and a fraction of the day-12 CFU-S: BM cells were labeled with rhodamine 123 (Rh123), a fluoresceing dye which accumulates exclusively in mitochondria of living cells. The cells were subsequently separated on fluorescence intensity with a cell sorter. Two third of the day-12 CFU-S and all day-8 CFU-S were brightly labeled with Rh123, while one third of the day-12 CFU-S was recovered from the weakly labeled fraction. Radioprotection, expressed per day-12 CFU-S transplanted was predominantly mediated by the weakly labeled fraction. The diminished protective capacity of the brightly fluoresceing fraction, containing the day-8 CFU-S, agrees with the loss of protective capacity by SAFBM. The difference in 30 day survival per transplanted day-12 CFU-S also shows that the day-12 CFU-S present in the weakly labeled fraction differ from those in the brightly labeled fraction or that another earlier stem cell, the pre-CFU-S, is also present in this fraction (Chapter 4).

Thymus repopulation after lethal irradiation and BMT was found to be caused by, at least two cell types: a progenitor cell committed to the T lineage (the prothymocyte) and the pluripotent hemopoietic stem cell. Prothymocytes cause transient thymus repopulation between 15 and 21 days after BMT. PHSC show thymus repopulation which is 3 to 4 days delayed (Chapter 5). Prothymocytes differ from the day-12 CFU-S in the expression of class I H-2 antigens, from the cell responsible for radioprotection in the ability to incorporate Rh123 (Chapter 5) and from the day-8 CFU-S in the expression of Thy-1 antigens (Chapter 4). In addition, BM cells cultured with SAF, which contain large numbers of day-8 CFU-S, show a profound loss of thymus repopulating ability. These results indicate that prothymocytes do not derive from day-8 CFU-S. This

is in agreement with the very low radioprotective capacity of day-8 CFU-S. The prothymocyte, therefore, stems from either the day-12 CFU-S or its predecessor, the pre-CFU-S (see Fig. 8.5).

The experiments described in Chapters 3-5 show that infrequent cells like hemopoietic stem cells can be dissected into subpopulations with the aid of fluorescence activated cell sorting, monoclonal antibodies and fluorescent dyes. The relationship between these cells could subsequently be studied with the use of in vivo assays. Further studies at the single cells and molecular level should reveal the processes that govern these differentiation processes. Comparison of these events with those in malignant cells should enhance our understanding of neoplasms and may give us a clue to their treatment.

Bone marrow cells injected into lethally irradiated recipients seed into many organs. It has been claimed by others that the BM cells that appear at 3 h after BMT in the thymus represent prothymocytes. In Chapter 6 it is shown that this is not the case. Using fluoresceing cell suspensions of different composition, it appeared that the number of thymus immigrants at 3 h after BMT related more closely to the total number of cells injected than with the ability to repopulate the thymus. Moreover, the thymic immigrants were smaller than the prothymocytes in the BM. This was determined by analysis of the laser light scatter of prothymocytes and thymus immigrants. Therefore, the assay which determines the number of cells that appear in the thymus shortly after BMT cannot be used for quantification of thymocyte progenitor cells.

In Chapter 7 a series of experiments is reported which elucidated the exact time of entry of prothymocytes into the thymus following grafting of BM cells. For this purpose the recipient mice were reirradiated at various intervals after BM transplantation on the whole body, with or without thymus shielding or reirradiated on the thymus only. Thymus repopulation was used as a functional assay for the prothymocyte. All reirradiation designs resulted in the same conclusion, i.e. that during the first 24 hours there is no entry of prothymocytes in the thymus. For instance, reirradiation of the thymus at 1 day after BMT did not affect thymus regeneration, but reirradiation at later times (until 4 days post BMT), caused a delay in thymus repopulation. The later the reirradiation the more delay in thymus repopulation. From these results it was concluded that prothymocytes enter the thymus from the second day post grafting

onwards.

