CHARACTERISATION

OF MOUSE

PROTHYMOCYTES

The work described in this thesis has been performed at the Radiobiological Institute of the Division for Health Research TNO, Rijswijk, The Netherlands.

CIP
ISBN 90-9000373-8

This thesis is available as a publication of the Radiobiological Institute and the Institute for Experimental Gerontology of the Division for Health Research TNO, Rijswijk, The Netherlands.

CHARACTERISATION OF MOUSE PROTHYMOCYTES

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. J. SPERNA WEILAND
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
VRIJDAG 26 NOVEMBER 1982 DES NAMIDDAGS
TE 2.00 UUR

DOOR

WILHELMUS JOHANNES ANTONIUS BOERSMA

GEBOREN TE AMSTERDAM

KRIPS REPRO B.V. - MEPPEL

TABLE OF CONTENTS

CHAPTER 1 INTRODUCTION

CHAPTER 2 MATERIALS AND METHODS

CHAPTER 1

INTRODUCTION

1 GENERAL INTRODUCTION

Under normal conditions, the hemopoietic system in adults in higher organisms, e.g., mammals, represents an organ in which a continuous production of cells compensates for cell loss due to limited life spans or utilization of the mature cells. The generation of different lines of blood cells from a single cell (the hemopoietic stem cell) represents the entire process of cellular differentiation (Fig.1).

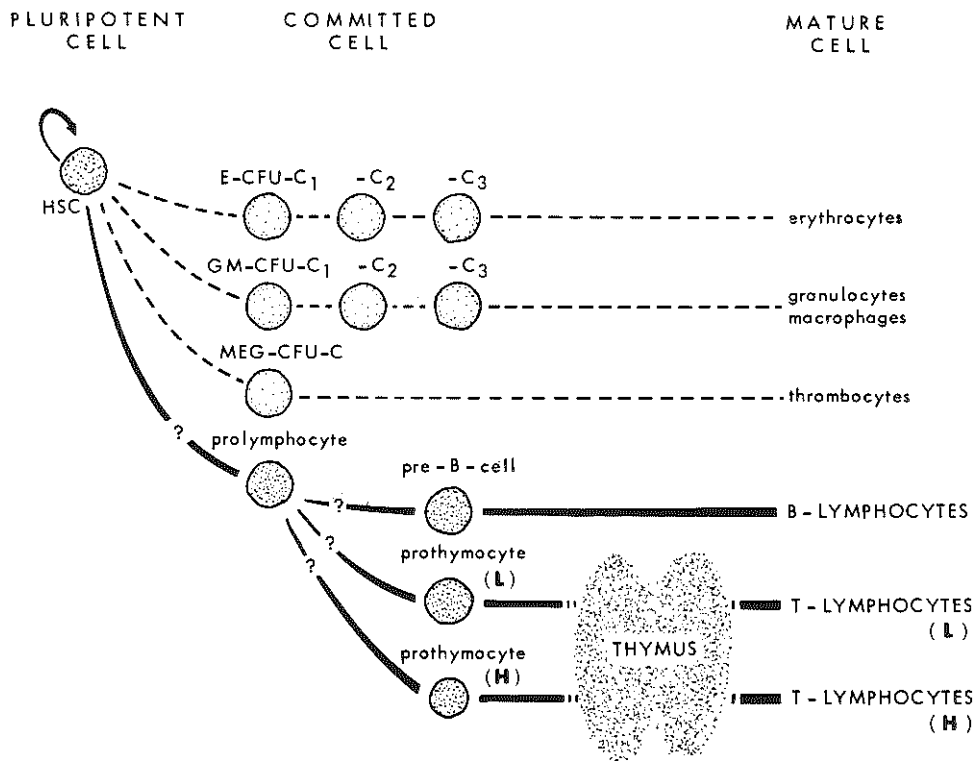

Fig. 1 Differentiation stages of mouse hemopoietic cells. Shaded cell types have been characterised in clonal in vivo or in vitro assays. (L) and (H) indicate cells of the tentative lineages of cells with "low" and "high" Thy-1 surface antigen density.

One requirement for the study of differentiation processes and mechanisms is the ability to recognize the different stages of development preceding the mature form of a cell type. In the more mature stages of development, the recognition of different stages is relatively easy on the basis of cell morphology, including cell surface markers and on the basis of cell functions. The recognition of cells in the early stages of development following the differentiation pathway of a cell lineage back to the stem cell is increasingly difficult because of the lack of characteristic morphological features of these cells. In addition, since the development of a cell lineage occurs via a process of exponential growth, the number of early progenitor cells needed for homeostasis is small.

In an investigation of the differentiation process of hemopoietic cells, the question arises whether a single precursor cell is indeed able to produce one or more than one type of progeny. The stem cell should be capable of self-renewal and differentiation. When there exists a pluripotent progenitor cell a second question arises as to what stage of differentiation does commitment to a certain lineage of blood cells occur. Finally, what is the nature of and the mechanism for the signal that induces the pluripotent cells into the differentiation process that leads to committed progenitors? Accurate characterisation or preferably isolation of the progenitor cells in the subsequent stages of development is required for analysis of the mechanism(s) of action of the factors influencing differentiation processes. In contrast to the progenitor cells of myeloid and erythroid cells, the earliest progenitor cells from which thymus processed cells (T cells) develop, were very poorly characterised until recently. There is even some doubt as to whether there exists at all a T-cell progenitor that is different from the pluripotent hemopoietic stem cell (HSC). Since T cells not only play a role in immunological processes, but are also able to influence the development of cells in other hemopoietic lineages, the investigation of early T-cell differentiation is of importance in understanding other features of hemopoietic differentiation.

An influence of the thymus has been demonstrated on the proliferation of stem cells (Hrsack, 1973; Lepault et al., 1979), on the development of cells of the erythroid lineage (Basford and Goodman, 1974; Goodman and Shinpock, 1968; Pritchard and Goodman, 1978) and on the development of myeloid cells (Goodman et al., 1979). Humoral factors produced by T lymphocytes have been shown to be effective in stimulating and maintaining hemopoiesis in various in vitro systems (Parker and Metcalf, 1974; Johnson and Metcalf, 1977; Fauser and Messner, 1979). It appears therefore that the thymus and thymus derived cells have a more important role in hemopoiesis than has been envisaged in the past (Miller, 1979).

2 DIFFERENTIATION OF PLURIPOTENT HEMOPOIETIC CELLS

As stated above, early progenitors are not easily identified because their low numbers and lack of distinctive morphological properties. Therefore, most of

the hemopoietic progenitor cells have been defined up to now in functional assay systems. In the following, the current state of knowledge of early hemopoietic cell differentiation, with special emphasis on development within the lymphoid cell compartment, will be discussed.

A protective effect of spleen shielding on mortality following X-irradiation was discovered by Jacobson et al. (1949). This observation led to the demonstration that lethally irradiated mice could be rescued from radiation death by transfusion of bone marrow cells (Lorenz et al., 1951). It was already shown in the early fifties that, after intravenous injection of syngeneic bone marrow cells into lethally irradiated mice, the thymus cell population was fully restored within a few weeks (Brown et al., 1953). After irradiation of mice for the purpose of inducing leukemia, enhanced regeneration of the thymus cell population was observed when one leg of the animal was shielded (Kaplan et al., 1954).

By means of cytological analysis with chromosomal markers (Ford et al., 1956) and serological and cytochemical analysis (Vos et al., 1956; Nowell et al., 1956), it was shown that all blood cell types in irradiated and bone marrow transfused mice were of donor origin. From this, it was postulated that all blood cells most probably had a common progenitor cell, the hemopoietic stem cell.

After transplantation, bone marrow cells are distributed over various hemopoietic organs. Till and McCulloch (1961) observed that in the spleens of lethally irradiated mice, macroscopically visible colonies of cells developed after transplantation of a few syngeneic bone marrow cells. Regeneration of the hemopoietic process starts from these foci, which also were observed in bone marrow. Using chromosomal markers, the bone marrow derived colony forming units in spleen (CFU-S) were shown to be single cells (Becker et al., 1963; Trentin and Fahlberg, 1963). After transplantation of colony derived cells into a second irradiated recipient, some of them gave rise to a spleen colony (Till et al., 1964; Lewis and Trobaugh, 1964). Further analysis revealed that the well developed spleen colonies beyond day 8 after transplantation contained cells of more than one lineage (Lewis and Trobaugh, 1964; Curry and Trentin, 1967). Before day 8, two types of cells predominated in spleen colonies; most such colonies contained cells of the erythroid lineage and undifferentiated cells (Curry and Trentin, 1967; Wu et al., 1967a). No lymphocytes were demonstrated in the spleen colonies. The pluripotential nature of the CFU-S was demonstrated when typical chromosomal markers observed in cells forming a colony were also found in cells of the lymphoid lineages (Trentin et al., 1967; Wu et al., 1967b; Moore and Owen, 1967a; Barnes et al., 1968; Nowell et al., 1970).

The size of the colonies, the various cell types present and the capacity of the CFU-S for self-renewal led to the conclusion that the CFU-S was a putative hemopoietic stem cell (for a review, see Metcalf and Moore, 1971).

In recent years, in vitro growth of mixed colonies has directly shown that multipotent cells exist in the fetal liver (Johnson and Metcalf, 1977; Metcalf et

al., 1979) and in the bone marrow (Iscove, 1978; Fauser and Messner, 1979; Johnson, 1980). However, these colonies also did not contain lymphoid cells (Johnson and Metcalf, 1977; Metcalf et al., 1979). Very recently, the presence of T cells in mixed colonies in assays for multipotent hemopoietic cells has been claimed by Messner et al. (1982).

At present, the role and even the existence of committed T-lymphocyte progenitors is relatively obscure. Thymology is the discipline that concerns the role of the thymus in hemopoiesis. The following sections are included as an introduction to thymology. Firstly the role of the thymus in the immune response and in early stages of development of thymus derived lymphocytes (T cells) from early progenitors is discussed in Sections 3-5. Precursors of T lymphocytes from various sources and their traffic into the thymus are discussed in Sections 6-7. Growth and education of T cells in the thymus is described in Sections 11-12. Different in vivo and in vitro systems currently used to study T-cell progenitors are briefly reviewed in Sections 13-15. Finally, the experimental chapters of this thesis are introduced in an outline of the study (Section 16).

3 THE ROLE OF THE THYMUS IN LYMPHOPOIESIS IN VIVO

A better understanding of thymic function in mice and other animals has been facilitated by analysis of the effect of thymectomy. Neonatal thymectomy led to various deficiences in the functions of the immune system. The number of circulating lymphocytes in blood, lymph and peripheral lymph nodes decreased (Miller, 1961; Miller et al., 1962; Parrot et al., 1966). In addition decreases in immunological functions such as skin graft rejection, delayed type hypersensitivity and tumor rejection over minor histocompatibility barriers were observed (Good et al., 1962; Armason et al., 1962). Antibody responses were also decreased after neonatal thymectomy in mice and rabbits (Miller et al., 1962; Good et al., 1962). Law et al. (1964) and Ting and Law (1965) observed that the decrease in antibody responses following neonatal thymectomy did not apply to all antigens. In addition, Parrot et al. (1966) found that the number of antibody forming cells was not changed but their function was severely affected in mice following neonatal thymectomy.

Cellular and antibody responses of the immune system could be restored by implantation of a thymus (Miller, 1962). When thymocytes were transfused into thymectomised irradiated mice, cellular and antibody responses could be restored for only a short period (Miller et al., 1967; Miller and Mitchell, 1968). This suggested the possibility that the thymic epithelial cells played a key role in the maturation of T lymphocytes. Since thymus grafts in thymectomised mice were repopulated by cells of host origin (Dalmasso et al., 1963), it was thought possible that the thymus function was mediated by a humoral factor. This was

supported by the finding that, thymus grafts in millipore diffusion chambers were able to restore the functional activity of T cells to some extent (Osoba and Miller, 1963,1964; Levy et al., 1964). Restoration of functional activity was not obtained when spleen or lymph node tissue was enclosed in diffusion chambers (Trainin et al., 1977). Experiments with tissues in diffusion chambers were not long lasting (up to 2 months) (Stutman, 1977). Whether supplementation of thymic factors allow restoration of T immune responses of a permanent character is questionable. When grafting of an enchambered thymus into newborn thymectomised mice was delayed for 40 to 60 days after birth, restoration of immune responses could not be achieved (Stutman et al., 1969). It was concluded that there existed a relatively long-lived population of cells that disappeared in the absence of a thymus and that these could not be restored by the humoral activity of the thymus alone. These cells were called "postthymic" cells. It was assumed that they acted as progenitors of the immunocompetent cells (Stutman et al., 1969; Stutman, 1975). According to Stutman, these postthymic precursor cells can be compared to "committed progenitor" cells of other hemopoietic lineages (Lajtha, 1975; Stutman, 1975, 1977).

By that time, it had already been demonstrated that, in lethally irradiated mice reconstituted with syngeneic bone marrow cells, the thymus lymphocytes carried the chromosome marker of the bone marrow donor after two to three weeks (Ford and Micklem, 1963). Tyan (1964) demonstrated that fetal liver cells require a thymus for maturation of T lymphocytes. In parabiotic mice it was established that lymphopoiesis in the thymus was initiated from bone marrow cells (Brumby and Metcalf, 1967). Ford et al. (1966) further showed that bone marrow cells are responsible for the continuity of thymus cell production in parabionts. Everett and Tyler (1969) confirmed the bone marrow origin of thymus cells in a rat system.

Transfusion of bone marrow cells into lethally irradiated thymectomised recipients did not lead to restoration of immune responsiveness (Miller et al., 1963; Cross et al., 1964). According to Van Bekkum (1967), in the absence of the thymus, the restoration of the responsiveness derived from the transplanted bone marrow is very low but not completely lacking. This is in contrast to transplantation in irradiated but nonthymectomised animals. Stutman et al. (1970a,b) and Doenhof et al. (1970) demonstrated that progeny of hemopoietic precursor cells could become immunologically competent T cells only after traffic through the thymus.

It can be concluded from the above summarised findings, that the presence of thymus is a prerequisite for the development of a subpopulation of lymphocytes (T cells) that plays a role in the generation of immune responses. Therefore, the thymus can be considered as a primary lymphoid organ (Miller and Osoba, 1967).

4 ONTOGENY OF FUNCTIONAL THYMUS CELLS

In an adult thymus, the analysis of cell differentiation meets specific difficulties due to the steady state system. The proportion of the different components there do not change considerably. In contrast, during ontogeny the analysis of hemopoietic cell differentiation is facilitated by the sequential appearance of the different cell types. However, the number of cells available, especially for functional studies, is often a limitation.

Development of mature T cells is dependent on the presence of an intact thymus and is under the control of the humoral function of the thymus (Stutman, 1977; Papiernik and Bach, 1979; Papiernik and Nabarra, 1981). During fetal life, the production of thymic factors (FTS) starts on day 12 to 13 of gestation and reaches adult levels at about the time of birth (Dardenne and Bach, 1976). This gradual development of the secretory function of the thymus is accompanied by a simultaneous development of thymus cell populations with Thy-1 and Lyt surface markers (Stobo and Paul, 1972; Mathieson, 1980). In a few days after birth, cell populations which express a cell surface marker distribution corresponding with that of the mature mouse thymocyte population develop (Haaijman et al., 1981) (Table I).

In most mammals, there is an immunological unresponsiveness during fetal life (Ashman, 1981) and, in the mouse, the expression of T-cell antigens that have been associated with T-cell functions precedes the immunological maturation which starts from birth and reaches maximum levels in young adults (Mosier and Cohen, 1975). Hoffman and Globerson (1973), however, found GvH reactive cells in the yolk sac. But in the thymus, some GvH activity develops from the day of birth and thereafter remains weak (Cohen et al., 1963; Ritter, 1971). The response to PHA and the MLR develop from one week after birth and reach adult levels between 30 and 60 days (Adler, 1970; Stobo and Paul, 1972; Wu, 1978). The sequence of development of functional T cells in the thymus during ontogeny is similar to that observed in radiation chimeras (Owen et al., 1977) and in grafted neonatal thymus (Dardenne and Tubiana, 1979). During ontogeny, the helper function of thymus cells in the antibody response to T dependent antigens shows a relatively slow development as compared with that observed for other functional T cells (Chiscon and Golub, 1972; Mosier and Johnson, 1975; Haines and Siskind, 1980). T cells active as suppressors of in vitro immune responses have been observed in neonatal as well as fetal thymus. According to Luekenbach et al. (1978), thymus cells from day 14 fetuses were active suppressors in in vitro anti-SRBC immune responses.

Thymus derived lymphocyte functions in general develop from the time of birth and lag somewhat behind phenotypical differentiation. In the following sections, the sources from which the progenitors of thymic lymphocytes are derived (5), their traffic into the thymus (6) and the development of differentiated thymocyte subpopulations (7-12) will be discussed.

TABLE I

T LYMPHOCYTE SUBPOPULATIONS* IN THYMUS, SPLEEN AND LYMPH NODES

	Thymocytes		Thymus migrants		Spleen		Lymph node	
I-A	30-50	dull[5]	30-50	dull[5]	15-20	dull[5]	8	dull[5]
H2D	10-20 80-90	bright[5] dull[5]	100	bright[5]	30-40	bright[5]	80	bright[5]
H2K	10-20 10-40	bright[5] dull[5]	100	bright[5]	30-40	bright[5]	80	bright[5]
TL	65-75	bright[5]	undetectable[5]		3-7	dull[5]	8-16	dull[5]
Thy-1	80-90 10-20	high[2,4,8,9] low[2,4,8,9]	98[3,6]		30-40	10-15 high[1,3,4,9] 20-25 low[1,3,4,9]	80[4,5]	
Lyt-1	20-40 60-80	bright[4,7,8,9] dull[4,7,8,9]	98[3,6]		30-35	bright[3,4,7,9]	80[3,4,7]	bright[3,4,7]
Lyt-2	85-90	bright[4,7,8,9]	31[3,6]		9-15	bright[1,3,4,7,9]	25	bright[3,4,7,9]

* Bright and dull subpopulations are expressed as a percent of the cells in the anatomically defined cell population; other cells are negative.

1 Cantor et al., 1975a,b
2 Fathman et al., 1975
3 Scollay et al., 1978
4 Ledbetter et al., 1980
5 Scollay et al., 1980
6 Scollay and Weissman, 1980
7 Ledbetter et al., 1980
8 Van Ewijk et al., 1981
9 Haaijman et al., 1981

5 SOURCES OF LYMPHOPOIETIC PROGENITOR CELLS

Progenitor cells of lymphocytes are found in various hemopoietic tissues which differ in their capacity to produce cells that contribute to lymphopoiesis by differentiation in the thymus. These differences have been ascribed in part to microenvironmental factors that influence early stages of hemopoiesis. Yolk sac derived hemopoietic progenitor cells are not able to directly repopulate the thymus of an irradiated mouse (Tyan, 1968; Owen, 1977; Stutman, 1978). Also for the chicken it has been thought that yolk sac cells do not directly differentiate into T cells in the thymus (Lassila et al., 1978, 1979). More recent experiments of Lassila et al. (1980) suggest that yolk sac cells in the chicken acquire their capacity for homing in the thymus with the age of gestation. Yolk sac cells of day 7 have lymphoid stem cells while day 2 yolk sac cells do not (Lassila et al., 1980). According to Stutman (1978), yolk sac cells following transplantation into lethally irradiated mice, develop into T-cell progenitors in the spleen.

Cells from the fetal liver, which is a hemopoietic organ in the mouse, can function as thymus cell progenitors (Vos et al., 1960; Tyan, 1964; Moore and Metcalf, 1970). When compared with bone marrow cells, however, fetal liver cells were shown to be relatively less efficient in repopulation of the thymus (Micklem et al., 1966, 1975). During development in the fetus, the thymus is colonized by prethymic hemopoietic cells. Work of the French group of LeDouarin has shown that in the chicken there is only a limited time span during which prethymic cells can enter the fetal thymus (LeDouarin, 1977; Jotereau et al., 1980). It has also been recently shown in vitro that cells from the yolk sac, spleen and bone marrow migrate into the thymus during a defined period of ontogenesis (Jotereau et al., 1980). It is thought that the "receptive" thymus produces regulating factors that influence the timed chemotaxis (Pyke and Bach, 1979; Jotereau et al., 1980). Haar and Loor (1981) have observed in an in vitro mouse system, chemotaxis of bone marrow and fetal liver cells through a 5.0 μ pore membrane towards a thymus derived humoral factor.

It has not been reported whether the entrance of lymphoid progenitor cells into the thymus of the mouse takes place at a definite time during gestation. During ontogeny, fetal liver becomes the primary hemopoietic organ from day 10 to 13 from gestation. In the mouse thymus, the first cells which can be recognized as being of the T-cell lineage (in that they express Thy-1 surface antigens) are found in the thymus on day 12 to 13 of gestation (Owen and Ritter, 1969; Owen and Raff, 1970; Ritter, 1978). Fetal spleen and bone marrow cells have not been extensively investigated for their capacity to produce lymphopoietic progenitor cells. On a morphological basis, it was concluded that large "lymphoid" blast cells in fetal spleen and bone marrow can be seen at all stages of hemopoiesis during ontogeny. Further functional identification has not been made as yet (Owen, 1977).

In adult life, thymus cell progenitors can be derived from bone marrow and spleen. Spleen cells are less efficient than bone marrow cells in the production of thymus cells (Muramatsu et al., 1976; Shisa et al., 1977). In the spleen, the proportion of pluripotent stem cells (CFU-S) is lower than in bone marrow. Even after transplantation of comparable numbers of CFU-S, there is a remarkable difference between the thymus regeneration capacity of bone marrow and spleen cells (Muramatsu et al., 1976). It is thought that the microenvironment from which the hemopoietic cells are derived influences the differentiation and proliferative capacities of these early hemopoietic cells (Lepault et al., 1979). The efficiency of spleen derived progenitor cells in the production of lymphocytes in an irradiated mouse thymus can be enhanced by cotransplantation of thymocytes, which themselves lack thymus repopulating capacity (Shisa et al., 1977). Therefore, the behaviour of spleen derived progenitors of the T-cell lineage could be based on a homing pattern which is different from that of bone marrow progenitors.

Thus, during ontogeny, T-cell progenitors are present mainly in the yolk sac and fetal liver. In the adult mouse, the bone marrow is the major source and spleen cells contribute only to a limited extent. All sources of lymphopoietic precursors, however, also contain pluripotent hemopoietic cells (CFU-S). In attempts to characterise T-cell progenitors, therefore, the contributions of pluripotent and committed progenitor cells to the production of T cells must be analysed separately. A direct approach to achieve this goal might be characterisation of the cells that immigrate into the thymus.

6 TRAFFIC OF PROGENITOR CELLS

Homeostasis in the T-lymphocyte system is a complex phenomenon of influx of progenitor cells, intrathymic differentiation, selection and cell death and finally efflux from the thymus.

Balner and Dersjant (1964) showed in irradiated recipient mice that only very few (0.1%) of radioactively labeled transfused bone marrow cells reach the thymus. These observations on specific seeding have been recently confirmed by Lepault and Weissman (1981), who used staining with fluorescein as a marker.

Progenitor cells enter the thymus via the bloodstream (Brumby and Metcalf, 1967; Owen and Ritter, 1969). Doenhoff and Davies (1971) and Lepault and Weissman (1981) observed that homing of cells in the thymus was proportional to the number of cells inoculated, at least up to 10^8 bone marrow cells transfused. Of transplanted bone marrow cells reaching the thymus (0.075%), about one fifth were transformed into Thy-1^+ cells within 24 h after infusion (Lepault and Weissman, 1981). If the latter cells are considered as putative T-cell progenitors, their

frequency in the bone marrow is at least in the order of 15 per 10^5 bone marrow cells transplanted. To estimate the total number of T cell progenitors more accurately, the seeding efficiency of these cells with respect to the thymus has to be determined. This has been shown to be impossible to analyse in serial transplantations since prothymocytes upon retransplantation loose their original characteristics (Boersma et al., 1981).

If it is assumed that the concentration of prothymocytes in bone marrow cells is in the same order of magnitude as observed for progenitor cells in other lineages (1%) (for a review, see Bol, 1980), based on the data of Lepault and Weissman (1981), it can be calculated that about 15 per 10^3 prothymocytes can be expected to produce progeny in the thymus.

It has been noted that the seeding of transfused bone marrow cells in the thymus is inversely related to the proportion of cycling cells in the grafts. Balner and Dersjant (1964) found that dividing cells in a bone marrow graft do not reach the thymus in significant numbers. From this, it was concluded that the homing pattern of thymus cell progenitors probably varies with their cell cycle status.

Thus, from a quantitative analysis of the traffic of bone marrow cells into the thymus (0.075%), it can be concluded that the number of immigrant prothymocytes (0.015%) is higher then would be expected on the basis of nonspecific homing. One might speculate that cell surface markers on immigrant cells in thymus tissue play a role in the recognition patterns that influence homing of T cell progenitors into the thymus. The lymphopoietic progenitor cells that enter the thymus have been characterised in various systems including ontogenetic thymic development and regenerating thymus in bone marrow chimeras. The phenotypic characteristics of these cells and their progeny will be discussed in the following sections.

7 EARLIEST STAGES IN T-CELL DIFFERENTIATION

Use of antibodies to their cell surface antigens is one of the possible approaches in characterising hemopoietic cells in a sequence of development. This type of work is based on the assumption that hemopoietic cells of one lineage will share one or more antigenic determinants, while the cells in the various stages of differentiation in the sequence of development are characterised by others.

The surface antigen Thy-1 has been demonstrated on mouse thymus cells, various leukemia cells, nerve tissue (Reif and Allen, 1964) and mouse epidermal cells (Scheid et al., 1972). Two Thy-1 alloantigenic determinants are known in mice: Thy-1.1 and Thy-1.2. These have been found to be present only on thymus processed lymphocytes (T cells) and not on antibody forming cells (Raff, 1971). Thy-1.1 antigenic determinants have also been detected on rat thymocytes with mouse alloantisera (Douglas, 1972; Williams, 1977).

The characterisation of cells that enter the thymus and of their differentiation products, the first recognisable T-lineage cells, was originally based on morphological criteria. Such studies have been made in the fetal thymus in vivo, in organ cultures of the thymus and in the regenerating thymus after total body irradiation in intact and parabiotic animals. For recognition of the successive differentiation stages, the thymic cells have been distinguished by classical cytology and by cell surface antigens (Thy-1, Lyt-1, Lyt-2, Lyt-3) (Table I and Fig.2).

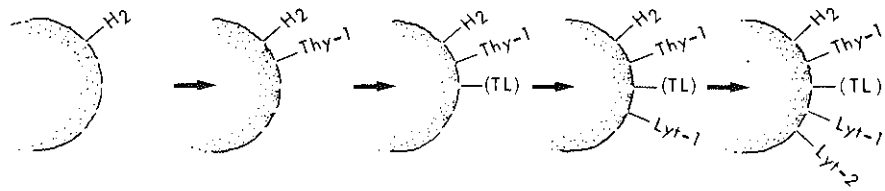

Fig. 2 Sequential development of thymocyte surface markers during ontogeny.

The first cells that enter the thymus have no known surface markers except H-2 (Owen and Raff, 1970; Ritter, 1978; Jotereau et al., 1980; Lepault and Weissman, 1981). These are large basophilic cells which go through a series of blast cell generations (Dukor et al., 1965; Moore and Owen, 1967a,b; Sato and Sakka, 1969). In grafted epithelial thymus (irradiated in vivo), the first typical small lymphocytes become apparent at day 5 to 6 after transplantation and at that time a cortical structure can be recognized (Dukor et al., 1965). In fetal thymus, the influx of the basophilic cells is observed at 11 days of gestation (Moore and Owen, 1967a,b; Owen and Ritter, 1969; Owen and Raff, 1970; Mandel and Kennedy, 1978; Ritter, 1978). Then markers are subsequently expressed on the thymus cell surface. In fetal thymus organ culture, the lymphoid cells with "low" Thy-1 antigen density acquire TL antigens between day 13 and 14 (Mandel and Kennedy, 1978). At day 16 to 17, cells which express TL have developed a "high" Thy-1 antigen density (Owen and Raff, 1970; Mandel and Kennedy, 1978). After bone marrow transplantation, the cells from the graft which enter the thymus show the same sequence of development of T-cell-specific antigens as is observed during ontogeny (Moore and Owen, 1967b; Owen and Ritter, 1969; Lepault and Weissman, 1981). These observations indicated that the radiation induced bone marrow chimera can be used as a model to mimic the sequence of events observed in ontogeny. The appearance of Thy-1 antigens marks the initial stage of the thymus lymphocyte development (Fig.2). A quantitative analysis of the development of subclasses of thymus lymphoid cells after transplantation of hemopoietic cells has been performed using Thy-1 as a marker. Results of these studies are presented in the Chapters 3 to 6.

8 THY-1$^+$ CELLS IN THE MOUSE THYMUS

Two subpopulations of Thy-1$^+$ thymus cells have been described (Shortman et al., 1973). One has a "low" Thy-1 antigen density and constitutes 10-20% of total cells. A second population (80-90%) has a "high" expression of Thy-1 (Shortman et al., 1973; Zeiller et al., 1974; Fathman et al., 1975) (Table II).

"Low" Thy-1$^+$ thymocytes are relatively cortisone resistant (Blomgren and Andersson, 1971; Jacobsson and Blomgren, 1974), express a relatively high level of H-2 antigens (Ishidate and Metcalf, 1963), have a relatively high electrophoretic mobility (Zeiller et al., 1974; Droege et al., 1974), low buoyant density and high sedimentation velocity (Shortman et al., 1975). "High" Thy-1$^+$ cells are cortisone sensitive, have low levels of H-2, low electrophoretic mobility and sedimentation velocity and a high buoyant density.

Among the "low" Thy-1 cells are at least the cells that are able to give a proliferative response upon mitogenic stimulation with ConA and PHA (Stobo and Paul, 1972; Blomgren and Andersson, 1974) and can generate a mixed lymphocyte reaction (Shortman et al., 1975). Progenitors for cytotoxic T cells also have a "low" Thy-1 phenotype (Hopper et al., 1978). The role of "high" Thy-1$^+$ cells in immunoresponsiveness until now has remained unclear. In general "low" Thy-1$^+$ cells are therefore thought to be more mature than the "high" Thy-1$^+$ ones.

In the thymus, the cells with "low" Thy-1 expression are located mainly in the thymus medulla and those with "high" Thy-1 expression are predominantly found in the cortical part of the thymus (Shortman and Jackson, 1974). The numbers of "low" Thy-1$^+$ cells account for the total number of cells in the medulla (~ 15% of all cells) and "high" Thy-1$^+$ cells for those in the cortex (~ 85% of all cells) (Metcalf, 1966; Shortman and Jackson, 1974; Droege and Zucker, 1975). Among both "low" and "high" Thy-1$^+$ cells, a subpopulation of cycling cells was demonstrated. In adult mice, the two subpopulations seem to develop simultaneously from large dividing lymphocytes (Shortman and Jackson, 1974). Shortman and Jackson (1974) concluded that "low" and "high" Thy-1$^+$ cells develop independently and represent two separate lines of development in the thymus.

From the distribution patterns of radioactive label (^{3}H-thymidine) applied to the outer surface of the thymus (Weissman, 1973; Weissman et al., 1975a,b; Fathman et al., 1975) and from the distribution of FITC injected into the thymus (Scollay et al., 1980, Scollay and Weissman, 1980), it was concluded that a gradually increasing level of differentiation exists from the outer cortex to the medulla. According to Scollay et al. (1981), all cells in the outer cortex are "high" Thy-1$^+$ cells. Therefore, it was assumed that "high" Thy-1$^+$ cells of the outer cortex represent the progenitor cells of "low" Thy-1$^+$ cells. Recently, however, Van Ewijk et al. (1981), using a different technique, did not confirm these findings and demonstrated that cells with different levels of Thy-1 expression are present in the outer cortex of the thymus. This leaves the possibility

open that cells with different Thy-1 antigen density may function as intrathymic progenitor cells. A development as two separate lineages of "low" and "high" Thy-1$^+$ cells remains an alternative mechanism.

9 THE FUNCTION OF DIFFERENT LYT$^+$ CELL POPULATIONS

A system of alloantigens on mouse lymphocytes has been identified by Boyse et al. (1968, 1971). These Lyt-1, Lyt-2 and Lyt-3 alloantigens were shown to be related to distinct functional subpopulations (Cantor and Boyse, 1975a,b; Kisielow et al., 1975). The T lymphocytes form a complex network of effector cells, regulating cells and factor producing cells that are characterised by expression of different combinations of Lyt antigens. In addition, these cells show different levels of Lyt antigen expression (Mathieson et al., 1979a, 1981; Al-Adra et al., 1980; Ledbetter et al., 1980; Nagy et al., 1981).

Helper effects of T cells on humoral immune responses were associated with cells that express Lyt-1 antigens. Lyt-2 and Lyt-3 alloantigens were associated with suppressor effects and cytotoxic cells (Cantor and Boyse, 1975a,b). Helper and suppressor functions of Lyt subpopulations were shown to be interdependent resulting in network like systems (Cantor and Boyse, 1977; Boyse and Cantor, 1978, Cantor et al., 1978).

10 EXPRESSION OF LYT ANTIGENS ON THYMOCYTE SUBPOPULATIONS

Almost all cells (99-100%) in the adult thymus express Thy-1 and Lyt-1 antigens (Ledbetter and Herzenberg, 1979). Only 0.5-1% of Thy-1$^+$ cells are negative for Lyt-1 (Ledbetter and Herzenberg, 1979; Mathieson et al., 1979a). Of all thymocytes, about 90% express Lyt-2 (Ledbetter and Herzenberg, 1979; Mathieson et al., 1979a). According to their location in the organ, thymus lymphocyte subpopulations show a different expression of Lyt antigens (Table II). Medullary cells which are "low" Thy-1$^+$ (10-20% of all thymocytes) express high levels of Lyt-1 and are mostly negative for Lyt-2 (Van Ewijk et al., 1981). Medullary Lyt-1$^+$ cells show no variation in antigen expression. Most cortical cells which are "high" Thy-1$^+$ show intermediate staining with Lyt-1; however, also bright and dull Lyt-1$^+$ cells can be observed in the cortex. Most cortical cells express high levels of Lyt-2, but a few dull Lyt-2$^+$ cells are also found in the cortex (Van Ewijk et al., 1981). In the outer cortical zone, cells express high levels of Thy-1 and variable levels of Lyt-2 antigens. In the subcapsular region, large lymphoblast like cells express Thy-1 antigen levels varying from "high" to "low"; some of these cells are bright Lyt-1$^+$ (Van Ewijk et al., 1981). Localisation of T-cell subpopulations in the thymus has been performed by fluorescent antibody staining of cells in frozen sections (Van Ewijk et al., 1981). It has not been described as

TABLE II

INTRATHYMIC ANATOMICAL DISTRIBUTION OF T-CELL SUBPOPULATIONS*

Antigen	All thymocytes		Subcapsular		Cortex		Medulla	
IA[5]	30-50	dull		n.d.**		dull		dull
H2D[5]	10-20 80-90	bright dull		bright		dull		
H2K[5]	10-20 20-40	bright dull		bright	20-50	dull		bright
TL[5]	65-75	bright	70-80	bright	70-80	bright	30-40	bright
Thy-1[2,4,8,9]	80-90 10-20	high low		negative		high		low
Lyt-1[4,7,,8,9]	20-40 60-80	bright dull		negative	40 60	bright dull		bright
Lyt-2[4,7,8,9]	85-90	bright		negative		bright		bright

* Bright and dull subpopulations are expressed as a percent of the cells in the anatomically defined cell population; other cells are negative.

** n.d.= not done.

1 Cantor et al., 1975a,b
2 Fathman et al., 1975
3 Scollay et al., 1978
4 Ledbetter and Herzenberg, 1979
5 Scollay et al., 1980
6 Scollay and Weissman, 1980
7 Ledbetter et al., 1980
8 Van Ewijk et al., 1980
9 Haaijman et al., 1981

to what extent the resolution of this method is comparable to that of flow cytofluorometry. In double labeling experiments, Ledbetter et al. (1980) showed that expression of "high" levels of Thy-1 and Lyt-2 on thymocytes is correlated with a low expression of Lyt-1, whereas "low" Thy-1^+ cells are generally bright Lyt-1 and negative for Lyt-2.

According to Scollay et al. (1978), 30% of thymic emigrant cells have a Lyt-1^+,2^+ phenotype. In the thymus, such a Lyt antigen distribution is found on "high" Thy-1^+ cells (Ledbetter et al., 1980). Since a similar proportion of spleen T cells express relatively high levels of Thy-1 antigens (Cantor et al., 1975; Haaijman et al., 1981), it might be concluded that the "high" Thy-1^+ subpopulation of thymus cells contributes directly to the pool of peripheral T cells.

Treatment of mice with cortisone leads to a decrease in the cortical area of the thymus. Cortisone resistant cells are enriched for cells which are "low" Thy-1^+, bright Lyt-1^+, Lyt-2^-; a few cells showing high levels of Thy-1 and being negative for Lyt-1 and Lyt-2 are located in the outer cortex (Mathieson et al., 1979a; Van Ewijk et al., 1981).

Several observations indicate that, analogous to the subdivision of Thy-1^+ cells, there are probably two independent lineages of cells with Lyt surface antigens. One is the separate development of functional cells in congeneic chimeric mice which were deprived of Lyt-1^+ or Lyt-2^+,3^+ subclasses of lymphocytes (Huber et al., 1976). In ENU induced T-cell tumours, expression of Lyt antigens is restricted to Lyt-1 or Lyt-2 only (Mathieson et al., 1978).

In the fetal thymus, Lyt antigen expression, which occurs on day 14 to 15 of gestation, follows the expression of Thy-1 (Mathieson et al., 1981, Kamarck and Gottlieb, 1977). The Lyt-1^+ cells which are detected first have a low expression of antigens, which increases to adult levels at around the time of birth. Lyt-1 expression precedes Lyt-2 (Mathieson et al., 1981) (Table II).

Using the method of subcapsular topical labeling, Scollay and Weissman (1980) observed that, in adult mice, two cell populations of thymic emigrants with Lyt-1^+, 2^- and Lyt-1^+,2^+ phenotypes developed simulataneously.

Most of the evidence emerged so far supports the existence of two lineages of Thy-1^+ cells and two lineages of Lyt$^+$ cells in the thymus. According to Betel et al. (1980a,b), the "low" Thy-1^+ subpopulation is predominantly Lyt-1^+, 2^-,3^-, but also contains Lyt-1^+,2^+,3^+ cells. The latter subpopulation has been shown to consist at least in part of precursors for functional T cells (Mathieson et al., 1979b). The "high" Thy-1^+ cells consist of Lyt-1^+,2^+,3^+ cells only (Betel et al., 1980a,b). These observations are best explained in a model for T-cell development based on simultaneous development of two lineages, one having "low" Thy-1^+ cells as progenitors, the other "high" Thy-1^+ progenitors.

The separate lineages of Lyt cells can already be detected in the fetal thymus. On this early observation, Cantor and Boyse (1975a,b) and Jandinsky et al. (1976) based their hypothesis that the generation of Lyt subpopulations is a

differentiation process independent of an encounter with exogenous antigens. The question then arose, whether a different progenitor or prethymic committed cell exists in the bone marrow for each lineage of Lyt^+ cells. There are as yet no definitive experimental data to support this hypothesis.

11 THE THYMUS AS A PRODUCTION UNIT FOR PERIPHERAL T CELLS

There is an important discrepancy between the proportions of the T-cell subpopulations found in the thymus as compared with those in the peripheral lymphoid organs. In the thymus, the "low" Thy-1^+ cells represent a minor subpopulation (± 15%), while almost all peripheral T cells have a low expression of Thy-1. "High" Thy-1^+ cells constitute the majority of thymocytes but are relatively rare among peripheral T cells. Considering these differences, the question of what can be the mechanisms underlying this difference arises (Table II). Quantitative aspects will be discussed in this section.

Most cells in the peripheral lymphoid organs have a low expression of Thy-1^+ antigens (Reif and Allen, 1964). According to Cantor et al. (1975), spleen T cells (30-40% in adult spleen) consist primarily (20-25%) of "low" and partly (10-15%) of "high" Thy-1^+ cells. With increasing age, the proportion of "low" Thy-1^+ cells in the spleen increases (Cantor and Weissman, 1976). Although different levels of Thy-1 expression can be observed, Haaijman et al. (1981) report that Thy-1 expression (on spleen and lymph node cells) never reaches levels comparable to that observed in "high" Thy-1^+ thymocytes.

Since the thymus appears to function as a production unit for peripheral T lymphocytes, several authors have investigated the relation between intrathymic differentiation and the influx and outflux of cells from the thymus. In young adult mice, an overall production of 40-50 x 10^6 cells/day has been calculated on the basis of radioactive label redistribution patterns (Bryant 1972; Joel et al., 1974; Claësson and Hartmann, 1976). The numbers of cells arising in the peripheral lymphoid tissues could account for only a small proportion of the cells produced (Bryant, 1972; Joel et al., 1977). Using subcapsular labeling, it was found that only 1% of the number of thymus cells in adult mice could be traced in the peripheral lymphoid organs. The number of emigrants from the thymus per day increases from 0.1×10^6 at one week of age to 2×10^6 at 4-5 weeks of age. Thereafter, a gradual decrease occurs to 0.1×10^6 at 6 months of age (Scollay et al., 1981). It has been concluded that most cells (60-95%) produced in the thymus die in situ (Bryant, 1972; Joel et al., 1977; McPhee et al., 1979), although there is no direct morphological evidence for this.

The phenomenon of intrathymic death is often explained as being the result of a selection system in T-cell differentiation and in regulation of T-cell reactivity. According to the clonal selection theory of Burnet (1959), the thymus has

been defined as that part of the immune apparatus that distinguishes between self and non-self by the elimination of "forbidden" clones.

The total net production of thymocytes per day is calculated at about 5 times the number of cells in the peripheral pool (Joel et al., 1974). The latter cells partly belong to a longer lived T-cell population which will contribute only little to the turnover of T lymphocytes. A tentative conclusion that can be drawn from studies on thymus cell traffic and production is that efflux from the thymus does not influence thymus cellularity to any great extent.

The questions that arise from above observations are the following: 1) which cells are selected to die in the thymus?; 2) which cells will go to the periphery?; and 3) what is (are) the responsible mechanism(s) involved? Hopper and Shortman (1976) and Hopper et al. (1978) have observed that, under the best available culture conditions, the "high" Thy-1^+ thymus cells rapidly die in vitro. The cells that survive in culture are cortisone resistant, "low" Thy-1^+ and "high" H-2 cells; they respond to mitogens and in MLR and give a CTL response. Culturing of thymus cells in thymus conditioned medium allowed growth of the "low" Thy-1^+ cell population (Hopper et al., 1978). The authors suggested that a large proportion of "high" Thy-1^+ cells which die in vitro will also die in vivo.

Jerne (1971) proposed that the T-cell repertoire would be dependent on germ line genes coding for structures complementary to MHC alleles. According to this hypothesis, it is to be expected that only cells with a low affinity for self-antigens are released into the periphery. The mechanism of selection for survival in the thymus concerns only a small proportion of the cells produced.

12 THE ROLE OF THE THYMUS IN EDUCATION OF T-CELL PROGENITORS

When a thymus was grafted in a thymectomised (Miller et al., 1962, 1963) or nude mouse (Loor et al., 1976a,b), the graft was in due time repopulated by progeny of host derived hemopoietic cells. The restoration of T-cell reponses in athymic animals receiving a thymus transplant was found to be dependent on histocompatibility of donor and recipient. Zinkernagel and Doherty (1974, 1977) and Zinkernagel (1978) first recognised this MHC restriction or, better, preferences of cytolytic T cells, in virus infected thymectomised and thymus grafted animals. The response specificity or preference was shown to be coded for by the major histocompatibility antigens of the thymus donor. In radiation chimeras, it was shown that the progeny of (a × b)F_1 stem cells maturing in an a thymus contains T cells that can respond to virus infected a cells but not to infected b cells. But progeny of a type stem cells differentiating in (a × b)F_1 thymus had anti-infected-a as well as anti-infected-b activity. MHC restriction of a virus specific response was not found in fully allogeneic chimeras (Bevan and Fink, 1978; Zinkernagel et al., 1978a,b; Blanden and Andrew, 1979). It was thought that viral antigens on infected cells were recognized as an entity, together with

histocompatibility antigens, which then gave rise to a cell surface modified as compared with "self". This so-called altered-self hypothesis was based on the assumption that the differentiated T cells were "educated" in the thymus to respond to "non-self".

Hünig and Bevan (1982) using cloned cytolytic T cells specific for self-H-2 confirmed the altered-self hypothesis. They demonstrated that restricted responsivenes is coded for by a one-receptor controlled process that recognises at the same time a structure that is called the self-H-2 restriction element and the expression of the target antigen. The K and D region structures of the H-2 complex form the restriction elements for cytolytic T cells that recognise cell surface antigens (Pfizenmaier et al., 1980; Wagner et al., 1980a,b; Hünig and Bevan, 1982).

The proposed MHC restricted mechanism for T-cell activity, however, does not account for the following observations. 1) Kindred and Loor (1974), Kindred (1976, 1977) and Isaak (1978) showed that, after transplantation of a thymus in nude mice, the T cells showed a preference but no absolute specificity in the response pattern with respect to the MLC determinants and skin grafts of the strain of the thymus donor. In strain a nude mice that received an (a × b)F_1 thymus, the cytolytic response to virus infected cells was restricted to a type cells. No response to infected b cells was observed (Zinkernagel et al., 1980). 2) In apparent contrast to earlier reports, Kreeb and Zinkernagel (1980) recently showed that for virus antigen induced altered self there is only a limited form of restriction of the cytolytic response. After depletion of alloreactive T cells in certain MHC haplotype combinations, still T cells were left that could respond to the hapten trinitrophenyl (TNP) (Wilson et al., 1979) or to viral antigen (Doherty and Bennink, 1979) present on allogeneic cells. 3) In fully allogeneic chimeras, the host MHC (thymus) coded for the restricted response of a proportion of the T cells. Most T cells showed restriction coded for by the MHC type of the bone marrow donor (Bevan, 1977; Matzinger and Mirkwood, 1978; Wagner et al., 1980a,b). The response of T cells to minor histocompatibility antigens in incompatible chimeras showed no selectivity for donor or host H-2 type (Matzinger and Mirkwood, 1978).

These data suggest that cells with different MHC restriction types can mature in the thymus. This allows speculation as to where and when during T cell differentiation the capability of discrimination between self and non-self is acquired (Matzinger and Waterfield, 1980). But a word of caution must be given with respect to the intercomparison of the foregoing data. Very complex in vivo and in vitro systems which possibly will lead to differences in antigen presentation, have been used to analyse the restriction phenomena. The quality and condition of laboratory animals, e.g., GvHD, the dose of irradiation and bone marrow transplanted as well as the time of investigation after establishment of the chimera varies in the above-mentioned studies (see also Wagner et al., 1981).