A wave of host thymocytes is present in mice during the second and third week after lethal irradiation and BMT. Reirradiation of the thymus in regenerating mice abolished this host repopulation, indicating that the precursor cells of host thymocytes are located in the thymus, but that they are not radioresistant cells as was reported previously by several authors. It was calculated that the radiosensitivity of the host intrathymic thymocyte precursor cells was similar to that of the CFU-S (Chapter 7).

The reirradiation experiments indicated that after the first week post-BMT the donor BM also had produced significant numbers of intrathymic thymocyte precursor cells which gave rise to cortical type thymocytes 14 days later. It was discussed that it is likely that the doubling time of the thymocyte precursor cell population is less than that of the total thymocyte population (e.g. due to loss of cortical thymocytes and exhaustion of precursor cells). Therefore, it was assumed that the doubling time of these precursor cells is close to the initial thymus regeneration following injection of FUBM or purified stem cells, which was considerably faster than after injection of normal BM cells (18.4 h vs. 30.4 h). From the thymus repopulation data obtained in the reirradiation experiments and a doubling time of 18 h for the thymocyte precursor population a Do of 0.94 Gy could be calculated. The similarity in thymus regeneration kinetics (i.e. 14 days after their presence in the thymus they give rise to thymocytes) and in radiosensitivity suggests that these donor and host intrathymic precursor cells represent similar cell types, which have the same radiosensitivity as other lymphoid cells.

The experiments described in Chapters 6 and 7 may help the study of the very first differentiation steps of prothymocytes in the thymus. Prothymocytes enter the thymus on and after the second day after BMT. In this period the thymus contains, due to the irradiation, very few nucleated cells. With the present separation and detection techniques it should be possible to follow the changes that occur to the relatively synchronized population of prothymocytes in the subsequent days.

SAMENVATTING

Hemopoietische cellen onstaan door differentiatie en proliferatie uit onrijpe voorlopercellen. Deze voorlopercellen, stamcellen, kunnen door middel van diverse in vivo en in vitro testen worden bestudeerd. De miltkolonie test, de bepaling van overleving na letale bestraling en beenmergtransplantatie (BMT), en de thymus repopulatie test zijn methoden om stamcellen in vivo te meten. Helaas is het onduidelijk of, en in welke mate, de verschillende testsystemen verschillende stamcellen meten. Hierdoor is het onmogelijk om te bepalen waar de splitsing tussen twee differentiatielijnen, bijvoorbeeld van granulocyten en lymfocyten, is ontstaan. De scheiding tussen de cellen die zich in de richting van thymocyten en T cellen ontwikkelen en die van de andere differentiatiereeksen vormt het eerste deel van dit proefschrift. Hiervoor werden diverse soorten en/of fracties van beenmerg cellen (BM) getest op de drie bovengenoemde in vivo meetmethoden. Uit de resultaten kon worden geschat in welke mate verschillende stamcellen bijdragen tot de drie in vivo testen en welke verschillen er tussen de stamcellen bestaan.

In de miltkolonie test worden stamcellen gemeten, die, na injectie in letaal bestraalde dieren, een kolonie vormen die op het oppervlak van de milt zichtbaar is. Een kolonie is een kloon en de stamcel wordt "colony forming unit-spleen" (CFU-S) genoemd. De kolonies, bestaande uit cellen van diverse differentiatie-lijnen, worden 6 tot 14 dagen na BMT macroscopisch zichtbaar. Miltkolonies welke op de achtste en twaalfde dag aanwezig zijn worden per definitie gevormd uit respectievelijk dag-8 en dag-12 CFU-S. Slechts het deel van de geinjiceerde stamcellen dat in de milt verschijnt vormt kolonies. De miltkolonie test is lange tijd beschouwd als een techniek waarmee de meest onrijpe stamcellen (pluripotente hemopoietische stamcellen; PHSC) quantitatief kunnen worden bepaald. De CFU-S populatie bleek echter uit een heterogene groep cellen te bestaan, want dag-8 en dag-12 CFU-S zijn verschillende cellen.