Furthermore, cytolytic responses on which most restriction data are based are probably not solely dependent on a single cell type response but consist of a complex cooperation of factor producing cells, antigen presenting cells, etc. Each of these functions will have its own time course of development during T-cell differentiation.

Kruisbeek et al. (1981) showed that parent→F_1 and F_1→parent chimeric thymocytes of donor type could give a cytotoxic response at four weeks after transplantation provided that IL2, a humoral T cell factor, was added to the culture. These relatively immature thymocytes of either chimera showed an allo-reactive response to third parties but were tolerant to the MHC haplotypes of both parents. The response to trinitrophenol (TNP) modified cells of donor and recipient was restricted to cells with MHC determinants syngeneic to the chimeric host (thymus). The same group, (Morrissey et al., 1982), found that chimeric thymocytes of thymectomised ($\underline{a} \times \underline{b}$)$F_1$ mice that received an $\underline{a}$ type thymus graft and were subsequently lethally irradiated and reconstituted with $\underline{a}$ type bone marrow cells were tolerant to the $\underline{b}$ MHC haplotype. They concluded that pre-thymic encounter of an antigen led to tolerance. However, the possibility that immunocompetent chimeric thymocytes were of extrathymic origin, e.g., by traffic via the spleen, was not excluded.

It has been recently demonstrated that, in radiation chimeras, T-helper activity is coded for by the thymus MHC determinants (Singer et al., 1981). Spleen cells in thymus grafted nude mice were of host origin but recognised a "help" situation only in the context of the thymus MHC haplotypes (Singer et al., 1982). In contrast, experiments of Kindred (1980, 1981) showed that T cells from parent → F_1 chimeras gave help only to B cells of their own H-2 genotype.

Some of these observations clearly demonstrate that the thymus microenvironment plays an important role in the "specificity education" of the T-cell response while others do not. This makes the situation far from clear. In addition, it has been demonstrated that the thymus determines the magnitude of the response. Kindred (1977) and Von Boehmer et al. (1978) employed combinations of genetically determined high and low responder strains for specific T-cell responses. They found that the host derived T cells in a nude mouse that received a thymus graft showed the response pattern of the thymus donor. However, observations of Fink and Bevan (1981) provided no such evidence. They showed that the thymus allows differentiation and maturation of bone marrow progenitor cells of different origin. When two thymuses of different genetic background were transplanted in one recipient, no selective differentiation in the H-2 compatible thymus as compared with allothymus grafts was observed.

The literature data summarised above illustrate the complexity of the educational processes in the thymus. The observation that so-called restriction phenomena are not absolute makes it more difficult to understand the selection criteria.

In fetal thymus, antigens of the K and I regions of the MHC have been demonstrated on the cells of the thymic stroma from day 14 of gestation. The I-A

antigens are present in limited amounts at that time (Jenkinson et al., 1980). Mouse thymus dendritic cells also express MHC antigens (Rouse et al., 1979). According to Van Ewijk et al. (1980), the stromal cells in the thymus medulla express H2K and I-A, whereas the cortex is negative for MHC antigens. Some areas in the thymus have specialised in expressing MHC antigens. This suggests that the antigens of the H-2 complex present on cells of the thymic microenvironment could play a role in the education of T cells to preference for "nonself" antigens.

Recently, a specific cellular structure has been found in the thymus. This so-called nurse cell is a large Thy-1$^-$, Ig$^-$, bag-like cell that contains up to 50 small and medium-sized lymphoid cells (Wekerle and Ketelsen, 1980). It has been assumed that nurse cells play a role in the very first processes that bone marrow derived progenitor cells undergo on their intrathymic maturation pathway. I-A as well as K and D region antigens of the H-2 complex which could play a role in education to self recognition have been demonstrated on or in the nurse cell.

The supposedly immediate progenitors of prothymocytes, the pluripotent stem cells have been shown to express H-2 determinants (Russel and Van den Engh, 1979; Trask and Van den Engh, 1980). The prothymocytes have not yet been characterised for MHC-antigens, but mapping of H-2 antigens on prothymocytes could, apart from providing tools for separation studies, give a clue to a better understanding of early intrathymic differentiation of functional T cells (Mathieson, 1980; Matzinger, 1981). Preliminary studies indicate that prothymocytes and CFU-S differ in the expression of K region antigens of the H-2 complex (A.H. Mulder, personal communication).

13 IN VITRO DIFFERENTIATION OF PRETHYMIC HEMOPOIETIC CELLS

The endocrine function of the thymus probably influences the differentiation of thymus lymphocytes. Thymus specific factors have been shown to induce T-cell specific surface antigens in vitro. (For a review of the influence of thymic factors on T-cell function and markers, see Kruisbeek (1978) and Pahwa et al. (1979). To determine the effect of thymic factors on prethymic cells, these cells have been operationally defined as those displaying no exclusive T cell feature. In practice, the transition of Thy-1 negative to Thy-1 positive cells has been accepted as the earliest recognisable event in T-cell differentiation (Komuro et al., 1975).

A serum derived thymus dependent factor, "Facteur Thymique Serique", (FTS) has been shown to induce T-cell markers on Thy-1 negative bone marrow cells in vitro (Dardenne et al., 1974).

Various thymic factors have also been isolated by biochemical purification of thymus gland preparations. Also with those preparations, induction of T cell markers Thy-1 and TL on previously negative bone marrow cells has also been

demonstrated (Bach et al., 1971; Komuro and Boyse, 1973a,b; Basch and Goldstein, 1974; Hooper et al., 1975). Thy-1 antigens were induced by the same factors on nude mouse spleen and bone marrow cells and on fetal liver cells (Komuro and Boyse 1973a; Scheid et al., 1975). According to Komuro and Boyse (cited by Stutman, 1977), yolk sac cells were not inducible with a thymus derived factor (thymopoietin). Basch and Goldstein (1974) found that, after induction of Thy-1 on bone marrow and spleen cells with thymopoietin, the surface Thy-1 antigen density is comparable to that observed in the "high" Thy-1$^+$ thymocyte population. It has been shown that many substances (hormones, cyclic AMP) are able to mimic the effect of thymus derived humoral factors (Scheid et al., 1973, 1978; Singh and Owen, 1975; Ahmed et al., 1978; Horowitz and Goldstein, 1978).

Most of the induction experiments discussed so far were performed before quantitative membrane fluorescence by cytofluorography became available. It cannot be excluded therefore that the induced bone marrow cells expressed a low level of T-cell antigens which could not be detected with conventional antisera and complement in cytotoxic assay systems. The cells that are sensitive to in vitro treatment with thymic factors were considered to be thymocyte precursor cells, i.e., prothymocytes. It still remains to be shown whether cells that can be induced to express T-cell-specific surface markers are the same ones that play a role in the in vivo generation of thymus cells or their progeny.

In mouse bone marrow and spleen, cells that can be induced to express T-cell surface markers have a low buoyant density on discontinuous albumin gradients (Komuro and Boyse, 1973b; Komuro et al., 1975; Scheid et al., 1978). This density trajectory coincides with the density of CFU-S and cells which have T-cell repopulating capacity (Komuro et al., 1975; Basch and Kadish, 1977). The thymus repopulating capacity of spleen cells after Thy-1 induction and subsequent elimination of Thy-1$^+$ cells with cytotoxic anti-Thy-1 sera and complement was only moderately decreased (Komuro et al., 1975). In the samples used for reconstitution, the number of pluripotent stem cells that actually contribute to the thymus regeneration was not determined. According to Basch and Goldstein (1974), CFU-S would not have been affected by the Thy-1 induction procedure. One explanation of these observations might be that only a proportion of thymocyte progenitors can be induced to express Thy-1 markers with the thymic factor (thymopoietin) used in this study. The results might also be explained by the presence of cells that express low levels of Thy-1 antigens not readily detectable by means of conventional cytotoxic antisera and complement and which are converted to cells that express high levels of Thy-1 (Dardenne et al., 1978). The experiments discussed in fact deserve confirmation by new ones with monoclonal reagents for cytolysis and for analysis of Thy-1$^+$ cells. The total number of surface marker inducible cells amounts to up to 11, 6 and 4%, respectively, in bone marrow, spleen and fetal liver, proportions that are 10-fold higher than those of CFU-S and committed stem cells (Komuro and Boyse, 1973a,b; Scheid et

al., 1973; Komuro et al., 1975). This indicates that the in vitro induction of T-cell markers on previously negative cells is probably not a very representative differentiation step. Considering the marginal changes in T-cell functions (mitogen responses) of bone marrow and spleen cells after treatment with thymic hormones, the proportion of cells that can be induced to express T cell antigens is rather high (Basch and Goldstein, 1974, 1975a,b).

Quantitative data on bone marrow cells that home into the thymus after bone marrow transplantation (Balner and Dersjant, 1964; Lepault and Weissman, 1981) are also in contrast with results obtained in the induction of T-cell surface markers with thymic factors. From the former experiments, it can be concluded that the number of thymocyte progenitor cells in bone marrow is far lower than that of cells that can be induced to bear T-cell markers. In recent experiments, it has been shown that the proportions of colony forming units of T cells (CFU-T), in vitro T-cell colony forming cells in bone marrow and spleen, are also much lower than the number of cells in these tissues that can be induced to express T-cell markers (Jacobs and Miller, 1979; Ching et al., 1981). These discrepancies, among others, have led us to the development of the quantitative assay for prothymocytes that will be discussed in Chapters 2 and 3.

During ontogeny, expression of cell surface markers in the thymus preceeds functional maturation (Section 4). Thus it is quite possible that the induction of surface marker expression on bone marrow cells mimics one stage in the sequence of T-cell differentiation. This step is expected to arise after the stage where progenitors become committed to the T lineage. From this, one might conclude that, for differentiation into mature T cells, multiple factors as well as a suitable microenvironment are required.

14 IN VITRO CULTURES OF T-CELL COLONIES

A culture system where normal and nude mouse bone marrow and spleen cells are used as sources of immature T cells, has recently been introduced (Jacobs and Miller, 1979; Ching et al., 1981). In this system, colonies of both T lymphoid (CFU-T) and of myeloid origin can be detected. Proliferation of T-cell colony forming units was dependent on one or more growth factors other than IL2 which are present in conditioned medium of PHA stimulated human leucocytes and in selected fetal calf serum batches. In bone marrow, the concentration of CFU-T was estimated to be about 300 per 10^5 cells (Ching et al., 1981). This frequency of CFU-T is in the same order of magnitude as the numbers of CFU-S (600-800 per 10^5) and GM-CFU and E-CFU in bone marrow cells (Bol, 1980). Using hydroxyurea, it was estimated that the CFU-T is a proliferating cell (Rusthoven and Phillips, 1980; Ching et al., 1981). It was concluded that the clonable cells are in a relatively immature stage of development, since no reduction in colony growth could be shown on culturing spleen and bone marrow cells after treatment with

anti-Thy-1 sera. Treatment of cells with cytotoxic antisera and complement, however, does not always guarantee the elimination of a specific subpopulation (Mathieson et al., 1979b). The major drawback which makes the system not useful for quantitative analysis of T-cell progenitors is the absence of a linear precursor-product relationship (Ching et al., 1981). Provided that linearity can be obtained in this system, it is one of few promising approaches to in vitro analysis of T-cell differentiaton.

15 PUTATIVE T-CELL PROGENITOR CELLS

There are various current views on the nature of thymocyte progenitor cells. A number of possibilities is listed in Table III. Although the stem cell is ultimately a thymus cell progenitor, there is a kind of consensus in the literature that committed prethymic cells exist in the T-cell differentiation pathway. This supposition is based on the observations of differentiation patterns of other cell lineages of the blood. Most hemopoietic differentiation schemes therefore include a pre-T-cell or a restricted T stem cell (Shortman and Jackson, 1974; Van Bekkum, 1974; Till and McCulloch, 1980). However, there is no definite proof at present for the existence of such cells. Direct evidence was derived only from experiments of Abramson et al. (1977). They transplanted bone marrow cells which contained chromosomes in which markers were induced by radiation into lethally irradiated mice. In two of 28 mice a chromosome marker was present on lymphocytes of the T-cell lineage (PHA induced lymphoblasts) but not in bone marrow or in spleen colonies; this suggested the existence of a separate committed T-cell progenitor cell. However, independent confirmation has not yet been made.

The bone marrow cells that can be induced to bear T-cell markers under the influence of various factors (as discussed in Section 13) have in fact not been reproducibly shown to be functional T-cell progenitors. The proportion of these cells in the bone marrow leads to considerable scepticism as to whether they can really function as pre-T-cells (Komuro et al., 1975).

The CFU-T detected in the colony assay of Jacobs and Miller (1979) is a candidate committed precursor cell but requires further characterisation and the assay itself needs to be verified. The CFU-T have not been isolated to be tested for thymus repopulation capacity.

Prothymocytes as defined in a functional way have been characterised in a qualitative way by Kadish and Basch (1976). This method has been adapted for use in a quantitative way (Boersma et al., 1981). The prothymocytes are defined as those cells in sources of hemopoietic cells that are able to restore the thymus of a lethally irradiated host (Komuro et al., 1975; Kadish and Basch, 1976; Boersma et al., 1981).

An approach supplementary to the characterisation of prothymocytes is the study of thymic immigrant cells. The latter, at least some of them, can be

TABLE III

PUTATIVE T-CELL PROGENITORS IN THE MOUSE

Cell type	Function or definition	Thy-1	Frequency
CFU-S	Pluripotent hemopoietic stem cell. Regenerates all lineages of blood cells in irradiated recipients (1-3)	-	0.6% of all bone marrow cells (1)
Restricted T lineage progenitor	Restricted T progenitor cells in mice transplanted with bone marrow cells with chromosomal markers (4); single report, not reproduced	-	unknown
T-cell marker inducible cell	Cells that are negative for T-cell markers that can be induced to be Thy-1$^+$ (5,6), TI$^+$ (7) Lyt$^+$ (8)	-	10-12% of all bone marrow cells (6,7)
CFU-T	Give rise to T-cell colonies in vitro when stimulated with appropriate factors present in human leucocyte conditioned medium and selected sera (10,9) (not reproduced by other groups)	-	0.6% of all bone marrow cells (10,9)
Prothymocyte	Functionally defined T-cell progenitor. Gives rise to donor derived thymus cells in irradiated recipients. (7,11,12)	-	0.03% of all bone marrow cells (12)
Thymus immigrant	Bone marrow cell that arrives in the thymus of an irradiated mouse after transplantation (13,14)	-	0.08% of all bone marrow cells. After 24h, 20% is Thy-1$^+$ 0.016% of all cells (13,14)

1 Till and McCulloch, 1961
2 Ford et al., 1966
3 Ford and Micklem, 1963
4 Abramson, 1977
5 Bach et al., 1971
6 Komuro and Boyse, 1973
7 Komuro, et al., 1975
8 Goldschneider et al., 1981
9 Jacobs and Miller, 1979
10 Ching et al., 1981
11 Kadish and Basch, 1976
12 Boersma et al., 1981
13 Balner and Dersjant, 1964
14 Lepault and Weissman, 1981

expected to represent the first stage of development that follows the prothymocytes. It remains to be determined which immigrants are functional prothymocytes in the sense that they can give rise to Thy-1$^+$ progeny in the thymus.

To study the differentiation pattern of early T-lineage cells, we have chosen to develop the in vivo prothymocyte assay as described by Komuro et al. (1975) and Kadish and Basch (1976). This system offers the possibility of a direct approach, since the assay characterises the T-cell progenitors in a functional way.

16 OUTLINE OF THE STUDY

The aim of the experiments supporting this thesis was to characterise prethymic T-lineage cells and to investigate their relationship to the pluripotent stem cell. Characterisation of such a cell would contribute to a better understanding of the early phases of T-cell differentiation.

Characterisation of sequential differentiation stages may permit the recognition of the level of hemopoietic cell differentiation at which a cell is committed to the T-cell lineage as well as the mechanism by which the thymus epithelial structures influence the immigrating commited progenitors to initiate the proliferation of T-cell subpopulations in the thymus.

Apart from the method recently described by Jordan et al. (1977) and Pyke and Bach (1979, 1981) in which the growth of hemopoietic cells in organ cultures of thymic tissue was analysed, the only way to study early T-cell differentiation up to now has been to determine the proliferation of T cells in the intact animal. This entails experimental conditions which can be controlled only to a limited extent. To permit reproducible quantitative analyses of physiological phenomena in intact animals, the condition and health of the experimental animal have to be maintained at a constant high level. Reproducible results can be expected only when mice of the same age, sex and intestinal microflora are used in all experiments.

The role of the thymus in the development of immunocompetent T cells has been studied in various systems. In intact adult mice, the steady state like situation in the hemopoietic system makes it relatively difficult to determine the relationship between different subpopulations of cells. The sequential appearance of cells in various stages of development during ontogeny offers a more favorable experimental situation. The restoration of the lymphoid system of lethally irradiated and bone marrow reconstituted mice as far as the cells of the T-cell lineage are concerned follows a similar pattern of development as has been observed for ontogenetic development (Owen, 1977). The use of regenerating chimeric laboratory animals to study T-cell differentiation has the advantage that the number of cells from which the hemopoietic system regenerates is under control. In addition, the cells used for functional studies can be collected more easily from adult mice than from fetuses.

In this thesis, the study of growth kinetics of thymocytes in lethally irradiated mice as a means to characterise the early T-cell progenitors is described. Normal bone marrow cells were used as the source of hemopoietic cells. The experiments show that it is possible to obtain information on T-cell differentiation from a complex experimental set-up in vivo. The quantitative relation between the thymus regeneration capacity and the CFU-S content of bone marrow cells was established. Kinetic studies of the regeneration process led to formulation of the framework for the in vivo thymus regeneration assay (Chapter 3).

From the study of erythroid and granulocyte-macrophage lineages, it had been established that at least these lineages start from early progenitor cells that are immediate successors of the pluripotent cells, but can be distinguished experimentally from CFU-S (Bol, 1980). However, apart from the cells postulated by Abramson et al. (1977), no such early progenitor cells are known for the T-cell lineage.

Our results obtained in thymus repopulation after transplantation of bone marrow from normal mice did not allow a distinction between the T-cell progenitor function of prothymocytes and that of the pluripotent stem cell determined as CFU-S.

To establish whether there was a prethymic precursor cell distinguishable from the stem cell, we have characterised cells that fulfill the prothymocyte and CFU-S functions according to their physical parameters as determined by separation procedures such as equilibrium centrifugation, velocity sedimentation and free flow electrophoresis (Chapter 4).

Furthermore it was attempted to distinguish between the CFU-S and prothymocyte in hemopoietic cells of various sources other than bone marrow of normal mice. Thus, the quantitative relation between prothymocytes and CFU-S was studied in fetal liver, long-term cultured bone marrow cells and regenerating bone marrow cells.

In fetal liver, the ratio of prothymocytes over CFU-S was found to be different from that in normal bone marrow. Prothymocytes disappeared more rapidly from long-term cultures of bone marrow than did CFU-S. In regenerating bone marrow, prothymocytes initially were present in small proportions (~ 1% of normal bone marrow levels). Prothymocytes and CFU-S showed different regeneration kinetics. Prothymocytes regenerate relatively slow (Chapter 5). The results of these studies suggested that prothymocytes and the great majority of CFU-S are present in different frequencies in these cell sources. Finally, the sensitivity of prothymocytes and CFU-S to γ-irradiation, S-phase specific ^{3}H thymidine suicide and cell surface modification by neuraminidase incubation was determined (Chapter 6). These criteria for cell characterisation were used to discriminate between the two cell types without separating them physically. Prothymocytes and CFU-S differed in sensitivity to γ-irradiation and neuraminidase treatment. Prothymocytes and CFU-S have a similar sensitivity to ^{3}H thymidine suicide. In addition, pro-

thymocytes in regenerating bone marrow are sensitive to treatment with cold thymidine while CFU-S are not. It is concluded that a separate prethymic precursor cell for the T-cell lineage exists in the mouse: the prothymocyte.

CHAPTER 2

MATERIALS AND METHODS

1 MICE

Male and female C3H and (C3HxAKR)F_1 mice were used to study thymus regeneration after bone marrow transplantation. Mice of the same sex were employed within the same experiment. Bone marrow donor mice were 6 weeks of age. Recipients were used when they were 8 weeks old. This age was choosen for bone marrow recipients, because, at that age, the first signs of thymus involution become apparent in intact mice and this can be interpreted as an indication for complete maturation of the thymus function. In addition, recipient mice of eight weeks of age can be conditioned with the same dose of irradiation that is applied to adult mice without changing the survival pattern when compared to older mice.

There are indications that the composition of the microflora under certain conditions may influence hemopoiesis. Total and selective decontamination of the gastrointestinal contents have been shown to prevent the development of graft versus host disease in mice (Van der Waaij et al., 1971; Van Bekkum et al., 1974; Heidt et al., 1981a,b). In addition, toxins which are produced in the host in case of bacterial infections have a stimulating effect on proliferative activity of CFU-S and granulocytic progenitor cells (Metcalf, 1971).

Therefore, as a precaution, specific pathogen free mice were used throughout this study to prevent ambiguity due to changes in microflora. The microflora of the mice consisted of a colonisation resistant population of anaerobic mouse-derived Clostridium species in combination with Staphylococcos albus, Streptococcus faecalis and Enterobacter cloacae.

2 IRRADIATION IN VIVO AND IN VITRO

2.1 In Vivo γ-Irradiation

In order to be able to study the regeneration of the lymphopoietic system after transplantation of hemopoietic progenitor cells, recipient mice were conditioned by lethal irradiation. Total body irradiation of C3H recipient mice was performed in a ^{137}Cs γ-irradiation apparatus, Gammacell 20 (Atomic Energy of Canada Ltd., Ottawa, Canada). The mice were placed in an animal container which is flushed with air during irradiation. A maximum number of about 25 mice can be irradiated simultaneously. Two ^{137}Cs γ-ray sources are located above and below the irradiation unit thus providing an approximately homogeneous radiation

dose distribution over the animal (maximum variation 2%). Absorbed dose measurements were made with a calibrated ionization chamber. The dose rate was at the position of the mice 0.9 Gy.min.$^{-1}$. All lethally irradiated mice received a γ-radiation dose of 8.65 Gy. Without bone marrow transplantation, they survived for 12 ± 2 days after irradiation. The number of bone marrow cells transplanted varied according to the purpose of the experiment. Transplantation of hemopoietic cells was performed within 3-5 h after γ-irradiation. Rescue of 100 percent of lethally irradiated mice was possible with a dose of bone marrow cells containing about 20 spleen colony forming units (CFU-S).

2.2 In Vitro γ-Irradiation of Hemopoietic Cells

To determine the radiation sensitivity of CFU-S and T-cell progenitors in mouse bone marrow, these cells were irradiated in suspension in a Gammacell 20 small animal irradiator as described in Section 2.1. Cells were suspended in a volume of 3 ml Eagle's Basal medium (EBH) (Gibco, Grand Island, NY, USA) containing 5 x 10^6 cells.ml^{-1} in 25 cm^2 flasks (model 3013; Falcon Oxnard, CA, USA) placed horizontally in the center of the irradiation chamber. Irradiation conditions were chosen such as to avoid hypoxia (Barendsen et al., 1966). Dependent on the dose of irradiation, 5-100 x 10^4 nucleated cells were transplanted per mouse to determine the CFU-S content of bone marrow inocula.

For in vivo as well as as for in vitro irradiation of cells, D_0 values were calculated from log-linear regression analysis of the dose-effect curves. Only the linear part of the curve was used (Anderson and Warner, 1976).

3 CELL SUSPENSIONS

3.1 Media

Ten mM HEPES (Merck, Darmstadt, B.R.D.) buffered EBH was used for isolation of cells. Media used for suspensions and cultures were adjusted to pH 7.1. The osmolarity was adjusted to 310-320 mOsm, to equal the serum of the mice used. Final suspensions were supplemented with 5% fetal calf serum (FCS) (Flow, Irvine, Scotland, UK).

3.2 Isolation of Cells

All mice were anaesthesized with ether before sacrifice. For removal of the thymus, the animals were decapitated and exsanguinated to prevent the thymus cells being contaminated with peripheral blood cells. Single cell suspensions from thymus and fetal liver were made by gently pressing the organs through a nylon

gauze premoistened with medium. The gauze was then thouroughly washed to minimise cell loss, which was of especial importance in quantitative experiments. Spleens were first cut into small pieces with scissors and then processed as described for thymus. After isolation, the cells were washed twice with medium.

Bone marrow cells were isolated by flusing the cells from the bone with medium injected through a needle on one end of the bone. The bone marrow cells were collected and washed through a nylon gauze to improve monocellularity of the suspension and to remove bone splinters.

4 FETAL LIVER

Mouse embryos were obtained by timed mating. A group of 20 females was placed with 3-4 males in a cage overnight; the following morning males were removed. The day after conception was counted as day 0. Pregnant mice were selected by palpation at the time they were to be used as embryo donors. Donor mice were anaesthesized with ether and killed by cervical dislocation. Uteri were removed and the embryos were dissected out. The fetal livers were removed from the embryos by careful manipulation with two pairs of smooth tweezers. The livers were pooled and disaggregated as described for the isolation of thymus cells.

5 REGENERATING BONE MARROW

Regenerating bone marrow cells were obtained from mice recovering from radiation damage. These mice were lethally irradiated (8.65 Gy) and transplanted with 3×10^6 isogeneic nucleated bone marrow cells at about 4 h after irradiation.

6 THYMECTOMY

One or two-days-old mice were cooled on ice. Six-weeks-old mice were anaesthesized with ether. Thymectomy was performed as described by Hudson and Hay (1978), in brief: The mice were fixed on a cork board in a harness made of adhesive tape. A small cut was made in the rib cage at the anterior end of the sternum and the thymus lobes were aspirated through a plastic pipet tip attached to a vacuum line. In newborn mice, the incisions were closed with two to three silk sutures; in adults, metal clips were used. Bone marrow of neonatally thymectomized mice was used 6 weeks after thymectomy. Adult thymectomized mice were used three weeks after thymectomy.

7 NEURAMINIDASE

Neuraminidase hydrolyses the α-glycosidic bonds between N-acetylneuraminic acid residues and the adjacent carbohydrate moieties of glycoproteins and glycolipids (Gottschalk and Drzhiek, 1972). The effect of enzyme treatment on mouse bone marrow hemopoietic cells has been extensively reported by Bol (1980) and Ploemacher et al. (1981a,b). It results in a change in the homing pattern of pluripotent hemopoietic stem cells and changes the electrophoretic mobility of CFU-S and GM-CFU. Neuraminidase from Vibrio cholerae was obtained from Behring Werke A.G., BRD in a concentration of 2×10^{-1} I.U.ml^{-1}. The units were defined according to Warren (1963). This preparation contains no measurable activities of proteases, aldolase and phospholipase C. According to the method described by Bol (1980), bone marrow cells were incubated with neuraminidase for 1 h at 37^{0} C. The bone marrow cells to be used in electrophoretic mobility studies were incubated with 2×10^{-2} I.U.ml^{-1} neuraminidase, because at this concentration the reduction CFU-S in the bone marrow cell suspension shows a plateau. During incubation, the cell concentration was 1 to 5 x 10^{7} cells.ml^{-1}. Control cells were incubated without neuraminidase. After incubation, cells were washed twice in Hank's balanced salt solution.

8 COUNTING THE CELLS

Thymus cells were counted in a Bio/Physics pulse cytophotometer model 6301 (Bio/Physics System Inc., Mahopac, NY, USA).

For viable cell counts, cells were stained with 0.75 ml Eagle's Basal Medium containing 12 µg.ml^{-1} fluorescein diacetate (FDA) (Sigma Chem. Co. St. Louis, MO, USA.) and 5-10 µg.ml^{-1} propidium iodide (PI)(Calbiochem., San Diego, CA, USA). Thymus cells were incubated for 15 minutes at room temperature before determination to allow equilibration. Living cells with intact membranes exclude PI but take up and hydrolyse FDA and are thus stained with intracellular free fluorescein (emission 530 nm). The DNA of cells with damaged membranes (dead cells) is stained with PI (emission 590 nm), while FDA hydrolysation products leak rapidly from these cells. Blue laser light (488 nm) was used for excitation. The Bio/Physics cytofluorograph allows the setting of electronic windows such as to discriminate between living and dead cells, 10^{4} cells were routinely analysed in duplicate.

Nucleated cell counts for bone marrow and spleen cell suspensions were made microscopically in a hemocytometer after staining with Türk's solution. Viable cell counts of spleen cell and bone marrow cell suspensions were based on trypan blue (0.2%) or eosin (0.2%) dye exclusion and counted microscopically.

9 CELL CYCLE ANALYSIS

9.1 Flow Microfluorometric Analysis of Cell Cycle Stages

Metachromatic staining of cellular DNA and RNA with acridine orange was used to analyse the distribution of cells throughout the cell cycle stages with flow microfluorometry. We used the Betel and van der Westen (Betel and Van der Westen, 1978) modification of the method originally reported by Darzynkiewicz et al. (1976).

Briefly, 0.2 ml of a cell suspension containing 2 to 5 x 10^6 cells.ml^{-1} were mixed with an ice-cold solution containing 0.1% Triton X-100, 0.1 M HCl and 0.15 M NaCl. After 15 seconds, 1.2 ml of a solution containing 10 µg.ml^{-1} Acridine Orange, 10^{-3} M EDTA, 0.15 M NaCl and phosphate (0.2 M) - citrate (0.1 M) buffer, pH 6.0, were added. Cells were processed within 10 min after staining. The Bio/Physics cytofluorograph laser excites at 488 nm. Red fluorescence of RNA-AO passed a O.G. 590 filter. A green interference band filter with 50% transmission at 520 nm and 550 nm was used to select the green DNA-AO fluorescence.

The instrument settings for discrimination of cells in different stages of the cell cycle were as described by Betel and Van der Westen (1978).

9.2 Spontaneous ^{14}C Thymidine Incorporation

Spontaneous ^{14}C TdR incorporation, which is a method used by Kadish and Basch (1976) to estimate the proportion of surviving cycling cells in a suspension after cytotoxic treatment, was used for comparison with cytofluorographic viable counting of FDA/PI stained cells. For this determination, 5 x 10^5 cells in 250 µl EBH supplemented with 5% FCS were incubated over a 3 hr period with 0.15 µCi ^{14}C TdR (specific activity: 50 µCi/nmol, Radiochemical Center, Amersham, UK). Cultures were harvested on glass fibre filters (Whatman GF/A Lab. Sates Ltd., Maidstone, Kent, UK) using an automated culture harvester (Cryoson, Midden-Beemster, The Netherlands). Incorporation was measured in a toluene based scintillation fluid in a Mark II Scintillation Counter (Nuclear Chicago, Chicago, IL, USA).

9.3 ^{3}H Thymidine Suicide

Incubation of cells with ^{3}H TdR of high specific activity leads to death of the cells which incorporate this precursor into their DNA in S phase. This so-called suicide phenomenon can be used as a rough measure for the proliferative

state of cells. A decrease in cell survival after treatment with increasing concentrations of highly labeled ^{3}H TdR has been observed (Iscove et al., 1972).

The proportion of cells killed with ^{3}H TdR when compared to control values was taken as the fraction of cells in S phase. The specificity of the suicide with ^{3}H TdR was verified by adding excess cold thymidine (0.2 mg TdR.ml^{-1}) to the labeled compound. Addition of cold thymidine should abolish the suicide effect of ^{3}H TdR. For comparison of the proliferative state of progenitor cells in different samples of hemopoietic cells it can be assumed that the total fraction of cycling cells was twice the proportion of S-phase cells (Mitchison, 1971).

Bone marrow cells were resuspended in HEPES buffered Eagle's Basal Medium (Gibco, Grand Island, NY, USA) in a concentration of 5 x 10^6.ml^{-1} at 2 ml per tube and incubated for 20 min at 37^0 C with or without ^{3}H TdR in the medium. The specific activity of ^{3}H TdR was 25 Ci.mmol^{-1}, which has been advised for use in suicide studies (Lord et al., 1974). After incubation, the cells were washed once with cold thymidine (2 ml; 0.2 mg.ml^{-1} in medium). They were then washed twice with medium only.

10 LYMPHOCYTE STIMULATION IN VITRO

For mitogenic stimulation cell suspensions were adjusted to 2 x 10^6 living cells per ml in RPMI 1640 (Flow Laboratories, Irvine, Scotland, UK), buffered with 10 mM HEPES, pH 7.2 and supplemented with 10% fetal calf serum and 5 x 10^{-5} M of 2-mercaptoethanol (Merck, Darmstadt, BRD). Triplicate cultures in Falcon microtiter plates (model 3040 Falcon, Oxnard, CA, USA) were incubated for a 72 hr period at 37^0 C in a humidified atmosphere of 95% with 5% CO_2.

Cultures were stimulated at optimum doses of lectins determined previously in dose response curves. For spleen cells this was 1.25 µg.ml^{-1} concentration of Concanavalin A (ConA; Pharmacia, Uppsala, Sweden) and 2.0 µl.ml^{-1} Phytohaemagglutinin (PHA; Wellcome, Beckenham, UK). Thymocytes were incubated with 5.0 µg. ml^{-1} ConA and with 12.5 µl.ml^{-1} PHA for optimum responses. During the last 24 h of the culture period, the cells were labeled with ^{14}C TdR (Radiochemical Centre, Amersham, UK) at 0.075 µCi per well and with a specific activity of 11.25 µCi.µmol^{-1}.

Mixed lymphocyte responses of C3H and (C3HxAKR)F_1 derived $H2^K$ cells were determined in cultures with irradiated (20 Gy) DBA ($H2^d$) spleen cells. The culture medium was the same as for mitogen stimulation. The cultures contained 0.5 x 10^6 irradiated DBA spleen cells and 0.5 x 10^6 responder cells in a total volume of 200 µl. The cells were cultured for a 96 h period. During the last 24 h, the cultures were labeled with ^{14}C TdR, as for mitogen stimulation. The cultures were harvested on glass fibre filters with an automatic culture harvester, see Section 9.2.

The ^{14}C TdR incorporation into the cellular DNA was determined in a toluene based liquid scintillation fluid in a Mark II Scintillation Counter.

11 LONG-TERM CULTURES

The long-term persistence of prothymocytes in vitro was studied in cultures established according to the method originally described by Dexter et al. (1977) and modified by Greenberger (1978). The cultures used for the experiments described in Chapter 5 were established by Dr. J.F. Eliason during his stay in the Radiobiological Institute TNO. Briefly, the contents of a single femur were flushed into 10 ml of Fischer's medium (Gibco, Europe, Glasgow, Scotland, UK) supplemented with 20% fetal calf serum (Flow Laboratories, Irvine, Scotland, UK) and 10^{-6} M hydrocortisone sodium succinate (Upjohn Co., Kalamazoo, MI, USA) in a 25 cm^2 tissue culture flask (Falcon). The cultures were gassed with 5% CO_2 in air, incubated at 33^0C and fed at weekly intervals by replacing half of the growth medium with fresh medium. After three weeks, the cultures were inoculated with the contents of a second femur and this time was considered to be week 0 for the cultures. The nonadherent cells from 40 flasks were pooled and used for the experiments described in the text.

In order to quantitate the effect of culture on prothymocytes, the technique was modified: 1) now the cultures were not reseeded, a single inoculation of marrow was flushed into 10 ml of Iscove's modified Dulbecco's medium (Gibco, Europe, Glasgow, Scotland, UK) supplemented with 10% fetal calf serum and 10^{-7} M hydrocortisone, (to be published); 2) small aliquots were removed from at least four flasks, pooled and assayed in order to determine the initial numbers of the following cells: CFU-S, CFU-GM, BFU-E and prothymocytes.

12 FLOW MICROFLUOROMETRIC ANALYSIS OF ANTIBODY LABELED CELLS

Cells were analysed on the basis of their light scattering properties and their fluorescence with FITC labeled specific antibodies with a fluorescence activated cell sorter (FACS II, Becton and Dickinson, Mountain View, CA, USA). The apparatus was used in the configuration described by Visser et al. (1980). The instrument was equipped with logarithmic amplifiers which were used to determine immunofluorescence distributions.

12.1 Immunofluorescence Reagents

Initially, for analysis of Thy-1.1^+ subpopulations in the experiments described in Chapter 3, Sections 2.1.2 and 2.2.2, a direct labeled monoclonal

anti-rat-Thy-1 serum obtained from Sera Lab (Copthorne, Sussex, UK) was used. This anti-rat-Thy-1 serum cross-reacts with mouse Thy-1.1 but not with mouse Thy-1.2 and was used until the monoclonal anti-mouse Thy-1.1 reagent discussed below became available.

The anti-mouse-Thy-1.1 reagent used in experiments described in Chapter 3, Sections 2.1.2 and 2.2.2 was a monoclonal antibody IgG from New England Nuclear (Dreieich, BRD) and was used in an indirect immunofluorescence assay with a, thymocyte absorbed, fluorescent rabbit-anti-mouse IgG (RAM-FITC; Nordic Immunological Laboratories, Tilburg, The Netherlands) as the second step.

The other monoclonal antibodies employed in experiments discussed in Chapter 3, Sections 2.1.2 and 2.2.2 and directed against the framework determinants of Thy-1 (clone 59-AD 2.2) as obtained through the courtesy of Dr. J. Haaijman (Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands) were used directly conjugated with fluorescein (Ledbetter and Herzenberg, 1979).

12.2 Staining Procedures

Cells were resuspended in Hanks Balanced Salt Solution (HBSS) without phenol red (Gibco Europe, Glasgow, Scotland, UK) and supplemented with 0.1% B.S.A. Fraction V (Sigma Chemical Co. Ltd., St. Louis, MO, USA) and 0.1% sodium azide. Cells (0.5×10^6/25µl) were incubated for 30 minutes on ice with the appropriately diluted reagents and then washed with 3 ml HBSS-BSA-azide and resuspended in the same medium. Cells incubated with anti-Thy-1.1 were then again incubated for 45 min with saturating amounts of RAM-FITC.

Propidium Iodide (5µg.ml^{-1}) was added in the final suspensions to stain dead cells which could then be gated out during analysis on the FACS (Crissman and Steinkamp, 1973).

12.3 Determination of Thymocyte Subpopulations According to Fluorescence Distributions

Fluorescence distributions of C3H and C3AKF$_1$ thymocytes stained with anti-Thy-1.1 and anti-Thy-1.2 are shown in Figure 1. Fluorescence histograms were transformed to cumulative curves to determine the relative numbers of "low" and "high" Thy-1$^+$ thymocytes. Normal thymocytes showed a typical biphasic cumulative fluorescence curve. The channel number where the cumulative curve bent to the steep slope indicating the "high" fluorescent cells was established for each experiment to discriminate between negative, "low" and "high" fluorescent cells. This same channel number was then used throughout that experiment (Fig.2). The channel number below which 99% of the fluorescence distribution of either un-

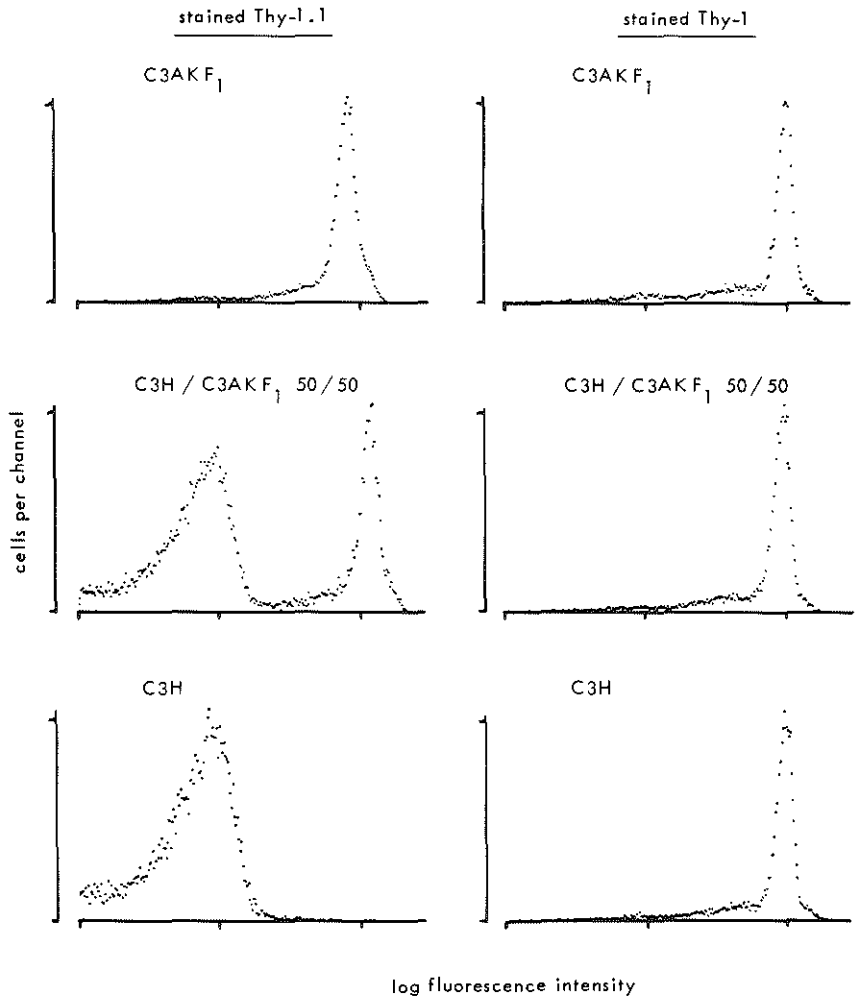

Fig. 1 Fluorescence distribution of 2 x 10^4 thymocytes stained with anti-Thy-1.1 or anti-Thy-1 antibodies. Similar fluorescence histograms were used to discriminate between negative, "low" and "high" Thy-1$^+$ cells in single suspensions and mixtures of C3H and C3AKF$_1$ thymocytes (see also Fig.2).

stained cells or cells stained with the second step reagent only was found, was used to determine the relative number of negatively stained thymocytes within the experimental groups.

Graded mixtures of C3H and C3AKF$_1$ thymocytes were prepared and subjected to analyses as described above. A good agreement was obtained between the composition of the mixture and the proportion of total Thy-1.1$^+$ and "low" Thy-1.1$^+$ cells that was determined according to our discrimination criteria (Fig.3). With this method as little as 1% Thy-1.1$^+$ cells could be detected in the

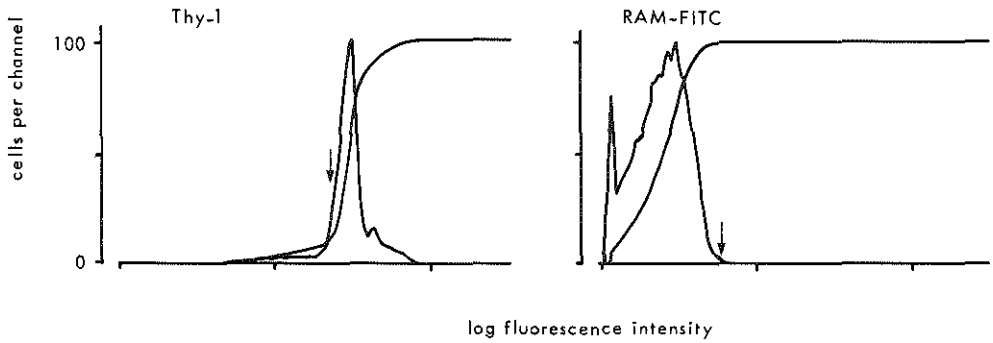

Fig. 2 Fluorescence distribution of C3AKF$_1$ thymocytes labeled with fluoresceinated anti-Thy-1 antibody. Distribution and cumulative curves are shown.
left : Thymocytes incubated with anti Thy-1 antibody. Indicated is the cut-off channel for discrimination between "low" and "high" Thy-1 cells.
right: Thymocytes incubated with second layer only (RAM-FITC). Indicated is the cut-off channel for discrimination between positive and negative cells.

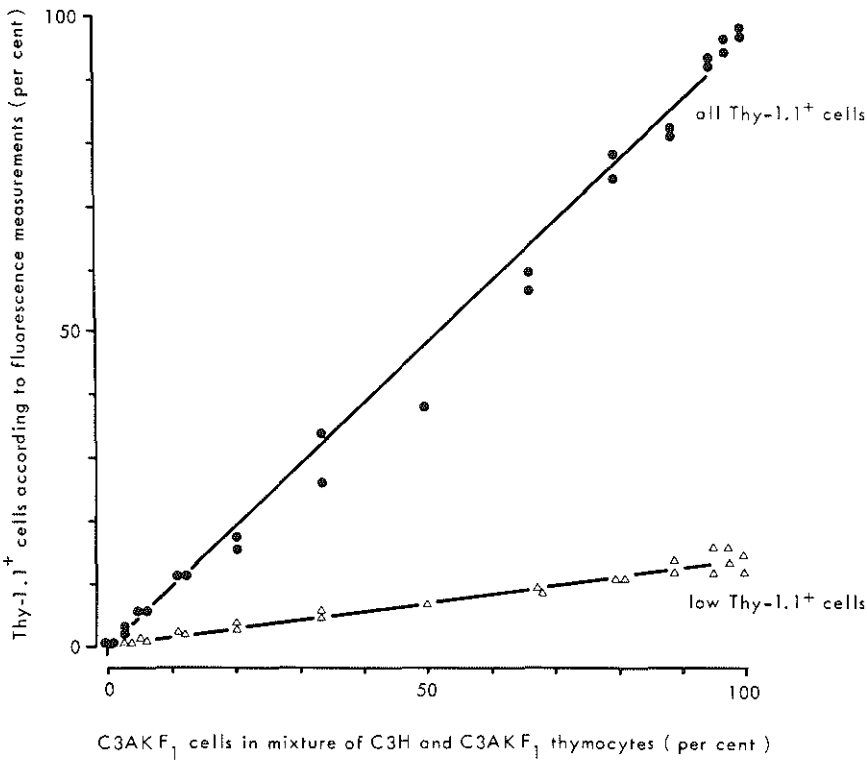

Fig. 3 Proportion of total Thy-1.1$^+$ and "low" Thy-1.1$^+$ cells in a mixture of (C3H x AKR)F$_1$ (Thy-1.1$^+$ and Thy-1.2) and C3H (Thy-1.2) thymocytes. Cells were stained with anti-Thy-1.1 and RAMIgG-FITC. Discrimination was performed in fluorescence histograms for which 2 x 10^4 cells were analysed. For total Thy-1.1$^+$ cells linear regression analysis gave a correlation coëfficient of 0.996 (n=27). For "low" Thy-1.1$^+$ cells a correlation coëfficient of 0.979 (n=23) was obtained.

mixture. The percentages of "low" and "high" Thy-1$^+$ subpopulations were calculated directly for donor type cells (Thy-1.1$^+$ cells). For host derived cells, we subtracted the proportion of donor derived cells of each subpopulation from the proportion of all cells that belonged to that subpopulation, e.g., "low" Thy-1$^+$, recipient derived cells = All "low" Thy-1$^+$ cells minus the number of donor derived "low" Thy-1$^+$ cells. Similar calculations were done for size distribution analysis.

13 CELL SEPARATION ACCORDING TO PHYSICAL PARAMETERS

In all fractionation procedures described below, the number of cells of each fraction that was transplanted depended on the assay to be performed. For the CFU-S assay a graded number of cells from a fraction was transplanted such that at least 5 to 10 colonies per spleen could be expected. For the thymus repopulation assay, 10^6 cells of a fraction were transplanted per recipient. In general, transplantation of such a cell dose allowed a donor thymocyte determination at 17 to 19 days after transplantation. The distribution of cells and cell function in the fractions was expressed as a percentage of the fraction with the peak value.

13.1 Isopycnic Density Centrifugation

Density centrifugation of hemopoietic cells was performed on continuous isoosmotic gradients according to the technique described by Shortman (1972). Bovine serum albumin (Fraction V, Sigma Chem. Co., St. Louis, MO, USA) was dialysed extensively against water, lyophylized, and dissolved in a balanced salt solution (310 m osm. kg^{-1}). The final solution had a pH of 5.1. The density of the solutions used for gradients as well as that of BSA solutions that were prepared as references was determined on a digital density meter (K. Paar, K.G., Graz, Austria). All procedures involving cells were carried out at 4^0C. Cells were suspended in the high density medium and mixed continuously with the low density solution while the mixture was simultaneously tapped of into a centrifuge tube. The maximum load of cells on a 12-14 ml gradient in Sorvall 00292 tubes was 3 x 10^8 cells. The gradient was centrifuged at 4000 g for 30 min in a Beckman J-21 centrifuge equipped with a JS-13 rotor.

Fractions were collected by upward displacement by pumping bromobenzene under the gradient. The density of the fraction was measured on a linear bromobenzene-petroleum ether gradient which was made in a burette. A small droplet of about 10 µl of each fraction was placed on this gradient. In addition, droplets of standard BSA solution were used as references. After the BSA droplets in the gradient reached equilibrium density, the burette readings were made. The mean density of the fractions was calculated according to the calibration based on standard BSA droplets.

The cell distributions in the gradient were calculated as the number of cells per density increment to correct for minor non-linearities. The cells in the fractions were washed with Eagle's Basal Medium. They were then used in various assays (see ahead). The cell recovery from continuous gradients was 65-85% counting viable cells only. Selective loss of functionally defined subpopulations of cells was not observed. For separation of bone marrow cells, the density trajectory from 1.05 $g.cm^{-3}$ to 1.10 $g.cm^{-3}$ was employed.

13.2 Sedimentation Velocity

The sedimentation velocity of cells in a solution is a function of cell size and cell density. The technique for measuring the sedimentation velocity has been described in detail by Miller and Phillips (1969). Briefly, cells were suspended in 0.2% w/v of BSA in Eagle's Basal Medium and placed into a cylindrical sedimentation chamber (diameter 19.5 cm) with a conical bottom overlaid with a layer of medium. A gradient from 0.2-2% w/v of BSA was then made under the cells through a baffle which prevented disturbance of the cell layer. The cells were allowed to sediment for 3 to 4 h in the cold (4^0C). Fractions were then collected and the sedimentation rate calculated as described by Miller and Phillips (1969). Following this, the cells were centrifuged at 400 g for 10 min and suspended in medium for counting and processing in different assays. The recovery of viable nucleated cells was better than 85%.

13.3 Electrophoretic Mobility

The electrophoretic mobility (EPM) of cells in a buffer system is mainly a function of the cell surface charge density, which can be influenced by the choice of the experimental conditions. We have separated cells according to the modifications of Bol (1980) of the continuous free flow electrophoresis method originally developed by Hannig et al. (1969, 1975). The separation was performed in a Elphor VaP 5 apparatus (Bender and Holbein, GmbH, Munich, BRD).

The buffers for electrode chambers and cell separation were prepared as described by Von Boehmer et al. (1975). The low ionic strength separation buffer allows a compromise between maintenance of cell mobility and limitation of heat production. The osmolarity was 308 $mOsm.kg^{-1}$ and the pH 7.2. The cells were suspended in the separation buffer. Aggregates were removed by filtration over a cotton wool plug in a siliconized pasteur pipet. The recovered suspension contained more than 95% viable cells. Cell recovery after resuspension in low ionic strength buffer was 75%-85% for fresh bone marrow cells. Neuraminidase treated cells showed a lower recovery (60%-70%) due to increased clumping in low ionic strength medium. The cells were introduced into a continuous flowing buffer

stream in the separation chamber. The electric field applied was 115 $V.cm^{-1}$. Under the experimental conditions described, a current of 210 mA was measured. The separation chamber was cooled to 6°C. Between the electrodes, the field was divided into 100 channels which were drained separately. Cells entered the field in channel 70. The EPM distribution for mouse erythrocytes has a peak in channel 30. A difference of one channel corresponds to a change in EPM of 0.0577 ± $0.0022.10^{-4}$ $cm^2.V^{-1}.s^{-1}$. Recovery of cells after the separation procedure was 80%-90% of viable cells. The fractionated cells were resuspended in Eagle's basal medium and counted and then used in different assay systems.

14 SPLEEN COLONY ASSAY

The number of spleen colony forming cells (CFU-S) was determined according to the method described by Till and McCulloch (1961). Appropriate cell numbers were injected intravenously in a volume of 0.3 ml into lethally irradiated mice. After 8 days, the mice were killed and their spleens removed and fixed in Tellyesniczky's solution. The colonies that appeared as white nodules on the spleens were counted. The average of colony counts on 8 to 10 spleens was used to calculate the CFU-S content of bone marrow inocula used in thymus regeneration studies. For CFU-S determinations in normal bone marrow, 5×10^4 cells were transplanted per mouse. For determination of CFU-S in regenerating marrow, in long-term cultured bone marrow, in neuraminidase treated bone marrow and in irradiated bone marrow or ^3H TdR suicided bone marrow cells, the number of cells to be inoculated was chosen such as to allow the counting of at least 5 to 10 colonies per spleen.

15 COLONY ASSAYS IN VITRO

Granulocyte/macrophage progenitors (GM-CFU) and early erythroid progenitors (BFU-E) were assayed in methylcellulose cultures as previously described (Wagemaker and Visser, 1980). Cells were plated at a concentration of 5×10^4 $cells.ml^{-1}$ in alpha medium supplemented with 5% fetal calf serum, 1.75% bovine serum albumin Fraction V (Sigma, Chemical Co.Ltd., St. Louis, MO, USA), 0.8% methylcellulose (Dow Chemical Co. Ltd., Indianapolis, IN, USA), 3×10^{-5} M egg lecithin (Sigma, Chemical Co.Ltd., St. Louis, MO, USA), 7×10^{-6} M human transferrin (Beringwerke A.G, Marburg, BRD), 10^{-7} M sodium selenite (Merck A.G., Darmstadt, BRD), and 10^{-4} M 2-mercaptoethanol (Merck). Assay cultures for CFU-GM contained colony stimulating factor isolated and purified from pregnant mouse uterine extract at a concentration which gave maximum colony numbers in titration experiments done with normal marrow (Wagemaker et al., 1979). Assay cultures for BFU-E contained both BFA, partially purified from Concavalin A stimulated mouse spleen cell conditioned medium, and 2 $units.ml^{-1}$ of sheep plasma erythropoietin (Wagemaker, 1980).

16 IN VIVO THYMUS REPOPULATION ASSAY

16.1 The Mouse Model

Thymus repopulation after transplantation of hemopoietic cells was investigated in lethally irradiated mice. In most experiments, (C3H x AKR)F_1 bone marrow cells were used to restore the hemopoietic system of irradiated C3H recipients (Fig.4).

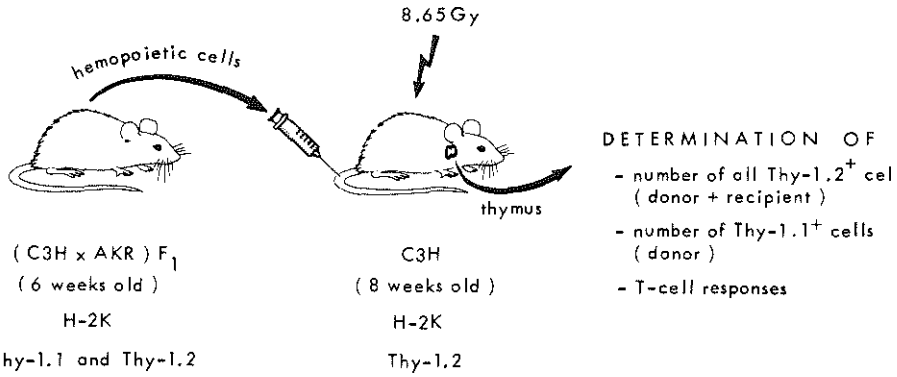

Fig. 4 Thymus repopulation assay. Hemopoietic progenitor cells from (C3xAKR)F_1 mice are transferred into lethally irradiated sex matched C3H recipients. Donor T cells have Thy-1.1 and Thy-1.2 antigens on their surface. Host cells carry only Thy-1.2 antigens on their surface.

This system has been set up as a modification of the transplantation model that had been introduced by Kadish and Basch (1976) as a tool to investigate early stages of T-cell differentiation. In this system the growth of donor derived thymocytes in the thymus of a lethally irradiated recipient, after transplantation of hemopoietic progenitor cells, was used as a measure to determine the number of T-cell progenitors. In order to use the system for quantitative analysis we had to demonstrate that, in contrast to the findings of Kadish and Basch (1976), it could be used in a quantitative way.

The mice we used have the same major histocompatibility antigens but differ at minor histocompatibility loci (C3H is Mls^c; (C3H x AKR)F_1 is $Mls^{a/c}$). The capacity of C3H and (C3H x AKR)F_1 bone marrow cells to rescue lethally irradiated mice was determined on the basis of 30-day survival of bone marrow transplanted mice. It was found that the survival of lethally irradiated (8.65 Gy) C3H mice transplanted with C3H or (C3H x AKR)F_1 bone marrow cells was similar when the inocula contained the same number of CFU-S (Fig.5). From this, we concluded that no adverse effects were to be expected from transplantation of semisyngeneic

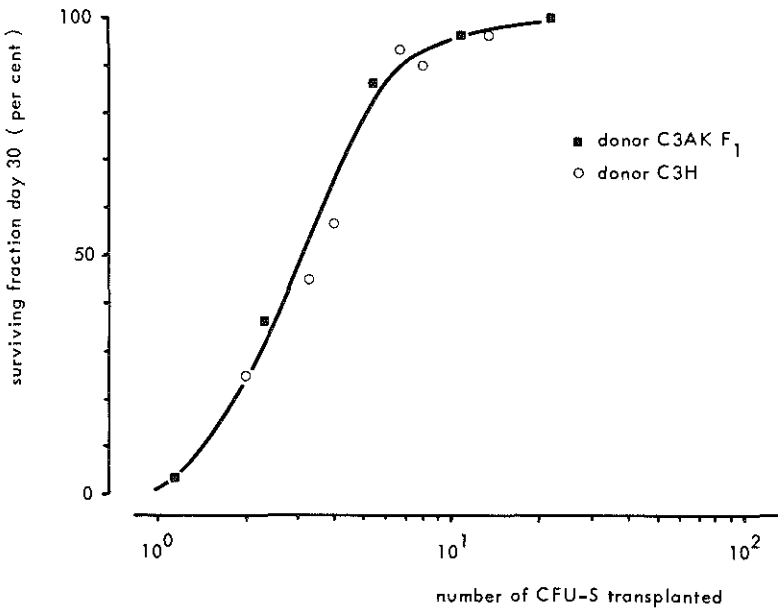

Fig. 5 Thirty-day survival of lethally irradiated C3H recipients transfused with bone marrow cells of C3H or $C3AKF_1$ mice (n=30 per point) as a function of the number of CFU-S transplanted.

cells in the above-mentioned combination with respect to hemopoietic and lymphopoietic reconstitution when the recipients were lethally irradiated.

16.2 Discrimination between Donor and Recipient Derived Thymocytes

Thymus derived lymphocytes in C3H mice carry Thy-1.2 surface antigens. In $(C3H \times AKR)F_1$ mice, T cells have both Thy-1.1 and Thy-1.2 alloantigens. In the thymus of bone marrow transplanted mice, some cells with donor and some with host T-cell phenotype will develop (Kadish and Basch, 1975, 1976; Boersma et al., 1981). These cells can be discriminated according to their Thy-1 surface marker. We used cytotoxic or fluorescent labelled anti-Thy-1.1 and anti-Thy-1.2 sera to determine the number of donor and host derived cells during hemopoietic regeneration after bone marrow transplantation. The growth curves of donor derived thymocytes were used to calculate relative numbers of thymocyte progenitor cells.

16.3 Cytotoxicity Assays

Thy-1 antisera, C3H anti-AKR thymocytes and AKR anti C3H thymocytes, were raised in young adult mice by seven injections of 10^7 thymocytes at weekly intervals. Antisera were not toxic without the addition of complement and showed no autoimmune activity. Antisera were occasionally purchased from Searl (UK). A complement mediated cytotoxicity assay was used to assess the number of donor and host derived cells in a regenerating thymus. To 50 µl of medium containing 0.5×10^6 cells, 50 µl of the appropriate antiserum dilution were added. After 30 min incubation at 4°C, 250 µl agarose absorbed guinea pig complement were added in a 1:12 to 1:15 dilution. The cell suspension was then incubated for a further 45 min at 37°C. After the incubation, the cells were washed and the living and dead cells were counted. Occasionally, living and dead cells were discriminated microscopically using trypan blue dye exclusion as a criterion for viability. A flow cytofluorographic method was mostly employed in a Bio/Physics cytofluorograph, making use of differential staining of living and dead cells with PI and FDA as described in Section 9. The sensitivity of this method allowed detection of 1-2% differences between dead cells in antiserum treated and nontreated cell suspensions.

Our thymus regeneration model described in Section 17.1 is a modification of that described by Kadish and Basch (1976). They, however, used depletion of spontaneous ^3H thymidine incorporating cells by treatment with donor or host specific antisera to determine the proportion of donor and host derived cells. This method was based on the assumption that during the rapid phase of thymus regeneration, day 14 after transplantation of 5×10^6 cells, all, or most thymocytes were in cycle so that ^3H thymidine incorporation would be a sensitive method to discriminate between the living and dead cells. This assumption of course only would be valid when the proportion of cycling cells within the donor and in the host subpopulations is the same. The poor reproducibility of the thymus regeneration assay (Kadish and Basch, 1976) led us to use a different method for the discrimination of living and dead cells in a cytotoxic assay. The flow cytofluorometric method we used for discrimination between living and dead cells with spontaneous ^3H TdR incorporation as used by Kadish and Basch (1976) was compared, to evidence of our modification of the in vivo thymus regeneration assay. For this, differential kill with specific Thy-1 antisera in a mixture of C3H (Thy-1.2) and (C3H x AKR)F_1 (Thy-1.1 and Thy-1.2) thymocytes was determined. A good correlation between the composition of the mixture and the differential kill was obtained with both methods (Fig.6). Neither method determines Thy-1 negative cells.

According to Ledbetter and Herzenberg (1979) and Shortman et al. (1979) the surface antigen density of the Thy-1 on mouse thymocytes is very well reflected in the susceptibility to lysis with cytotoxic antisera of those cells.

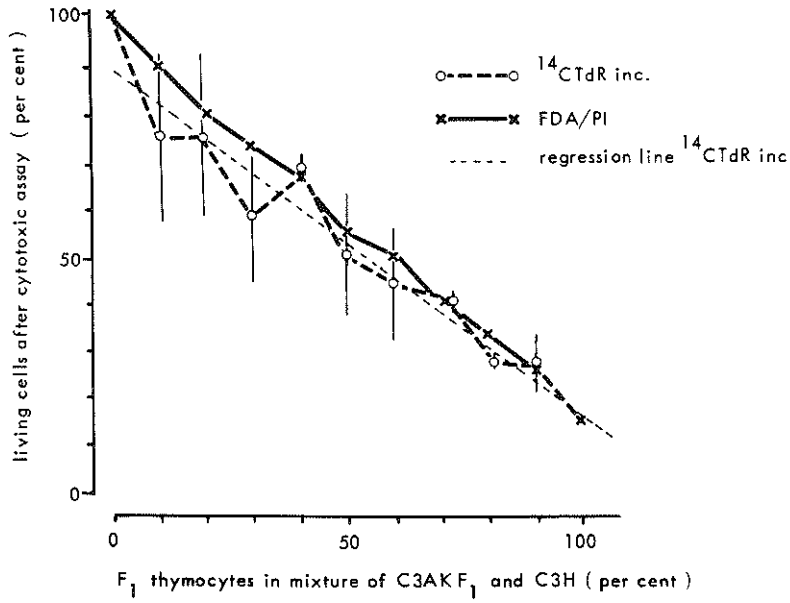

Fig. 6 Comparison of methods to determine the endpoint in cytotoxicity assays. Mixtures of C3H and C3AKF_1 thymocytes were treated with anti-Thy-1.1 serum and complement. Flowmicrofluorometry using PI and FDA staining was compared with spontaneous ^{14}C thymidine incorporation. For FDA/PI staining $2x10^4$ cells were counted in a flow microfluorometer with an estimated standard error of 0.7%. Incorporation of ^{14}C thymidine was measured in triplicate cultures for which S.D. are shown.

The fraction of Thy-1.1$^+$ cells in all experiments was defined as:

$$\text{Thy-1.1}^+ \text{ cells} = \frac{(\% \text{ kill with Thy-1.1} + c') - (\% \text{ kill with } c')}{(\% \text{ kill with Thy-1.2} + c') - (\% \text{ kill with } c')} \times 100\%$$

Spontaneous thymidine incorporation and the FDA/PI based flow cytofluorometric method also gave similar results when they were used to discriminate Thy-1.1$^+$ and Thy-1.2$^+$ cells in regenerating thymus after normal bone marrow transplantation (Fig.7). There is a tendency to overestimation of donor cells by ^{14}C TdR incorporation at low proportions of donor cells e.g. during the early phase of thymus regeneration. The flow cytofluorometric method was thus shown to be a better method than measuring spontaneous ^{14}C TdR incorporation. We prefer the flow cytofluorometric method for the counting of living and dead cells over spontaneous thymidine incorporation because it takes into account not only the cycling Thy-1$^+$ cells, which represent only a minor proportion of all thymocytes, but also the resting cells. This could be the reason that, for donor cell proportions lower than 10%, the flow cytofluorometric method gave better reproducibility than did ^{14}C TdR incorporation.

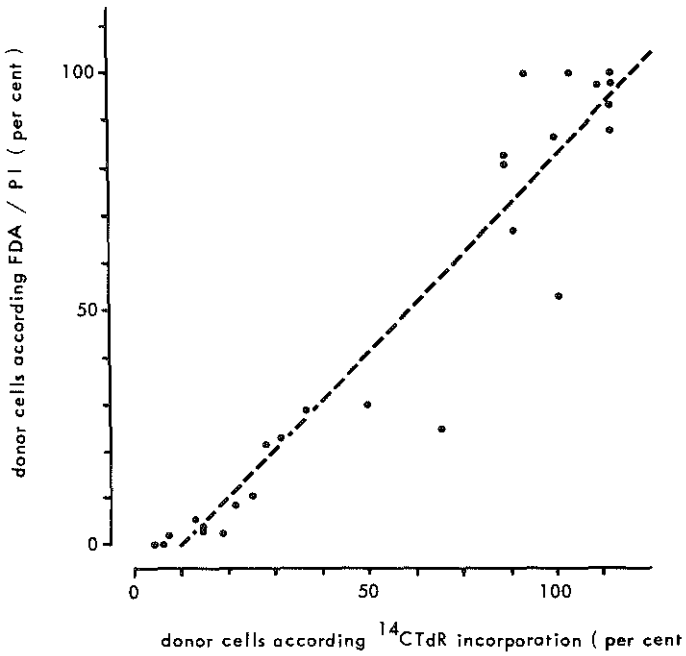

Fig. 7 Correlation of donor cell determination using FDA/PI flow microfluorometry and spontaneous ^{14}C thymidine incorporation. Donor cell determinations in a number of experiments 12 to 20 days after bone marrow transplantation are shown. The correlation coefficient of the linear regression is 0.956 (n=26).

17 PROTHYMOCYTES: DEFINITION AND QUANTIFICATION

17.1 Definition

Prothymocytes were defined as the cells which when transplanted into a lethally irradiated animal give rise to thymus cells of donor origin.

17.1.1 Extrapolation of growth curves

The growth curve of the donor cell population in a regenerating thymus is extrapolated to the day of transplantation using a regression analysis on the log-linear (cell number-time) curve. The offset value determines the number of donor cells, most probably T-cell progenitors, that are present after the inoculation of bone marrow cells.

In thymus repopulation experiments with regenerating bone marrow, it was found that these cells represent a rather poor source for prothymocytes, as will be discussed in Chapter 5 (Fig.9). This indicates that during the period after

lethal irradiation there is such a minute production of prothymocytes in the bone marrow that these will not influence the thymocyte production by the initially inoculated progenitor cells, even if some of these secondary committed cells homed into the thymus. From this, we concluded that extrapolation of thymocyte growth curves is a legitimate quantification procedure for prothymocytes.

17.1.2 The estimation of relative prothymocyte numbers

After transplantation of different numbers of bone marrow cells into lethally irradiated recipients, the growth curves for donor derived thymocytes were parallel from the day donor derived cells can be detected with a cytotoxic assay (day 10 to 11) to about 18 to 19 days after transplantation. Therefore, donor cells develop at the same rate independent of the number of cells transplanted. The delay determined between these growth curves can be used as a measure for the relative number of progenitor cells.

17.2 Considering the Accuracy of the Prothymocyte Assay

The assay is initiated with irradiation of the recipients of the progenitor cells. The variation of the dose distribution over the animals is at most 2%. The number of thymus cells in a thymus changes with age. Variation in the age of animals in a group influences the maximum number of donor cells that can be detected at a certain time after irradiation. In each experiment the mice were of the same week of birth therefore, at the age of 8 weeks they are 53 to 60 days old.

Cell counting in a haemocytometer or machine counting in a pulse cytophotometer, cytofluorograph or coulter counter follows a Poisson distribution. The standard deviation then can be estimated to be $N^{-1}.\sqrt{N}$ expressed in percent, without having to do replicate observations (Colquhoun, 1974; Blacket, 1974). For cell counts in a haemocytometer between 100 and 200 cells were counted with an accuracy of 7.1-10.0%. For cell counts in machines 10^4-2.10^4 cells were counted with an accuracy of 0.7-1.0%.

Under experimental conditions, the capacity of hemopoietic cells from various sources to restore the thymus cell population was always compared with that of fresh normal bone marrow of 6-week-old mice. The log-linear growth curves obtained with experimental samples and normal marrow, the latter serving as a reference, were required to run parallel.

Donor cell production in the thymus was measured after transplantation of 2.10^4 - 20.10^6 cells in 0.3-0.5 ml per mouse in a group of four mice. Machine counting was used to determine the number of thymocytes in the pooled thymuses. The population of cells killed with anti-Thy-1.2 serum (all thymocytes) and of cells killed with anti-Thy-1.1 serum (donor cells) was counted in duplicate samples in a cytofluorograph. Since all thymocytes were Thy-1.2^+ the accuracy of

Thy-1.2^{+} cell counts is in the order of 0.7-1.0%. The proportion of donor cells varied between 2 and 100%, therefore the accuracy of donor cell counts is 7 and 1%, respectively. This indicates that donor cell determinations at later time points during the regeneration period has a greater accuracy than counts at an earlier time. In most experiments at least three to four donor cell determinations were made during the rapid phase of regeneration soon after transplantation of progenitor cells. The log-donor cell number versus linear-time plot showed correlation coefficients between 0.91 and 0.98. There was a direct relationship between the number of CFU-S in the inoculum and the number of donor cells produced at a certain time point after transplantation of normal bone marrow (Fig.8).

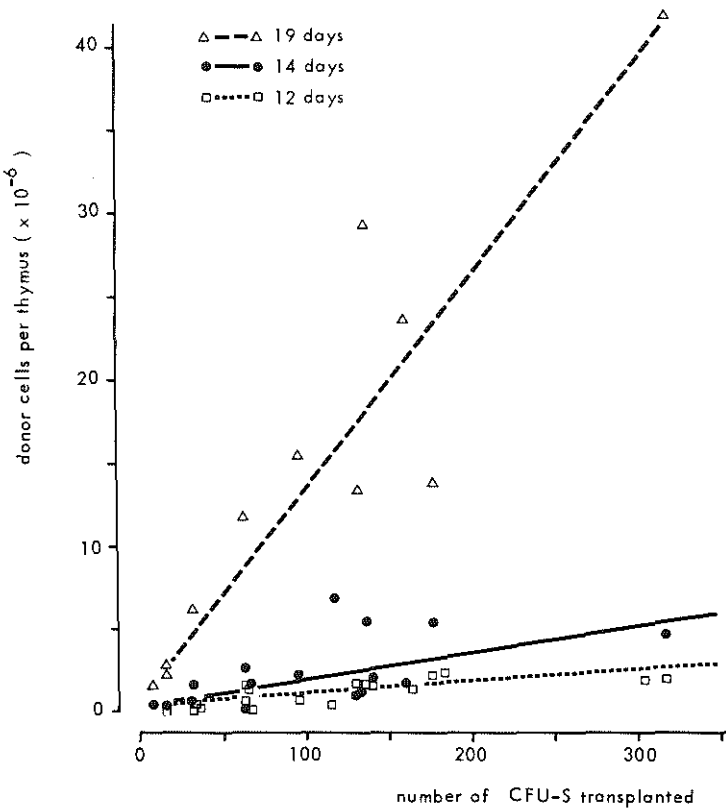

Fig. 8 Quantification of thymocyte progenitor cells in bone marrow of normal mice. For seventeen experiments, the relation between the number of CFU-S transplanted and thymocyte regeneration is shown. Linear regression correlation coefficients were for 12 days, 0.61 (n=16), 14 days, 0.90 (n=17) and 19 days after transplantation 0.94 (n=11).

Let us consider one example of donor cell progenitor calculation. Thymus repopulating ability of fetal liver cells (Chapter 5, Fig.2) was compared to normal bone marrow cells. In six different experiments, donor cell regeneration curves

(log-donor cells/10^6 bone marrow cells transplanted vs time) each consisting of 3-4 donor cell determinations between day 12 and 19 after transplantation of bone marrow were calculated. The mean slope of these growth curves was 0.2988 with S.D. 10.7%. This means that individual doubling time determinations will show a similar variation. Quantification of prothymocytes on the basis of extrapolation of the growth curve to the day of transplantation has only limited accuracy since at most four observations per experiment were used to calculate offset values for regression curves. Extrapolation beyond the range of observation introduces a grave statistical uncertainty because of the combined uncertainty in the slope and in the offset value (Colquhoun, 1974). When the 20 donor cell determinations of 6 experiments were fitted in one growth curve the slope showed a S.E. of 6.8% whereas the offset value showed a S.E. of 126%. When the donor cell growth was expressed per CFU-S transplanted, offset values of individual growth curves generally showed less variation. In the case of our example the offset value of the donor cells/CFU-S vs time curve had a S.E. of 41%. The number of prothymocytes per CFU-S was 0.8 ± 0.3.

After transplantation of bone marrow cells from gradient fractions it was found that the slope of the growth curve for donor derived thymocytes was independent of the cell density and cell sedimentation velocity. The number of cells in the fractions of analytical gradients did not allow more than one donor cell estimate. Calculation of the relative number of thymocyte progenitor cells was based on the following reasoning. The single donor cell determinations were assumed to be part of parallel growth curves. The relative number of prothymocytes could thus be calculated as the ratio of the donor cell numbers determined at the same time after transplantation.

During thymus regeneration after fetal liver cell transplantation as described in Chapter 5, growth curves of donor cells per CFU-S transplanted were obtained that were compared to similar growth curves after bone marrow transplantation.

For the curves obtained after transplantation of day 16 fetal liver the accuracy of the relative donor cell estimate was evaluated. The pairs of these donor cell growth curves (logarithmic regression lines) obtained after fetal liver cell transplantation were (when tested in pairs) identical according to testing at the 5% level for equal slope and equal offset. The same was found for the donor cell growth curves obtained after transplantation of bone marrow (Fig.9). When within individual experiments growth curves obtained after fetal liver and after bone marrow transplantation were compared, the lines were found to be only parallel. The growth delay between the pairs of growth curves was 0.85 ± 0.10 (S.E.) doubling time which leads to a number of prothymocytes per CFU-S which is 55 ± 7% (S.E.) of that in normal bone marrow. Since growth curves for donor derived Thy-1^+ cells/CFU-S obtained after fetal liver transplantation and after bone marrow transplantation were statistically parallel but not identical, it can be concluded that for day 16 fetal liver transplantation a difference of a factor two

in the ratio of prothymocytes over CFU-S is detectable with the prothymocyte assay.

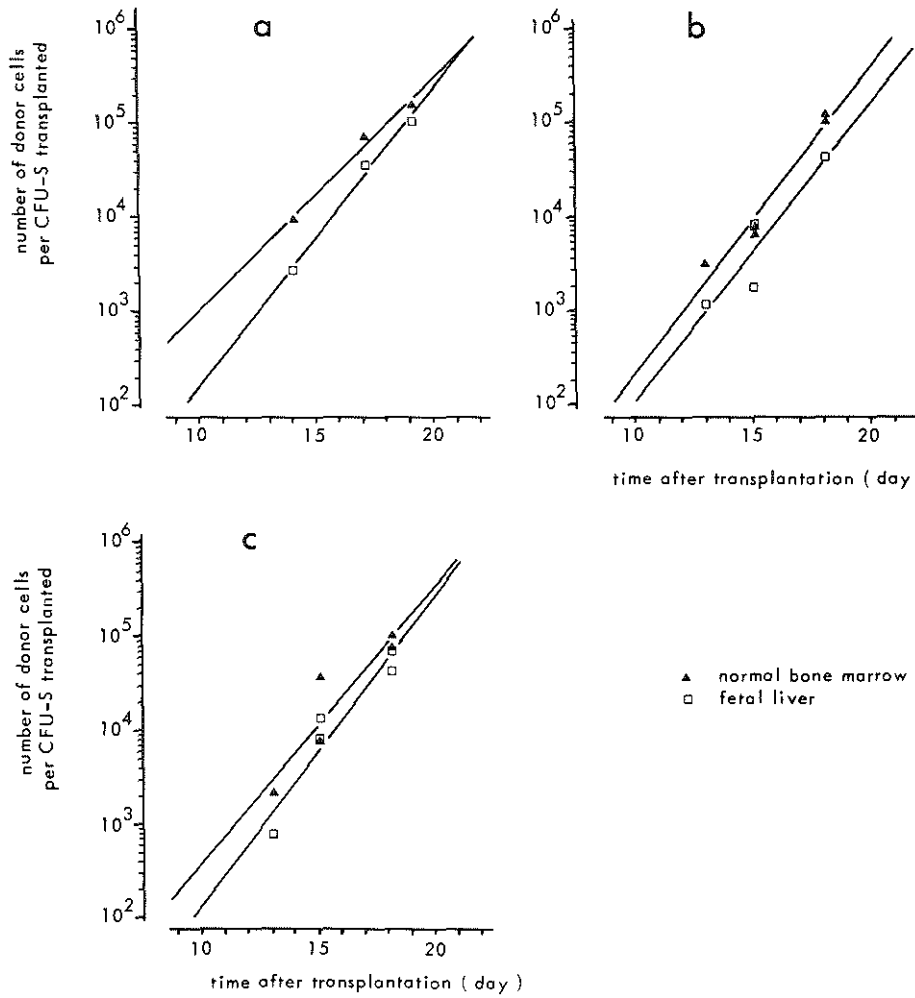

Fig. 9 Comparison of prothymocytes per CFU-S in fetal liver cells and normal bone marrow. The pairs of logarithmic regression lines can be accepted as parallel at a 5% test level. Equal slopes can be accepted based on p values: a:p = 0.117; b:p = 0.908; c:p = 0.721; equal offsetts are rejected a:p = 0.0012; for b:p = 0.0039; for c:p = 0.025. The mean delay time between the growth curves was estimated to be 1.0 ± 0.2 days. This means that a difference of a factor 2 in the prothymocyte or a CFU-S ratio can be estimated with a standard error of 20%.

CHAPTER 3

THE PROTHYMOCYTES IN NORMAL BONE MARROW

1 INTRODUCTION

The thymus is the specific site where development of progenitor cells into the T-lymphocyte lineage is initiated and controlled (Ford et al., 1966; Everett and Tyler, 1969; Takada and Takada, 1972).

The available literature data do not make it possible to determine whether a distinct prothymocyte is indeed more than a hypothetical entity (Kadish and Basch, 1977; Basch and Kadish, 1977). Since no in vitro assay systems are available for T progenitor cells, an in vivo system using the mouse as a "test tube" was used for investigation of T-cell differentiation.

The regenerating thymus as a model system for the study of T-cell development offers some advantages over the analyses of T-cell differentiation in other systems, e.g., 1) the development during fetal life, 2) hemopoietic regeneration in sublethally irradiated animals, 3) regeneration of the thymus lymphocytes in grafted thymus.

1. In the embryonic state, the numbers of cells available for analyses are low and not easily accessible.
2. The development of thymocytes in sublethally irradiated mice has been described by Takada et al. (1971) and Kadish and Basch (1975). A major disadvantage of this model system is that the regeneration of the thymus simultaneously starts from T-cell progenitors of distinct stages of development. The number of progenitor cells in such a system can only be controlled by the dose of irradiation. In sublethally irradiated animals, there is a biphasic postirradiation regeneration pattern for thymocytes which has been interpreted as being produced by different progenitor cells (Takada et al., 1971; Kadish and Basch, 1975).
3. Transplantation of a thymus graft in thymectomised or nude mice offers the possibility to study regeneration of the thymus by host derived progenitor cells (Dalmasso et al., 1963). Practical objections against such a system were based on the laborious aspect of the operations (thymectomy and thymus grafting) when large numbers of experimental animals are needed. In addition, the number of progenitor cells in the bone marrow of the recipients is rather high and not under control.

The transplantation system that we have modified (Chapter 2) was developed by Kadish and Basch (1976) to study thymus regeneration in a qualitative way. In this system, the number of progenitor cells is variable and can be choosen in

a way which is most appropriate for the experimental demands. Lethally irradiated mice receive a transplant of hemopoietic cells containing some, which were able to produce T cells with a Thy-1 alloantigen phenotype different from that of the host. The development of donor and host derived thymocytes after the transplantation was described in a qualitative way. Quantitative aspects of these phenomena were less well explored.

The aim of our studies with the transplantation system described (Chapter 2) was to investigate the relationship between the cells that developed in the thymus and the progenitor cells from which they were derived. Accurate quantification of the cellular kinetics in the thymus had to provide the data required for a better characterisation of these thymocyte progenitor cells.

For this purpose, the cell in which we were mostly interested, the prothymocyte, was defined as the one that gave rise to Thy-1^+ cells in the thymus of irradiated animals. A quantative relation was established between the number of progenitor cells and the progeny produced. The definition used for prothymocytes was the same as that of Kadish and Basch (1976). According to their findings, the prothymocyte in the bone marrow is Thy-1 negative and relatively resistant to cortisone and radiation (Basch and Kadish, 1977). They did not compare these sensitivities to cortisone and radiation of the prothymocyte with those of known progenitor cells for the T-cell compartment, i.e., pluripotent stem cells (CFU-S).

In nude mouse bone marrow, but especially in nude spleen, cells with a low expression of Thy-1 antigens have been found (Loor and Roelants, 1975). Similar findings have been made in thymectomised irradiated and bone marrow reconstituted mice. It was suggested by Roelants et al. (1975) and Loor et al. (1976a,b) that such cells could be thymus cell progenitors, since, in athymic mice, they disappeared after a thymus graft was implanted. "Low" Thy-1 positive cells were also found in normal bone marrow and spleen. Most of the weakly Thy-1 positive cells could be detected only with anti-mouse brain reagents (Loor and Roelants, 1975; Roelants et al., 1975). These reagents, however, have also been shown to detect antigens expressed on stem cells and possibly other early hemopoietic progenitor cells (Golub, 1971, 1972; Adler et al., 1978; Van den Engh et al., 1978).

Two lineages of cells with different expression of Thy-1 antigen have been described in the thymus (Shortman et al., 1973; Shortman and Jackson, 1974). According to these authors, these two cell populations develop independently in the adult thymus. From this, one might conclude that there possibly exist two types of progenitor cells, one for each sublineage, in the bone marrow.

In the experiments described in this chapter, the capacity of normal bone marrow cells to produce progeny in the thymus of lethally irradiated mice was determined. The quantification methods (Chapter 2) which were based on theoretical assumptions were tested and applied in different experiments. From the evaluation of the results, it was concluded that the growth kinetics of the rege-

nerating thymus cell population after bone marrow transplantation was in agreement with the assumptions.

Fluorescent anti-Thy-1 reagents were used to establish the regeneration kinetics of the "low" and the "high" Thy-1^+ thymus subpopulations. This was done in order to investigate whether there was a sequential relationship between the two subpopulations or whether they developed independently.

2 RESULTS

2.1 Donor Derived Thymocyte Growth after Transplantation of Normal Bone Marrow

2.1.1 Dose-effect relationships

After transplantation of normal bone marrow cells in lethally irradiated host mice, the time delay between transplantation and the first detection of donor cells with the cytotoxicity assay increased with decreasing numbers of cells in the inoculum (Fig.1). After transplantation of 1×10^6 bone marrow cells, the first

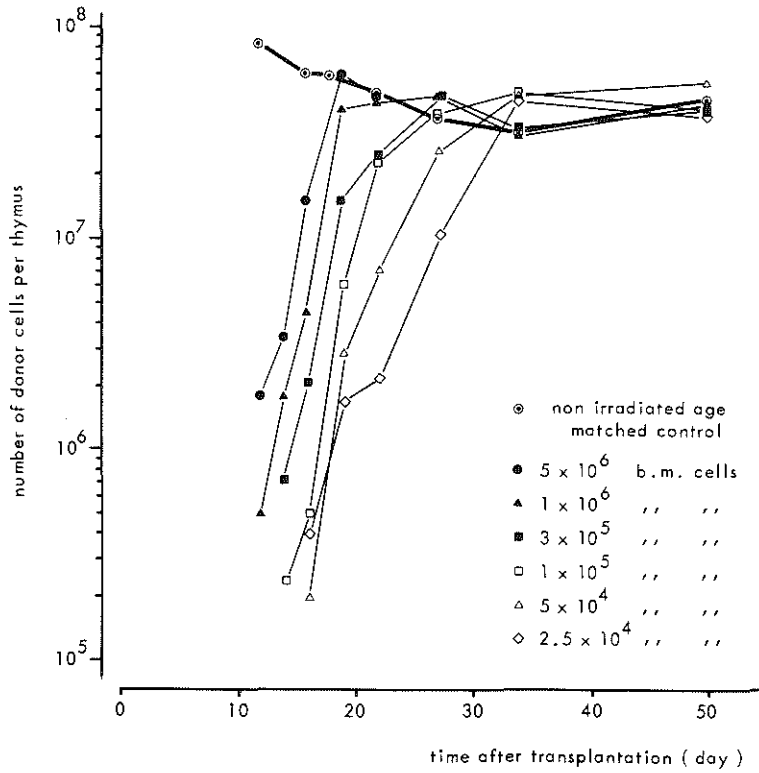

Fig. 1 Donor derived thymus cells in mice after transplantation of bone marrow cells. The plateau level of donor cell regeneration is reached when the number of donor cells is the same as that of cells in age-matched host type control thymus.

donor cells in the thymus were detected in a cytotoxity assay around day 10 to 11 after transplantation. Following transplantation of 2.5×10^4 bone marrow cells, first detection was at fifteen days. A rapid phase of development with a doubling time of 28 h lasted until 17 to 19 days after transplantation. The time delay between the parallel growth curves determined during the rapid phase of development after transplantation of different bone marrow cell numbers was in agreement with the assumptions that we made for quantification of prothymocytes (Chapter 2, Section 17). A decrease by a factor 2 in the number of cells transplanted gave an estimated delay of one doubling time between the growth curves.

When less than 1×10^6 bone marrow cells were injected, a biphasic development of the donor cells was found. After the rapid first phase, a second phase of slower development was observed (Fig.2). The time between transplantation and

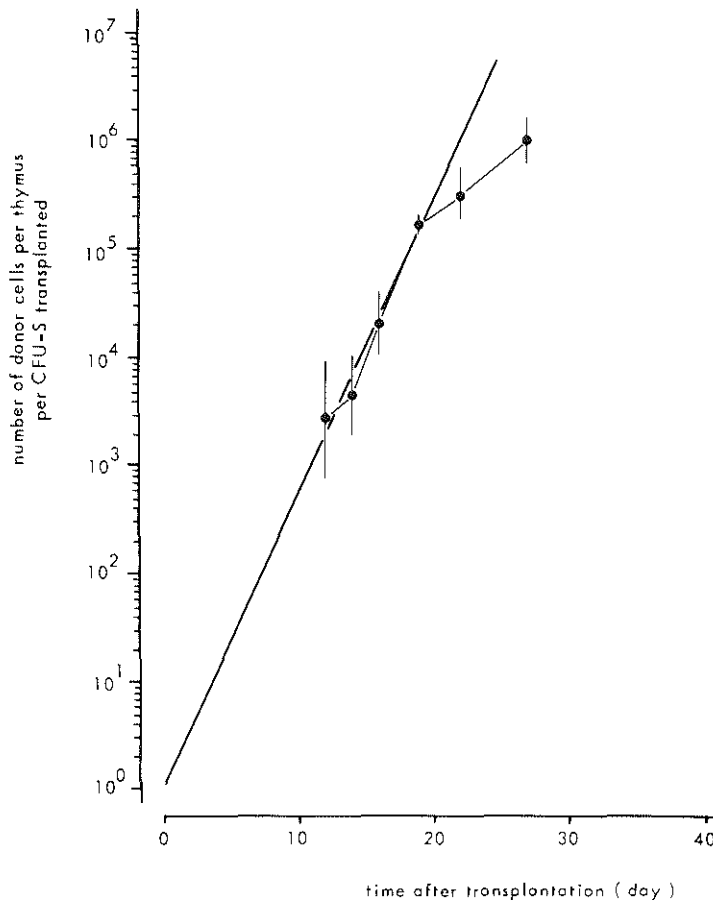

Fig. 2 Donor cell regeneration in bone marrow transplanted mice. Extrapolation of the rapid growth phase shows that one progenitor cell arrived in the thymus per CFU-S transplanted. day 12: n=3; day 14: n=7; day 16: n=6; day 19,22,27: n=5.

the onset of the second phase was independent of the bone marrow cell number transfused. In this latter phase, the apparent doubling time increased when the number of cells in the inoculum was smaller. The expansion of the donor cell population ended when the number of cells per thymus approached that of untreated control mice of the host type. Transplantation of 0.5-1 x 10^6 bone marrow cells allowed three to four donor cell determinations by cytotoxic detection methods during the rapid growth phase.

If it is assumed that the donor cells develop from precursor cells in the transplanted bone marrow which home into the thymus only shortly after transplantation, the number of thymocyte precursor cells can be extrapolated from the growth curve of the rapid phase. When calculated in such a way, about 300 progenitor cells initially homed into the thymus after transplantation of 10^6 nucleated cells. In the experiment shown (Fig.2), this means that about one progenitor cell arrived in the thymus per unit of bone marrow cells containing one CFU-S.

2.1.2 Analysis of donor cell regeneration with fluorescent anti-Thy-1 reagents

The relationship between subpopulations of "low" Thy-1^+ and "high" Thy-1^+ cells of donor origin during regeneration of the thymus was studied in mice which were transplanted with 10^7 bone marrow cells. The development of the cells with donor alloantigens (Thy-1.1) detected by flow cytofluorometry (Chapter 2) is shown in Figure 3. The results shown in this figure are expressed as a percent

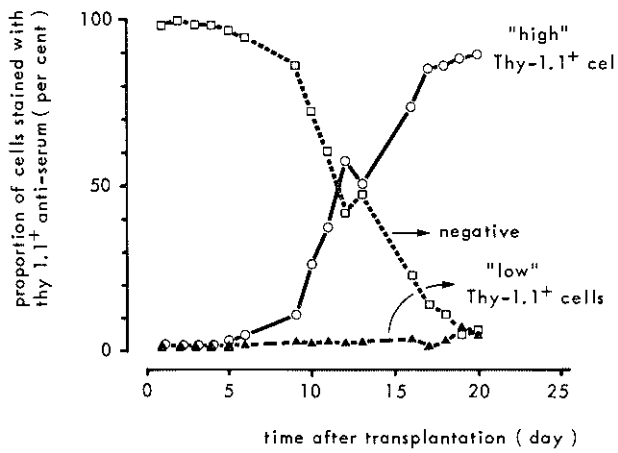

Fig. 3 Regeneration of donor derived cells as determined by fluorescent anti-Thy-1.1 in flow microfluorometry after transplantation of 10^7 bone marrow cells.

of normal values to allow combination of results of two experiments. Early during regeneration, donor thymocytes represent only a small proportion of total cell numbers. This is mainly due to the high number of residual host derived Thy-1$^+$ cells after irradiation and transplantation.

The relative distribution of "low" Thy-1.1$^+$ and "high" Thy-1.1$^+$ cell populations showed an inverse relationship with time (Fig.4). The proportion of "low"

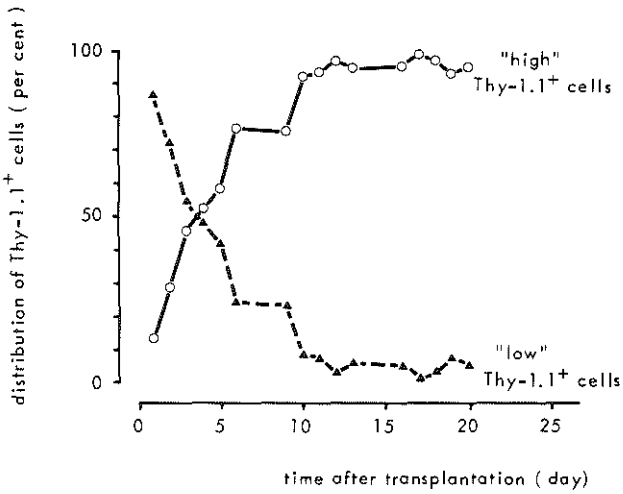

Fig. 4 Relative distribution of "low" and "high" Thy-1.1 donor derived thymocytes after transplantation of 10^7 bone marrow cells.