De relatie tussen de dag-8 en dag-12 CFU-S werd onderzocht met BM van muizen welke van te voren waren ingespoten met 5-fluorouracil (5-FU; FUBM). 5-FU doodt voornamelijk delende cellen. Dag-12 CFU-S zijn minder gevoelig voor 5-FU dan de dag-8 CFU-S en dag-12 CFU-S regenereren eerder dan de dag-8 CFU-S. Dit suggereert dat de dag-12 CFU-S een onrijpere cel is dan de dag-8 CFU-S. In

andere experimenten werd normaal BM en FUBM gekweekt met een stamcel activerende factor (SAF, hetgeen identiek is aan interleukine-3). Deze substantie zet stamcellen aan tot deling. Kweek van normaal BM met SAF veroorzaakt een relatieve toename van de dag-8 CFU-S t.o.v. de dag-12 CFU-S (Hoofdstuk 3). Na stimulatie van FUBM, hetgeen aanvankelijk geen dag-8 CFU-S bevatte, met SAF werd eerst een toename van de dag-12 CFU-S gevonden. Dit celtype verdween vervolgens langzaam uit de kweken. De groei van de dag-8 CFU-S in vitro was, evenals in vivo, enkele dagen vertraagd t.o.v. de dag-12 CFU-S. Zij waren echter langer aanwezig in de kweken dan de dag-12 CFU-S (Hoofdstuk 3). Deze resultaten geven aan dat de dag-12 CFU-S gevoelig is voor de werking van SAF en dat dag-8 CFU-S de nakomeling kunnen zijn van de dag-12 CFU-S.

Twee verschillen in de aanwezigheid van membraanantigenen op de dag-12 en de dag-8 CFU-S zijn beschreven in Hoofdstuk 4. Op de dag-8 CFU-S zijn minder klasse I H-2 antigenen aanwezig dan op de dag-12 CFU-S. Bovendien zijn alle dag-12 CFU-S positief voor het Thy-1 antigen, terwijl ongeveer 50 % van de dag-8 CFU-S geen Thy-1 bevatten. In Hoofdstuk 4 zijn eveneens scheidingsproeven met rhodamine 123 (Rh123) beschreven. Deze fluorescerende stof wordt opgenomen in de mitochondria van levende cellen. Na scheiding van de gekleurde cellen op basis van fluorescentie werd gevonden dat alle dag-8 CFU-S en tweederde van de dag-12 CFU-S relatief veel rhodamine 123 opnemen. Eenderde van de dag-12 CFU-S nemmt echter weining Rh123 op (Hoofdstuk 4). Met behulp van een licht geactiveerde cellensorteerder konden dag-8 en dag-12 CFU-S op bovengenoemde karakteristieken worden gescheiden. Deze resultaten steunen eerdere bevindingen, waarin verschillen tussen de dag-8 en dag-12 CFU-S werden aangetoond.

Diverse BM suspensies of BM fracties werden getest op het vermogen om letaal bestraalde dieren te beschermen tegen het ontstaan van een beenmergsyndroom. BM, gekweekt in de aanwezigheid van SAF en dus rijk aan dag-8 CFU-S, beschermde letaal bestraalde dieren minder goed dan het oorspronkelijke BM. Hieruit blijkt dat de dag-8 CFU-S in deze kweken niet de pluripotente stamcel vertegenwoordigt (Hoofdstuk 3). Met behulp van de reeds hierboven beschreven scheiding op basis van rhodamine123 opname kon hetzelfde worden aangetoond: De zwak met Rh123 gekleurde BM cellen, welke vrijwel geen dag-8 CFU-S bevatten, bleken letaal bestraalde muizen aanzienlijk beter te beschermen dan de sterk fluorescerende BM fractie. Overleving na letale bestraling, uitgedrukt per getransplanteerde dag-12 CFU-S, was eveneens minder na transplantatie van de sterk fluorescerende fractie dan van de zwak

fluorescerende BM-populatie. Deze resultaten duiden erop dat of de dag-12 CFU-S een heterogene populatie is, waarvan slechts een deel het onstaan van een stralingsziekte kan voorkomen, of dat de zwak met Rh123 gekleurde fractie nog een andere stamcel bevat die deze ziekte voorkomt, de pre-CFU-S (Hoofdstuk 4).