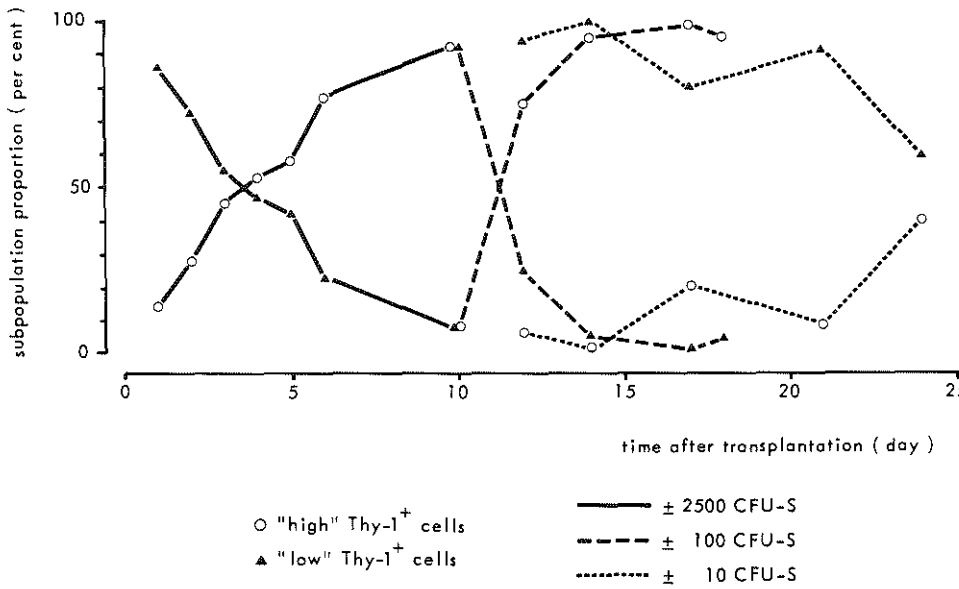

Fig. 5 Relative distribution of "low" and "high" Thy-1.1 donor derived thymocytes after transplantation of different numbers of bone marrow cells containing 10, 100 and 2500 CFU-S.

Thy-1.1$^+$ cells changed from 90 to 100% during the first days after transplantation to 5 to 7% of the donor cell number around 20 days after transplantation. This is below the level of "low" Thy-1.1$^+$ cells in normal age matched donor mice (12 to 15%). Also, after transplantation of smaller numbers of bone marrow cells containing 10 and 100 CFU-S, the sequence of appearance of "low" and "high" Thy-1$^+$ cells and their subsequent development showed the same time course as was observed after transplantation of 10^7 cells (~ 2500 CFU-S)(Fig.5). After transplantation, both "high" and "low" Thy-1.1$^+$ cells showed a rapid growth phase. The decrease in the proportion of "low" Thy-1.1$^+$ cells during thymus regeneration is due to the differences in growth rate between the two donor derived subpopulations. "High" Thy-1.1$^+$ cells had a doubling time of 26 h while "low" Thy-1.1$^+$ cells increased with a doubling time of 40 h. The "high" Thy-1.1$^+$ subpopulation overgrowed the "low" Thy-1.1$^+$ cells (Fig.6). The doubling time for

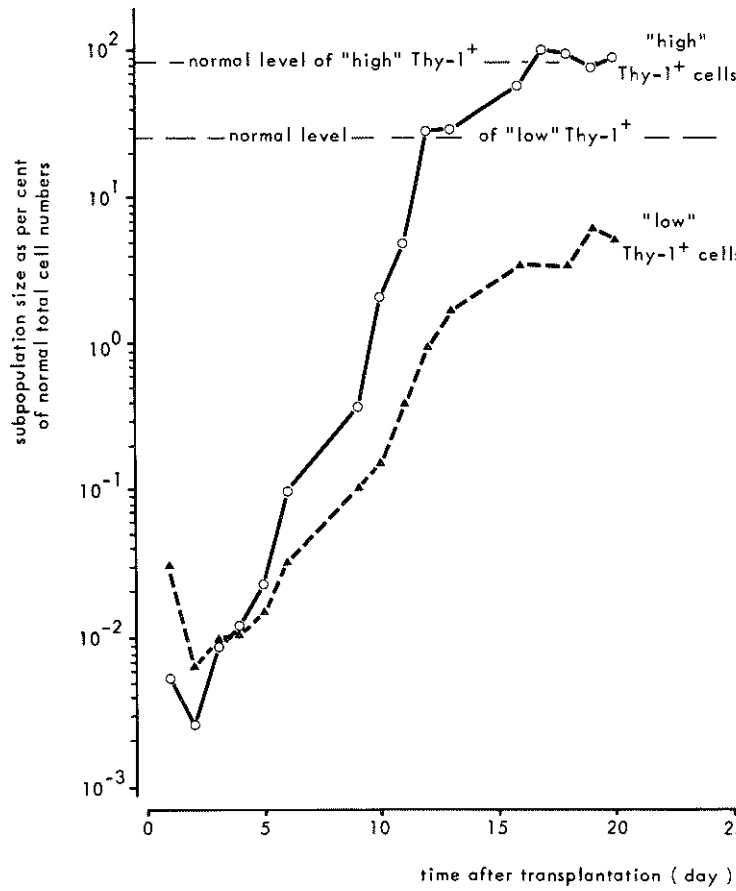

Fig. 6 Growth curves for "low" and "high" Thy-1.1 donor derived cells after transplantation of 10^7 bone marrow cells. "Low" Thy-1.1 cells have a doubling time of 40 h. "High" Thy-1.1 cells have a doubling time of 26 h.

the total donor cell population was the same as observed in the experiments where donor and host cells were distinguished according to lysis with anti-Thy-1 antisera and complement.

2.1.3 Size distribution of donor cells

After transplantation of 10^7 cells, the Thy-1^+ donor cells were initially of large size according to analysis of low angle forward light scatter. These cells remained in the majority for up to 16-18 days after transplantation. The number of small cells of donor phenotype then increased rapidly to normal proportions. The first typical small thymocyte developed on day 6-8 after transplantation. These cells belonged to the "high" Thy-1.1^+ subpopulation (Fig.7a).

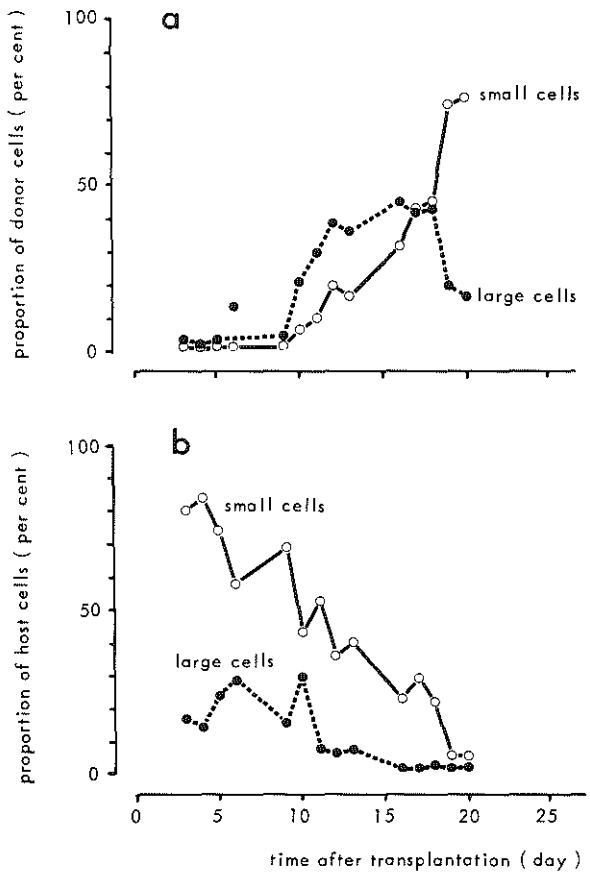

Fig. 7 a. Forward light scatter analysis of regenerating thymocytes. Changes in relative size distribution in donor derived thymus cells after transplantation of 10^7 bone marrow cells.

b. Changes in relative size distribution of host derived cells after transplantation of 10^7 bone marrow cells.

2.2 Host Derived Cells after Irradiation and Bone Marrow Transplantation

2.2.1 Development of cells with host Thy-1 allotype

A typical growth curve for host derived Thy-1$^+$ thymocytes is shown in Figure 8. The expansion of this population was limited. The host cells reached a

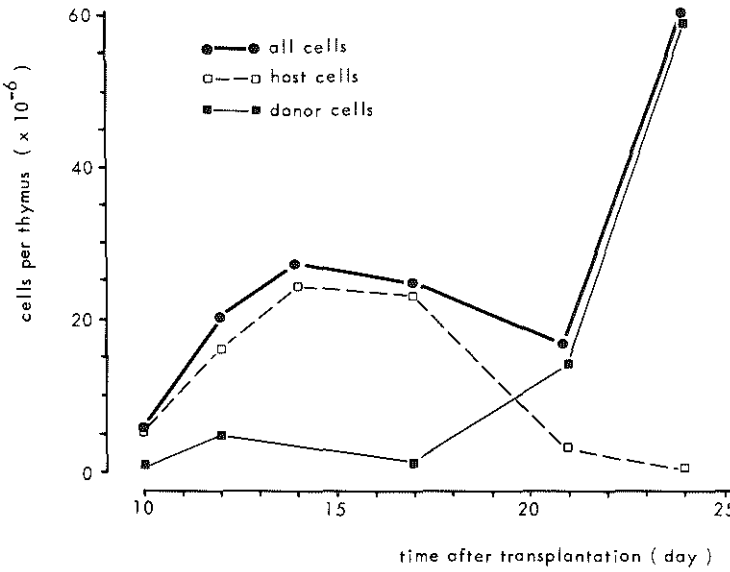

Fig. 8 Regeneration of thymocyte subpopulations after transplantation of 2.5 x 10^5 bone marrow cells.

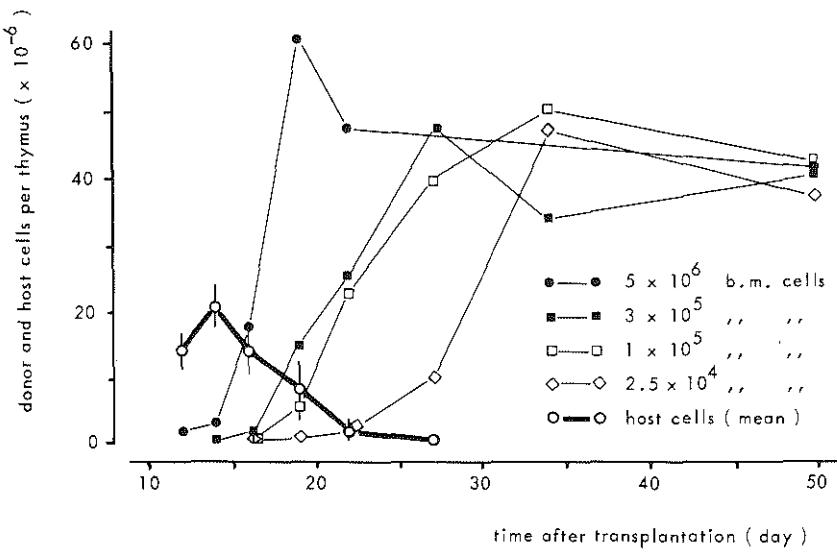

Fig. 9 Regeneration of donor and host cells after bone marrow transplantation. The mean host cell regeneration after transplantation of bone marrow concentrations ranging from 2.5 x 10^4 to 5 x 10^6 nucleated cells is shown.

peak at between 12 and 14 days after transplantation. Then, the host thymocyte number declined. Based on the use of the cytotoxic assay for donor/host thymocyte discrimination, no host cells were detectable after 25 days posttransplantation. The host cell population grew out independently of the number of bone marrow cells transplanted (Fig.9).

2.2.2 Independent development of "low" and "high" Thy-1$^+$ host cells

During regeneration of the thymus host Thy-1$^+$ subpopulations were also analysed with fluorescent anti-Thy-1 reagents. It was found that, early after irradiation and transplantation, most cells (about 90%) with the host Thy-1 alloantigen were "low" Thy-1$^+$ cells (Fig.10). The number of these cells remained

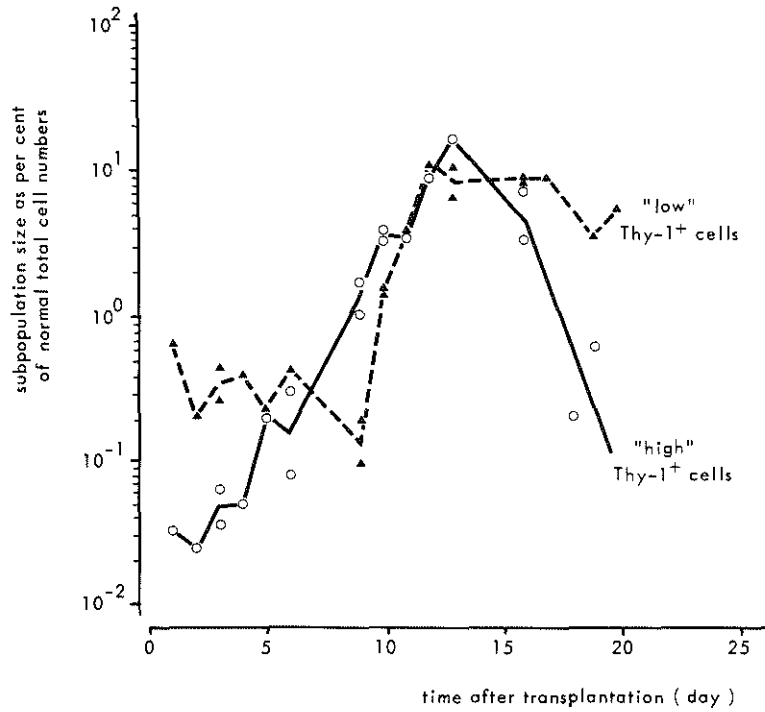

Fig. 10 Regeneration of host derived "low" and "high" Thy-1$^+$ cells after transplantation of 10^7 bone marrow cells.

rather constant in number during the first 10 days after irradiation and transplantation. From day 10 onwards, the number of "low" Thy-1$^+$ cells increased rapidly until day 12 to 14 after transplantation. Host cells with "high" Thy-1$^+$ fluorescence already showed a rapid growth phase at the beginning of the regeneration process. The expansion of the latter subpopulation lasted until 12 to 14 days after irradiation. The doubling time of these "high" Thy-1$^+$ cells was 34 h.

Extrapolation of the growth curve is valid if the subpopulation is assumed to develop independently. Such an extrapolation indicated that the "high" Thy-1^+ host derived cell population starts from 0.005% ($5x10^3$) radiation resistant precursor cells.

2.2.3 Size distribution of host cells

During the first stage of the regeneration process, according to low angle forward light scatter, most (85%) of the host cells were of relatively small size (Fig.7b). The proportion of large host cells increased up to day 8 to 10 and then decreased to normal proportions (10-20%).

2.3 Cycling Status of Regenerating Thymus Cells

The ^3H TdR incorporation assay that was employed by Kadish and Basch (1976) to distinguish between donor and host derived cycling cells was based on the assumption that the majority of regenerating thymocytes was dividing. According to the experiments discussed above, regenerating donor and host derived cells showed different growth kinetics. In order to determine the relative contributions of the donor and recipient cell populations to the pool of proliferating thymus cells, the distribution of cycling cells over these subpopulations was determined during the regeneration period.

The proportion of cycling cells in the thymus was determined by flow cytofluorometry using metachromatic staining with acridine orange as described in Chapter 2. In the rapid phase of development of donor derived cells after transplanation of 10^6 cells (day 12-17), the proportion of cycling cells, including the G_1 phase, was about 25% (Table I). There was almost no change in proportion of cycling cells with the time after transplantation. In this regeneration phase, a decrease of ^{14}C-thymidine incorporation into the regenerating host thymocytes was observed (Fig.11). At the same time still, the donor cell population was relatively small (Table I). From the ^{14}C TdR incorporation studies, we concluded that this decrease in cells in cycle was due to the decreased contribution of host cells to the pool of cycling thymocytes.

When the proliferative state of the thymus cells was determined by the metachromatic staining at one time point after transplantation of different numbers of bone marrow cells (day 17), no differences were observed in the proportion of cycling cells, although varying proportions of donor and host derived cells were present in the thymus (Table II). From the doubling time (Fig.2) observed for the total donor derived thymus cell population and the fraction of cycling cells determined, a cycle time of 8 h was calculated.

TABLE I

FLOW CYTOFLUOROMETRIC DETERMINATION OF THE PROPORTION OF 'CYCLING CELLS'

Number of bone marrow cells inoculated	Donor cells* as percent of all cells			Cycling cells** as percent of all cells		
	Time after transplantation			Time after transplantation		
	12 d	14 d	16 d	12 d	14 d	16 d
5 x 10^6	13	15	58.2	30	27	30
1 x 10^6	4	8	25.2	30	22	27
0.3 x 10^6	6	3	9.5	22	32	24
0.2 x 10^6	-	3.5	14	-	23	19
0.1 x 10^6	-	1	3	-	29	19
0.05 x 10^6	-	-	1.5	-	21	19
0.025 x 10^6	-	-	4	-	31	19

* Proportion of donor cells determined with flow cytofluorometry after staining with FDA and PI.

** Proportion of cycling cells determined with flow cytofluorometry after metachromatic staining with acridine orange.

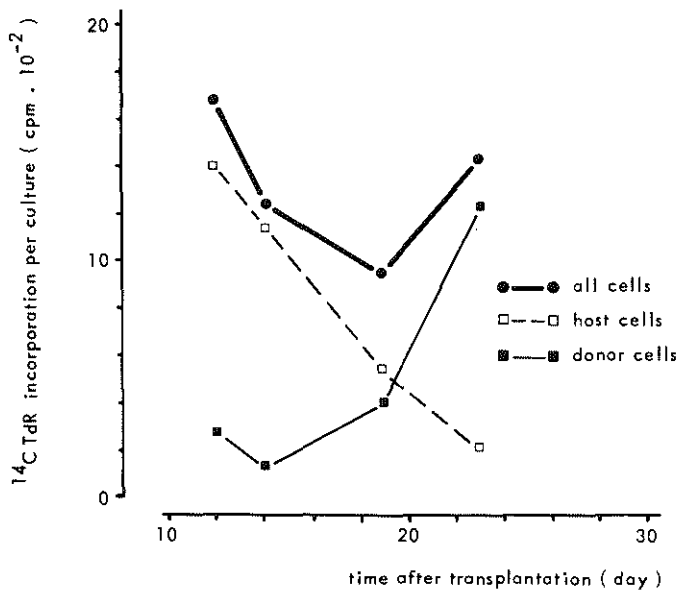

Fig. 11 Spontaneous post-irradiation ^{14}C-thymidine incorporation into thymocytes after transplantation of 10^6 bone marrow cells.

TABLE II

DISTRIBUTION OF DONOR AND HOST CELLS THROUGH THE CELL CYCLE

Subpopulation* analyzed	Day 17 after transplantation of different numbers of bone marrow cells: Donor cells (%)	G_0 (%)	G_1 (%)	S + G_2 + M (%)
Total		79.0	8.2	12.8
host + negatives	2.0	80.0	8.0	12.0
negatives		80.7	7.3	12.0
Total		78.0	11.0	11.0
host + negatives	58.0	81.5	7.0	11.5
negatives		80.3	7.2	12.5
Total		80.5	8.0	11.5
host + negatives	87.0	85.0	7.0	8.0
negatives		79.0	8.0	13.0

* Distribution of cells in cycle in total cells, including donor (Thy-1.1$^+$, Thy-1.2$^+$), host (Thy-1.2$^+$) and Thy-1 negative cells, was determined after incubation with complement only.
Host cells and Thy-1 negative cells were determined after incubation with complement and anti-Thy-1.1 serum.
Thy-1 negative cells only were determined after incubation with complement and anti-Thy-1.2 serum.
The proportion of Thy-1 negative cells according to cytotoxic treatment varied between two and four percent of total cells.
The proportion of cycling cells can be calculated from the differences observed between cell populations incubated with anti Thy-1.1 and complement, and the cell populations incubated with complement only.

2.4 Immune Capacity of T Lymphocytes in Thymus and Spleen after Bone Marrow Transplantation

When the donor cell population in the thymus developed to levels that were detectable in the cytotoxic assay, a response to T-cell specific stimulation was found. The responses to ConA, PHA and allogeneic cells showed a parallel development. After 30 days, responses of thymocytes reached the same values as were determined for normal age-matched donor type control thymus cells (Fig.12a). In the spleens of bone marrow transplanted animals, the first detectable response to T-cell stimulation was observed from about 18 to 20 days after transplantation of 10^6 bone marrow cells. The regeneration of the ConA response was most rapid and reached about 80% of control values in 60 days. PHA and MLR responses developed more slowly (Fig.12b).

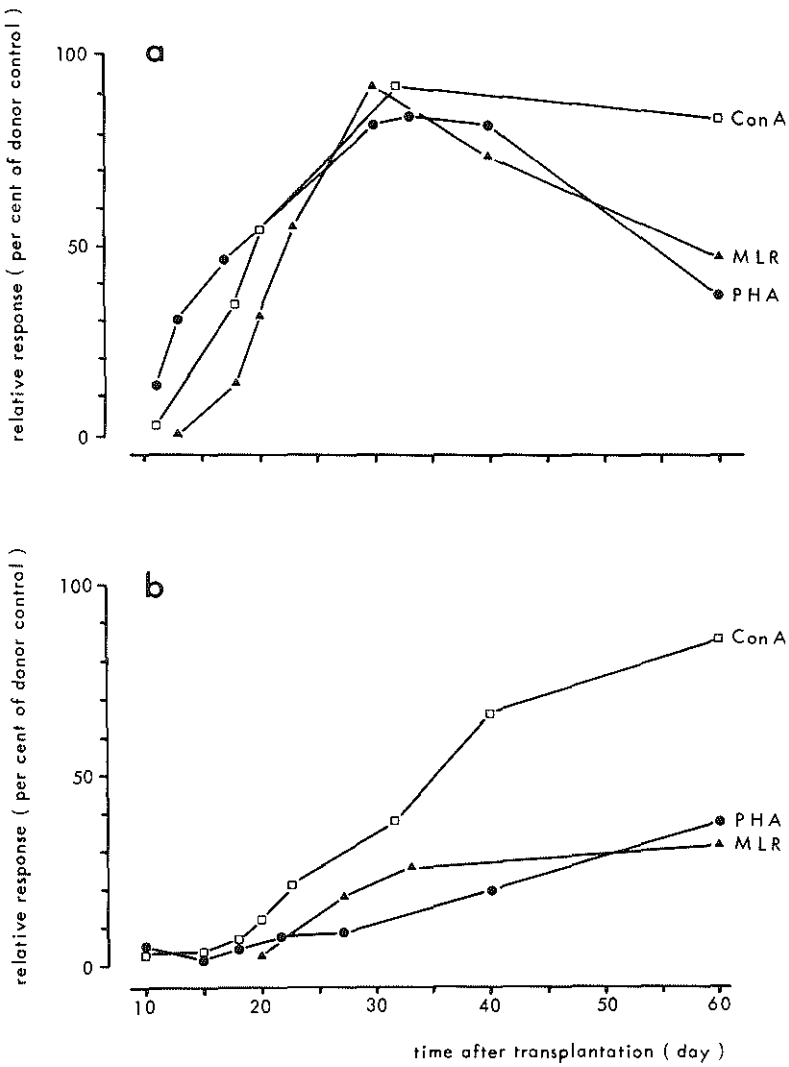

Fig. 12 Regeneration of T-cell function after transplantation of 10^6 bone marrow cells. The response was expressed as a percentage of that in normal donor type thymocytes/spleen cells of age-matched animals.
a. Regeneration of ConA, PHA and MLC responses in thymus cells after transplantation.
b. Regeneration of ConA, PHA and MLC responses in spleen cells after transplantation.

2.5 Effect of Radiation Damage of Thymus on Seeding of Prothymocytes

Since the thymus of the recipient mice used had been heavily irradiated (8.65 Gy), the question arose as to whether or not the capacity of the thymus to be colonised by infused T-cell progenitor cells had changed. During the first two

days after lethal irradiation and bone marrow transplantation, the thymus contained many dead and fragile cells (50% of total). The total number of cells decreased rapidly in two to four days to about 0.5% of the number of cells in the thymus before the irradiation. It was investigated whether these changes in the thymic cellular environment and the decreasing size of the thymus influenced the seeding of T-cell progenitors. If so, it was expected that eventual influences of recovery would be measurable at the end of the period in which major changes were observed.

Three groups of lethally irradiated C3H mice were studied. On the day of irradiation, group I received a syngeneic bone marrow transplant of 3×10^4 cells to rescue them from irradiation death. Group II received 1.5×10^6 (C3H x AKR)F_1 bone marrow cells on the day of irradiation only. Group III received 3×10^4 syngeneic bone marrow cells and an additional 1.5×10^6 (C3H x AKR)F_1 bone

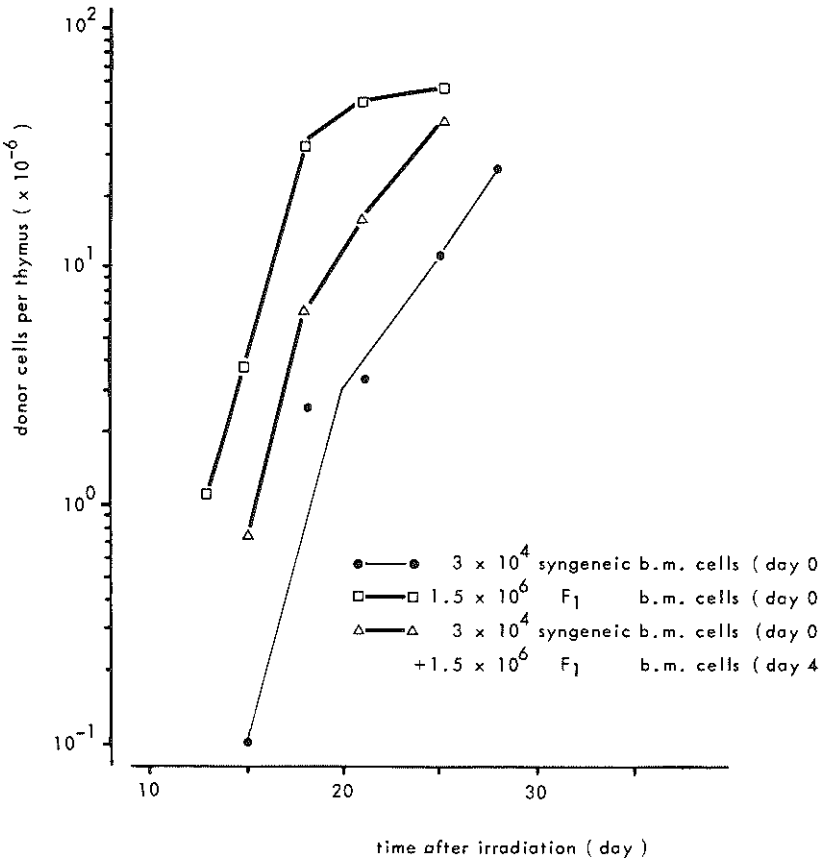

Fig. 13 Effect of delayed bone marrow transplantation following lethal-irradiation of C3H recipients.
(I) 0.03 x10^6 nucleated cells (C3H) injected on day zero.
(II) 1.5 x10^6 nucleated cells (C3AKF_1) injected on day zero.
(III) 0.03 x10^6 nucleated cells (C3H) injected on day zero. A second transplant of 1.5×10^6 nucleated cells (C3AKF_1) was transfused on day zero + 4. The net effect of the second transplant is shown.

marrow cells on day four after the first transplant. The development of the F_1 derived donor thymocytes from marrow transplanted on day four (III) after irradiation showed no difference in kinetics when compared with transfusion of F_1 bone marrow cells within 4 h after irradiation (II) (Fig.13). From this, we concluded that, at least during the first four days after irradiation, the receptive state of the thymus with respect to prothymocytes did not change.

2.6 Basic Characteristics of Prothymocytes in Normal Bone Marrow

2.6.1 The Thy-1$^-$ prothymocyte develops independently of the presence of a thymus in a bone marrow donor

From thymus regeneration experiments in which normal bone marrow was used for reconstitution of lethally irradiated animals, it could be concluded that a T-cell progenitor is present in the bone marrow. In addition, a strong quantitative relation was observed between the thymocyte progenitor cells and CFU-S.

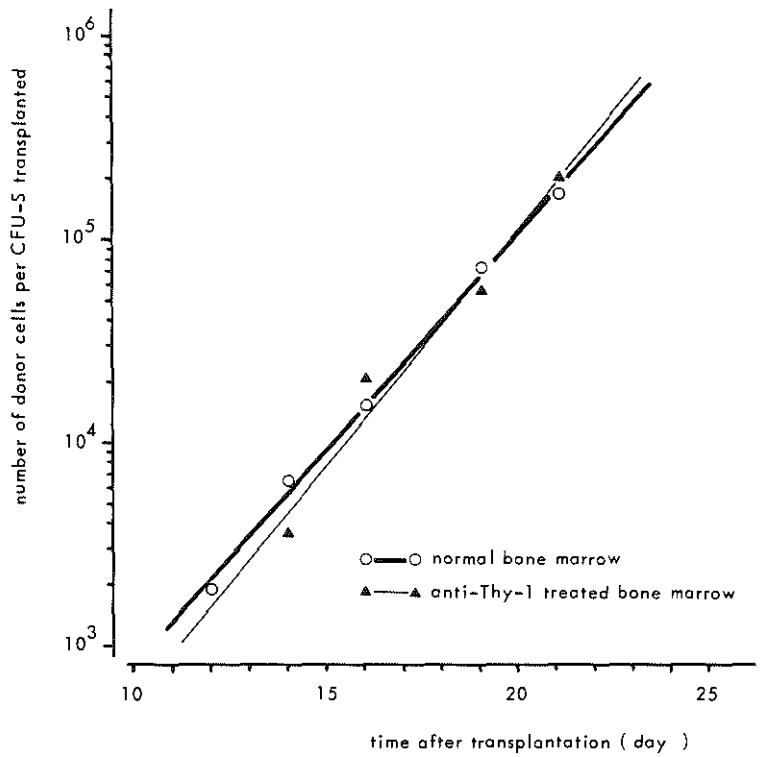

Fig. 14 Growth curves of donor derived cells after transplantation of anti Thy-1 and complement treated bone marrow and of normal bone marrow.

To characterise prothymocytes in normal bone marrow, we determined the effect of the presence of a thymus in the donor mouse on the growth curve for donor derived thymocytes. Therefore, the capacity of normal bone marrow cells and bone marrow cells of neonatal and adult thymectomised mice to restore the thymus cell population was determined. No differences were found between the growth curves for donor cells in the thymus after transplantation of cells from the sources mentioned. In vitro treatment of bone marrow cells with high concentrations of anti-Thy-1 sera (sufficient to kill over 96% of all thymocytes) in a cytotoxic procedure such as used for discrimination between donor and host derived thymocytes did not change the growth of donor derived thymocytes after transplantation of the bone marrow cells (Fig.14).

2.6.2 Bone marrow cells after seeding in the thymus lose the capacity to form spleen colonies

Since we could not discriminate between prothymocytes and CFU-S, the thymus was tested for the presence of both cell types after bone marrow transplantation.

In a normal thymus cell suspension, only very few CFU-S are present. For each 2×10^7 thymocytes, 1.2 ± 1.0 CFU-S were found. It was determined that no significant numbers of E-CFU, E-BFU, GM-CFU-c_1 or GM-CFU-c_2 were detectable in the thymus (Table III).

TABLE III

HEMOPOIETIC PROGENITOR CELLS IN THYMUS, REGENERATING THYMUS AND BONE MARROW

	Number of cells assayed	Normal thymus colonies/ 10^6 cells	Regenerating thymus colonies/ 10^6 cells	Normal bone marrow colonies/ 10^6 cells
CFU-S	$(0.05 - 10) \times 10^6$	0.06 ± 0.05	0.05 ± 0.11	273 ± 64
E-CFU-c	$(0.05 - 10) \times 10^6$	0	0	1517 ± 194
E-BFU	$(0.05 - 10) \times 10^6$	0	0	260 ± 15
GM-CFU-c_1	$(0.05 - 10) \times 10^6$	0.8 ± 0.7	0	1589 ± 147
GM-CFU-c_2	$(0.05 - 10) \times 10^6$	0.5 ± 0.6	0	795 ± 88

In vitro colony assays were performed in quadruplicate. Regenerating thymocytes were obtained from animals at two days after transplantation of $14.5 \pm 1.8 \times 10^6$ bone marrow cells.

The cell content of one $C3AKF_1$ femur, ($14.5 \pm 2.5 \times 10^6$ cells), containing 2790 ± 210 CFU-S was transplanted into a lethally irradiated C3H recipient. The thymuses of 5 recipient mice were pooled at different times after transplantation and inoculated into one secondary recipient in which CFU-S were counted on day 8 after transfusion. Almost no CFU-S (0 - 0.25 CFU-S per thymus) were determined in the second recipients at any time between 2 and 120 h after transplantation of the regenerating thymus cells (Table IV).

From these results, it was concluded that, when the CFU-S functioned as a prothymocyte, it had lost the capacity to form spleen colonies, at least within 2 h after homing into the thymus.

TABLE IV

CFU-S IN THYMUS AFTER TRANSPLANTATION OF NORMAL BONE MARROW CELLS*

	Experiment I				
Time after transplantation (h)	2	6	15	25	45
Cells per thymus ($\times 10^{-6}$)	18.0	8.9	0.7	0.6	0.3
Thymocytes transplanted in second recipients ($\times 10^{-6}$)	81.0	40.1	3.2	2.7	1.4
CFU-S per thymus	0.11	0.13	0.04	0.11	0.04
	Experiment II				
Time after transplantation (h)	24	48	72	96	120
Cells per thymus ($\times 10^{-6}$)	4.1	0.8	0.9	1.0	0.8
Thymocytes transplanted in second recipients ($\times 10^{-6}$)	19.1	3.6	3.8	4.2	3.5
CFU-S per thymus	0.25	0.04	0	0.07	0.2

* Lethally irradiated C3H recipients received cells from one $C3AKF_1$ femur. The CFU-S content per femur was about 2000. The thymuses of 5 mice were pooled and transferred into a second recipient in which the number of CFU-S was counted on day 8 after transfer.

2.6.3 Bone marrow cells lose the capacity to function as prothymocytes after seeding in the thymus

Since cells that homed into the thymus lost any capacity they had to form spleen colonies, it was assumed that it should be possible to recover prothymocytes from the thymus early after transplantation of high numbers of bone marrow cells.

Cells from a regenerating thymus two days after bone marrow transplantation (10^7 cells) were transfused into a second lethally irradiated recipient. These thymus cell suspensions did not contain CFU-S. Therefore, in these experiments, thymocytes were transfused together with a few syngeneic bone marrow cells (5×10^4) to allow the recipient mice to survive (Fig.15). Thymocytes with the Thy-1 allotype of the bone marrow donor were only temporarily detectable in the thymus of the second recipient.

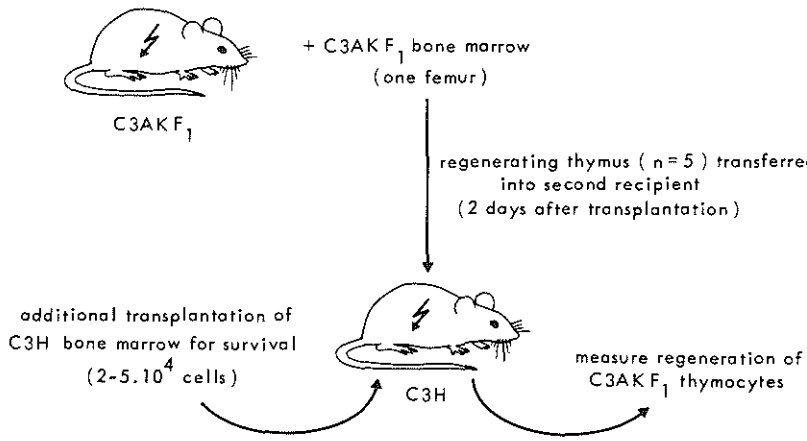

Fig. 15 Schematic representation of the transplantation model used for the study on commitment of bone marrow thymocyte progenitor cells.

The maximum number of thymocytes produced was 5×10^4 to 10^5 cells per 10^6 thymocytes infused. This maximum was reached at around 12 to 15 days after transplantation. A characteristic quantitative difference was observed between the thymocyte production after transplantation of intrathymic progenitor cells from normal and from regenerating thymus, for which the reasons are not yet clear. The time course of development of these cells is similar as was found after transplantation of normal thymocytes (Fig.16). In addition, this time course of donor cell development after transplantation of normal and regenerating thymus cells showed the same characteristics as determined for the development of host derived cells in lethally irradiated mice.

From the differences observed in the growth kinetics of donor cells derived from transplanted bone marrow progenitor cells and those derived from transplanted intrathymic precursor cells, it can be concluded that prothymocytes from the bone marrow lose the capacity to act as prothymocytes soon after they have arrived in the thymus.

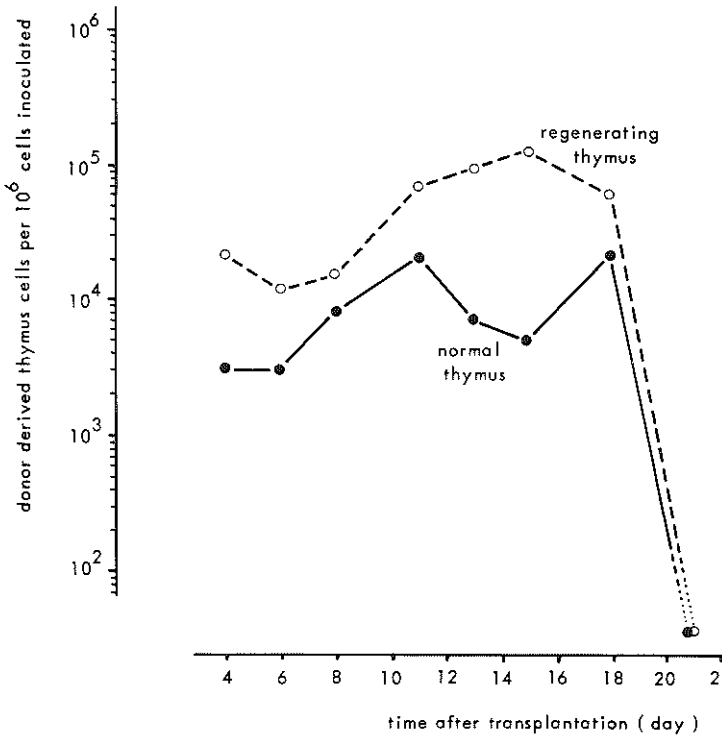

Fig. 16 Regeneration of donor derived thymocytes after transplantation of cells from normal and regenerating thymus (2 days after transplantation of 10^7 bone marrow cells); 5 x 10^6 cells were infused per recipient.

3 DISCUSSION

When normal bone marrow cells are transplanted into lethally irradiated recipient mice, the growth of the donor derived thymus cells is directly related to the number of cells transplanted. As a consequence, there is a direct relation with the number of CFU-S transplanted, although this does not necessarily mean that the CFU-S is a direct T-cell progenitor. The rate of development of the total cell population with donor Thy-1 allotype is independent of the number of cells transplanted up to 17 to 19 days after transplantation. This indicates that a new kinetic parameter is possibly introduced into the system at that time. It seems that the influence of the efflux of cells from the thymus at that time begins to play a more important role. For this reason, observations up to 19 days after transplantation only were used for quantitative interpretation in our kinetic studies. Since the regenerating bone marrow does not contain significant numbers of prothymocytes (see Chapter 5), CFU-S derived thymocyte progenitor cells probably will not contribute significantly to the development of donor derived

thymocytes during the first three weeks after bone marrow transplantation. This allows the assay system to determine only injected numbers of prothymocytes.

From the studies in which fluorescent reagents were used for donor cell recognition, it may be concluded that donor cells begin to develop from the time of inoculation. This means that the up to now puzzling lag phase in donor cell development observed in experiments where cytotoxic antibodies were employed for donor cell discrimination was due to the detection limits of the experimental technique used (Kadish and Basch, 1976; Boersma et al., 1981). Donor cell analysis with fluorescent reagents proved to be a more sensitive method. According to Shortman et al. (1979) and Ledbetter and Herzenberg (1979) susceptibility of Thy-1 alloantigens to lysis with anti Thy-1 sera increases with antigen density. The abundantly present "low" Thy-1 cells in the initial phase of the regeneration probably escape the consequences of cytotoxic treatment with conventional anti Thy-1 sera. These observations suggest that homing of prothymocytes into the thymus takes place early after transplantation and that these cells do not require processing in other organs before homing into the target organ.

The results of the experiments where the donor and host derived cells were distinguished by use of fluorescent antibodies completely confirmed the results obtained by cytotoxic determination of the two cell types. Therefore, extrapolation of donor cell growth curves to the day of transplantation was permissable. The number of progenitor cells in bone marrow in our experiments - about $300/10^6$ bone marrow cells - is larger than was calculated by Wallis et al. (1975). These authors calculated that the thymus is repopulated from very few progenitor cells and that this number - 2-14 cells per thymus - was almost independent of the number (5×10^6-5×10^7) of bone marrow cells transplanted. These low values are in apparent contrast to their observation that the length of the period in which the thymus repopulation by donor cells was completed, was inversely related to the number of progenitor cells transplanted. (A delay of 10 days corresponded to a 1000-fold decrease in the number of bone marrow cells transplanted.) In our experiments, at least for up to 10^7 cells transplanted, there was a direct relationship between the number of cells in the thymus early after transplantation and the number of cells transplanted. These findings are in agreement with the observations of Lepault and Weissman (1981).

At the end of the rapid regeneration phase, the number of thymus cells reaches the levels observed in age-matched unirradiated recipients and then decreases, as observed for these latter mice. This indicates that there is an age dependent, possibly hormonally regulated, maximum number of cells per thymus.

After treatment of bone marrow with cytotoxic anti-Thy-1 serum, it retains its normal capacity for regeneration of the thymus. Therefore, it can be concluded that the prothymocytes do not have Thy-1 antigens on their surfaces. This confirms previous observations of Komuro et al. (1975) and Kadish and Basch (1977). Furthermore, the number of prothymocytes does not seem to be

under feedback control of the thymus. This was already expected, since bone marrow cells of nude mice had been shown to be capable of regenerating the lymphoid tissue of lethally irradiated euthymic mice to the same extent as did normal bone marrow cells (Pritchard and Micklem, 1973; Tao and Floersheim, 1976). Also Kraal and Boden (1981), after transplantation of bone marrow from thymectomised mice, did not find a change in thymic immigrant cells up to 12 months after adult thymectomy. This indicates that the thymus does not influence the total number of thymocyte progenitor cells. Time-restricted immigration of thymocyte progenitors has been demonstrated directly during ontogeny in chickens (Le Douarin, 1977). Thus, at least during fetal life, the actual number of immigrating precursor cells seems to be regulated by the thymus.

Soon after arrival in the thymus, the prothymocytes lose their property to repopulate the thymus of an irradiated host. Neither CFU-S nor prothymocytes could be recovered from the thymus after transplantation of bone marrow cells. Progenitor cells for other lineages were also absent. This rapid disappearance of the prothymocyte characteristics after arrival in the thymus could be related to the induction of T-cell specific markers on the cell surface. According to Lepault and Weissman (1981), the surface membrane Thy-1 antigen begins to be expressed within 3 h after homing of transplanted fluorescein labeled bone marrow cells into the thymus. In our experiments, the cells which were recovered from the thymus early after bone marrow transplantation had a limited capacity for regeneration of the thymus in a second recipient similar to that observed after transplantation of normal thymocytes (Fig.14). For these experiments (Section 2.6.3), mice were transplanted with 10^7 bone marrow cells. According to data from the literature (Balner and Dersjant, 1964; Lepault and Weissman, 1981), about 0.1% of these cells (equivalent to 60 CFU-S when for spleen the seedings efficiency is 5%) arrive in the thymus. The cell content of 5 regenerating thymuses (2-120 h after bone marrow transplantation) was transplanted into a second recipient but less than 1 spleen colony was found in its spleen. This indicates either that CFU-S never arrived in the first recipient's thymus or, alternatively, that CFU-S differentiated immediately upon entrance into the thymus. The latter possibility would mean that the CFU-S homing in the thymus functions as a prothymocyte.

Homing of bone marrow cells into the thymus occurs more frequently than the homing of cells from peripheral lymphoid organs (Lepault and Weissman, 1981). After transplantation of bone marrow cells Lepault and Weissman (1981) found that, within 3-24 h after infusion, 8 per 10^4 bone marrow cells homed into the thymus. Of these cells, increasing numbers (9% at 3 h and 20% at 24 h) expressed Thy-1 surface markers (Lepault and Weissman, 1981). Since thymocyte progenitor cells were not found in the thymus shortly after transplantation, it is possible that the Thy-1^+ cells observed in the thymus after bone marrow transplantation are the intrathymic T-cell progenitors (Lepault and Weissman, 1981). The number of Thy-1^+ cells detected in the thymus shortly after transplantation

[0.6-1.6 per 10^4 bone marrow cells (Lepault and Weissman, 1981)] is of the same order of magnitude as we have calculated from thymus regeneration kinetics [2-3 per 10^4 bone marrow cells, (Boersma et al., 1981)].

From in vitro Thy-1 induction on bone marrow cells with thymic "hormones", it is known that this phenomenon takes place within minutes (Bach et al., 1971; Komuro and Boyse, 1973a,b). But, the proportion of CFU-S is not decreased after Thy-1 induction in bone marrow cells (Basch and Goldstein, 1974). Only a limited decrease of prothymocyte activity in spleen cells after Thy-1 induction and subsequent depletion of Thy-1^+ cells with cytotoxic anti Thy-1 sera has been reported up to now (Komuro et al., 1975); this suggests that only a subpopulation of prothymocytes is possibly recruited from the Thy-1 inducible cells. However, in those studies no attempts were made to establish a quantitative relationship between thymus cell regeneration and the various thymocyte progenitor cells, such as CFU-S and prothymocytes. Therefore, the conclusion of Komuro et al. (1975), that cells that can be induced to express T-cell markers are prothymocytes requires additional experimental support.

After transplantation of bone marrow cells, both "high" and "low" Thy-1^+ donor derived cells grow out simultaneously. The observation that the "low" Thy-1^+ donor cells are initially in the majority in the regenerating thymus might be explained in two ways.

First, one might assume that there are two precursor cells in the bone marrow, one for each lineage of thymus cells which arrive in the thymus after transplantation. The progenitor cells of the slow growing "low" Thy-1^+ cells are then in the majority and there are only very few progenitors for the "high" Thy-1^+ cell population.

Alternatively, the early "low" Thy-1^+ cells can be assumed to be the intrathymic progenitor cells for the two subpopulations of thymocytes. We are in favour of the last explanation because "low" Thy-1^+ intrathymic cells are also found to constitute the major population among the radiation resistant cells that are the progenitors of both "low" and "high" Thy-1^+ host derived cells.

Radiation chimeras can be used as models to mimic the sequence of events that take place in the thymus during ontogeny (Owen et al., 1977; Cohen and Scott, 1980). The sequence of events we observed in the regenerating thymus, namely that the independent development of "low" Thy-1^+ cells precedes "high" Thy-1^+ cells, is in agreement with similar observations on Lyt subpopulations during ontogeny (Mathieson et al., 1981; see also Chapter 1). In contrast, topical labeling experiments of Weissmann's group have shown that the first cells labeled in a normal adult thymus belong to the large subcapsular "high" Thy-1^+ cells (Fathman et al., 1975). The label then spreads to small "high" Thy-1^+ and medium-sized "low" Thy-1^+ cells. This was thought to be evidence for the large subcapsular cells being intrathymic precursor cells. The topical labelling used in these experiments has the drawback that every soluble substance applied to the

outside of the thymus in vivo will show a labeling pattern from outside to inside due to a diffusion process. As a result, the subcapsular cells will be labeled first. Therefore, it is our opinion that these data cannot be used to establish the sequential development of thymus cell populations.

The doubling time for the total population of donor Thy-1$^+$ cells is 28 h. The growth rate is different for "low" Thy-1$^+$ cells and "high" Thy-1$^+$ cells in the thymus, the "low" Thy-1$^+$ ones have a long doubling time (40 h), while the "high" Thy-1$^+$ cells have a short one (26 h). For the host (C3H), "high" Thy-1$^+$ cells showing a relatively long doubling time of 34 h were found. This doubling time agrees well with that of 37.5 h which we determined for the total population ("high" and "low") of Thy-1$^+$ cells that developed after transplantation of syngeneic cells into lethally irradiated C3H mice (data not shown). As shown by Metcalf, the proliferation rate of thymic cell populations is genetically determined (Metcalf et al., 1961; Metcalf, 1963). "Low" Thy-1$^+$ cells that developed from radiation resistant precursor cells showed a growth delay followed by a rapid growth phase. From the above observation, it was concluded that, "high" Thy-1$^+$ cells develop at the same rate while "low" Thy-1$^+$ cells develop in a different way. This suggested that "low" and "high" Thy-1$^+$ cells develop independently, which does not implicate that these cells are derived from separate progenitor cells. These findings are in agreement with the data provided by labeling experiments of Shortman and Jackson (1974) on which their concept of independent development of thymus cells belonging to the different Thy-1$^+$ subpopulations was based.

During the regeneration phase of thymus cells after transplantation of 10^6 bone marrow cells, the proportion of cycling cells of the total population remained the same or decreased only slightly after day 10 (Table I). The distribution of cycling cells in the donor and recipient derived cells remained the same. This suggested that the intrathymic development of donor and host cells was quite similar. The proportion of cycling cells beyond day 10 after transplantation of 10^6 bone marrow cells is independent of the proportion of donor and host derived cells. According to the metachromatic staining with acridine orange, the proportion of cycling cells in the thymus showed good agreement with cytokinetic data of Bryant (1972). The proportion of cycling cells is therefore much less than often assumed (Kadish and Basch, 1976). For the total population of donor cells, an overall cell cycle time of 8 h was calculated from the doubling time of the population and the proportion of cells in cycle. This cell cycle time is of the same magnitude as the 7-8 h found by Metcalf (1969), 8.5-9.5 h by Bryant (1972) and 9.5 h by Claësson and Hartman (1976) who obtained these values from labeling studies.

Size distributions for donor derived Thy-1$^+$ cells early during regeneration revealed that the typical small thymocytes did not develope until day six. From these observations, it was concluded that, early during the regeneration phase all

donor cells are relatively large; this is an indication that both subpopulations of Thy-1^+ cells initially contain a relatively high proportion of cycling cells which confirms the results based on labeling data (Shortman and Jackson, 1974).

After transplantation of 10^6 nucleated bone marrow cells, the proliferative responses of thymocytes to ConA, PHA and allogeneic cells were not different from background values until day 10-12 after bone marrow transplantation. This indicated that the cells derived from host type radiation resistant progenitor cells, which constituted the largest proportion of cells at that time, were not capable of responding. After about 10 days after transplantation of 10^6 bone marrow cells, the proliferative responses of thymus cells to mitogenic stimuli and MLC began to increase. The regeneration of the responsive cells reaches the age matched donor control values about 30 days after transplantation.

A separation in time was observed between acquisition of Thy-1 antigens and the capacity to respond to T-cell mitogens which has also been observed during ontogenetic development (Stobo and Paul, 1972; Mosier, 1977).

After transplantation of 10^6 bone marrow cells, the T-cell response of spleen cells appear only from day 18 to 20 following transplantation onwards. The increase in spleen T-cell responses is evident at about the same time that the second phase of the donor cell growth starts in the thymus. This suggests that the cells that appeared in the spleen were possibly the result of an efflux of matured cells from the thymus. Histological observations of Viktora et al. (1978) and Kraal et al. (1979) are in accordance with the observed time course. The first signs of lymphoid development in spleen were found on day 19 to 20 after bone marrow transplantation. The mechanism or signal responsible for the initiation of the efflux is not known. After transplantation of 10^6 bone marrow cells, the response of spleen cells to PHA and in the MLR reaches 30 to 40% of control values as late as 30 days after transplantation, while the ConA response is the same as in controls at that time. In the same time period, the "low" Thy-1^+ cells in the thymus remained at a lower level than normal values, even after transplantation of 10^7 bone marrow cells.

The sequence of development of immunocompetent cells in thymus and spleen as observed in our experiments is in agreement to those of Cohen and Scott (1980). The question arises whether irradiation damage to the thymus which influenced the production of thymic factors but left the morphology of the thymus intact had an effect on thymus cell maturation (Trowel, 1961; Sharp and Watkins, 1981). The effect of irradiation on the thymus after bone marrow transplantation has been studied by Van Bekkum and coworkers (Van Bekkum, 1967). Mice that received an isologous bone marrow graft after supralethal irradiation (11.0 Gy) showed a marked mortality up to 70% in the 2nd and 3rd month after transplantation. After irradiation with doses of 9.0 Gy only 10% of the recipients died within 100 days (Van Bekkum, 1967). The pathological changes in various tissues of non surviving animals were very similar to those observed in neonatally thymectomised

animals (De Vries et al., 1964). In a semi-quantitative histopathological evaluation of damage to the thymus epithelial cells it was found that after irradiation with a dose of 8.0 Gy the thymus epithelium was recovered from the initial radiation damage within 2 to 4 weeks. After irradiation with 11.0 Gy no recovery was observed within the same period (Van Bekkum, 1967). Newborn thymus epithelial cells seemed to be more sensitive to irradiation than those from adults. The regeneration of lymph node and spleen cells after isologous bone marrow transplantation in supralethally irradiated mice (11.0 Gy) was impaired at least during four weeks after transplantation when compared to regeneration in 8.0 Gy irradiated recipients (Van Bekkum, 1967). A negative effect on the restoration of T-cell responses after transplantation of irradiated as compared to nonirradiated neonatal thymus in nude mice has also been observed (Pritchard and Micklem, 1973; Loor and Hägg, 1977). Completely defective thymus dependent activity, however, has been reported only after application of extremely high radiation doses (~ 20.0 Gy) (Dukor et al., 1965; Miller et al., 1966). From these observations it was concluded that the radiation dose that we have applied in our transplantation model (8.65 Gy) probably did not cause irrepairable damage to the thymus epithelium. It cannot be excluded, however, that the regeneration kinetics of thymus epithelial cells influences to some extent the development of thymus cell populations after irradiation and transplantation.

Regeneration of host derived thymocytes in lethally irradiated animals takes place to a considerable extent and independently of the number of bone marrow cells transplanted. The regeneration of these host derived cells as such had previously been described by Kadish and Basch (1975) and Sharp and Thomas (1975, 1977) and others. At those times, the origin of the radiation resistant cells that produced such progeny was unclear. The kinetics of development of the Thy-1$^+$ donor derived subpopulation after transplantation of intrathymic precursor cells is similar to the observed growth of Thy-1$^+$ cells from radiation resistant precursor cells. This suggests that the latter are indeed of intrathymic origin. Characterisation of the host derived thymocytes after bone marrow transplantation by means of fluorescence labeled anti-Thy-1 sera revealed that most of the radiation resistant cells had a "low" Thy-1$^+$ phenotype. These cells were probably intrathymic precursor cells that gave rise to the transient growth of the host cells. The radiation resistant precursor cells are capable only of a limited regeneration. This can be best explained by a limited self-renewal capacity of the intrathymic progenitor cells. The transient aspect of the host cells could also be due to the rather short time that these cells remained in the thymus.

On the basis of the experiments discussed, a tentative intrathymic differentiation pattern may be formulated. It indicates that a Thy-1$^-$ progenitor cell with a very short half-life enters the thymus. It is then rapidly converted to a "low" Thy-1$^+$ intrathymic precursor cell which in turn gives rise to the "low" and

"high" Thy-1$^+$ cell lineages in the thymus. Both "low" and "high" Thy-1$^+$ cells contain a subpopulation of cycling cells (Fig.17).

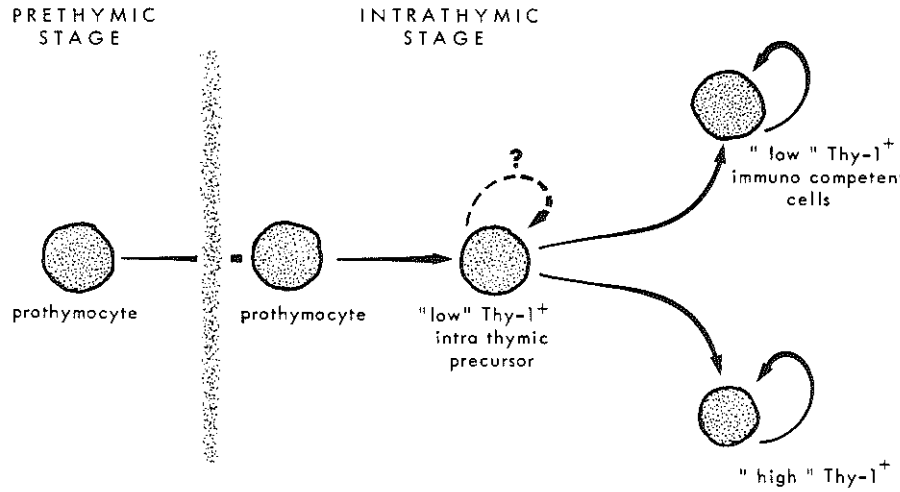

Fig. 17 Tentative model for intrathymic T-cell differentiation. An intrathymic low Thy-1 progenitor cell gives rise to two independent lineages of thymocytes.

4 CONCLUSIONS

1. There is a strong quantitative relation between the number of bone marrow cells transplanted and the growth of donor cells in the thymus of an irradiated mouse.

2. Prothymocytes are Thy-1 negative and not under feedback control of the thymus.

3. After bone marrow transplantation, neither CFU-S nor prothymocytes are found in the thymus.

4. Cells that develop from intrathymic precursor cells and from prothymocytes show different proliferation patterns.

5. The intrathymic precursor cell is probably a "low" Thy-1^+ cell.

6. "Low" and "high" Thy-1^+ cells in the thymus can develop simultaneously and independently.

CHAPTER 4

CHARACTERISATION OF PROTHYMOCYTES AND PROGENITORS OF IMMUNOCOMPETENT CELLS ACCORDING TO PHYSICAL PARAMETERS

1 INTRODUCTION

The application of cell separation methods in experimental hematology serves two major purposes. One is the analytical use that leads to a better characterisation of the cells in complex mixtures such as bone marrow cells, fetal liver cells, blood and thymocytes. The second is to enrich or isolate certain cell types for a variety of purposes e.g., transplantation, functional assays and morphological studies. The development of different fractionation techniques using physical parameters was based on the premise that cell populations that are homogeneous in many parameters are likely to be homogeneous in function. As pointed out by Shortman et al. (1975), most of the physical parameters do not reflect biological specificities. However, cell separation methods combined with functional assays based on factors controlling cell differentiation have contributed considerably to a better characterisation of erythroid (Health et al., 1976; Wagemaker et al., 1977) and myeloid precursor cells (Williams & Van den Engh, 1975; Van den Engh, 1976; Bol et al., 1977, 1979; Visser et al., 1977; Williams & Pluznik, 1978; Bol, 1980).

In Chapter 3, it was shown that there is a quantitative relationship between the number of bone marrow cells transplanted and the thymocytes that develop from these cells. Obviously, the search for distinct T lymphocyte progenitor cells, had to concentrate on attempts to separate them from other early progenitor cells in the bone marrow.

Basch and Kadisch (1977) reported enrichment of functional thymocyte progenitor cells in low density fractions of discontinuous albumin gradients. They did not determine any other hemopoietic progenitor cells in the various fractions. The same method of cell separation combined with other enrichment procedures e.g., anti Ig and anti Thy-1 treatment, glass wool filtration, depletion for cells with complement receptors was applied to bone marrow and spleen cells by Basch et al. (1978). They obtained an enrichment of 10 to 15-fold for thymocyte progenitor cells in bone marrow cells and up to 40-fold in spleen cells.

El Arini and Osoba (1973) applied a continuous ficoll gradient according to Gorcinsky et al. (1970) to separate T precursor cells and CFU-S. They defined T

precursors as those cells that can give rise to MLC responsive spleen cells in irradiated and reconstituted mice 14 days after transplantation of 4×10^6 syngeneic bone marrow cells. They observed a difference between the peak of the density distribution of CFU-S and that of the T precursor cell, but there was considerable overlap between the distributions of the two cell types.

Bone marrow cells that can be induced to express T-cell surface antigens and CFU-S both sediment in the low density fraction of discontinuous density gradients. We have used continuous albumin density gradients to fractionate normal bone marrow cells. The equilibrium density centrifugation performed according to the method of Shortman (1972) was employed to characterise the cells that are active in the T cell differentiation process. The results obtained with this method, may be compared directly with the results obtained by Wagemaker et al. (1977) and Bol et al. (1977) for CFU-S and other early progenitor cells.

According to Gorcinsky and McRae (1977), the Thy-1$^-$ bone marrow progenitors for cells in the spleen that respond to T-cell-specific stimuli 30 days after transplantation of 15×10^6 bm cells have a sedimentation velocity range of 6-8 $mm.h^{-1}$. Worton et al. (1969a,b) and Visser et al. (1977) have shown that CFU-S in fresh bone marrow have a sedimentation velocity of 4.0 $mm.h^{-1}$. Therefore, we envisaged that it should be possible to separate the thymus dependent T-cell progenitors and the pluripotent stem cells (CFU-S) according to their differences in sedimentation velocity only.

Free flow electrophoresis is mainly a function of cell surface charge density when the appropriate conditions are chosen. As was shown by Bol (1980), it is possible to characterise different early myeloid progenitor cells according to differences in electrophoretic mobility (EPM). This observation led us to try to separate the cells of the early stages of T cell differentiation from CFU-S with this technique.

2 RESULTS

2.1 Fractionation of T Cell Progenitors According to Buoyant Density

2.1.1 Buoyant density distributions of prothymocytes and CFU-S

After transplantation of fractionated bone marrow cells, the first donor derived thymocytes could be detected in mice transplanted with low buoyant density cells (Fig.1). High density fractions were relatively poor sources of prothymocytes. In animals injected with 10^6 cells from high density fractions, it required 60 days before the number of cells in the thymus equalled the number in age-matched control mice. At that time, the thymus of animals injected with bone marrow cells from low density and high density fractions contained the same number of cells because of the age-dependent decline in the thymus cell number in animals injected with low density bone marrow cells (Fig.2).

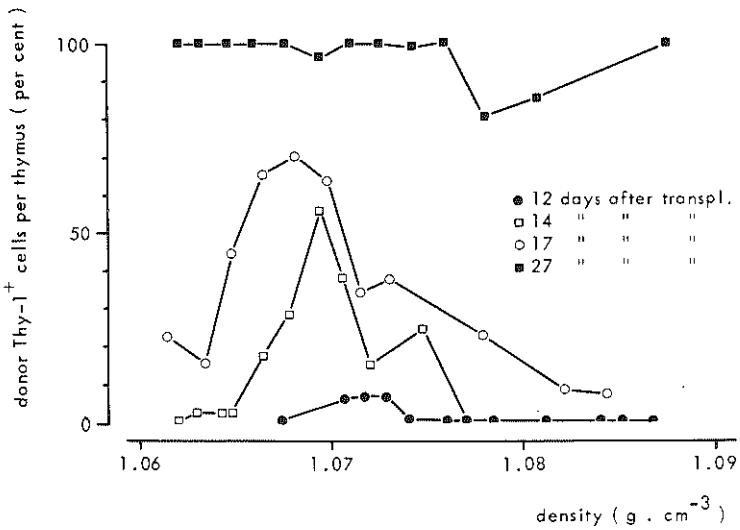

Fig. 1 Thy-1$^+$ donor cells (percent) in the thymus after transplantation of 10^6 bone marrow cells of indicated buoyant density.

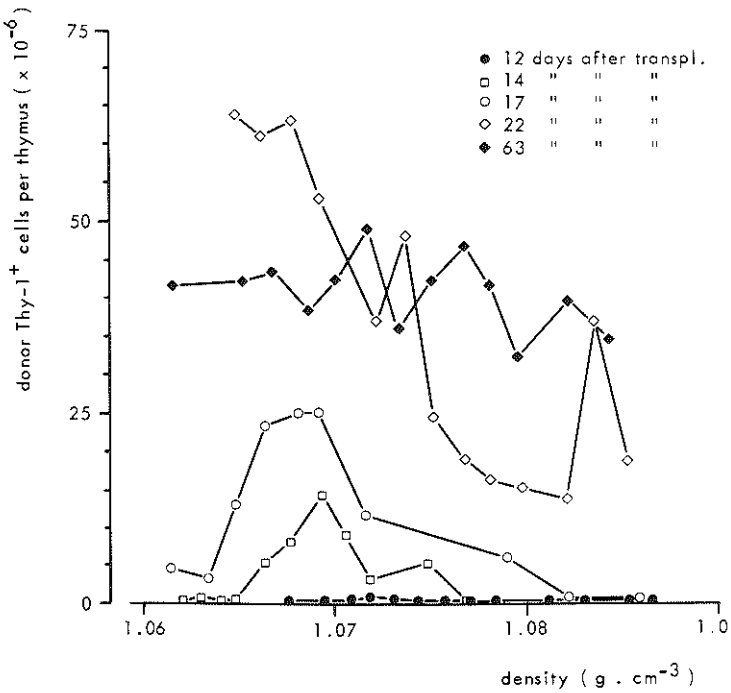

Fig. 2 Number of Thy-1$^+$ donor cells in the thymus after transplantation of 10^6 bone marrow cells of indicated buoyant density.

In the preceeding chapter, it was concluded that the development of host cells seemed to be independent of the number of total bone marrow cells transplanted. From the development of host cells after transplantation of fractionated bone marrow cells, it became apparent that, the growth of these cells was inversely related to the number of donor derived cells that developed. A typical distribution of donor and host cells after transplantation of density fractionated bone marrow cells is shown in Figure 3. Regeneration of the host type cells was

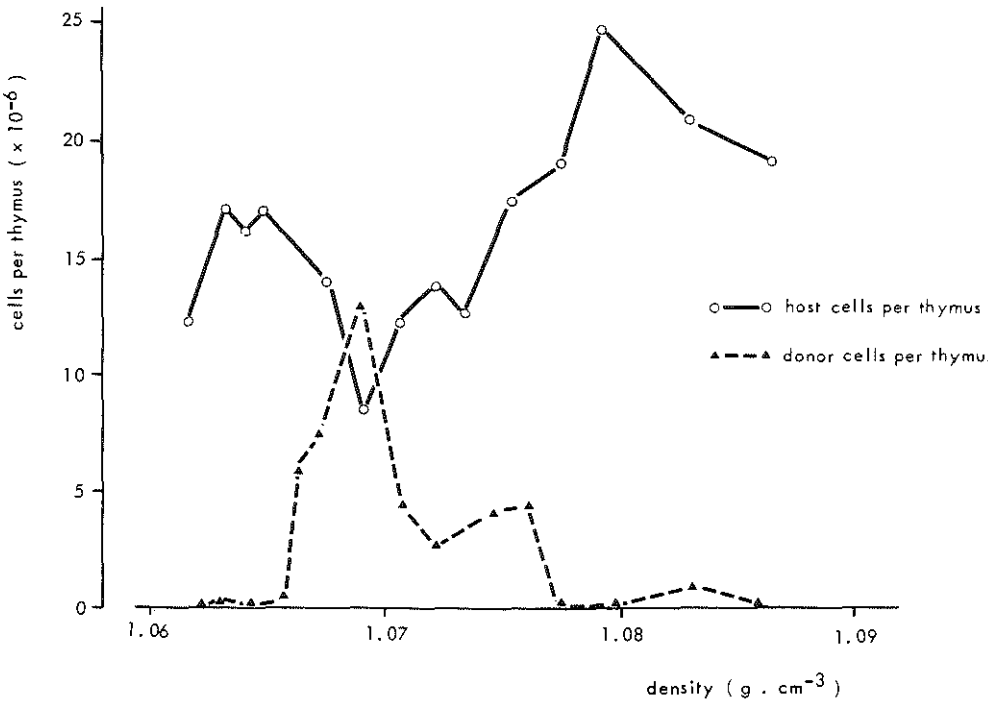

Fig. 3 Number of donor and host Thy-1$^+$ cells in the thymus 14 days after transplantation of 10^6 cells from fractions of indicated buoyant density.

most pronounced after transplantation of bone marrow cells of higher density ($d \geq 1.078$ g. cm^{-3}). When bone marrow cells of low density were transplanted (~ 1.070 g.cm^{-3}), the regeneration of the host cells was limited to about 30% of the maximum capacity to produce thymocytes from radiation resistant progenitor cells.

It seems that, in competition for space in the thymus, the donor cells prevent their host derived counterparts from proliferation. It could be concluded that bone marrow derived T-cell progenitor cells, at least those of low density, interfere with the development of host thymocytes.

The density distributions of prothymocytes had a mean modal value of 1.071 $g.cm^{-3}$ (mean of 3 experiments). For CFU-S, the mean modal density was 1.070 $g.cm^{-3}$ in the same experiments. The band width at 50% of the peak height for the two cell types determined was 0.0083 $g.cm^{-3}$. This meant that the density distributions almost completely overlapped. Prothymocytes and CFU-S therefore have a similar buoyant density (Fig.4).

A minor difference between the two cell distributions was observed in the high density region of the distribution between 1.074 and 1.080 g. cm^{-3}. This is an indication that the two functions could be performed by different cells. The number of donor cells produced in the recipient thymus per CFU-S transplanted was larger in the relatively low density region of the gradient ($1.065 g.cm^{-3} \leq d \leq 1.075$ $g.cm^{-3}$) when compared to higher density fractions (Fig.4).

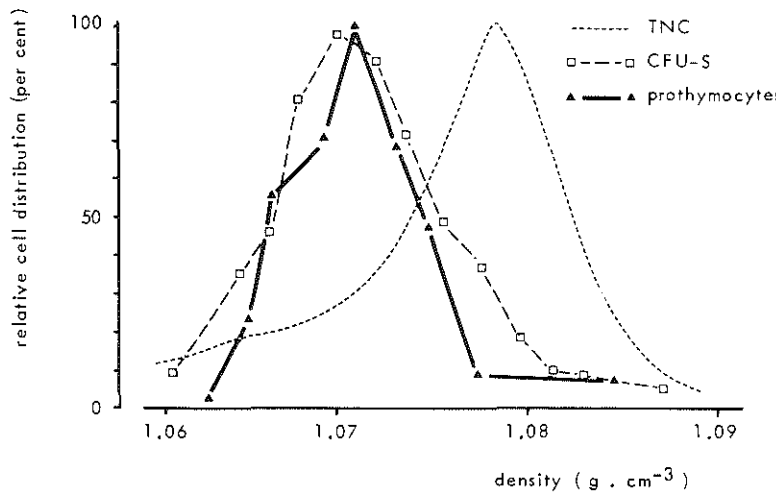

Fig. 4 Relative buoyant density distribution of nucleated cells, CFU-S and prothymocytes in mouse bone marrow. The distribution of prothymocytes was calculated as the number of donor cells produced in the thymus after transplantation of 10^6 cells of a fraction at a certain time after transplantation x the number of cells per density increment of that fraction and expressed as a percentage of the highest value (see Chapter 2). CFU-S was expressed as the number of spleen colonies per 10^5 cells x the number of cells per density increment of the fraction from which the cells were obtained. Mean of four distributions is shown. Standard deviation for each point varied between 3 and 16 percent.

2.1.2 Comparison of the density distribution of prothymocytes and functional T cells in the bone marrow

There are cells in the bone marrow which are capable of giving a proliferative response to T-cell-specific stimuli. To investigate whether these cells or their progeny interfere with the prothymocyte assay we used, the density distribution of these cells was determined. It could be shown that, although there is some

overlap between the density distribution of MLC responsive bone marrow cells and CFU-S, there was a considerable difference between the peak values of the distribution of the two cell types. The MLC responsive bone marrow cells had a high buoyant density (peak ~1.080 $g.cm^{-3}$) as compared to CFU-S and prothymocytes (Fig.5).

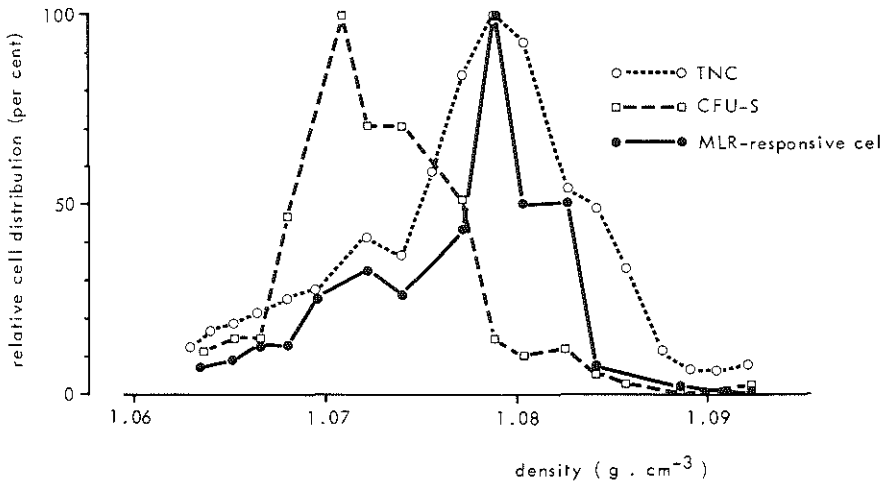

Fig. 5 Relative density distribution of nucleated cells, CFU-S and MLC responsive cells in mouse bone marrow. Calculations were performed as for Fig.4.

2.1.3 Comparison of the density distribution of prothymocytes and progenitors for functional T cells in various tissues

After transplantation of bone marrow, the cells that have been processed by the thymus will give rise to immunocompetent T cells in various lymphoid tissues i.e., thymus, spleen and lymph nodes. Functional T cells can be regarded as a later stage in T-cell differentiation than expression of Thy-1 surface markers (see Chapter 1). It was investigated whether differences between progenitors for $Thy\text{-}1^{+}$ cells in the thymus: prothymocytes, and progenitors for functional T cells could be distinguished on the basis of their density distribution. When it is assumed that in the regenerating tissue the requirements for a response to PHA or response to MLC e.g., presence of non-T cells as macrophages and possibly other accessory cells are fulfilled, the distribution of progenitors of the functional cells in these tissues can be derived from a comparison of the response after transplantation of different fractions of bone marrow cells. After transplantation of density fractionated bone marrow cells, the most rapid regeneration of thymocytes having the capacity to respond to PHA was found when low density cells (d = 1.072 $g.cm^{-3}$) were transplanted (Fig.6). The density distribution of the

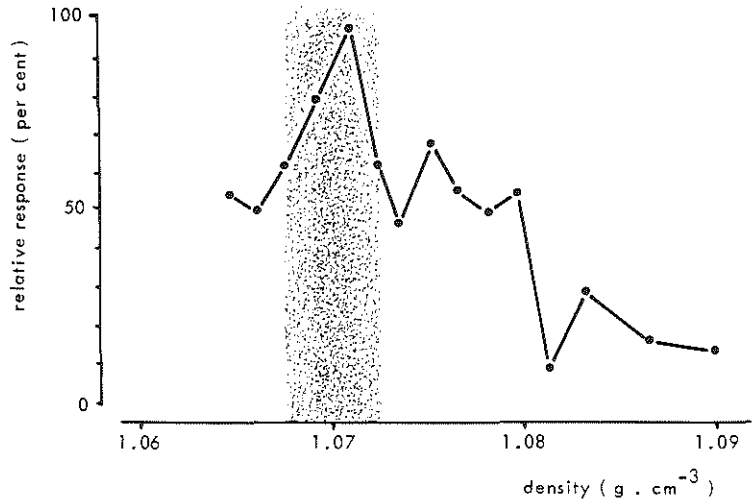

Fig. 6 PHA response in thymocytes after transplantation of 10^6 bone marrow cells of indicated density. The response was measured as described in Chapter 2. and was expressed as a percentage of the peak value. The results of three curves obtained at 12, 14 and 22 days after transplantation were pooled.
The density of prothymocytes is indicated by a bar.

precursor cells had a shoulder at a density of 1.075 g.cm^{-3}. This shoulder was consistently observed in the density region of the gradient where the CFU-S distribution also had a shoulder; in contrast, only relatively few prothymocytes were present in this region (Figs.4,6). After transplantation of 10^6 bone marrow cells from the fraction that gave the peak response to PHA in thymocytes, donor control levels were reached as early as 22 days after infusion of the bone marrow cells. Apart from the shoulder, the density distribution of the progenitor cells for the PHA responsive thymocytes was similar to that of the progenitors of Thy-1$^+$ cells in the thymus.

In the spleen, the regeneration of cells responding to PHA was observed at days 12 and 14 after transplantation of bone marrow cells from fractions with a density below 1.065 g.cm^{-3} or higher than 1.072 g.cm^{-3}. There was a minimum in the relative density distribution of progenitors for PHA responsive cells at 1.071 g.cm^{-3} (Fig.7). From day 18 after transplantation, the spleen cell progeny of bone marrow cells of the intermediate density (1.065 g.cm^{-3} $\leq$ d $\leq$ 1.072 g.cm^{-3}) were also capable of responding to PHA stimulation. After 40 days, the PHA response of spleen cells was independent of the density of the bone marrow cells transplanted.

It seemed that the density distribution of the precursor cells for early PHA response in spleen cells was inversely related to that of the progenitors for Thy-1$^+$ thymocytes. Therefore, the PHA response of spleen cells early after

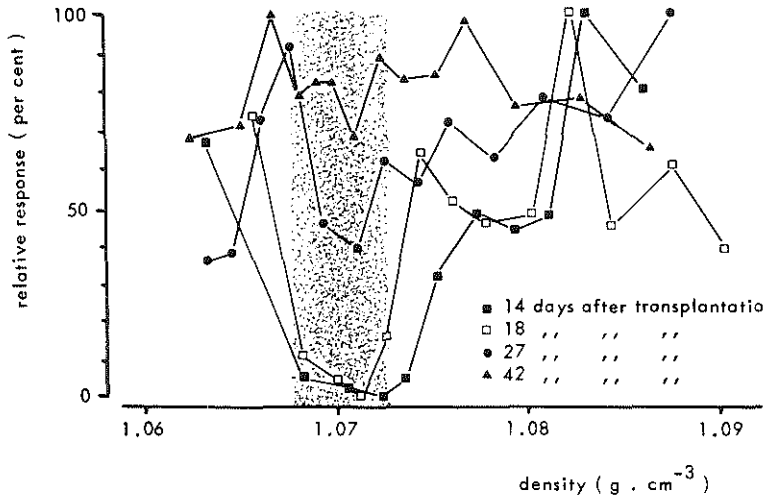

Fig. 7 PHA response in spleen cells after transplantation of 10^6 bone marrow cells of indicated density. The responses were measured and calculated as for Fig.6. 14 days, 18 days, 27 days and 42 days after bone marrow transplantation. The density of prothymocytes is indicated by a bar.

transplantation of bone marrow cells could be a function of mature donor bone marrow derived T cells that homed into the spleen of the recipient. On the other hand, it cannot be excluded that radiation resistant host spleen cells were responding to PHA stimulation, since the contribution of donor and host derived cells was not distinguished in these experiments.

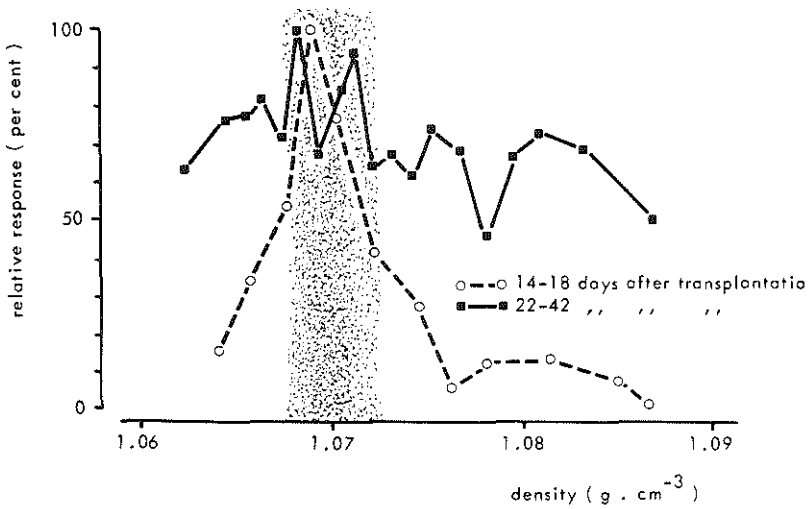

Fig. 8 MLC response in spleen cells after transplantation of 10^6 bone marrow cells of indicated density. The responses were determined and calculated as for Fig.6 . The results of experiments 14 days (2 exp.) and 18 days (1 exp.) after transplantation were pooled. Three other experiments at 22, 27, 30 and 42 days after transplantation are shown in a second curve. The density of prothymocytes is indicated by a bar.

MLC reponsive cells were already present in the spleen 14 to 18 days after bone marrow transplantation. The response of the peak fractions, however, did not exceed 7% (range 4-7%) of the response of spleen cells of normal untreated donor mice (see also Chapter 3). The density distribution of the progenitor cells for the splenocytes that respond to MLC 14 to 18 days after transplantation of fractionated bone marrow cells is similar to that of prothymocytes (Fig.8). The peak of the relative density distribution is found at 1.069 $g.cm^{-3}$. After 18 days, in spleen cells of mice transfused with high density bone marrow cells, the number of cells that respond in MLC is increasing. But, up to about 40 days after transplantation, there was still an optimum response of spleen cells to MLC after transplantation of bone marrow cells with a density of 1.067 $g.cm^{-3}$ to 1.071 $g.cm^{-3}$.

2.2 Sedimentation Velocity of Prothymocytes and CFU-S

Cells which are allowed to sediment at 4°C in a medium with a relatively low density have a sedimentation velocity that is influenced by the cell size and the cell density. Within a homogeneous population of cells, the distribution of size is influenced by the cell cycle state (Miller and Phillips 1969, 1970). Bone marrow prothymocytes and CFU-S have very similar sedimentation velocity distributions (Fig.9). The distribution of CFU-S showed a peak at a sedimentation rate of

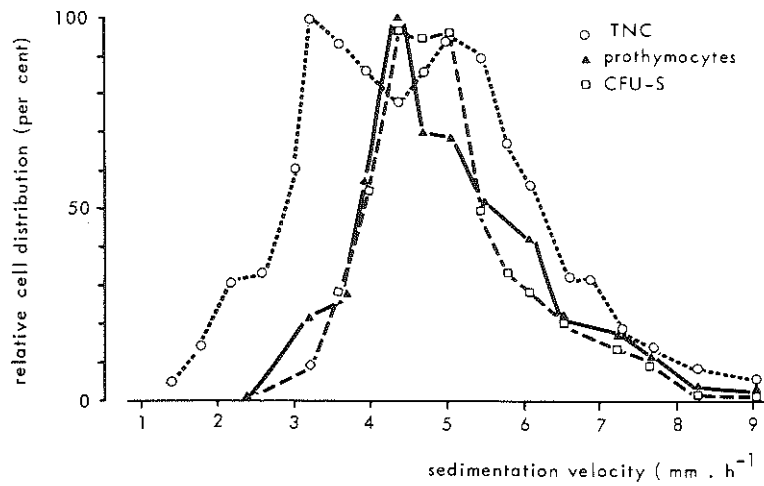

Fig. 9 Relative distribution of sedimentation velocity of nucleated cells, CFU-S, and prothymocytes in mouse bone marrow. Calculations were performed as for Fig.4, instead of density increments velocity sedimentation increments were used.

4.4-5.0 mm.h^{-1} (mean of three exp.). Although the distributions of CFU-S and prothymocytes showed a strong overlap, the ratio of prothymocytes to CFU-S tended to increase with increasing sedimentation rate. The observed slight differences in sedimentation velocity rates were not sufficient to allow separation of the prothymocytes and CFU-S according to this method.

2.3 EPM Distribution of Prothymocytes and CFU-S

The distribution of normal bone marrow cells according to their mobility in an electric field shows two peaks. The first peak in the low electrophoretic mobility (EPM) trajectory of the distribution is found at -1.88 x 10^{-4} .cm^2 .V^{-1} .s^{-1}

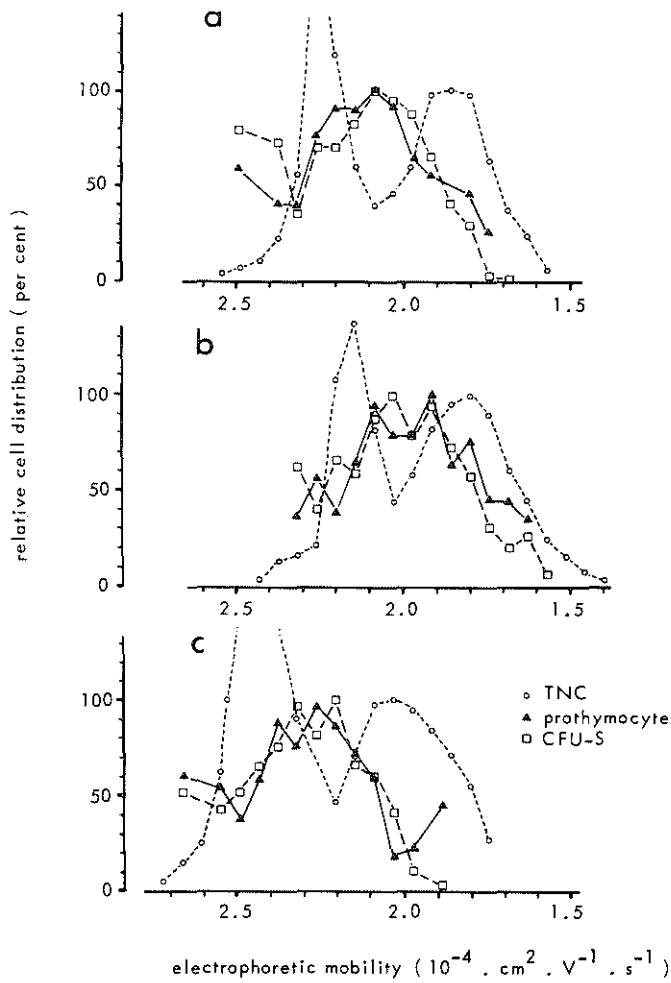

Fig. 10 Relative distribution of electrophoretic mobility in free flow electrophoresis of nucleated cells, CFU-S and prothymocytes in mouse bone marrow. Three experiments are shown. The curves represent the distribution of prothymocytes/10^5 cells and CFU-S/10^5 of each fraction transplanted.

(mean of 3 exp.) and the second peak of high electrophoretic mobility is found at $-2.26 \times 10^{-4}.cm^{2}.V^{-1}.s^{-1}$ (mean of 3 exp.). In experiments where normal bone marrow cells were separated some clumping of cells was observed in the fractions of the high EPM peak, even though the cells in the low ionic strength buffer were prefiltered before separation. (Bol, 1980). Therefore, relative distributions of prothymocytes and CFU-S were calculated with the low EPM peak as a reference instead of using the highest peak (high EPM). The CFU-S/10^5 distribution had a low EPM peak at $-2.10 \times 10^{-4}.cm^{2}.V^{-1}.s^{-1}$ (mean of 3 exp.). At this EPM, the enrichment of CFU-S cells was about twofold. The low EPM peak for the prothymocytes cells was also found at $-2.10 \times 10^{-4}.cm^{2}.V^{-1}.s^{-1}$. For these cells, we determined a similar enrichment as for CFU-S (Fig.10). There was a tendency to a relative enrichment of prothymocytes over CFU-S in the lowest EPM fractions of the cell distribution. These fractions, however, contained only small numbers of cells so that a more refined characterisation of these cells was difficult. A rather high number of cells is needed for a reliable determination of the prothymocyte numbers in the in vivo assay. Thus, it was not feasible to use differences in relative cell surface charge for the separation of CFU-S and prothymocytes (see Chapter 2). It can be concluded that these two cell types have a rather similar net cell surface charge.

3 DISCUSSION

In this chapter, we have shown that prothymocytes and CFU-S have a number of physical characteristics in common. The distributions of the two cell types in buoyant density, sedimentation velocity and electrophoretic mobility were very much alike.

In total bone marrow, the contributions of prothymocytes and CFU-S to thymus regeneration cannot be distinguished (Chapter 3). In the electrophoretic mobility distributions, there is a tendency to a relative enrichment of prothymocytes over CFU-S in the low EPM fractions. In one or two fractions of the EPM distribution, the ratios of prothymocytes over CFU-S was about seven times that observed in total marrow. CFU-S/10^5 decreased to about 3.5% of the value in total marrow, whereas prothymocytes in those fractions were decreased only to 25% of the value in unseparated cells. When such an "enrichment" was achieved, fractions contained between 1 and 5 percent of all cells separated. This meant that the numbers of cells that could be collected in those fractions were rather low as compared to the numbers needed in our in vivo assays.

In velocity sedimentation fractions containing larger cells, the ratio of prothymocytes to CFU-S tends to increase with increasing sedimentation rates. The ratio of prothymocytes to CFU-S in fractions of continuous albumin gradients changes slightly with density. A decrease in the prothymocyte to CFU-S ratio is

observed in the medium density region of the gradient where according to Visser et al. (1977) the cycling CFU-S can be expected. The differences in prothymocyte and CFU-S density distributions between 1.074 $g.cm^{-3}$ and 1.080 $g.cm^{-3}$ do not prove that different cell types are involved but could be due to the incapability of the cycling pluripotent progenitor cells to play a role in the regeneration of the thymus after bone marrow transplantation or to the fact that prothymocytes might form a subpopulation of resting CFU-S.

Basch and Goldstein (1974) have separated bone marrow cells on discontinuous albumin gradients. CFU-S as well as cells that could be induced to bear T-cell-specific surface markers after incubation with thymic hormone (thymopoietin) were enriched in the low density fractions of the gradients. Induction of Thy-1 surface markers on Thy-1$^-$ spleen cells does lead to a limited decrease in thymus repopulating ability (Komuro et al., 1975; Chapter 1). The fractions enriched in thymocyte progenitor cells also showed an enrichment in cells that could be induced to bear T-cell-specific markers after induction with thymopoietin (Basch et al., 1978). The number of CFU-S in the bone marrow is not influenced by incubation with thymic factors that induce T-cell-specific surface markers (Basch & Goldstein, 1975a). CFU-S are sensitive to treatment with rabbit anti mouse brain serum (RAMB) to the same extent as has been shown for cells that can be induced to express T-cell markers (Van den Engh & Golub, 1974; Basch et al., 1978). This makes it difficult to distinguish between prothymocytes and CFU-S.

Our results show that the physical characteristics of prothymocytes and CFU-S are clearly similar, but because of the small differences observed between the two cell types it cannot be excluded that the two functions are performed by different cells.

A similarity in physical characteristic also has been found between E-CFU-c_1 and CFU-S. E-CFU-c_1 and E-BFU have a density distribution and velocity sedimentation pattern similar to CFU-S (Wagemaker et al., 1977; Wagemaker, 1980).

According to Visser et al. (1977) and Bol (1980), GM-CFU-c_1, have the same modal buoyant density as CFU-S, but GM-CFU-c_1 have a slightly higher sedimentation velocity then CFU-S. In addition, GM-CFU-c_1 have a lower modal EPM than was determined for CFU-S but their distributions show a considerable overlap (Bol, 1980).

The density distributions of progenitors for T cells in the thymus that can respond to PHA overlap to a large extent with that of prothymocytes.

The density distribution of precursor cells for PHA response in the spleen early after bone marrow transplantation is similar to that of immunocompetent cells in the bone marrow (Figs.5,7). The bone marrow cells used in our separation studies were not subjected to anti-Thy-1 treatment. Therefore, it is likely that the observed PHA response in spleen early after bone marrow transplantation is due to homing of immunocompetent bone marrow cells into the recipient spleen.

From experiments of El Arini and Osoba (1973) and Gorcinsky and McRae (1977), one can conclude that host cells do not contribute significantly to T cell responses after a lethal dose of γ-radiation. Bone marrow cells treated with cytotoxic anti-Thy-1 serum and complement show the same thymus cell regeneration as nontreated cells (Chapter 3).

As shown previously (Chapter 3), during the first 10 days after transplantation, the response of spleen cells to certain T-cell-specific stimuli is rather low, ≦ 10% of control values (Boersma et al., 1981). This is in agreement with observations of Gorcinsky and McRae, they found responses that did not exceed 10-20% of control values (Gorcinsky & McRae, 1977). These authors also provided evidence that, at least up to 15 days after bone marrow transplantation, the T-cell response observed in spleen cells is to a large extent independent of the presence of the host thymus; it has therefore been attributed to post-thymic precursor cells that are not readily susceptible to lysis with anti-Thy-1 serum. A thymus dependent T-cell response could be determined only after 20 days following transplantation (Gorcinsky & McRae, 1977). This suggested that the T-precursor cell as defined by El Arini and Osoba (1973) was not a bone marrow derived prothymocyte.