Repopulatie van de thymus na letale bestraling en beenmergtransplantatie wordt veroorzaakt door, tenminste twee, stamcellen: een voorlopercel, beperkt tot de T cel reeks (de prothymocyt), en de pluripotente hemopoietische stamcel. De thymus wordt door de prothymocyt tijdelijk (tussen 15 en 21 dagen na BMT) gerepopuleerd. Pluripotente stamcellen veroorzaken echter een permanente thymusrepopulatie, die 3 tot 4 dagen na de repopulatie door de prothymocyt begint. Prothymocyten verschillen van de dag-12 CFU-S in de dichtheid van klasse I H-2 antigenen op de celmembraam, van de cel die bescherming biedt na letale bestraling in de opname van Rh123, en van een deel van de dag-8 CFU-S in de dichtheid van het Thy-1 antigeen op de celmembraan (Hoofdstukken 4 en 5). Bovendien werd gevonden dat BM, gekweekt met SAF en waarin zich veel dag-8 CFU-S bevinden, de thymus aanzienlijk minder goed repopuleert (Hoofstuk 3). Deze resultaten wijzen erop dat de dag-8 CFU-S niet een voorlopercel is van de prothymocyt en steunen de hypothese dat de prothymocyt is ontstaan uit de dag-12 CFU-S of een nog vroegere voorlopercel, de pre-CFU-S.

De experimenten beschreven in de Hoofdstukken 3-5 tonen aan dat weinig voorkomende cellen, zoals de hemopoietische stamcellen, kunnen worden onderverdeeld in subpopulaties met behulp van de licht geactiveerde cellen-sorteerder, monoclonale antilichamen en fluorescerende kleurstoffen. De relatie tussen deze subpopulaties kon vervolgens worden bestudeerd met behulp van in vivo testen. Verder onderzoek met technieken waarby een cel kan worden bestudeerd op moleculair niveau zijn nodig om de differerentiatiestappen tussen de diverse stamcellen te bestuderen. Vergelijking van deze ontwikkelingsstappen tussen normale en maligne cellen kan ons begrip van maligniteit verhelderen en mogelijk nieuwe therapeutische gezichtspunten aan het licht brengen.

Beenmergcellen, getransplanteerd in een letaal bestraalde muis, komen in vele organen terecht. Men heeft beweerd dat de BM cellen, die zich 3 uur na BMT in de thymus bevinden, prothymocyten zijn. De experimenten beschreven in Hoofdstuk 6 tonen aan dat deze stelling niet klopt. Met behulp van diverse suspensies van fluorescerende BM cellen bleek het aantal thymus immigranten

beter te correleren met het totaal aantal ingespoten cellen dan met het thymus repopulerend vermogen. Verder bleek o.a. dat de thymusimmigranten kleiner waren dan de prothymocyt. De voorwaartse verstrooiing van laserlicht, welke toeneemt bij grotere cellen, van de thymusimmigrant was namelijk minder dan dat van de prothymocyt. Een test, waarin het aantal BM cellen, dat kort na bestraling in de thymus verschijnt, wordt bepaald, kan dus niet gebruikt worden om het aantal thymusvoorlopercellen te bepalen.

In Hoofdstuk 7 staat beschreven op welk tijdstip na letale bestraling en BM transplantatie de prothymocyten in de thymus verschijnen. Hiervoor werden ontvanger muizen op verschillende tijdstippen na BM-transplantatie herbestraald op de thymus of op het hele lichaam met of zonder afscherming van de thymus. De aanwezigheid van prothymocyten in de thymus werd gemeten als repopulatie van dethymus. Herbestraling van de thymus 1 dag na BMT beinvloedde de thymus-repopulatie niet. Herbestraling op latere tijdstippen leidde echter tot een toenenende vertraging van de thymusrepopulatie. Hieruit blijkt dat op de eerste dag na BMT de prothymocyt nog niet in de thymus aanwezig is en dat vanaf de tweede dag prothymocyten in de thymus komen.

Een golf van thymocyten, afkomstig van de ontvanger, wordt gevormd gedurende de tweede en derde week na letale bestraling en BMT. Herbestraling van de thymus voorkomt dit en toont aan dat deze voorlopercellen zich reeds in de thymus bevinden. Deze intrathymische voorlopercellen zijn beschreven als radioresistente cellen. Het niet verschijnen van de thymocyten uit ontvanger voorlopercellen na herbestraling van de thymus is hier niet mee in overeenstemming.

De herbestralingsproeven lieten verder zien dat een week na BMT aanzienlijke aantallen donorvoorlopercellen in de thymus aanwezig zijn. Er werd bediscussieerd dat de verdubbelingstijd van deze voorlopercellen sneller moet zijn dan dat van de totale thymocytenpopulatie, welke pas een week later kan worden gemeten. Op dat tijdstip treedt nl. reeds verdwijning van thymocyten op en uitputting van de eerste golf van prothymocyten. Herbestraling van de thymus op zeven dagen na BMT leidt tot een vertraging in thymus repopulatie. Uit de meetgegevens kon voor de gastheer en de donorvoorlopercel een bestralingsgevoeligheid met een Do van, respectievelijk 0.90 and 0.94 Gy worden berekend. Deze waarden zijn vrijwel eender aan die van de hemopoietische stamcel, de prothymocyt en de meeste lymphocyten. Deze resultaten bestrijden de opvatting in de literatuur waarin radioresistente voorlopercellen zijn beschreven.

De experimenten beschreven in de Hoofdstukken 6 en 7 kunnen bydragen tot de studie van de eerste differentiatiestappen van de prothymocyt in de thymus. Prothymocyten verschijnen vanaf de eerste dag na BMT in de thymus. Op dit tijdstip bevat de thymus, als gevolg van de letale bestraling zeer weinig kernhoudende cellen. Het moet mogelijk zijn om de min of meer gesynchroniseerde populatie van prothymocyten met de huidige celscheidings - en detectietechnieken te bestuderen.

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CURRICULUM VITAE

Andries Hans Mulder werd in 1954 in Delfzijl geboren. Na het Atheneum B werd in 1972 begonnen met de medische studie aan de Erasmus Universiteit te Rotterdam. Na het kandidaatsexamen werd gedurende een jaar onderzoek verricht aan de Purdue University, W-Lafayette, In. onder leiding van Prof.Dr. E.S. Golub. Eind 1976 werd de medische studie te Rotterdam voortgezet en in november 1980 afgesloten met het artsexamen. Hierna vond aanstelling plaats in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek in het kader van het Zwaartepunt Programma Hemopoietische Regulatie in het Radiobiologisch Instituut TNO te Rijswijk onder leiding van Prof.Dr. D.W. van Bekkum. Hier vond het in dit proefschrift beschreven onderzoek plaats. Vanaf januari 1985 tot maart 1986 volgde aanstelling bij het Integraal Kankercentrum Rotterdam met als standplaats het Radiobiologisch Instituut. In deze periode werd medewerking verleend aan een project van het Koningin Wilhelmina Fonds. Per 1 maart 1986 is A.H.M. werkzaam als arts-assistent op de afdeling Klinische Pathologie aan de Erasmus Universiteit te Rotterdam onder leiding van Prof.Dr. R.O. van der Heul.