In the experiments described above, 14 days post-irradiation and transplantation of density fractionated marrow, MLC reponsive spleen cells were observed in animals that received low density bone marrow cells. The response levels, however, were low. The early MLC responsive spleen cells have a similar density distribution as have prothymocytes and CFU-S. This in contrast to the results obtained by El Arini and Osoba (1973), who applied a different separation technique. Thus, progenitors for MLC responsive spleen cells and prothymocytes have similar buoyant density. The results presented in this chapter suggest that the prothymocytes and the progenitors for functional T cells in thymus and spleen are the same cells.

4 CONCLUSIONS

1. Prothymocytes and CFU-S cannot be separated according to differences in size, buoyant density and cell surface charge.

2. The minor differences observed in the distribution of the prothymocytes and CFU-S in buoyant density and sedimentation velocity can be explained by differences in cell cycle state of one cell type or by presence of two different progenitor cells.

3. Bone marrow progenitor cells for immunologically mature T cells have a similar buoyant density distribution as determined for prothymocytes.

CHAPTER 5

DIFFERENCES BETWEEN PROTHYMOCYTES AND PLURIPOTENT STEM CELLS DERIVED FROM VARIOUS SOURCES OF HEMOPOIETIC CELLS

1 INTRODUCTION.

1.1 General Introduction

Recent reports suggest that cells forming spleen colonies (CFU-S) represent a heterogenous pool of hemopoietic progenitor cells (Worton et al., 1969a; Haskill et al., 1970; Sutherland et al., 1971; Visser et al., 1977; Magli et al., 1982). The CFU-S compartment contains cells that are pluripotent. Although physical parameters for prothymocytes and CFU-S are almost the same, it can be speculated from the data presented in Chapter 4 that the prothymocyte function and the formation of spleen colonies in irradiated recipients are properties of different cells. However, it proved to be impossible to separate the two cells according to size, density or cell surface charge. Therefore, it was attempted to distinguish between CFU-S and prothymocytes in systems which are not in a steady state e.g., regenerating bone marrow. In such systems, the concentrations of CFU-S and progenitor cells for the myeloid series were found to differ from those in normal bone marrow (Moore et al., 1970; Metcalf and Moore, 1971; Visser et al., 1977; Dexter and Testa, 1980). In fetal liver cells and regenerating bone marrow, the concentration of CFU-S is lower than in normal bone marrow. The stem cells in those systems are also in a different proliferative state (Becker et al, 1965; Lahiri & Van Putten, 1969; Duplan, 1970; Vassort et al, 1973; Blacket et al., 1974).

Systems not in a steady state which were selected for our studies were bone marrow cells from long-term cultures and from bone marrow reconstituted animals and fetal liver. The prothymocytes and CFU-S were determined according to the assays described in Chapter 2 in order to establish their relationship.

1.2 Fetal Liver Cells as Source of Prothymocytes

The embryonic liver is a major site of hemopoiesis during prenatal development in the mouse (Silini et al., 1968; Niewisch et al., 1970; Metcalf & Moore, 1971). Transfusion of fetal liver cells is effective in restoring the hemopoietic

system of lethally irradiated mice. Since the concentration of pluripotent stem cells and early committed progenitor cells in fetal liver is lower than in normal bone marrow, a relatively high number of fetal liver cells is needed for reconstitution (Moore et al., 1970; Vogel et al., 1970; Löwenberg, 1975; Löwenberg et al., 1975). Nevertheless, much attention has been paid to the possibility of using fetal liver cells as a source of hemopoietic cells in clinical settings because of the observation that, after transplantation of embryonic hemopoietic liver cells in mice, no acute GvHD occurred and mortality from delayed GvHD was decreased (Upphoff, 1958; Crouch, 1959; Micklem and Loutit, 1966; Van Putten et al., 1968). The absence of acute GvHD is due to the very low number of immunocompetent T cells in fetal liver (Löwenberg, 1975; Löwenberg et al., 1977). Regeneration of the peripheral T-lymphocyte pool after infusion of fetal liver cells is similar to that observed after bone marrow transplantation; this indicates that the grafted cells in the two specimens contain T precursor cells with the same functional properties (Löwenberg, 1975). The altered pattern of development of GvHD after transplantation of fetal liver cells (Upphoff, 1958; Crouch, 1959; Van Putten et al., 1968; Löwenberg, 1975), according to the hypothesis of Löwenberg (1975) is based on the distinct differentiation pattern of pluripotent stem cells derived from fetal liver cells when compared to the same cells in bone marrow.

Spleen colony forming cells in fetal liver also differ from those in normal bone marrow in that they are to a larger proportion in cycle (Becker et al., 1965; Duplan, 1970). In addition, fetal liver CFU-S have a higher radiation sensitivity than CFU-S in bone marrow (Duplan, 1970). Fetal liver cells were used as a source of prothymocytes in the experiments here because it was thought that, in a source of hemopoietic cells where differentiation of stem cells is distinct from that observed in bone marrow cells, the differences between prothymocytes and CFU-S might be more pronounced and therefore easier to detect.

1.3 Prothymocytes in Long-Term Cultures

Long-term maintenance of progenitor cells for all lineages of hemopoietic cells (Dexter et al., 1973; Dexter and Testa, 1980), including T and B lymphoid lines (Dexter and Lajtha, 1974), has been described. These cultures do not contain mature T or B lymphocytes after the second week of culture (Schrader and Schrader, 1978; Dexter and Testa, 1980). This apparent block in terminal lymphoid differentiation makes these long-term cultures potentially useful systems for studying the control mechanisms involved in early lymphopoiesis. Since the cultures are capable of repopulating the thymus of a lethally irradiated recipient (Schrader and Schrader, 1978), they should contain prothymocytes, although it cannot be excluded that the repopulation stems from the pluripotent progenitor cells (CFU-S) present in the cultures. Previous studies did not provide quantitative information on prothymocyte levels in the cultures. We have used the prothy-

mocyte assay to determine the number of prothymocytes in long-term cultures. The cycling state of early progenitor cells in the cultures was also investigated.

1.4 Prothymocytes in Regenerating Bone Marrow

In the bone marrow of irradiated and bone marrow reconstituted mice, the number of CFU-S one week after transplantation was low in comparison with normal bone marrow (Lahiri and Van Putten, 1969; Vassort et al., 1973; Blacket et al., 1974; Visser et al., 1977). After several weeks, CFU-S levels in the bone marrow returned to normal values. Therefore, the regenerating bone marrow cells were considered to be an excellent source of hemopoietic cells for investigating to what extent the thymus cell regeneration was directly dependent on CFU-S or whether it was dependent on other cells. From the donor derived thymus cell regeneration kinetics after transplantation of regenerating bone marrow cells, it was concluded that prothymocytes regenerated more slowly than did CFU-S. Therefore, it was concluded that, the pluripotent stem cells (CFU-S) and the prothymocyte are different cells.

2 RESULTS

2.1 Prothymocyte Levels in Mouse Fetal Liver

2.1.1 Quantification of prothymocytes in fetal liver

The number of cells per fetal liver increases linearly with time during pregnancy in the mouse (Fig.1a), while the proportion of stem cells does not change significantly (Fig.1b). The mean number of CFU-S in fetal liver cells was found to be 7.6 ± 3.1 per 10^5 cells, which was much lower than observed in bone marrow, 31.2 ± 7.9 per 10^5 cells (Table I). Prothymocyte proportions were not significantly different when fetal liver cells of different gestational ages were compared (Fig.1c).

As there were no significant differences between fetal liver cells obtained at different gestational ages, the data for donor cell regeneration after transplantation were pooled. From the growth curves in Figure 2, it can be concluded that there is a growth delay after fetal liver cell transplantation as compared with transfusion of normal bone marrow cells in irradiated recipient mice. The growth curves were parallel. From the delay observed, we calculated that the relative number of prothymocytes is 10.7 percent of the value determined in bone marrow cells. When expressed per CFU-S transplanted, it was calculated that in fetal liver cells the ratio of prothymocytes to CFU-S was decreased to 38 percent of the normal bone marrow value (Table I).

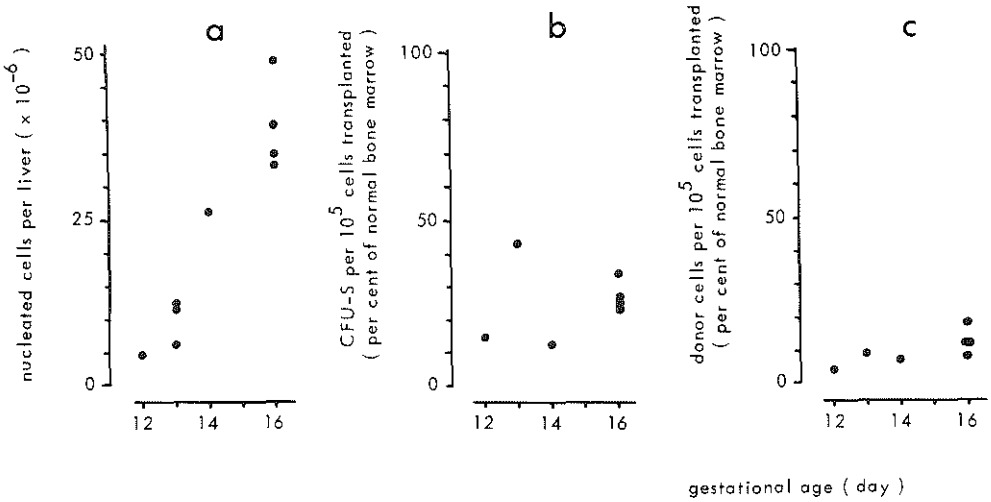

Fig. 1 Nucleated cells, CFU-S, and prothymocytes in fetal liver during gestation.

a. Cellularity in fetal liver. Each point represents the mean of 15-25 pooled fetal livers of the gestational age indicated.
b. CFU-S in fetal liver during gestation.
c. The capacity of fetal liver cells to produce donor thymocytes.

TABLE I

RELATIVE CONCENTRATION OF PROTHYMOCYTES AND PROPORTION OF CFU-S IN FETAL LIVER

	CFU-S per 10^5 cells	Prothymocytes per 10^5* cells	Prothymocytes* per CFU-S
Fetal liver	7.6 ± 3.1 (n=7) [24.4%]	10.7% (n=22)	38.0% (n=22)
Bone marrow	31.2 ± 7.9 (n=5) [100%]	100.0% (n=20)	100.0% (n=20)

* Relative prothymocyte concentrations were calculated from the delay between donor thymocyte development after transplantation of fetal liver cells and bone marrow. Calculations on thymocyte growth curves were performed separately for donor cell numbers expressed per 10^5 cells and per CFU-S transplanted.

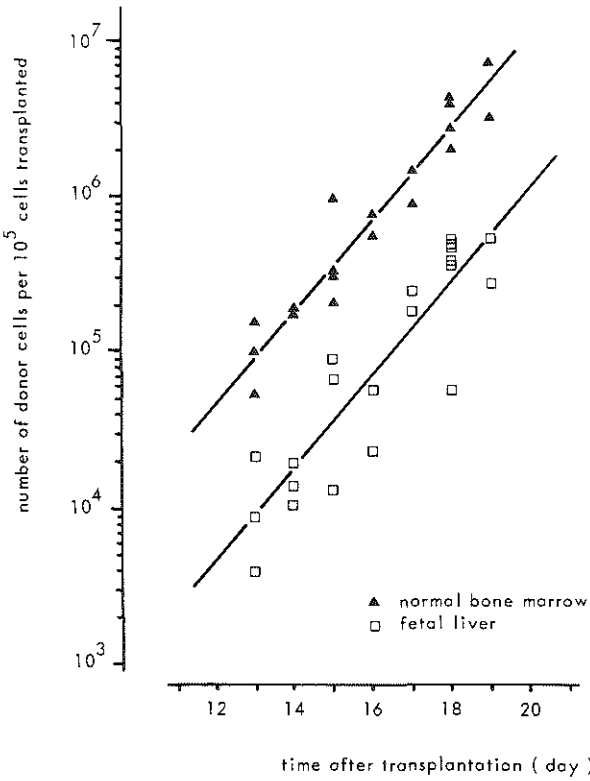

Fig. 2 Donor cell regeneration in the thymus after transplantation of bone marrow and fetal liver cells. Log-linear regression lines are shown. For bone marrow cells, 20 observations are shown. Corr. coëff., 0.96; $p \leq 0.005\%$. For fetal liver cells, 22 observations are shown. Corr. coëff., 0.89; $p \leq 0.005\%$. Comparison of regression lines showed that they were parallel ($p = 90.66\%$). The delay observed can be expressed as 3.22 doubling times. This indicates that the concentration of prothymocytes in fetal liver is 10.7% of that determined in normal bone marrow.

2.1.2 Density distribution of fetal liver derived prothymocytes and CFU-S in continious albumin gradients

It was shown in the previous chapter that prothymocytes and CFU-S in fresh normal bone marrow have buoyant density distributions that show a strong similarity. In addition, they have features such as cell surface charge and cell size in common.

The quantitative differences between the prothymocytes and CFU-S suggested the possibility that, due to the different physiological condition of the cells, they could be separated and/or purified according to physical characteristics.

The density distribution of nucleated fetal liver cells is shown in Figure 3 (upper panel). The peak in the relative density distribution was found at 1.065 $g.cm^{-3}$ which is low, when compared to normal bone marrow cells. As can be seen

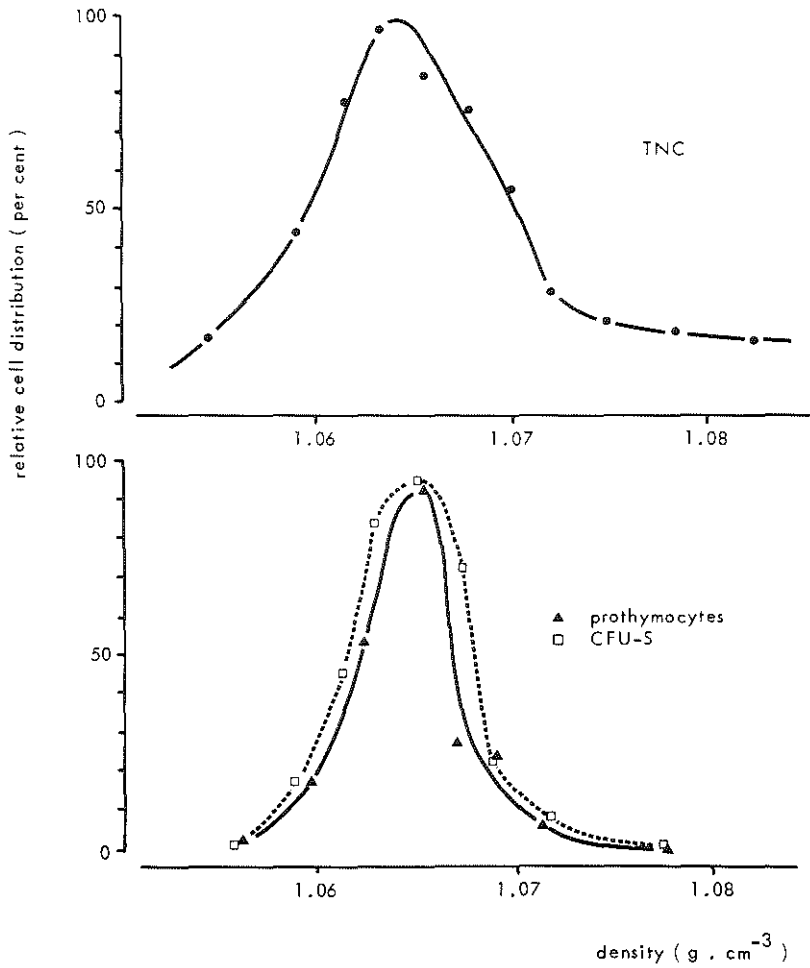

Fig. 3 Relative density distribution of nucleated cells (upper panel), CFU-S and prothymocytes (lower panel) in fetal liver cells at a gestational age of 13 days. The mean distribution of three experiments is shown.

in Figure 3 (lower panel), the relative density distributions of prothymocytes and CFU-S almost completely overlap. For both cell types, the mean buoyant density is 1.065 $g.cm^{-3}$.

It can be concluded from this that, under physiological conditions different from those in normal bone marrow, the buoyant density of CFU-S is changed, and that the density of the prothymocytes is changed in a similar way.

2.2 Prothymocyte Levels in Long-Term Culture

2.2.1 Quantification of long-term cultured prothymocytes

After transplantation of bone marrow cells from long-term cultures, the growth rate of the donor derived thymus cells was the same as observed after transplantation of fresh bone marrow cells (Fig.4).

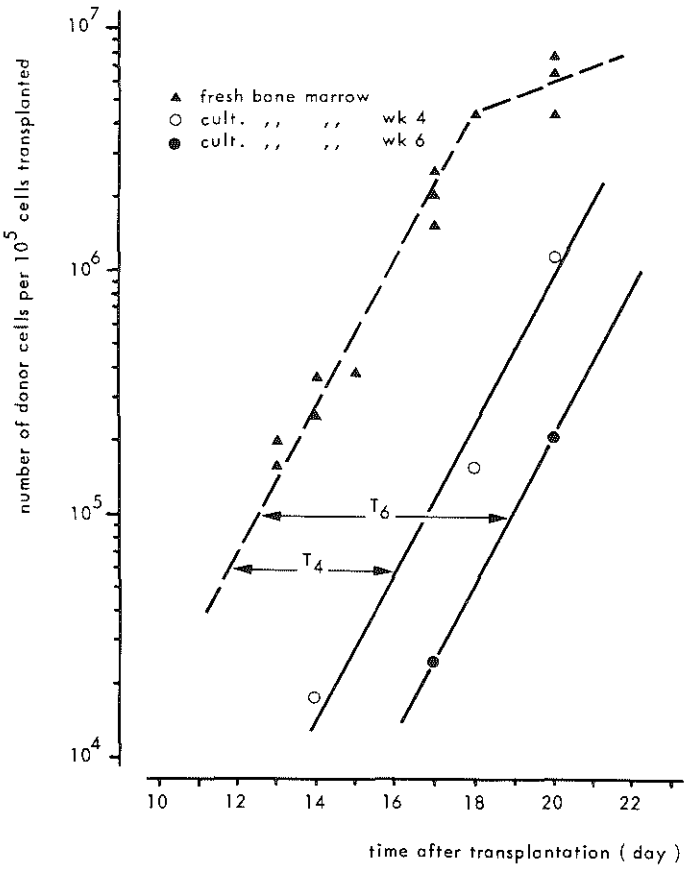

Fig. 4 Donor cell growth after transplantation of fresh and long-term cultured bone marrow cells. The growth curves are statistically parallel. The delay between similar growth curves was used to calculate relative concentrations of prothymocytes in the long-term cultures. For data see Table II.

In these experiments, CFU-S and prothymocytes could be maintained in culture through 17 weeks of incubation. Prothymocytes (6 ± 4%) and CFU-S (25 ± 13%) were present at lower levels than were observed in fresh normal bone marrow (Table II). During the last five weeks of a 17-week culture period, the

TABLE II

LONG-TERM MAINTENANCE OF PROTHYMOCYTES IN VITRO

Week of culture *	CFU-S/10^5 cells percent of nbm	Prothymocytes/10^5 cells percent of nbm **	Prothymocytes/CFU-S percent of nbm **
3	n.d.	13.3	-
4	55.2	6.9	12.5
5	34.2	3.8	11.1
6	9.7	2.3	23.6
7	35.0	8.4	24.0
9	31.0	1.8	5.8
11	33.7	8.4	24.9
12	11.1	3.0	27.1
13	n.d.	0.8	-
17	4.7	0.24	5.1

* The cultures were established with two inoculae of bone marrow as originally described by Dexter et al. (1977).

** Compared to normal bone marrow (nbm) = 100% assayed at the same time.

ratio of prothymocytes to CFU-S in the cultures decreased rather rapidly. Prothymocytes disappeared from the cultures more rapidly than did CFU-S. Although the cultured cells were still able to produce colonies in spleens of irradiated mice, they had apparently lost the capacity to reconstitute the hemopoietic system. After transplantation of the equivalent of 100-250 CFU-S from the cultures, the mice died even before the number of donor cells in the thymus could be assessed. Under the same conditions, only 20 to 30 CFU-S derived from normal bone marrow are necessary to rescue mice from radiation death.

In the cultures of 11 to 14 weeks after inoculation of bone marrow, the proportion of CFU-S in S phase was increased as compared to normal bone marrow values (Table III). Progenitor cells for the erythroid lineage, BFU-E, were also killed in a ^{3}H TdR suicide experiment in a larger proportion than observed in normal marrow. The cycling state of the GM-CFU appeared to be the same as in fresh marrow cells. This showed that the CFU-S as well as committed precursors in the culture were in a high cycling state.

Cultures were also established with a single inoculum of bone marrow cells.

TABLE III

CYCLING STATE OF PROGENITOR CELLS IN LONG-TERM CULTURES AS DETERMINED BY KILL WITH TRITIATED THYMIDINE

Week of culture		CFU-S/10^5 cells	% kill	GM-CFU/10^5 cells	% kill	E-BFU/10^5 cells	% kill
11	culture	6.8 ± 0.4	39	216 ± 72	50	56 ± 26	21
	nbm	28.1	n.d.	344 ± 57	24	66 ± 18	12
12	culture	3.8 ± 0.6	37	88 ± 37	32	32 ± 20	50
	nbm	21.7	9	344 ± 52	40	52 ± 8	23
13	culture	n.d.	n.d.	84 ± 28	24	40 ± 18	30
	nbm	n.d.	n.d.	296 ± 52	44	46 ± 16	13
14	culture	11.1 ± 1.2	37	138 ± 37	48	n.d.	n.d.
	nbm	21.1	13	278 ± 33	45	n.d.	n.d.
Mean:	culture		38 ± 1		38 ± 11		34 ± 12
	nbm		11 ± 2		38 ± 8		6 ± 5

n.d.: not determined

TABLE IV

THE EFFECT OF CULTURE* ON CFU-S AND PROTHYMOCYTES**

Week	Cells percent of input	CFU-S percent of input	Prothymocytes (PT) percent of input	PT/CFU-S percent of input
0	100	100	100	100
1	25.9	4.1	2.4	57
2	4.1	0.9	-	-
3	7.6	0.7	0.3	45
4	10.0	1.5	0.3	21
5	8.8	1.5	0.03	1.8
6	7.0	1.3	0	0
7	4.7	0.2	0	0
8	2.9	0.01	0	0

* Cultures were inoculated only once.

** PT (prothymocytes) compared to normal bone marrow assayed at the same time.

There the number of CFU-S decreased within the first two weeks to about few percent of the proportion inoculated and remained at this low level during the next five weeks (Table IV). Prothymocytes also decreased with the time in culture but more rapidly than CFU-S.

From the differences in growth kinetics of prothymocytes and CFU-S in the cultures, it is suggested that these functions represent different cell types.

2.2.2 Buoyant density distribution of prothymocytes in long-term cultures

The CFU-S in long-term cultures were in a different proliferative state than found in fresh normal bone marrow cells. The quantitative relationship between prothymocytes and CFU-S was also changed. The buoyant density distribution of prothymocytes and CFU-S because of practical reasons was determined at weeks 7 and 9 of culture.

The buoyant density distribution of nucleated cells was markedly different in the two experiments (Fig.5a). The peak value determined at weeks 7 and 9 was

1.0733 g.cm^{-3} and 1.0694 g.cm^{-3}, respectively. The density distribution of CFU-S did not change with time. The mean peak value in the relative density distribution for CFU-S (peak as 100%) was found to be 1.070 g.cm^{-3}. The 50 percent bandwidth was 0.0070 g.cm^{-3} (Fig.5b). Also for prothymocytes we found the same density distribution as was determined in the two experiments. For these cells, a mean buoyant density peak value of 1.0690 g.cm^{-3} at a bandwidth of 0.0055 g.cm^{-3} was determined (Fig.5c). The density distribution of CFU-S and prothy-

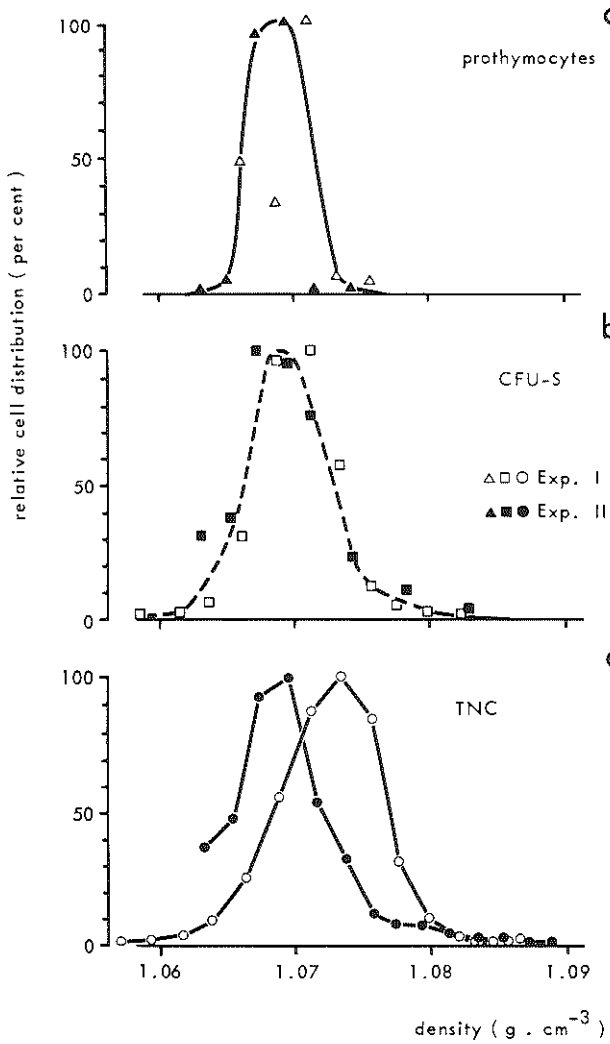

Fig. 5 Relative density distribution of prothymocytes (a) and CFU-S (b) in long-term cultured bone marrow. Nucleated cell distributions (c) at 7 (I) and at 9 (II) weeks of culture.

mocytes from long-term cultures therefore showed overlap to a great extent. In addition these density distributions were similar to those determined for freshly isolated bone marrow cells.

2.3 Transplantation of Regenerating Bone Marrow

2.3.1 Quantification of prothymocytes in regenerating bone marrow

In the bone marrow of lethally irradiated mice reconstituted with 3 x 10^6 bone marrow cells, one week after transplantation the proportion of stem cells was reduced to 25% of that in normal marrow. The CFU-S concentration slowly increased and reached the 60% level (compared to normal bone marrow) three weeks after transplantation (Fig.6).

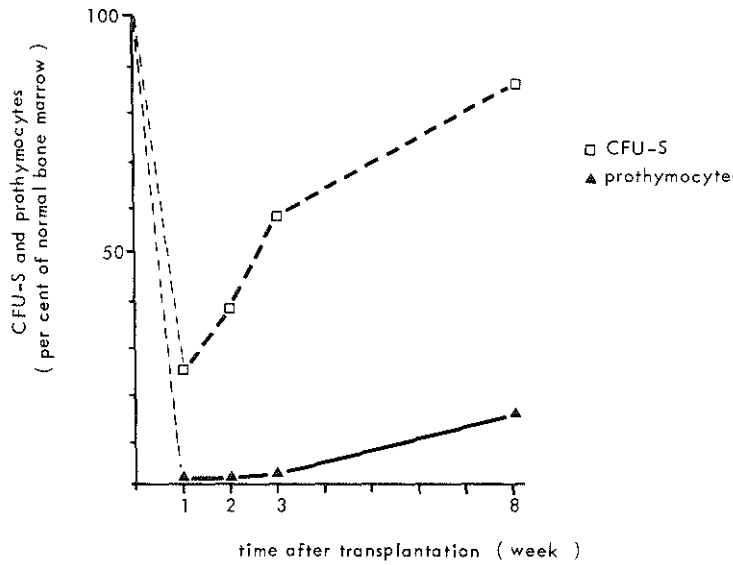

Fig. 6 Regeneration of prothymocytes and CFU-S in bone marrow of lethally irradiated and bone marrow transplanted mice. After irradiation, (day zero) the mice received 3 x 10^6 bone marrow cells. CFU-S and prothymocyte concentration in regenerating bone marrow are expressed as a percentage of the fresh marrow concentration. Calculated as described in Section 2.16.

After lethal irradiation and reconstitution with 3 x 10^6 bone marrow cells, mice were used as bone marrow donors at different times after the reconstitution. The proportion of prothymocytes was determined as described above (Chapter 2). A relatively large number of regenerating bone marrow cells had to be transplanted in order to initiate a regeneration process that had the same time course as observed after transplantation of normal bone marrow (Fig.7).

The growth curves of donor derived thymocytes after transplantation of regenerating bone marrow were parallel to those determined after transplantation of normal marrow cells in the same experiment. Donor and host derived cells in a regenerating thymus after transplantation of normal and regenerating bone marrow cells showed a similar spontaneous ^{14}C TdR incorporation. From this it was

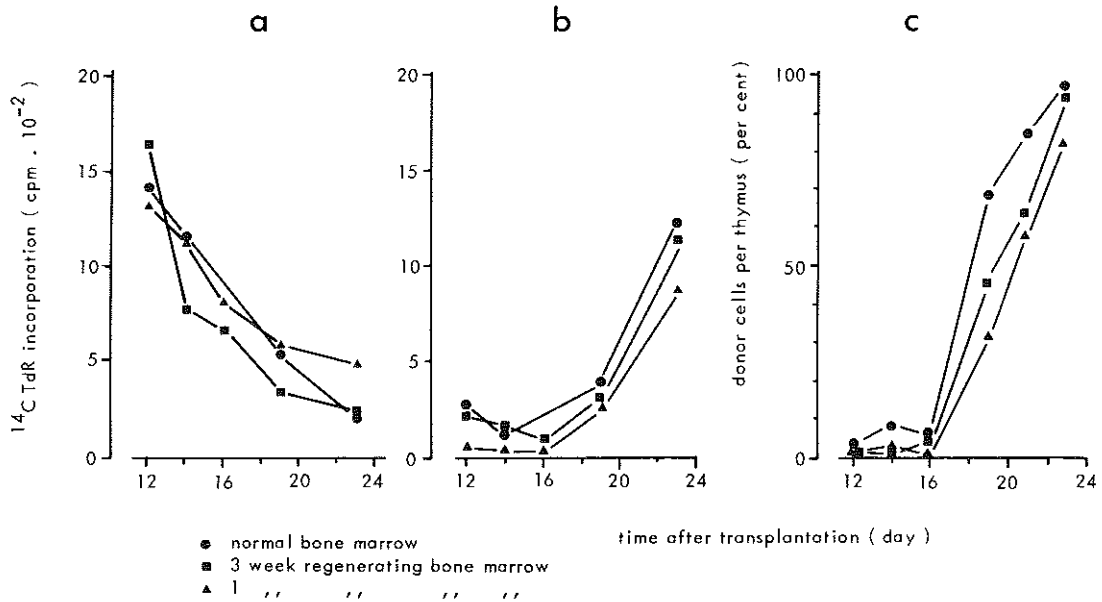

Fig. 7 Spontaneous ^{14}C TdR incorporation into thymocytes (a = host cells, b = donor cells) after transplantation of normal and regenerating bone marrow cells, and proportion of donor cells according to flow cytofluorometry (c). Normal bone marrow: 5×10^5 cells transplanted (176 CFU-S). Three-week regenerating bone marrow: 10×10^5 cells transplanted (204 CFU-S). One-week regenerating bone marrow: 20×10^5 cells transplanted (184 CFU-S).

concluded that the proportions of cycling cells in the donor and in the host derived thymocyte subpopulations after transplantation of one week and three-week regenerating bone marrow were the same as determined after normal bone marrow transplantation. This suggested that the thymus cells that develop from T-cell progenitors present in regenerating bone marrow as far as it concerns their rate of development and proportion of cycling cells, behave like those that are derived from normal bone marrow progenitor cells (Fig.7).

When expressed per 10^5 bone marrow cells, the delay observed between the growth curves determined after transplantation of 1, 3 and 8 week regenerating bone marrow cells in comparison with normal marrow could be expressed as a decrease in thymocyte progenitor cells to 1%, 2.5% and 15%, respectively (Fig.8).

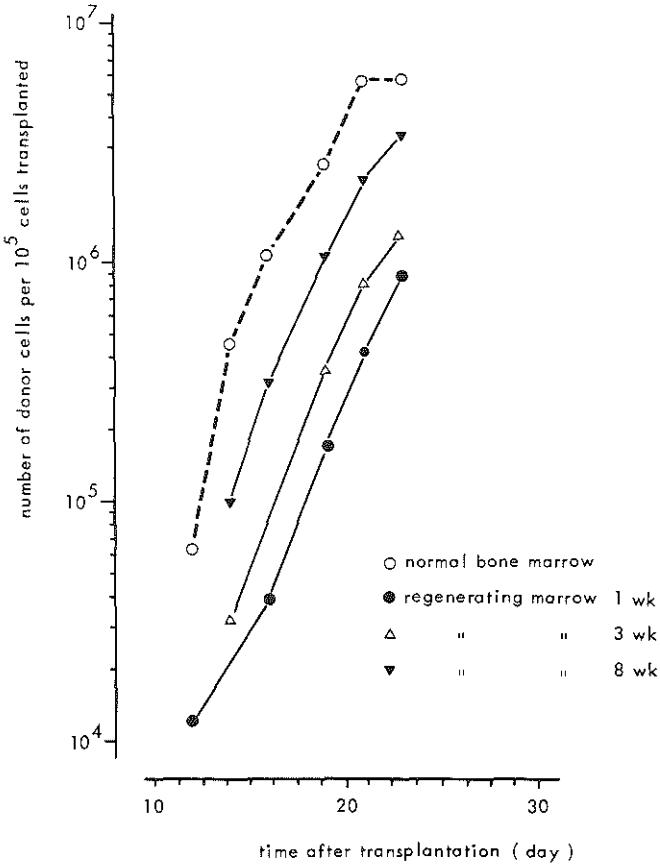

Fig. 8 Donor thymocyte growth after transplantation of normal and regenerating bone marrow cells expressed per 10^5 cells transplanted. Normal marrow: 5×10^5 cells transplanted. Eight-week, 3-week and 1-week regenerating marrow: respectively 7.5×10^5, 10×10^5 and 20×10^5 cells transplanted.

In a linear plot of the same data the difference in regeneration kinetics for Thy-1$^+$ cells from primary and secondary progenitor cells is accentuated (Fig.9).

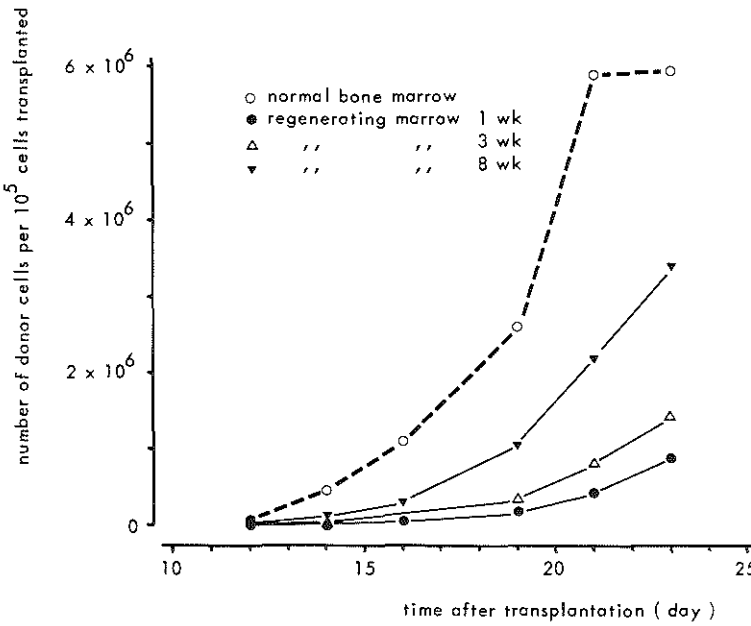

Fig. 9 Donor thymocyte growth after transplantation of normal and regenerating bone marrow expressed per 10^5 cells transplanted. Linear plot of the data shown in Fig.8.

When the production of donor cells was expressed per equivalent of bone marrow cells containing one CFU-S, the observed delay in regeneration was the same for 1 and 3-week regenerating marrow (Fig.10). This indicated that there was no measurable change in the ratio of the number of thymocyte progenitor cells to CFU-S. When 1, 3 and 8-week regenerating bone marrow cells were transplanted, the number of thymocyte progenitor cells per CFU-S transplanted was reduced to 7%, 7% and 23%, respectively. The relative number of prothymocytes and CFU-S during regeneration in an irradiated animal is shown in Figure 6. It can be concluded from this that CFU-S and prothymocytes have different regeneration kinetics. The delay observed in the thymus cell growth after transplantation of regenerating bone marrow may be due to initiation of the regeneration process from CFU-S instead from prothymocytes.

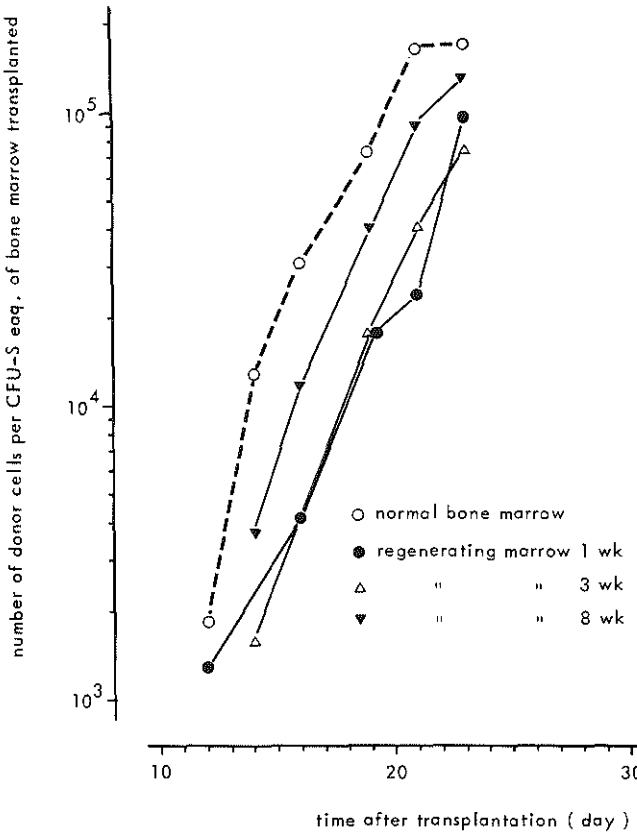

Fig. 10 Donor thymocyte growth after transplantation of normal and regenerating bone marrow cells expressed per CFU-S transplanted. Normal bone marrow: 176 CFU-S transplanted. Eight-week, 3-week and 1-week regenerating bone marrow respectively 200 CFU-S, 204 CFU-S and 184 CFU-s transplanted.

2.3.2 Relative density distributions of prothymocytes and CFU-S in regenerating bone marrow

Regenerating bone marrow cells contained a rather low proportion of prothymocytes. Since the ratio of prothymocytes to CFU-S changed only slowly during the first weeks of the regeneration and was still markedly different from that in normal bone marrow, the relative density distribution of prothymocytes and CFU-S was determined in three- week regenerating bone marrow cells. At that time, the relative number of prothymocytes was 3% of the normal bone marrow value. Both prothymocytes and CFU-S showed a peak in the relative density distribution at 1.070 $g.cm^{-3}$, which was similar to that observed in normal bone marrow (Fig.11). The density distribution for CFU-S had an asymmetric appearance which was more pronounced than generally observed in normal bone marrow. The shoulder was found between 1.072 and 1.076 $g.cm^{-3}$. This value was similar to the buoyant

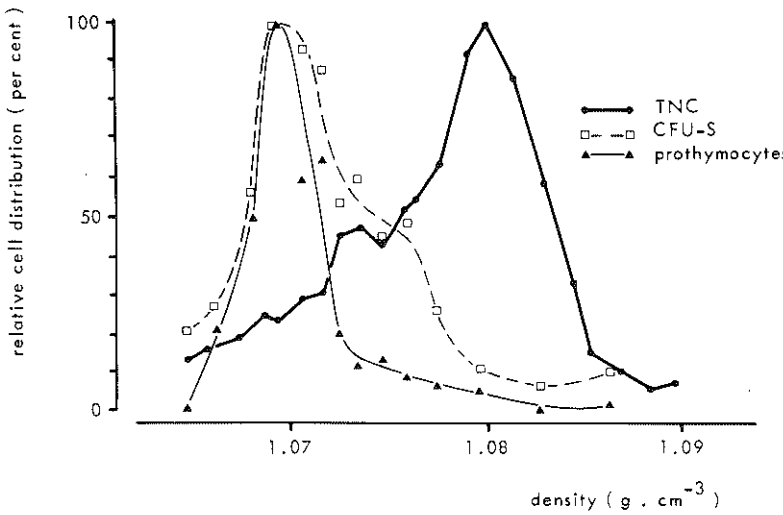

Fig. 11 Relative density distribution of nucleated cells, CFU-S and prothymocytes in 3-week regenerating bone marrow.

density of CFU-S in one week regenerating bone marrow (Visser et al., 1977). On the other hand, the density distribution for prothymocytes in three-week regenerating bone marrow (which had the same peak value as determined for CFU-S) was rather homogeneous and had a 50% bandwidth of 0.0050 g.cm^{-3}. This bandwidth is smaller than determined in normal bone marrow (0.0083 g.cm^{-3}) but of the same magnitude as has been found for prothymocytes in fetal liver (0.0050 g.cm^{-3}) and in long-term cultured bone marrow cells (0.0055 g.cm^{-3}).

3 DISCUSSION

In three sources of hemopoietic precursor cells, fetal liver, long-term cultured bone marrow and regenerating marrow, it has been shown that the prothymocyte to CFU-S ratio is decreased as compared to normal bone marrow cells. Fetal liver cells and regenerating bone marrow contain a pool of rapidly proliferating hemopoietic progenitor cells which allow the rapid growth of the donor thymocyte cell population. In the long-term cultures, it was shown that there is a larger proportion of CFU-S in cycle than is found in fresh bone marrow cells. The prothymocytes in the hemopoietic cell sources that were described in this chapter appeared to be normal according to the observed growth kinetics for donor derived thymocytes. The growth curves for donor thymocytes after transplantation of any of these sources revealed the same growth rate as that determined after transplantation of fresh normal bone marrow.

The density distribution of prothymocytes and of CFU-S from the three cell sources analysed were similar to a great extent. In all three cases, the peak value for the relative density distribution was the same for prothymocytes and CFU-S.

The low buoyant density that we observed for prothymocytes and CFU-S in fetal liver cells is in agreement with the findings of Moore et al. (1970) and Haskill and Moore (1970). That the density distributions of the two cell functions in fetal liver show a comparable similarity as observed for the prothymocytes and CFU-S in normal bone marrow is an additional indication for the close relationship between the two cell types. The great degree of overlap between the density distribution of prothymocytes and CFU-S makes that the two functions cannot be separated on the basis of this physical parameter. When compared to normal bone marrow prothymocytes, the density distribution of prothymocytes in fetal liver, regenerating bone marrow and long-term cultured bone marrow is more homogeneous. First, this might suggest that prothymocytes in normal bone marrow possibly show a greater variation as compared to prothymocytes from the other sources described. Secondly, this phenomenon could be explained by prothymocytes being part of the subpopulation of resting CFU-S. This latter possibility will also be dealt with in Chapter 6.

From the density distributions in normal and one-week regenerating bone marrow, it was concluded by Visser et al. (1977) that CFU-S which sediment at 1.070 g.cm^{-3} represent resting CFU-S, while those sedimenting at 1.075 g.cm^{-3} represent the cycling cells within the CFU-S population. If this were true then, from the distribution of CFU-S in 3-week regenerating bone marrow, one may conclude that the resting compartment of CFU-S has recovered to about 80% of the normal value at that time. If prothymocytes were a subpopulation of resting CFU-S, it would be expected that they would be recovered along with the CFU-S. However, three weeks after bone marrow transplantation of 3×10^6 cells, prothymocytes are still at about 3% of normal levels. In fetal liver cells and in bone marrow cells from long-term cultures the density distributions of CFU-S and prothymocytes completely overlap, while CFU-S in these sources are rapidly proliferating. These results are best explained in a two cell model.

In fetal liver, the numbers of prothymocytes, CFU-S and progenitor cells for the myeloïd lineage are lower than determined in normal bone marrow. However, the ratio of GM-CFU-c over CFU-S remains the same, but the ratio of prothymocytes to CFU-S is lower than in normal bone marrow. The numbers of CFU-S that we determined in fetal liver were in agreement with those observed by others in the same mouse strain (Schofield, 1970). The decrease in the relative concentration of prothymocytes to 38% of the bone marrow value will lead to a delay in development of thymocytes of only 1.2 doubling times.

There are indications that following allogeneic transplantation delayed type GvHD is caused by lymphocytes that originate from donor T lymphocyte precursors

(Van Putten, 1964). As has been shown by Heidt et al. (1981b), delayed type GvHD can also be evoked by adding low numbers of immunocompetent cells to lymphocyte poor grafts. Delayed type GvHD after fetal liver and after bone marrow transplantation leads to different survival rates even when T lymphocyte poor, stem cell enriched fractions are transplanted (Löwenberg, 1975). This difference could have its origin in the lower proportion of fetal liver prothymocytes as compared to bone marrow prothymocytes. A lower proportion of prothymocytes per CFU-S transplanted will lead to a relatively low and delayed production of immunocompetent T lymphocytes after fetal liver transplantation as compared to that after bone marrow transplantation.

In the long-term cultures, the cells capable of repopulating the thymus of a lethally irradiated host could be maintained for at least 17 weeks after an initial decrease. Not only the proportion of prothymocytes but also the ratio of prothymocytes to CFU-S decreased. Prothymocytes were lost from our long-term cultures before the disappearence of CFU-S was apparent (Tables II,IV). The finding that a high proportion of CFU-S in our cultures were killed with tritiated thymidine is in disagreement with the results of Dexter et al. (1979b), who found that the CFU-S in their cultures were not cycling 7 days after feeding. This difference might be due to the fact that we used nonselected fetal calf serum supplemented with hydrocortisone in our cultures, whereas they used selected horse serum. These conditions were chosen because cortisone had a positive effect on the maintenance of granulocyte-macrophage and of erythroïd progenitor cells (Eliason et al., 1977; Greenberger, 1978; Toogood et al., 1980; Suda and Dexter, 1981).

According to Reiman and Burger (1979) and Dexter et al. (1979a), bone marrow cells of mice which have H2k histocompatibility antigens - in their work CBA mice - were difficult to maintain in the type of cultures that have been used. This could at least in part explain the relatively poor maintenance of the bone marrow cells after 12 weeks of culture. Culturing of C57BL/Ka (H2b) mouse bone marrow cells under identical conditions as described above, revealed that in those cultures prothymocytes and CFU-S were present in proportions similar to that in normal bone marrow. In addition, CFU-S were predominantly in the resting state (Eliason, Boersma, Knaan, unpublished observations).

The differences between the two cell types in disappearance rate from the cultures provide additional evidence that two different cell types are involved in the CFU-S and prothymocyte functions.

As far as it concerns the proportion of prothymocytes, the regenerating bone marrow cells resemble very much the long-term cultured bone marrow cells. At one week after transplantation of 3×10^6 bone marrow cells, the concentration of CFU-S is reduced to 25% and prothymocytes to about 1% of the original values. This suggests that the differentiation into prothymocytes does not contribute to the exhaustion of the stem cell pool. The low number of prothymocytes in regenerating bone marrow could be explained in at least two ways. First, it could be

due to their immediate differentiation into thymocytes. But then one would expect a rapid growth of donor thymocytes. The relatively impaired regeneration of the thymus indicates that only a limited number of prothymocytes will be involved. Secondly, the low concentration of prothymocytes could be due to the selective differentiation of pluripotential hemopoietic cells in a regenerating phase into the nonlymphoid cell lineages. Morphological observations of Viktora et al. (1978), Trentin (1978) and Kraal et al. (1979) show that after transplantation of normal bone marrow cells into lethally irradiated recipients, T-lymphoid development is the slowest in the sequence of differentiation detectable in the spleen. Lymphoid development is preceded by erythroid, myeloid and megakaryocytic regeneration.

The low prothymocyte to CFU-S ratios in long-term cultures, where the ratio of myelopoietic progenitor cells to CFU-S remains stable, supports the view that differentiation of the cycling CFU-S in the cultures and regenerating bone marrow is directed towards myelopoiesis at the expense of lymphopoiesis (Boersma and Eliason, 1982). It seems that regeneration of T lymphocytes is delayed until myelopoiesis has reached a steady state. In addition, if the number of prothymocytes is decreased to extremely low levels (zero), the thymus cell repopulation stems directly from the pluripotent cell (CFU-S). This will also lead to a delay in thymocyte regeneration.

In Figure 12, we present an idealised picture of the kinetics of development of thymocytes from progenitor cells in various stages of differentiation. Intrathymic progenitor cells do not possess an unlimited selfreplicating capacity. Thymocytes derived from radiation resistant cells have kinetics of development similar to that observed after transplantation of intrathymic progenitor cells (Chapter 3).

Prothymocytes from fresh normal bone marrow have a relatively high capacity to produce thymocytes. From the regeneration pattern of prothymocytes in the bone marrow, it was concluded that a subpopulation of the bone marrow cells inoculated into lethally irradiated recipients is directly responsible for the early regeneration of the thymus (Chapter 3). Therefore, extrapolation of the rapid phase of thymocyte regeneration to day zero, on which quantification of the prothymocyte is based, gives a reliable estimate of the number of prothymocytes present in the graft.

The prothymocyte to CFU-S ratio which was decreased in the cell sources analysed shows a broad range of values. The delay in thymocyte development after transplantation of hemopoietic cell suspensions containing low numbers of prothymocytes ($\leq$ 1%) as compared to normal bone marrow almost meets the criteria for the situation where T cell differentiation starts from CFU-S. The intermediate stages between the CFU-S and the thymocyte will then have short half-lives because of the differentiation "pressure" in such a system. The prothymocytes in the regenerating marrow, only to a limited extent, will contribute to the growth of the thymus cell population in the first three weeks after transplantation. The

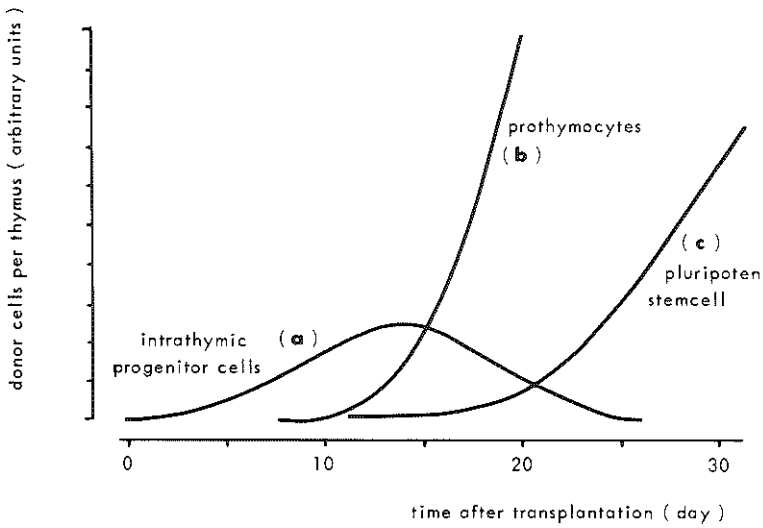

Fig. 12 Thymocyte production scheme. Three important phases of thymocyte production are distinguished, see also Chapter 3, Figure 9 and this chapter, Figure 9:

a. Intrathymic progenitors have a limited capacity to produce Thy-1$^+$ progeny. Intrathymic progenitors are found in normal thymocytes, radiation resistant thymocytes and regenerating thymocytes 2 days after bone marrow transplantation.

b. Prothymocytes, present in various tissues, restore the thymus cell population. Regeneration starts from the time of transplantation at a constant rate.

c. In absence of prothymocytes the restoration of the thymus cell population is initiated from CFU-S. This explains the delay observed after transplantation of hemopoietic progenitors that do not comprise prothymocytes.

changes in the prothymocyte to CFU-S ratio can be explained by a balance between the contribution to thymocyte differentiation by prothymocytes directly, on the one hand and via the indirect pathway by CFU-S on the other.

4 CONCLUSIONS

1. In hemopoietic systems which are in a high proliferative state, the relative number of prothymocytes is low and the prothymocyte to CFU-S ratio is decreased as compared to normal bone marrow levels.

2. The difference in the rate of disappearance of cells capable of the prothymocyte function and CFU-S from long-term cultures is an indication that these functions are performed by different cells.

3. The rate of regeneration of prothymocytes and CFU-S after bone marrow transplantation is markedly different and this can be a reflection of selective differentiation of hemopoietic stem cells in early phases of regeneration into the non-T-cell lineages of hemopoietic cells.

4. Prothymocytes in fetal liver, regenerating bone marrow and long-term cultures represent a homogeneous population of T-cell progenitors.

5. After transplantation of normal bone marrow, the contribution of the regenerating bone marrow cells to the regeneration of the thymus cell population is negligible as compared to the repopulating capacity of the cells that directly home into the thymus.

CHAPTER 6

DIFFERENCES BETWEEN MOUSE BONE MARROW PROTHYMOCYTES AND THE PLURIPOTENT STEM CELL

1 INTRODUCTION

From the analysis of the differences in kinetics of regeneration of progenitor cells for thymocytes and CFU-S after bone marrow transplantation, it appeared likely that the prothymocytes and the CFU-S are different hemopoietic cells. However, prothymocytes and CFU-S could not be distinguished on the basis of size, density and electrophoretic mobility even when they were derived from different sources of hemopoietic cells. It could not be excluded therefore that the prothymocytes belong to the noncycling subpopulations of CFU-S, at least on the basis of their buoyant density distributions (Chapter 4,5). Thus, it was decided to carry out investigations of the cycling state of prothymocytes and CFU-S in normal and regenerating bone marrow. In addition, the sensitivity of prothymocytes and CFU-S to gamma radiation and neuraminidase was determined.

According to suicide experiments with ^{3}H TdR, most CFU-S in normal bone marrow are out of cycle (G_0) (Lahiri and Van Putten, 1969; Vassort et al., 1973; Visser et al., 1977). Similarly, earliest progenitor cells of the myeloid (GM-CFU-c_1) and of the erythroid lineages (E-BFU) also have a limited sensitivity to ^{3}H TdR exposure. Other committed progenitor cells (E-CFU, GM-CFU-c_2 and pre-B cells) have been shown to be rapidly proliferating (Udupa and Reissman, 1978; Byrne et al., 1979; Osmond and Nossal, 1974).

Bol (1980) obtained indications that the differential effect of neuraminidase treatment on CFU-S reflects heterogeneity of these cells with respect to their capacity to form spleen colonies in lethally irradiated mice. Following neuraminidase treatment, only 20 to 30 percent of CFU-S are able to form spleen colonies (Bol, 1980). Treatment of bone marrow cells with neuraminidase changes the homing pattern of the CFU-S population. It was investigated whether thymus cell progenitors have a similar dependency on the presence of sialic acids on surface glycoproteins for their homing into the thymus.

After neuraminidase treatment of bone marrow cells, the change in electrophoretic mobility (EPM) of cells of the myeloid lineages is dependent on their stage of differentiation. The EPM of the earliest progenitors e.g., CFU-S and GM-CFU-c_1, is decreased most while that of the more differentiated progenitor cells, GM-CFU-$c_{2,3}$ is less affected by neuraminidase treatment. Therefore, it seemed of interest to investigate whether it was possible to separate prothymo-

cytes and CFU-S according to differences in their EPM after neuraminidase treatment.

In this chapter, we shall show that CFU-S and prothymocytes in normal bone marrow have a similar sensitivity to ^{3}H TdR exposure. Unexpectedly, prothymocytes in regenerating bone marrow are affected severely by exposure to cold thymidine. The two cell types were shown to have a different radiation sensitivity. Furthermore, the homing pattern of prothymocytes and CFU-S are affected differently by neuraminidase modification of the cell membrane.

2 RESULTS

2.1 In Vitro Exposure to 3H-Thymidine

After treatment of bone marrow cells with ^{3}H TdR, a plateau phase for the surviving fraction of CFU-S was expected between 25 and 200 $\mu Ci.ml^{-1}$ (Iscove et al., 1972; Van den Engh, 1976). Especially at higher doses of ^{3}H TdR (100-400 $\mu Ci.ml^{-1}$) we did not observe a plateau, but instead, a continuous decrease of the surviving fraction of progenitor cells (CFU-S) with increasing doses of ^{3}H TdR (not shown). It was decided to investigate the ^{3}H TdR sensitivity of prothymocytes and CFU-S for concentrations between 0 and 100 μCi ^{3}H TdR.ml^{-1}.

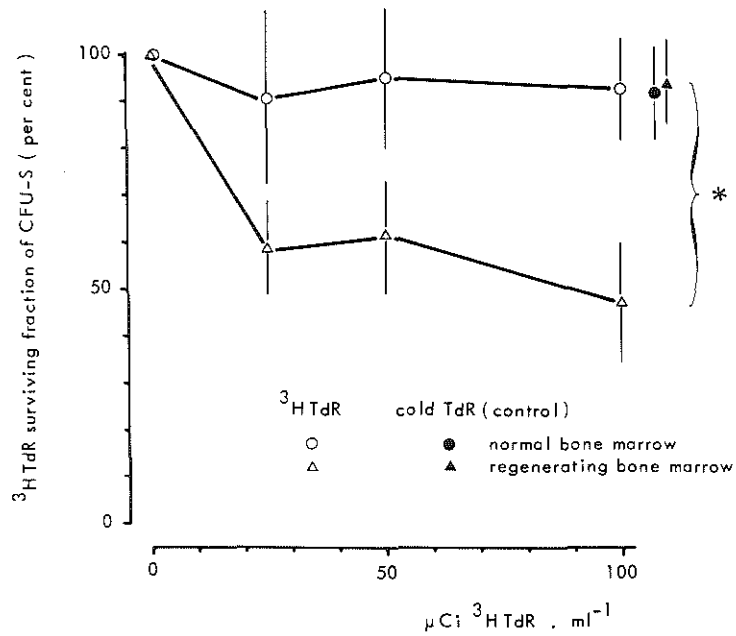

Fig. 1 Relative concentration of CFU-S in normal and in regenerating bone marrow after in vitro treatment with ^{3}H Thymidine. For regenerating bone marrow the differential effect of incubation with excess cold thymidine is indicated *.

The concentration of CFU-S in normal bone marrow after 20 min incubation at 37°C slightly decreased with increasing doses of ^{3}H TdR (0-100 $\mu Ci.ml^{-1}$) in the incubation medium. After incubation with 100 $\mu Ci.\ ml^{-1}$, the CFU-S decreased to 92 ± 10 percent of the initial concentration in the bone marrow sample. In regenerating bone marrow, CFU-S decreased to 48 ± 11 percent of the initial value upon incubation with 100 $\mu Ci.\ ml^{-1}$. This indicated that, in regenerating marrow, nearly all CFU-S are in cycle and this confirmed the observations of Van den Engh (1976) and Visser et al. (1977) (Fig.1).

The prothymocytes in normal bone marrow were moderately affected by incubation with ^{3}H TdR. At 100 $\mu Ci.ml^{-1}$, the prothymocytes decreased to 78 ± 18 percent of the initial value (Fig.2). This is lower than was determined for CFU-S. The difference determined, however, was not statistically significant.

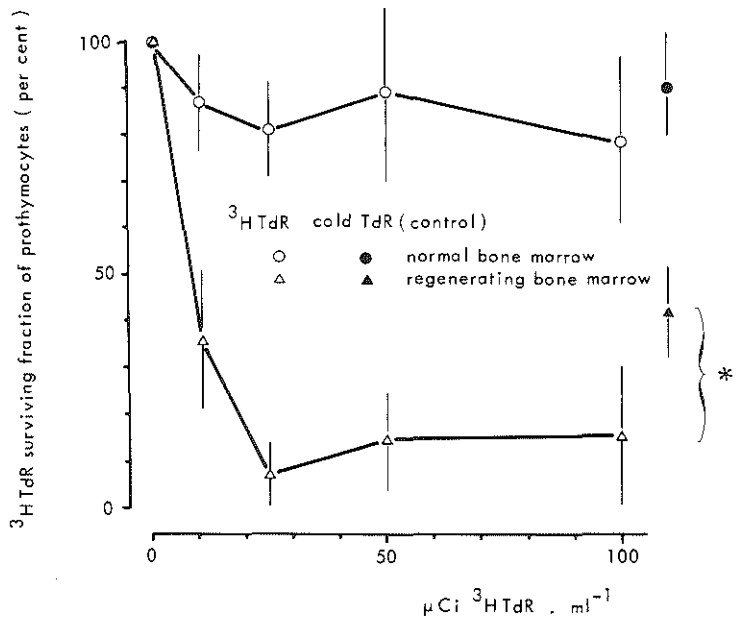

Fig. 2 Relative concentration of prothymocytes in normal and in regenerating bone marrow cells after in vitro treatment with ^{3}H Thymidine. For regenerating bone marrow the differential effect of incubation with excess cold thymidine is indicated *.

From the determinations described above, it was calculated that after exposure of normal marrow to ^{3}H TdR (25-100 $\mu Ci.ml^{-1}$) the ratio of prothymocytes to CFU-S decreased to 90 percent of the value determined in untreated normal marrow.

In regenerating bone marrow, the prothymocytes were found to be sensitive to incubation with excess cold thymidine (800 µmolar). Only 40 percent of pro-

thymocyte activity was observed. After incubation with 10-100 $\mu Ci.ml^{-1}$ ^3H TdR, the concentration of prothymocytes in regenerating marrow was reduced to 7-16 percent of the initial value when calculated as percent of cells incubated without ^3H TdR. Even when all prothymocytes are in cycle this reduction is beyond the decrease of about 50% which might be expected, unless the result of cold thymidine incubation is taken as a control value. In that case the prothymocytes in regenerating bone marrow are reduced to 18-40% of the value observed for non ^3H TdR exposed cells. Interpretation of these results will be further elaborated on in the discussion.

2.2 In Vitro Gamma Irradiation

Prothymocytes and CFU-S showed a different sensitivity to treatment with gamma radiation. The surviving fraction of both cell types decreased logarithmically with increasing doses of radiation (Fig.3). Prothymocytes decreased more

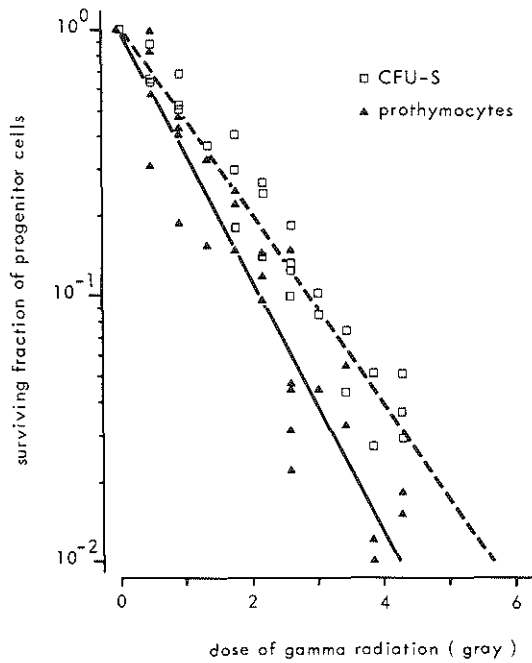

Fig. 3 Radiation sensitivity of prothymocytes and CFU-S in vitro. Both cell functions were measured in the same bone marrow cell suspension. The D_0 values were calculated from log-linear regression analysis. D_0 prothymocytes = 0.92 Gy and D_0 CFU-S = 1.22 Gy.

rapidly than CFU-S. For the calculation of the radiation sensitivity, we pooled the data of four experiments in which prothymocytes and CFU-S were studied in the same bone marrow samples. The D_0 values were calculated from log linear regres-

sion analyses. Statistical analysis of log-linear regression lines showed that the sensitivity of prothymocytes (D_0 = 0.92 Gy) and CFU-S (D_0 = 1.22 Gy) to γ-radiation was significantly different. The results are summarised in Table I.

TABLE I

SENSITIVITY OF CFU-S AND PROTHYMOCYTES TO IN VITRO GAMMA IRRADIATION

	Observations*	corr.coëf.	p	D_0		
CFU-S	33	-0.9795	≤0.005%	1.22 Gy	S.E.M.	3.7%
Prothymocytes	35	-0.9500	≤0.005%	0.92 Gy	S.E.M.	5.7%

* Data of 4 experiments were pooled.
Log-linear regression lines are significantly different in slope (p = <0.001) and have different variances (p = <0.002). Offset values (n = 1.06 for CFU-S and n = 0.99 for prothymocytes) are not significantly different.

In vivo γ-irradiation of prothymocytes (D_0 = 0.85 Gy) and CFU-S (D_0 = 1.01 Gy) revealed a similar difference in sensitivity as was observed in vitro. The differences in vitro were not significant, due to larger variation in the results, which probably is inherent to the method (Fig.4).

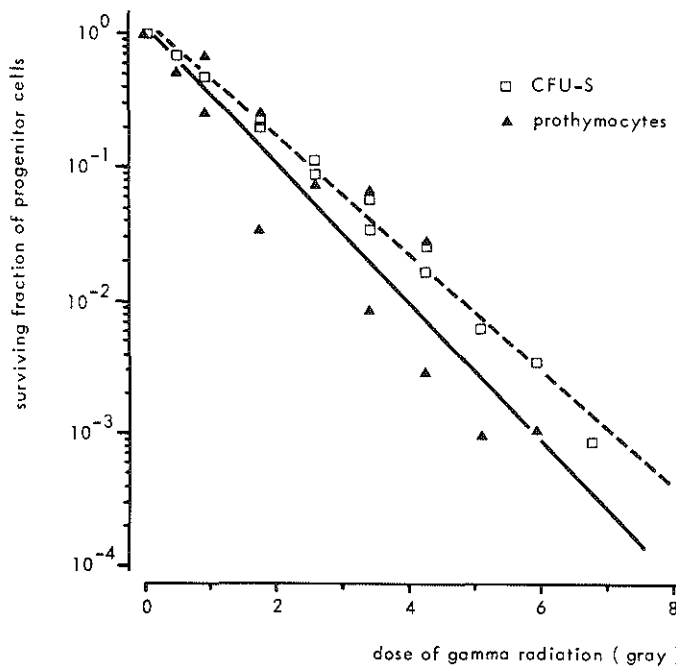

Fig. 4 Radiation sensitivity of prothymocytes and CFU-S in vivo. Both cell functions were measured in the same bone marrow cell suspension. The D_0 values were calculated from log-linear regression analysis. D_0 prothymocytes = 0.85 Gy and D_0 CFU-S = 1.01 Gy.

2.3 Neuraminidase Treatment

2.3.1 Differential sensitivity of prothymocytes and CFU-S

The sensitivity of CFU-S to treatment with neuraminidase reaches a plateau at a concentration of 1×10^{-2} I.U.ml^{-1} (Bol, 1980). In our experiments, the number of CFU-S decreased to 28 percent of the nontreated value when incubated with 1 to 3×10^{-2} I.U.ml^{-1} of neuraminidase. In the range between $1\text{-}2 \times 10^{-2}$ I.U.ml^{-1} neuraminidase, prothymocytes had a lower sensitivity than CFU-S, but at 3×10^{-2} I.U.ml^{-1} the sensitivity of prothymocytes approached that of CFU-S (Fig.5).

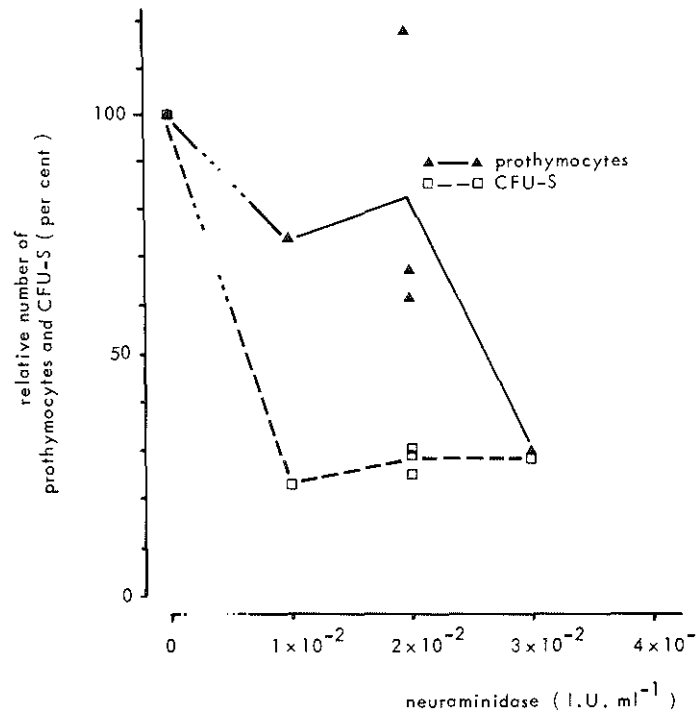

Fig. 5 Sensitivity of prothymocytes and CFU-S to <u>in vitro</u> neuraminidase treatment.

Since the objective of these experiments was to employ differential sensitivity of prothymocytes and CFU-S to neuraminidase to separate these cells on the basis of differences in electrophoretic mobility after treatment, we chose to incubate the bone marrow cells before separation with 2×10^{-2} I.U.ml^{-1}. Detailed results of such incubations are summarised in Table II.

TABLE II

DIFFERENTIAL SENSITIVITY OF PROTHYMOCYTES AND CFU-S TO NEURAMINIDASE TREATMENT

		CFU-S/10^5	Donor cells/10^5
Exp. I	nontreated	25.3 (100)	2.95×10^6 (100)
	treated	7.6 (30)	1.82×10^6 (62)
Exp. II	nontreated	38.7 (100)	2.01×10^6 (100)
	treated	9.7 (25)	1.35×10^6 (67)
Exp. III	nontreated	17.3 (100)	0.64×10^6 (100)
	treated	5.0 (28.2)	0.74×10^6 (117)
Mean percentage treated groups		27.8 ± 2.5	81.8 ± 30.1

- neuraminidase 2×10^{-2} I.U.ml^{-1} during incubation
- donor cells determined 17 days after transplantation
- figures in parentheses represent percent of nontreated values

2.3.2 Free flow electrophoresis

When neuraminidase treated bone marrow cells (2×10^{-2} I.U.ml^{-1}) were separated according differences in EPM, the relative distribution of all cell types were shifted to low EPM as compared to values for normal marrow. The low EPM peak for CFU-S was found at -1.29×10^{-4} $cm^2.V^{-1}.s^{-1}$. Surprisingly, the same peak value was determined for prothymocytes. Although the homing patterns of prothymocytes and CFU-S are differentially affected by neuraminidase treatment, these cells cannot be separated on the basis of differences in their behaviour in an electric field, even after exposure of their sialated surface glycoproteins to neuraminidase (Fig. 6).

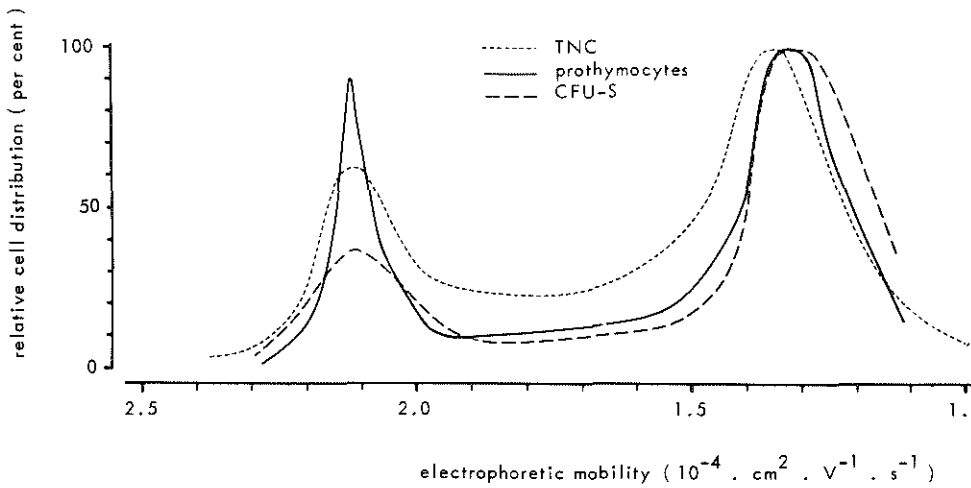

Fig. 6 Relative electrophoretic mobility distributions of prothymocytes and CFU-S and nucleated cells after in vitro neuraminidase treatment (mean of two experiments is shown).

3 DISCUSSION

According to Lord et al. (1974) and Yen et al. (1981), the sensitivity to proper in vitro treatment with ^{3}H TdR gives a good estimate of the fraction of cells that are initially in S phase in a cell population. The values that we observed for the fraction of CFU-S in cycle in normal (15-20%) and in regenerating bone marrow (85-100%) are in agreement with the observations of Lahiri and van Putten (1969), Vassort et al. (1973) and Visser et al. (1977). With this method, we have shown that, in normal bone marrow, the frequency of cycling cells in the prothymocyte cell population differs only slightly from that observed for CFU-S. In this respect, the prothymocyte behaves in a way similar to the earliest progenitor cells of the erythroid and myeloid lineages and pre-B cells. According to Mitchison (1971) one might expect to kill about 50 percent of a population of cycling cells by S-phase specific treatment of cells with ^{3}H TdR. Possibly, the process that leads to a severe reduction (84-93%) of prothymocytes after ^{3}H TdR exposure of regenerating bone marrow consists of two components. First is the killing due to ^{3}H TdR incorporated into DNA during S-phase. Secondly, although thymidine concentrations were rather low (0.5-5 μ molar) during exposure to ^{3}H TdR, in view of the negative effect of excess cold thymidine (800 μ molar) on prothymocytes in regenerating bone marrow, it is likely that these cells are sensitive to the inhibitive effect of thymidine on DNA synthesis. A high sensitivity to thymidine inhibition of DNA synthesis is a distinctive property of T lymphocytes which is most apparent in thymocytes (Whittle, 1966; Harris, 1977). In regenerating bone marrow, cold thymidine and ^{3}H TdR were not toxic to CFU-S when applied to the same concentrations as used for prothymocytes.

Phosphorylation is involved in incorporation of thymidine into DNA. High levels of dTTP, however, have a negative effect on the production of deoxycytidine. In this way, treatment of cells with thymidine can inhibit DNA synthesis. Hemopoietic progenitor cells are differentially sensitive to exposure to thymidine. The inhibitive action of thymidine even at low concentrations (1-5 μ molar) has been described by Hasthorpe and Harris (1979) for CFU-E. In most cells the inhibitive effect of thymidine is reversible by addition of deoxycytidine. The inhibitive effect of thymidine on prothymocytes in regenerating bone marrow could be explained by a change in the thymidine metabolism that leads to in vitro accumulation of this DNA precursor.

For prothymocytes one would expect that in case of an inhibitive action of thymidine, this effect would be reversed by supply of extracellular dCTP after transplantation in the recipient animal. This might lead to a delay in development of prothymocytes which in our kinetic assay is interpreted as a lower number of progenitor cells present in the bone marrow inoculated. This reasoning, however, does not account for the differences in sensitivity to thymidine exposure that have been observed between prothymocytes from normal and from regenerating

bone marrow. Yet, the effect of ^{3}H TdR on prothymocytes in regenerating bone marrow might be explained by the high cycling state of these cells.

The low proliferative state of prothymocytes in normal bone marrow differs from that reported for the in vitro T-cell colony forming unit (CFU-T) derived from phytohaemagglutinin stimulated nude mouse spleen cells (Jacobs and Miller, 1979; Ching et al., 1981). CFU-T derived from nude mouse spleen were killed up to 65-83% on treatment of these cells with hydroxyurea (HU) (Rusthoven and Phillips, 1980). Whether the cells determined in the CFU-T assay are T progenitor cells from the same differentiation level as prothymocytes has not been shown. The CFU-T could be a clonogeneic assay for more differentiated cells than prothymocytes.

The sensitivity of CFU-S (D_0 = 1.22 Gy) and prothymocytes' (D_0 = 0.92 Gy) to in vitro irradiation has been shown to be significantly different. The value obtained for CFU-S is in agreement with similar results as reviewed by Lajtha (1965) for stem cells in normal mouse bone marrow (D_0 = 0.7-1.05 Gy of X rays). Comparison of D_0 values for haemopoietic cells obtained by others is complicated by differences in methodology and mouse strains used. This is illustrated by the wide range of D_0 values reported for CFU-S (Lajtha, 1965). Therefore, accurate comparison between prothymocytes and CFU-S requires the determination to be done under exactly the same conditions. A directly determined D_0 value for prothymocytes has not been previously published. The value found is lower than generally observed for cortical thymocytes, but in rather good agreement with the D_0 value for thymic precursor cells as calculated by Sharp and Watkins (1981) from the thymus regeneration data of Kadish and Basch (D_0 = 0.7-0.8 Gy of X rays) (1976).

Neuraminidase treatment with doses up to 2×10^{-2} $I.U.ml^{-1}$ affects the capacity of prothymocytes to colonise an irradiated host thymus only to a limited extent. The same dose of neuraminidase strongly decreases the capacity of CFU-S to form spleen cell colonies in lethally irradiated mice. This is not due to elimination of CFU-S but neuraminidase apparently changes the distribution pattern of CFU-S (Bol, 1980). Trapping of neuraminidase treated cells in non-hematological organs might play an important role therein (Bol, 1980; Ploemacher et al., 1981b). Identical treatment of prothymocytes does only alter the homing pattern of these cells to a limited extent. This suggests that prothymocytes are only moderately dependent on the amount of sialated glycoproteins on the cell surface for their homing to the environment where they will differentiate.

In view of the above differences, one would expect that neuraminidase treatment of bone marrow cells would affect the EPM pattern for CFU-S and prothymocytes in a different way. This was tested in the free flow electrophoresis system. CFU-S and prothymocytes in normal untreated bone marrow have the same electrophoretic mobility. Surprisingly, it was found that after neuraminidase treatment the EPM for prothymocytes and CFU-S was changed to the same

extent. Prothymocytes and CFU-S cannot be separated according to differences in electrophoretic mobility. In addition, this provides evidence that the prothymocytes belong to the compartment of very early hemopoietic progenitor cells known. For GM-CFU-c_1, the behaviour in an electric field with and without neuraminidase treatment has also been found to be similar to that of CFU-S, whereas more differentiated GM-CFU showed smaller changes in EPM after neuraminidase treatment than was observed for the early precursor cells of this lineage (Bol, 1980).

The characteristics of prothymocytes and CFU-S that were discussed in this chapter provide evidence that the prothymocytes represent a very early stage in the T-cell lineage that is different from CFU-S. It is tentatively concluded that the prothymocyte is a single lineage committed stem cell.

4 CONCLUSIONS

1. According to experiments with ^{3}H TdR only a minor proportion of prothymocytes and CFU-S in normal bone marrow are in cycle. In regenerating bone marrow, both cell types are fully in cycle. Prothymocytes in regenerating bone marrow are sensitive to treatment with cold thymidine whereas CFU-S are not.

2. Prothymocytes and CFU-S in normal bone marrow have a different γ-radiation sensitivity.

3. Prothymocytes are less sensitive to treatment with neuraminidase than are CFU-S.

4. Prothymocytes and CFU-S cannot be separated according to differences in electrophoretic mobility with or without previous treatment with neuraminidase.

5. Prothymocytes and CFU-S are different cells.
   Prothymocytes are single lineage committed stem cells.

CHAPTER 7

GENERAL DISCUSSION

Repopulation of the thymus after transplantation of hemopoietic precursor cells of various sources into lethally irradiated recipients has been used to characterise T-cell progenitors and to investigate their relationship to the pluripotent stem cell.

Fetal liver cells were known to be less efficient in repopulation of the thymus than adult bone marrow cells. Spleen and bone marrow only to a limited extent contribute to fetal hemopoiesis. Therefore, the adult bone marrow was primarily used as a source of T-cell progenitors. In addition, bone marrow cells are easily available and investigation of T cell-progenitors therein allows comparison of these cells to well defined progenitors for other lineages of hemopoietic cells.

Hemopoietic stem cells upon transfer into lethally irradiated mice form colonies in the spleen. There is also a direct relation between the spleen weight (the number of cells) at day 10 after transplantation and the number of bone marrow cells transplanted (Van Bekkum and Schotman, 1974).

During repopulation of the thymus, after whole body irradiation and bone marrow transplantation, no focal cell growth is observed. Therefore it is not possible to investigate the proliferation of individual cells into colonies. The prothymocytes were defined as those cells that are able to produce Thy-1^+ progeny in the thymus of a lethally irradiated recipient.

The investigation of the growth of donor derived Thy-1^+ cells meets following drawbacks.

First, the growth of donor derived Thy-1^+ cells is a time consuming process. Using cytotoxic detection methods, the first donor cells can be found in the thymus from day 10 to 12 after lethal irradiation. Thymus regeneration can only be observed if the recipients are grafted with a sufficient number of bone marrow pluripotent stem cells to allow the animals to survive the lethal irradiation. The HSC could produce progeny which in turn might repopulate the thymus. Therefore, we had to distinguish between primary and secondary growth of donor derived Thy-1^+ cells which in principle are initiated at the same time. Primary growth is considered to be due to prothymocytes present in the transplanted bone marrow cells. Secondary growth results from the progeny of the CFU-S. In Chapter 5 (see Figs. 8, 9 and 12), however, it was demonstrated that the progeny derived from grafted normal bone marrow HSC will contribute to the thymus regeneration only to a limited extent as compared to the prothymocytes under the conditions of the assay as defined in Chapter 2.

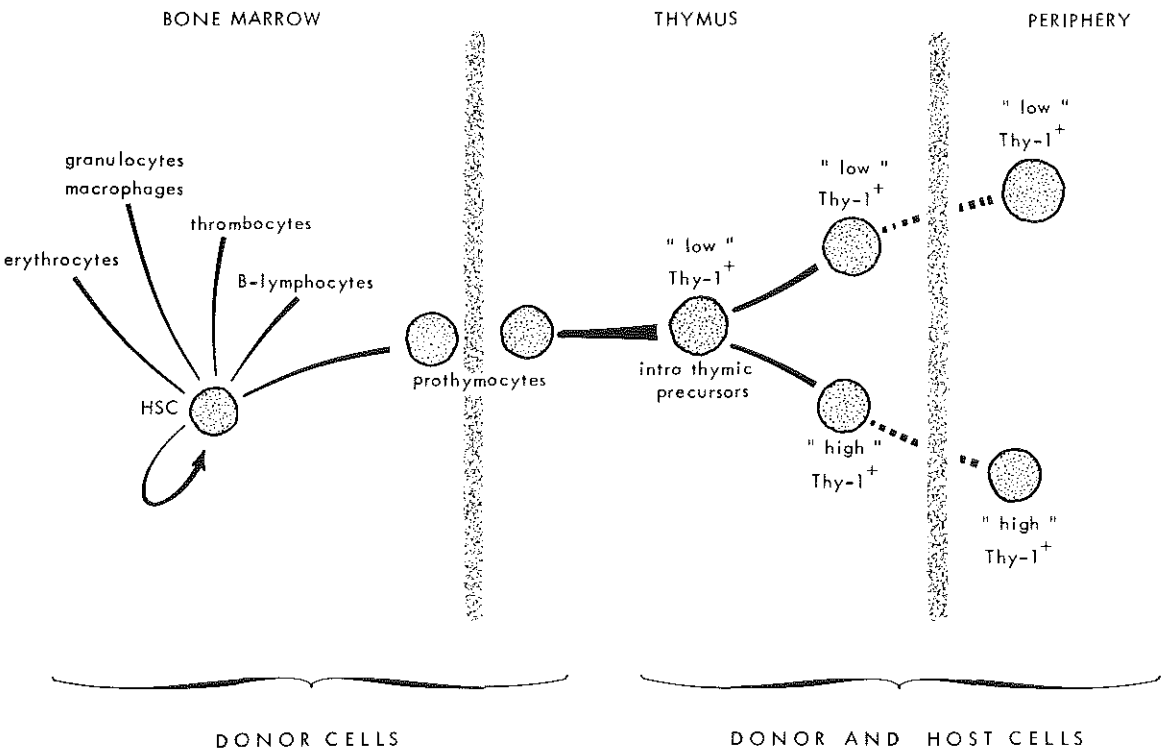

Fig. 1 Tentative T-cell differentiation scheme.

Secondly, the establishment of the relation between the number of progenitor cells transplanted and the growth of donor derived thymocytes was complicated by the development of a transient population of host derived thymocytes. Donor and host derived cells were discriminated according to differences in the Thy-1 allo-antigen on their surface.

The repopulation of the thymus with thymocytes may be negatively influenced by irradiation of the epithelial cells of the thymus. According to Van Bekkum (1967) the regeneration of thymic epithelial cells after radiation doses of about 8.0 Gy occurs within four weeks from irradiation. That is very similar to the time course of donor derived thymus cell development that we have observed after transplantation of bone marrow cells. Since there is a direct relationship between donor cell growth and the number of progenitor cells up to a dose of 10^7 bone marrow cells transplanted, most probably the regeneration of thymus epithelial cells does not act as a limiting factor in regeneration of the lymphoid thymocytes.

Our data, based on cytotoxic discrimination of donor and host derived bone marrow cells, confirmed the findings of Komuro et al. (1975) and Kadish and Basch (1976). However, the design of these latter studies only to a limited extent allowed to draw quantitative conclusions. In addition, thymus regeneration was only investigated from day 10 after transplantation which was inherent to the method of donor cell detection that was employed. The analysis of donor cell development with fluorescein labeled antibodies and the FACS as described in Chapter 3, permitted to extend the observations to the period shortly (0-2 days) after transplantation. From the kinetics of development of the "low" and "high" Thy-1^+ subpopulations it may be concluded that "low" Thy-1^+ cells always preceed the development of "high" Thy-1^+ cells (Fig.1). Due to the low numbers of donor cells in the thymus in the early days after transplantation the accuracy is low. Although in general Thy-1^+ cells do not home into the thymus we cannot exclude that bone marrow derived "low" Thy-1^+ cells (0.5-1.5%) contribute to the "low" Thy-1^+ cell population of the thymus early after transplantation, in particular, when relatively high doses of bone marrow cells (10^7) are transplanted. But the excess of "low" Thy-1^+ cells was also demonstrated in the thymus early after transplantation of much lower cell numbers in the graft ($5x10^4$ - $5x10^5$). The kinetics of the two Thy-1^+ cell populations are best explained when a "low" Thy-1^+ cell is considered to be the first recognisable T cell in the thymus. This cell therefore might be an intrathymic progenitor cell for "low" and "high" Thy-1^+ cells. During the preparation of this manuscript Goldschneider et al. (1982) identified distinct subsets of proliferating "low" Thy-1^+ cells in the thymus after in vitro labeling with ^{3}H TdR. They also have presented the hypothesis that all thymocytes are descendents from "low" Thy-1^+ precursors and that probably separate intrathymic precursors exist for "high" Thy-1^+ (cortical) and "low" Thy-1^+ (medullary) thymocytes.

A similar relationship between "low" and "high" Thy-1$^+$ cells, as was demonstrated for donor derived Thy-1$^+$ cells, has been observed for host derived cells a few days post irradiation and transplantation (Chapter 3, Figs. 6 and 10). The kinetics of development of "high" Thy-1$^+$ cells of donor and host origin are very much alike. "Low" Thy-1$^+$ cells of donor and host derived cells show a different time course of development. From this, one might conclude that at least under conditions as discussed here "low" and "high" Thy-1$^+$ cells develop independently.

In regenerating thymus a simultaneous development of "low" and "high" Thy-1$^+$ cells is observed. But at the time the number of donor cells reaches a maximum, the proportion of "low" Thy-1$^+$ cells still has not reached the levels of "low" Thy-1$^+$ cells in a normal thymus. These "low" Thy-1$^+$ cells are associated with the immunocompetent thymocytes. An incomplete recovery of a specific subpopulation of thymus cells also has been observed by Droege (1976) using cell size and cell density characterisation and by Daculsi et al. (1982) using H-2 surface antigens as a marker. The probably defective mechanism underlying this phenomenon is not yet known.

"High" Thy-1$^+$ host derived cells disappear completely within 25 days after irradiation. "Low" Thy-1$^+$ host derived thymocytes, though few in absolute numbers, therefore show a relative increase with time. Recently this has also been reported by Ceredig and MacDonald (1982).

In the previous chapters it has been demonstrated, that the prethymic T progenitor cell in many aspects resembles cells that are able to form spleen colonies.

The density distributions of the prothymocytes and of CFU-S from various sources overlap to a great extent. Prothymocytes have a rather symmetrical density distribution. Splinter and Reiss (1974) demonstrated for lymphoid cell lines that buoyant cell density within a homogeneous density distribution is not dependent on the cell cycle state. The density distributions of CFU-S in normal and regenerating bone marrow are typical for a heterogeneous cell population. The density of prothymocytes is the same as observed for the low density ($d=1.070\ g.cm^{-3}$) subpopulation of CFU-S. The different density subpopulations of CFU-S might be a reflection of the heterogeneity of colony forming cells as recently described by Magli et al. (1982).

Similar sedimentation velocity profiles were obtained for prothymocytes and CFU-S which implies that the cell size of the two cell types must be the same.

The electrophoretic mobility of prothymocytes and CFU-S in normal bone marrow was not significantly different.

In Chapter 2 the accuracy of the prothymocyte assay has been discussed. It was demonstrated that within one experiment at least a difference of a factor two in the ratio of prothymocytes over CFU-S could be detected. The degree of reproducibility that we obtained in rather complex experiments e.g., those with

fractionated cells suggest that even smaller changes in the ratio of prothymocytes over CFU-S can be detected (Chapter 4).

Donor derived thymocyte growth in the thymus of lethally irradiated mice is directly related to the number of bone marrow cells transplanted. Therefore, thymus cell regeneration shows a direct relation with the number of CFU-S in the inoculum. Inevitably, we have to adress the crucial question whether the progenitors of the thymus cells are committed progenitor cells from the marrow graft that migrate to the thymus or that prothymocytes are non-committed pluripotent stem cells that upon migration into the thymus differentiate into lymphoid thymus cells. According to our functional definition we would call them prothymocytes in both cases. A direct identification of the thymocyte progenitor cells was not possible. After transplantation, neither CFU-S nor prothymocytes could be detected in the thymus. Therefore, a determination of seeding efficiency of prothymocytes in the thymus cannot be performed.

The following characteristics suggest that the prothymocytes exist as a separate entity in the bone marrow.

First: In regenerating bone marrow the prothymocyte pool is almost completely depleted. The regeneration of the thymus which in that case will be initiated from CFU-S is delayed considerably when compared to regeneration from normal bone marrow prothymocytes. This suggests at least one differentiation step between prothymocytes and CFU-S. The regeneration of prothymocytes and CFU-S in bone marrow shows a different time course.

Second: The quantitative relationships (ratio) between prothymocytes and CFU-S are different in fetal liver and bone marrow.

Third: The differences in the rate of disappearance of prothymocytes and CFU-S from long-term cultures of bone marrow can be best explained by a two cell model.

Fourth: The prothymocytes have a different radiation sensitivity .

Fifth: Prothymocytes in regenerating bone marrow are very sensitive to incubation with cold thymidine while CFU-S are not.

Because prothymocytes and CFU-S also have many physical properties in common we have not succeeded in separating the two cell types. Other methods or approaches are clearly needed.

The development of monoclonal antibodies against surface determinants of CFU-S and of other early hemopoietic cells may lead to further identification of the prothymocytes.

Prothymocytes nor their immediate progenitors (CFU-S) can be recovered from the thymus. This suggests that prothymocytes require a special microenvironment for proliferation and differentiation. The in vitro system that has been developed by Pyke and Bach (1979, 1981) might lead to a better characterisation of the relation between microenvironment and differentiation of the prothymocytes.

Identification of early T-cell progenitors will be especially of interest in the investigation of thymic leukemias. The target cell of the transformation process is expected to be among these early precursors.

Once the characterisation of the prothymocytes can be established it will also be possible to find out whether the prothymocyte in our functional assay is a single cell or if separate progenitors for the two major lineages of Thy-1^+ cells exist.

SUMMARY

All blood cells are derived from a common progenitor cell, the pluripotent hemopoietic stem cell. The differentiation of hemopoietic progenitor cells into the different types of end cells is a continuous process. This differentiation process gives rise to the cells that compensate for cell loss due to a limited life span or utilisation of the cells.

In the adult mouse, the major sites of hemopoiesis are the bone marrow and the spleen. The recognition of cells in the early stages of development becomes increasingly difficult when tracing a differentiation pathway of a cell lineage from the mature cell back to the early progenitor stem cell. This is due to the lack of characteristic morphological features of the early progenitor cells and due to their low frequency. For the study of early hemopoietic progenitor cells, special assay systems based on the analysis of in vivo and in vitro clonogenic growth have been developed. From the growth kinetics and the composition of the cell colonies, the numbers and differentiation stages of their progenitor cells can be deduced.

In the in vivo clonogenic assay, multipotent hemopoietic stem cells (CFU-S) form colonies that can be recognised as nodules in the spleens of lethally irradiated mice. No lymphocytes are present in these nodules. Lymphocytes are also absent from the colonies which have been observed in the in vitro clonogenic assay for multipotent cells. Therefore, analysis of these colonies does not contribute to knowledge about T-cell progenitor cells. Very recently, however, growth of T cells in such cultures has been claimed by Messner et al. (1982). Committed progenitor cells of the erythroid cells, the granulocytes and macrophages and the thrombocytes do form colonies in vitro upon stimulation with the appropriate regulating factors. Until very recently, no clonogenic assay system was available for the investigation of T-cell differentiation from early, primitive progenitor cells. Therefore, an in vivo assay system is developed which allows quantification and analysis of T-cell progenitors, although it is not a clonogenic assay. In this assay, which is described in Chapter 2, Section 17, bone marrow cells were transplanted into lethallly irradiated H-2 compatible mice. T cells of donor and recipient origin have different Thy-1 allotype surface antigens. This allows a quantitative determination of the donor derived thymus cells. The rate of growth of the thymocytes of donor allotype in the exponential growth phase, which lasts to 19 to 20 days after transplantation, is independent of the number of bone marrow cells transplanted, until the thymus cell population reaches its age dependent maximum size. In this assay, the prothymocytes are defined as those cells that are capable of producing Thy-1$^+$ cells in the thymus of a lethally irradiated host.

From the growth kinetics of the thymus cells, two methods for quantification of the prothymocytes have been derived. The first is based on the assumption

that donor cells in the thymus after bone marrow transplantation are derived from the marrow cells that home into the thymus early after infusion. The number of progenitor cells can then be calculated by extrapolation of the donor thymocyte growth curve to the day of transplantation.

Relative quantification has been achieved by comparison of the growth curves for donor derived thymocytes after transplantation of different numbers of thymocyte progenitor cells. A delay of one doubling time between two parallel growth curves is then explained as a decrease of 50 percent in the number of progenitor cells.

There is a direct relationship between the number of normal bone marrow cells transplanted and the relative number of prothymocytes in the inoculum. Prothymocytes are Thy-1$^-$ cells (Chapter 3, Section 2.6).

Analysis of donor derived thymocytes with fluorescein labeled monoclonal anti-Thy-1 antibodies in a fluorescence activated cell sorter provides evidence that two donor derived cell populations with different expression of Thy-1 are present in the thymus as early as 24 h after transplantation of bone marrow cells. The major subpopulation (90-100%) of donor derived cells at that time shows a "low" Thy-1 expression. In a normal thymus, cells with "low" Thy-1 antigen density form a minor subpopulation (10-20%) (Chapter 3, Section 2.1).

Subpopulations with "high" and "low" Thy-1 antigen density from day two after transplantation develop simultaneously but with different growth rates. Rapidly growing (T_d= 26 h) "high" Thy-1$^+$ cells outnumber the slowly growing (T_d= 40 h) "low" Thy-1$^+$ cells from day 4 after transplantation. The development of both subpopulations reaches a plateau phase at a time after transplantation that is dependent on the number of progenitor cells transplanted.

Following lethal irradiation and bone marrow grafting, a transient thymocyte population develops which is derived from host radiation resistant committed T progenitors. The regeneration initially starts with a majority of "low" Thy-1$^+$ cells, then "high" Thy-1$^+$ cells rapidly develop. A maximum population size is reached at day 12 to 14 after transplantation. From that time on, the host cells decrease in number and they cannot be detected after day 25 following irradiation (Chapter 3, Section 2.2).

In the normal thymus, CFU-S are very rare. Even after transplantation of 10^7 bone marrow cells containing about 3000 CFU-S, almost no CFU-S were traced in the thymus. But, surprisingly, in a normal thymus and in regenerating thymus early (2-120 h) after transplantation of 10^7 bone marrow cells, almost no prothymocytes were detected (Chapter 3, Section 2.6).

Cell suspensions from both normal and regenerating thymus (early, 2-120 h after transplantation) when transplanted into lethally irradiated recipients produce only a small and transient population of Thy-1$^+$ cells in the thymus. The kinetics of development of this thymus cell population are very similar to what is observed for the development of host derived cells in the thymus following lethal irradia-

tion. It is concluded that, upon arrival in the thymus, early progenitor cells (prothymocytes) are rapidly committed to the T-cell lineage and loose their self-replicating capacity (Chapter 3, Section 2.6).

After transplantation of one week regenerating bone marrow cells, restoration of the thymus cell population is delayed as compared with that observed after transplantation of normal bone marrow. The growth rate of the Thy-1^+ donor derived thymocytes is similar in both cases. From these observations the relative concentration of prothymocytes in regenerating bone marrow has been calculated to be about 1% of the value observed in bone marrow of normal mice (Chapter 5, Section 2.3).

It is assumed that in the absence of more differentiated cells such as prothymocytes, or when these cells are present in very low numbers only, the thymus regeneration may be initiated from more primitive cells, probably pluripotent cells. It is not possible, however, to discriminate between the contribution of primary and secondary progenitors to the thymus cell regeneration. Initiation of thymus cell regeneration from CFU-S explains the delay in thymocyte regeneration after transplantation of regenerating bone marrow. It is concluded that, after transplantation of a sufficient number of bone marrow cells from normal mice, the regeneration of the thymus will not be initially influenced by the progeny of regenerating bone marrow cells. Thymus cell regeneration after transplantation of bone marrow from normal mice, is therefore initiated from prothymocytes present in the bone marrow inoculum. Extrapolation of donor cell growth curves may be used for quantification of the number of prothymocytes in the inoculum. In regenerating bone marrow, the proportion of prothymocytes increases with the time after transplantation (Chapter 5, Section 2.3).

In regenerating bone marrow differences between prothymocytes and CFU-S are observed. In one-week regenerating bone marrow, CFU-S are reduced to about 25% of the number found in normal bone marrow. Prothymocytes and CFU-S in the bone marrow show different regeneration kinetics after bone marrow transplantation. Prothymocytes regenerate more slowly than do CFU-S (Chapter 5, Section 2.3).

In long-term cultures of (C3HxAKR)F_1 bone marrow cells, the ratio of prothymocytes over CFU-S was decreased as compared with fresh marrow. The rate of disappearance of prothymocytes and CFU-S from the cultures is different. The ratio of prothymocytes over CFU-S decreases with time (Chapter 5, Section 2.2).

In fetal liver cells, proportions of CFU-S and committed progenitor cells of the myeloid and erythroid lineages are about 20% of the proportions in bone marrow cells. Prothymocytes are also present in lower proportions (~ 11%). The ratio of prothymocytes over CFU-S in fetal liver cells is about 40% of that in bone marrow (Chapter 5, Section 2.1).

It has been observed that, in hemopoietic systems where cells in the most primitive compartment were rapidly proliferating such as in regenerating marrow, in long-term cultures of bone marrow and in fetal liver, prothymocytes are relatively depleted.

In normal bone marrow, prothymocytes and CFU-S have similar physical characteristics: the distribution of the two cell types according to buoyant density, sedimentation velocity and electrophoretic mobility are very much alike. Progenitor cells for immunologically competent spleen cells and thymocytes have a density distribution similar to that observed for prothymocytes and CFU-S (Chapter 4, Section 2).

In buoyant density gradients of normal bone marrow, the distribution of prothymocytes is homogeneous. For CFU-S the peak in the density distribution ($1.070\ g.cm^{-3}$) is the same as for prothymocytes. The CFU-S distribution has a shoulder between 1.074 and $1.080\ g.cm^{-3}$. In normal bone marrow, the density characteristics for prothymocytes are the same as those observed for resting CFU-S (Visser et al., 1977) (Chapter 4, Section 2). The buoyant density distribution for CFU-S and for prothymocytes in long-term cultured bone marrow ($d=1.070\ g.cm^{-3}$) and in fetal liver cells ($d=1.065\ g.cm^{-3}$) are homogeneous and have the same peak values (Chapter 5). In both sources of hemopoietic progenitor cells, the CFU-S population is in a high proliferative state (Chapter 6).

In three-week regenerating bone marrow, the proportion of prothymocytes is still lower than in bone marrow of normal mice. Their buoyant density distribution completely overlaps the low density subpopulation of CFU-S. It has been concluded from this that prothymocytes have density characteristics in common with the low density subpopulation of CFU-S, independent of the cycling state of the latter cells (Chapter 5).

Since prothymocytes and CFU-S have similar physical characteristics, the former cells may be considered as early hemopoietic progenitors. The earliest progenitors for the erythroid lineages E-CFU-c_1, also have similar buoyant density and sedimentation velocity as CFU-S. GM-CFU-c_1 have a similar density distribution as that of CFU-S but have a slightly higher sedimentation velocity than CFU-S. GM-CFU-c_1 have a lower electrophoretic mobility than CFU-S. Increasing buoyant density, increasing sedimentation velocity and decreasing electrophoretic mobility in general are considered to be characteristic of an increasing level of differentiation of hemopoietic cells, as shown for E-CFU-$c_{1 \rightarrow 2}$ and GM-CFU-$c_{1 \rightarrow 3}$.

Prothymocytes and CFU-S are found to have a different radiation sensitivity according to both *in vitro* and *in vivo* determinations. According to 3H TdR suicide experiments, prothymocytes and CFU-S in normal bone marrow are in a low, but slightly different cycling state. In regenerating bone marrow, prothymocytes and CFU-S are in cycle. Prothymocytes in regenerating bone marrow were sensitive to an inhibitive effect of *in vitro* incubation with cold thymidine. CFU-S and normal bone marrow prothymocytes were not affected by cold thymidine

(Chapter 6).

From experiments in which bone marrow cells were treated with neuraminidase, it has been concluded that at relatively low concentrations of neuraminidase (2×10^{-2} I.U.ml^{-1}), prothymocytes are to a limited extent sensitive to the exposure. CFU-S are strongly reduced upon incubation with the same concentration of neuraminidase. From this, it has been concluded that prothymocytes are less dependent on sialic acid groups on their surface for their homing in the thymus than are CFU-S for their homing into the spleen of lethally irradiated animals. Although prothymocytes and CFU-S had different sensitivities to neuraminidase with respect to their homing pattern in irradiated hosts, they could not be separated according to differences in EPM, even after neuraminidase treatment (Chapter 5).

Differences and similarities of CFU-S and prothymocytes are listed in Table I. These characteristics are best explained if, in early T-cell differentiation, prothymocytes and CFU-S are considered to be different cells.

TABLE I (a)

SUMMARY OF SIMILARITIES BETWEEN PROTHYMOCYTES AND CFU-S

	Source	Prothymocytes	CFU-S
Frequency	normal bm*	~ $300/10^6$ cells	~ $300/10^6$ cells
Buoyant density (peak value)	normal bm	1.071 $g.cm^{-3}$	1.070 $g.cm^{-3}$
" "	regenerating bm	1.070 "	1.070 "
" "	long-term bm cultures	1.069 "	1.070 "
" "	fetal liver	1.065 "	1.065 "
Velocity sedimentation (modal)	normal bm	4.7 $mm.h^{-1}$	4.7 $mm.h^{-1}$
Electrophoretic mobility	normal bm	$-2.10.10^{-4}$ $.cm^2.V^{-1}.s^{-1}$	$-2.10.10^{-4}$ $.cm^2.V^{-1}.s^{-1}$
Electrophoretic mobility	neuraminidase treated bm	$-1.29.10^{-4}$ $.cm^2.V^{-1}.s^{-1}$	$-1.29.10^{-4}$ $.cm^2.V^{-1}.s^{-1}$
Thy-1 surface markers	normal bm	not detectable	not detectable
Proliferative state	normal bm	low (20-40%)	low (10-20%)
" "	regenerating bm	high	high

* bm = bone marrow

TABLE I (b)

SUMMARY OF DIFFERENCES BETWEEN PROTHYMOCYTES AND CFU-S

	Source	Prothymocytes	CFU-S
Frequency	regenerating bm*	~ $3/10^6$ cells	~ $75/10^6$ cells
Frequency	spleen **	almost absent	$30/10^6$ cells
Buoyant density distribution	normal bm	homogeneous	heterogeneous
" " "	regenerating bm	homogeneous	heterogeneous
Proportion of cells as compared to bm	fetal liver	~ 11%	~ 24%
Regeneration kinetics	regenerating bm	slow	rapid
Rate of disappearance	long-term bm cultures	rapid	slower
Radiation sensitivity in vitro	normal bm	D_0=0.92 Gy	D_0=1.22 Gy
Sensitivity to neuraminidase (2×10^{-2} I.U.ml^{-1})	normal bm	low (~ 20%)	high (75%)
Sensitivity to cold thymidine	regenerating bm	high	none

* bm = bone marrow
** Muramatsu et al., 1976; Shisa et al., 1977.

SAMENVATTING

Alle bloedcellen ontstaan uit een gemeenschappelijke voorlopercel: de pluripotente bloedvormende stamcel. Differentiatie van bloedvormende voorlopercellen in de verschillende typen eindcellen vindt plaats in een ononderbroken proces. Dit differentiatie proces levert voortdurend cellen, die nodig zijn als vervanging voor celllen, die verloren zijn gegaan ten gevolge van verbruik, of ten gevolge van de beperkte levensduur van de cellen.

In volwassen muizen vindt de aanmaak van bloedcellen plaats in het beenmerg en de milt. Het herkennen van vroege ontwikkelingsstadia van bloedcellen wordt moeilijker naarmate men verder teruggaat langs de ontwikkelingslijn van de rijpe eindcel naar de stamcel. Dit is het gevolg van het ontbreken van een kenmerkende uiterlijke verschijningsvorm en van de lage frequentie waarmee de vroege stadia voorkomen. Voor het onderzoek aan bloedvormende voorlopercellen zijn bijzondere systemen ontwikkeld, die gebaseerd zijn op het laten groeien van kolonies uit afzonderlijke voorlopercellen, zowel in proefdieren, als in een kunstmatige omgeving (in vitro). Uit de wijze van groeien en de samenstelling van de celkolonies kan het aantal en het ontwikkelingsstadium van de voorlopercel worden afgeleid.

Pluripotente bloedvormende stamcellen (CFU-S)* vormen kolonies in de milt van lethaal bestraalde muizen. Deze kolonies worden als kleine knobbeltjes zichtbaar op de gefixeerde milt. In deze kolonies zijn geen lymfocyten aanwezig. Ook in in vitro kweeksystemen voor multipotente kolonievormende cellen van muizen, werden geen lymfocyten gevonden in de kolonies. Het onderzoek, dat tot nu toe is gedaan aan deze kolonie vormende bloedcellen heeft daardoor niet bijgedragen tot de kennis van de voorloper cellen van de T lymfocyten. Zeer recent echter hebben Messner et al. (1982) mededeling gedaan van hun waarneming van T lymfocyten in culturen voor multipotente bloedvormende humane cellen. Voorlopercellen van rode bloedcellen, granulocyten en macrofagen en van trombocyten vormen wel kolonies in vitro als ze, door toevoegen van de juiste regulerende factoren, daartoe worden aangezet.

Tot voor kort was er geen in vitro systeem beschikbaar voor kwantitatief onderzoek naar T-cel differentiatie vanuit vroege primitieve voorlopercellen van muizen. Daarom is een proefdiermodel ontwikkeld waarmee de T-cel voorlopers kunnen worden onderzocht en hun aantal vastgesteld. Dit systeem, beschreven in hoofdstuk 2, berust niet op het vormen van kolonies, maar op analyse van de celgroei in de thymus na beenmergtransplantatie. Lethaal bestraalde muizen dienen als ontvangers van beenmergcellen van donoren met dezelfde histocompatibiliteits-

---

* CFU-S: kolonievormende cel in milt

antigenen. De T-cellen van donor en ontvanger verschillen in het T-cel oppervlakte alloantigeen Thy-1. Op verschillende tijdstippen na beenmergtransplantatie kan daardoor in de thymus van de lethaal bestraalde ontvanger worden vastgesteld hoeveel thymus lymfocyten afkomstig zijn van donor voorlopercellen. De groeisnelheid van de thymocyten met het donor allotype is tijdens de exponentiële groeifase (tot dag 19 à 20 na transplantatie) onafhankelijk van het aantal beenmergcellen dat is getransplanteerd. De exponentiële groeifase eindigt ook indien de celpopulatie in de thymus een omvang bereikt gelijk aan die van normale dieren van het ontvanger type van dezelfde leeftijd.

De prothymocyten, de voorlopers van de thymuslymphocyten, worden in dit systeem gedefinieerd als: die cellen, die in staat zijn om in de thymus van een lethaal bestraalde ontvanger Thy-1$^+$ cellen te vormen. Van de groeicurven voor thymocyten kan op twee manieren het aantal prothymocyten worden afgeleid. De eerste is gebaseerd op de aanname dat de donor type thymocyten in de thymus afkomstig zijn van de voorlopercellen, aanwezig in het beenmerg transplantaat, die direkt naar de thymus gaan. Het aantal voorlopercellen wordt dan berekend door extrapolatie van de groeicurven naar de dag van transplantatie. De tweede methode is gebaseerd op bepaling van het relatieve aantal prothymocyten op grond van het vergelijken van groeicurven voor thymocyten afkomstig van de donor na transplantatie van verschillende aantallen voorlopercellen. Een verschil van een populatieverdubbelingstijd tussen twee parallelle groeicurven wordt verondersteld te zijn veroorzaakt door een verschil van 50% in het aantal voorlopercellen.

Er is een direkte relatie gevonden tussen het aantal beenmergcellen dat wordt getransplanteerd en het relatieve aantal prothymocyten in het transplantaat. Prothymocyten zijn Thy-1$^-$ (Hoofdstuk 3, sectie 2.6).

Twee subpopulaties van donor afkomstige thymocyten met een verschillende expressie van Thy-1 antigenen kunnen al 24 uur na transplantatie van beenmergcellen in de thymus worden aangetoond met behulp van fluorescerende monoclonale anti-Thy-1 antilichamen en de FACS. Op dat moment hebben de meeste donor thymuscellen (90-100%) een lage Thy-1 oppervlakte dichtheid. In de normale thymus vormt dit type cellen altijd een minderheid (10-20%) (Hoofdstuk 3, sectie 2.1).

Subpopulaties met een sterke en met een zwakke expressie van Thy-1 antigenen ontwikkellen zich gelijktijdig, maar met een verschillende groeisnelheid. Cellen met een sterke expressie van Thy-1 groeien snel (T_d = 26 uur) en zijn vanaf 4 dagen na transplantatie talrijker dan cellen met een zwakke expressie van Thy-1 antigenen (T_d = 40 uur). Beide subpopulaties bereiken een plateaufase op een tijdstip dat afhankelijk is van het aantal voorlopercellen in het transplantaat.

Na lethale bestraling van de ontvanger ontwikkelt zich tijdelijk in de thymus van een met beenmerg getransplanteerde muis een van de ontvanger afkomstige lymfocytenpopulatie. Deze, van de ontvanger afkomstige thymocyten, worden waarschijnlijk geproduceerd door T-cel voorlopers, die de voor de muis lethale

bestraling overleven. Ook deze thymocytenpopulatie bestaat initieel hoofdzakelijk uit cellen met een zwakke Thy-1 expressie. Daarna ontwikkelt zich snel een ontvanger type thymuscellen met een sterke expressie van Thy-1. De totale, van de ontvanger afkomstige, thymuscelpopulatie bereikt zijn maximale omvang tussen dag 12 en 14 na transplantatie. Daarna neemt ze af in aantal en vanaf dag 25 na transplantatie is ze niet meer aantoonbaar (Hoofdstuk 3, sectie 2.2).

In een normale thymus zijn CFU-S zeldzaam. Zelfs na transplantatie van 10 x 10^6 beenmergcellen, die ongeveer 3000 CFU-S bevatten, is er minder dan één CFU-S per thymus gevonden. Maar, zeer verrassend, zijn er in de normale thymus en in de regenererende thymus, 2 dagen na transplantatie van 10 x 10^6 beenmergcellen, ook nauwelijks prothymocyten (Hoofdstuk 3, sectie 2.6). De cellen in suspensies van de normale en van de regenererende thymus, vroeg (2-120 uur) na transplantatie, hebben een beperkt vermogen tot zelf-replicatie. Voorlopercellen afkomstig uit de suspensie van thymuscellen van een muis die kort tevoren een beenmergtransplantatie heeft ondergaan, geven in de thymus van een tweede bestraalde ontvanger aanleiding tot een kleine, slechts tijdelijk aanwezige thymuscelpopulatie. De groeiwijze daarvan is dezelfde als waargenomen voor de nakomelingen van thymuscel voorlopers, die de lethale bestraling overleven. Hieruit kan worden geconcludeerd dat vroege voorlopercellen, de prothymocyten, na aankomst in de thymus zeer snel differentiëren in de richting van de T-cel en hun zelfreplicerend vermogen verliezen (Hoofdstuk 3, sectie 2.6).

Na transplantatie van regenererend beenmerg (een week na beenmergtransplantatie) is regeneratie van de thymuspopulatie vertraagd in vergelijking met de ontwikkeling na transplantatie van normaal beenmerg. De groeisnelheid is in beide gevallen dezelfde. De prothymocytenconcentratie in beenmerg van muizen, die een week tevoren zijn transplanteerd met 3 x 10^6 cellen, is ongeveer 1% van de concentratie in beenmerg van normale muizen (Hoofdstuk 5, sectie 2.3). Aangenomen wordt, dat indien prothymocyten in zeer lage aantallen voorkomen of geheel afwezig zijn, de regeneratie van de thymuscelpopulatie door primitieve cellen, die uit de CFU-S ontstaan, wordt geïnitieerd. Dit kan de vertraging in de regeneratie van de thymuscelpopulatie na transplantatie van regenererend beenmerg verklaren. Het is echter niet mogelijk onderscheid te maken tussen de bijdrage aan de thymusregeneratie door primaire (CFU-S) en secundaire (prothymocyten) voorlopercellen. Desalniettemin is gebleken, dat na transplantatie van een voldoende aantal beenmergcellen van normale muizen, de initiële regeneratiefase van de thymuscellen niet wordt beinvloed door voorlopercellen, die in het regenererende beenmerg worden geproduceerd. Regeneratie van thymuscellen na transplantatie van beenmerg van normale muizen vindt dus plaats vanuit de prothymocyten, die aanwezig zijn in het beenmergtransplantaat. Daarom kan het aantal prothymocyten in een transplantaat inderdaad worden berekend uit de extrapolatie van de thymocyten groeicurve. In regenererend beenmerg neemt het aantal prothymocyten toe met de tijd na transplantatie (Hoofdstuk 5, sectie 2.3).

Een week na transplantatie van 3 x 10^6 beenmergcellen is de concentratie van CFU-S in regenererend beenmerg ongeveer 25% van de concentratie in normaal beenmerg. In het regenererend beenmerg herstellen de prothymocyten en CFU-S populaties zich op verschillende wijzen. Prothymocyten regenereren langzamer dan CFU-S (Hoofdstuk 5, sectie 2.3).

Als (C3H x AKR)F_1 beenmergcellen langdurig in vitro gekweekt worden, is het aantal prothymocyten per CFU-S in de culturen lager dan in normaal beenmerg. Prothymocyten en CFU-S verdwijnen uit de culturen, met de duur van de kweek. Prothymocyten verdwijnen sneller dan CFU-S (Hoofdstuk 5, sectie 2.2).

In foetale levercellen is de concentratie van CFU-S en voorlopercellen van de myeloïde en erythroïde reeks ongeveer 20% van die in beenmerg. Prothymocyten komen in een nog lagere concentratie voor (~ 11%). Het aantal prothymocyten per CFU-S in foetale lever is ongeveer 40% van dat in beenmerg (Hoofdstuk 5, sectie 2.1).

In die bloedvormende systemen waar de meest primitieve voorlopercellen snel delen, zoals in regenererend beenmerg, langdurig gekweekt beenmerg en in foetale lever wordt in het algemeen een relatief lage concentratie van prothymocyten waargenomen.

In normaal beenmerg hebben prothymocyten en CFU-S vrijwel dezelfde fysische eigenschappen. De distributies van de twee celtypen volgens zweefdichtheid, sedimentatiesnelheid en elektroforetische mobiliteit, vertonen sterke overeenkomst. Voorlopercellen van immuuncompetente milt- en thymuscellen hebben vrijwel dezelfde dichtheidsdistributie als prothymocyten en CFU-S (Hoofdstuk 4, sectie 2). In dichtheidsgradiënten is de distributie van prothymocyten homogeen. Prothymocyten hebben in de dichtheidsverdeling dezelfde piekwaarde als CFU-S. In de CFU-S distributie zit een schouder tussen 1.074 en 1.080 $g.cm^{-3}$. In normaal beenmerg is de dichtheidsdistributie voor prothymocyten dezelfde als voor CFU-S in rust (d = 1.070 $g.cm^{-3}$) (Visser et al., 1977).

De dichtheidsverdelingen van CFU-S en van prothymocyten in langdurig gekweekt beenmerg (d = 1.070 $g.cm^{-3}$) en foetale levercellen (d = 1.065 $g.cm^{-3}$) zijn homogeen en hebben eenzelfde piekwaarde (Hoofdstuk 5). CFU-S in deze bronnen van bloedvormende cellen zijn snel delend. In drie weken regenererend beenmerg is het aantal prothymocyten nog steeds lager dan in beenmerg van normale muizen. De dichtheidsdistributie is dezelfde als die van de CFU-S subpopulaties met lage zweefdichtheid. Prothymocyten hebben dus dezelfde zweefdichtheid als de lichtere CFU-S onafhankelijk van de mate waarin de CFU-S prolifereren. Omdat CFU-S en prothymocyten vrijwel dezelfde fysische kenmerken hebben, kunnen deze laatsten als relatief vroege voorlopercellen worden beschouwd. De vroegste voorlopers van de erythroïde reeks (E-CFU-c_1) hebben ook eenzelfde zweefdichtheid en sedimentatiesnelheid als CFU-S. GM-CFU-c_1 hebben wel dezelfde dichtheid als CFU-S maar hebben een hogere sedimentatie snelheid. GM-CFU-c_1 hebben een lagere elektroforetische mobiliteit dan CFU-S.

Toenemende dichtheid, toenemende sedimentatie snelheid en afnemende elektroforetische mobiliteit worden algemeen beschouwd als kenmerkend voor een toenemend differentiatie niveau van hemopoietische cellen, zoals aangetoond voor E-$CFUc_{1 \rightarrow 2}$ (Wagemaker et al., 1977), en voor GM-$CFUc_{1 \rightarrow 3}$ (Bol and Williams, 1980; Bol, 1980).

Prothymocyten en CFU-S hebben een verschillende stralingsgevoeligheid zowel volgens in vitro als in vivo bepalingen. Prothymocyten en CFU-S in normaal en regenererend beenmerg hebben dezelfde gevoeligheid voor ^{3}H TdR. De frequentie van delende cellen zal voor beide celtypen onder dezelfde omstandigheden gelijk zijn. Prothymocyten in regenererend beenmerg worden in hun functie geremd door in vitro incubatie met koude thymidine. CFU-S en prothymocyten in normaal beenmerg hebben deze gevoeligheid niet (Hoofdstuk 6).

In vitro behandeling met relatief lage concentraties neuraminidase (2×10^{-2} I.U.$ml.^{-1}$) heeft slechts beperkte invloed op prothymocyten in beenmergcellen van normale muizen. CFU-S nemen sterk in aantal af na incubatie met dezelfde concentratie neuraminidase. Geconcludeerd wordt dat prothymocyten voor hun beweging naar de thymus minder afhankelijk zijn van de siaalzuurgroepen op hun oppervlak dan CFU-S voor hun transport naar de milt van bestraalde dieren (Hoofdstuk 6).

Ofschoon prothymocyten en CFU-S met betrekking tot het verkeer naar hun "doel"-orgaan verschillend gevoelig zijn voor neuraminidase kunnen ze niet worden gescheiden naar verschillen in elektroforetische mobiliteit, zelfs niet na neuraminidase behandeling.

Verschillen en overeenkomsten tussen CFU-S en prothymocyten zijn in een tabel bijeengezet (pag.136,137). Deze kenmerken kunnen het best worden verklaard wanneer prothymocyten en CFU-S als twee verschillende cellen worden beschouwd.

ABBREVIATIONS

AO	Acridine Orange
B-(cell) lymphocyte	lymphocyte which is part of the humoral immune system
bm	bone marrow
BSA	bovine serum albumine
CFU-S	colony forming unit-spleen
CFU-T	colony forming unit-T cells
cpm	counts per minute
ConA	concanavaline A
^{14} CTdR	^{14}C labeled thymidine
D_0	dose of radiation that kills 63% of cells at risk
dCTP	deoxycitidine triphosphate
dTTP	deoxythymidine triphosphate
E-BFU	erythroid burst forming unit
EBH	Eagle's Basal Medium with HEPES
E-CFU-c	erythroid colony-forming-unit in culture
EPM	electrophoretic mobility
ENU	1-ethyl-1-nitroso urea
F_1	first generation hybrid
FACS	fluorescence activated cell sorter
FDA	fluorescein diacetate
FITC	fluorescein isothiocyanate
FLS	forward light scatter
FMF	flow microfluorometry
GM-CFU-c	granulocyte-macrophage colony-forming-unit in culture
G_0	cell cycle phase, cells are in rest
GvHD	graft versus host disease
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
^{3}H TdR	tritiated Thymidine
HSC	hemopoietic stem cell
Lyt 1,2,3	T-cell alloantigen, specifying subpopulations
MHC	major histocompatibility (H-2) complex in the mouse. H-2 subregions are a.o.: D, K, I-A, I-E.
MLC	mixed lymphocyte culture
PHA	phytohaemagglutinine
PI	propidium iodide
S.D.	standard deviation
S.E.	standard error

T_d	doubling time
Thy-1	T-cell surface antigen (common)
TL	thymus leukemia antigen
T-(cell) lymphocyte	thymus processed (cell) lymphocyte ($Thy\text{-}1^+$)
TNC	total nucleated cells

ACKNOWLEDGMENTS

The work described in this thesis was made possible through the cooperation and support of my colleagues at the REPGO-Institutes in Rijswijk.
I am especially indebted to:
Prof.Dr. D.W. van Bekkum for inspiring me during the investigation and his criticism during the preparation of this and other manuscripts.
Prof.Dr. C.F. Hollander for giving me the opportunity to complete the investigations in his institute.
Prof.Dr. V.P. Eysvoogel and Prof.Dr.O. Vos for their critical reading of this thesis.
The late Dr. I. Betel for his encouraging and stimulating enthusiasm at the initiation of the studies presented in this thesis.
Dr. R. Daculsi for his cooperation and enthusiastic participation in discussing the preliminary and basic studies.
Dr. J. Eliason for his kind cooperation in the analysis of long-term cultured bone marrow.
Dr. S. Bol for his helpful assistance in doing cell separations in free flow electrophoresis.
Dr. G.J. van den Engh and Dr. J.J. Haaijman for their helpful criticism during the initial stages in the preparation of the manuscript.
The members of the Friday Afternoon Experimental Hematology Club for their active participation in discussing experimental results in all stages of development.
Mr. G van der Westen, Mr. H. Janse, Mrs. E. Blauw and Mr. F. Steinmeier for their excellent technical assistance.
Dr. J. Zoetelief for providing the information on dose distributions in the gamma-irradiation chamber.
Mrs. A. Wittwer and Mr. P. de Wit for their kind assistance in the irradiation of numerous mice.
Mrs. G.A. Beitsma, Mrs. M. Roggenkamp, Mrs. L. Vermeer-Greven, Mrs. A.H. Walop and Mr. J.Ph. de Kler for the preparation of the manuscript.
Dr. A.C. Ford for editing the English text.

The work described in this thesis is part of a study on "The Regulation of Haemopoietic Cell Differentiation" that was supported in part by a program grant of the Dutch Foundation for Medical Research (FUNGO).

Part of the data and figures in this monograph have been or will be published elsewhere. The figures listed below are reprinted with kind permission of the publishers.
Chapter 2, figures 6 and 8, Chapter 3, figures 1, 9, 12 and 13, Chapter 5, figures 6, 8 and 9 are reprinted from Cell Tissue Kinetics, 1981, 14, 179-196.
Chapter 4, figures 1-9 are reprinted from Cell and Tissue Kinetics, 1981, 14, 197-210.

Chapter 5, figures 4 and 5 are reprinted from Experimental Hematology, 1982, 10, 494-503. Chapter 2, figures 1 and 2, and Chapter 3, figures 5, 6, 7 and 10 are reprinted from European Journal of Immunology, 1982, 12, 615-620.

REFERENCES

Abramson, S., Miller, R.G. & Phillips, R.A. (1977) The identification in adult bone marrow of pluripotent and restricted stem cells of myeloid and lymphoid systems. J. Exp. Med. 145, 1567.

Adler, W.H.T., Takiguchi, T., Marsch, B. & Smith, R.T. (1970) Cellular recognition by mouse lymphocytes in vitro. II Specific stimulation by histocompatibility antigens in mixed lymphocyte culture. J. Immunol. 105, 984.

Adler, J.S., Kuznetsky, R.D. & Trobaugh Jr., F.E. (1978) Anti-CFU-S activity of rabbit anti-mouse brain serum: mechanism of action. In: Experimental Hematology Today. S.J. Baum & G.D. Ledney, (eds.). Springer-Verlag, New York, 23.

Ahmed, A., Smith, A.H., Wong, D.M., Thurman, G.B., Goldstein, A.L. & Sell, K.W. (1978) In vitro induction of Lyt surface markers on precursor cells incubated with thymosin polypeptides. Cancer Treat. Rep. 62, 1739.

Al Adra, A.R., Pilarsky, L.M. & McKenzie, I.F.C. (1980) Surface markers on the T cells that regulate cytotoxic T cell responses. I. The Ly phenotype of suppressor T cells changes as a function of time and is distinct from that of helper or cytotoxic T cells. Immunogenetics 10, 521.

Anderson, R.E. & Warner, N.L. (1976) Ionizing radiation and the immune response. Adv. Immunol. 24, 215.

Armason, B.G., Jankovic, B.D. & Waksman, B.H. (1962) A survey of the thymus and its relation to lymphocytes and immune reactions. Blood 20, 167.

Ashman, R.B. (1981) Immunologic unresponsiveness in fetal and neonatal mammals; a paradigm for immune deficiency diseases? In: Immunologic Defects in Laboratory Animals, Vol.I. M.E. Gershon, & B. Merchant, (eds.). Plenum, London, New York, 3.

Bach, J.F., Dardenne, M., Goldstein, A.L., Guha, A. & White, A. (1971) Appearance of T cell markers in bone marrow rosette forming cells after incubation with thymosin a thymic hormone. Proc. Natl. Acad. Sci. USA 68, 2734.

Balner, H. & Dersjant, H. (1964) Early lymphatic regeneration in thymectomized radiation chimearas. Nature 204, 941.

Barendsen, G.W., Koot, C.J., Van Kersen, G.R., Bewley, D.K., Field, S.B. & Parnell, C.J. (1966) The effect of oxygen on impairment of the proliferative capacity of human cells in culture by ionizing radiations of different LET. Int. J. Rad. Biol. 10, 317.

Barnes, D.W.H., Evans, E.P., Ford, C.E. & West, B.J. (1968) Spleen colonies in mice: karyotypic evidence of multiple colonies from single cells. Nature 219, 518.

Basch, R.S. & Goldstein, G. (1974) Induction of T cell differentiation in vitro by Thymin a purified polypeptide hormone of the thymus. Proc. Natl. Acad. Sci. USA 71, 1474.

Basch, R.S. & Goldstein, G. (1975a) Antigenic and functional evidence for the in vitro inductive activity of thymopoietin (thymin) on thymocyte precursors. Ann. N.Y. Acad. Sci. USA 249, 290.

Basch, R.S. & Goldstein, G. (1975b) Thymopoietin-induced acquisition of responsiveness to T cell mitogens. Cell. Immunol. 20, 218.

Basch, R.S. & Kadish, J.L. (1977) Hematopoietic thymocyte precursors. II. Properties of the precursors. J. Exp. Med. 145, 405.

Basch, R.S., Kadish, J.L. & Goldstein, G. (1978) Hematopoietic thymocyte precursors. IV. Enrichment of the precursors and evidence for heterogeneity. J. Exp. Med. 146, 1843.

Basford, H.L. & Goodman, J.W. (1974) Effects of lymphocytes from the thymus and lymphe nodes on differentiation of hemopoietic spleen colonies in irradiated mice. J. Cell. Physiol. 84, 37.

Becker, A.J., McCulloch, E.A. & Till, J.E. (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse bone marrow cells. Nature 197, 452.

Becker, A.J., McCulloch, E.A., Siminovitsch, L. & Till, J.E. (1965) The effect of different demands for blood cell production on DNA synthesis by haemopoietic colony-forming cells of mice. Blood 26, 269.

Betel, I. & Van der Westen, G. (1978) Cytofluorographic multiparameter analysis of mitogen induced activation and proliferation of mouse thymocytes. In: Pulse Cytophotometry, D. Lutz, (ed.), 3rd Int. Symposium, Vienna, 1977. Ghent, European Press, 289.

Betel, I., Mathieson, B.J. & Sharrow, S.O. (1980a) Differential agglutination of thymocytes by peanut agglutinin: Phenotypic characterization of the subpopulations by flow microfluorometry. In: Protides of the Biological Fluids. Proc. 27th Colloquium, H. Peeters (ed.), Pergamon Press, Oxford, 27, 529.

Betel, I., Mathieson, B.J., Sharrow, S.O. & Asofsky, R. (1980b) Distribution of Lyt phenotypes in thymocyte subpopulations as measured by flow microfluorometry: selective enrichment of Lyt-1$^+$2$^-$3$^-$ thymocytes. J. Immunol. 124, 2209.

Bevan, M.J. (1977) In a radiation chimaera host H-2 antigens determine immune responsiveness of donor cytotoxic cells. Nature 269, 417.

Bevan, M.J. & Fink, J.P. (1978) The influence of the thymus H-2 antigens on the specificity of maturing killer and helper cells. Immunol. Rev. 42, 3.

Blacket, H.M. (1974) Statistical accuracy to be expected from cell colony assays with special reference to the spleen colony assay. Cell Tissue Kinet. 7, 407.

Blacket, H.M., Milliard, R.E. & Belcher, H.M. (1974) Thymidine suicide in vivo and in vitro of spleen colony forming and agar colony forming cells of mouse bone marrow. Cell Tissue Kinet. 7, 309.

Blanden, R.B. & Andrew, M.E. (1979) Primary activated cytotoxic T-cell response in semi allogeneic chimeras are not absolutely restricted to H-2 type. J. Exp. Med. 149, 535.

Blomgren, H. & Andersson, B. (1971) Characteristics of the immunocompetent cells in the mouse thymus. Cell population changes during cortisone-induced atrophy and subsequent regeneration. Cell. Immunol. 1, 545.

Boersma, W., Betel, I., Daculsi, R. & Van der Westen, G. (1981) Postirradiation thymocyte regeneration after bone marrow transplantation. Regeneration and quantification of thymocyte progenitor cells in the bone marrow. Cell Tissue Kinet. 14, 179.

Boersma, W. & Eliason, E. (1982) Quantification of prothymocytes in long-term bone marrow cultures. Exp. Hematol. 10, 494.

Bol, S.J.L. (1980) The recognition of early developmental stages in haemopoiesis. Thesis, Erasmus University Rotterdam, The Netherlands.

Bol, S.J.L., Visser, J., Williams, N. & Van den Engh, G.J. (1977) Physical characterisation of haemopoietic progenitor cells by equilibrium density centrifugation. In: Cell separation methods, H. Bloemendal (ed.), Elsevier/ North Holland Biomedical Press, Amsterdam, 39.

Bol, S.J.L., Visser, J.W.M. & Van den Engh, G. (1979) The physical separation of three subpopulations of granulocyte-macrophage progenitor cells from mouse bone marrow. Exp. Hematol. 7, 548.

Bol, S.J.L. & Williams, N. (1980) The maturation state of three types of granulocyte-macrophage progenitor cells from mouse bone marrow. J. Cell. Physiol. 102, 233.

Boyse, E.A., Miyazawa M., Aoki, T. & Old, L.J. (1968) Ly-A and Ly-B: two systems of lymphocyte antigens in the mouse. Proc. R. Soc. (London) Ser. B. Biol. Sci. 170, 175.

Boyse, E.A., Itakura, K., Stockert, E., Iritani, C.A. & Miura, M. (1971) Ly-C: a third locus specifying alloantigens expressed only on thymocytes and lymphocytes. Transplantation 11, 351.

Boyse, E.A. & Cantor, H. (1978) Immunogenetic aspects of biologic communication: A hypothesis of evolution by program duplication. In: Birth Defects: Original Article Series. The National Foundation (ed.). Vol. XIV 2, 249.

Brown, M.B., Kaplan, H.S., Weymouth, P.P. & Paull, J. (1953) Effect of intravenously injected bone marrow cell suspensions on thymic regeneration in irradiated C57 Black mice. Science 117, 693.

Brumby, M. & Metcalf, D. (1967) Migration of cells to the thymus demonstrated by parabiosis. Proc. Soc. Exp. Biol. 124, 99.

Bryant, B.J. (1972) Renewal and fate in the mammalian thymus: Mechanisms and influence of thymocytokinetics. Eur.J. Immunol. 2, 38.

Burnet, F.M. (1959) The clonal selection theory of acquired immunity. VanderBilt University Press, Nashville, Tennessee.

Byrne, P.V., Heit, W. & Kubanek, B. (1979) The proliferative status of density subpopulations of granulocyte-macrophage progenitor cells. Exp. Hematol. 7, 105.

Cantor, H. & Boyse, E.A. (1975a) Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. J. Exp. Med. 141, 1376.

Cantor, H. & Boyse, E.A. (1975b) Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly^+ cells in the generation of killer activity. J. Exp. Med. 141, 1390.

Cantor, H., Simpson, E., Sato, V.L., Fathman, C.G. & Herzenberg, L.A. (1975) Characterisation of subpopulations of T lymphocytes. I. Separation and functional studies of peripheral T-cells binding different amounts of fluorescent anti-Thy-1.2 (Theta) antibody using a fluorescence-activated cell sorter (FACS). Cell. Immunol. 15, 180.

Cantor, H. & Weissman, I. (1976) Development and function of subpopulations of thymocytes and T cells. Progr. Allergy 20, 1.

Cantor, H. & Boyse, E.A. (1977) Lymphocytes as models for the study of mammalian cellular differentiation. Immunol. Rev. 33, 105.

Cantor, H., McVay-Boudreau, L., Hugenberger, J., Naidorf, K., Shen, F.W. & Gershon, R.K. (1978) Immunoregulation circuits among T cell subsets. II. Physiologic role of feed back inhibition in vivo: Absence in NZB mice. J. Exp. Med. 147, 1116.

Ceredig, R. & MacDonald, H.R. (1982) Phenotypic and functional properties of murine thymocytes. II. Quantitation of host and donor derived cytolytic T lymphocyte precursors in regenerating radiation bone marrow chimeras. J. Immunol. 128, 614.

Ching, L.M., Muroaka, S. & Miller, R.G. (1981) Differentiation of T cells from immature precursors of murine T cell colonies. J. Immunol. 127, 2156.

Chiscon, M.O. & Golub, E.S. (1972) Functional development of the interacting cells in the immune response. I. Development of T and B cell function. J. Immunol. 108, 1379.

Claësson, M.H. & Hartmann, H.R. (1976) Cytodynamics in the thymus of young adult mice: A quantitative study on the loss of thymic blast cells and non-proliferative small thymocytes. Cell Tissue Kinet. 9, 273.

Cohen, M.L. & Scott, D.W. (1980) Functional differentiation of T-cell precursors. II. Recovery of immunocompetence in lethally irradiated bone marrow reconstituted mice. Thymus 2, 61.

Cohen, M.W., Thorbecke, G.J., Hochwald, G. & Jacobson, E.B. (1963) Induction of Graft versus Host reaction in newborn mice by injection of newborn or adult homologous thymus cells. Proc. Soc. Exp. Biol. Med. 114, 242.

Colquhoun, D. (1974) Lectures on biostatistics, Clarendon Press, Oxford.

Crissman, H.A. & Steinkamp, J.A. (1973) Rapid simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. J. Cell. Biol. 59, 766.

Cross, A.M., Leuchars, E. & Miller, J.F.A.P. (1964) Studies on the recovery of the immunoresponse in irradiated mice thymectomized in adult life. J. Exp. Med. 119, 837.

Crouch, B.G. (1959) Transplantation of fetal hemopoietic tissues into irradiated mice and rats. In: Proc. 7th Congr.Eur. Soc. Haematol., London, 973.

Curry, J.L. & Trentin, J.J. (1967) Hemopoietic spleen colony studies. I. Growth and differentiation. Dev. Biol. 15, 395.

Daculsi, R., Astier, T., Legrand, E. & Duplan, J.F.(1982) Terminal deoxynucleotidyl transferase activity in the regenerating thymus of X-irradiated mouse. Thymus 4, 45.

Dalmasso, A.P., Sjodin, K. & Good, R.A. (1963) Studies on the role of the thymus in immunobiology: Reconstitution of immunologic capacity in mice thymectomized at birth. J. Exp. Med. 118, 1089.

Dardenne, M., Papiernik, M., Bach, J.F. & Stutman, O. (1974) Studies on thymic products. III. Epithelial origin of the serum thymic factor. Immunology 27, 299.

Dardenne, M. & Bach, J.F. (1976) Ontogeny of thymic factor secretion. Ann. Immunol. 127, 963.

Dardenne, M., Charreire, J. & Bach, J.F. (1978) Alteration in thymocyte surface markers after in vivo treatment by serum thymic factor. Cell. Immunol. 39, 47.

Dardenne, M. & Tubiana, N. (1979) Neonatal thymus grafts. II. Cellular events. Immunology 36, 215.

Darzynkiewicz, D., Traganos, F., Sharples, S.T. & Melamed, M.R. (1976) Lymphocyte stimulation: A rapid multiparameter analysis. Proc. Natl. Acad. Sci. 73, 2881.

De Vries, M.J., Van Putten, L.M., Balner, H. & Van Bekkum, D.W. (1964) Lésions suggérant une réactivité autoimmune chez des souris atteintes de la "runt disease" après thymectomie neonatale. Rev. Fr. Etudes Clin. Biol. 9, 381.

Dexter, T.M., Allen, T.D., Lajtha, L.G., Schofield, R. and Lord, B.I. (1973) Stimulation of differentiation and proliferation of haemopoietic cells in vitro. J. Cell. Physiol. 82, 461.

Dexter, T.M. & Lajtha, L.G. (1974) Proliferation of haemopoietic stem cells in vitro. Br. J. Haematol. 28, 525.

Dexter, T.M., Allen, T.D. & Lajtha, L.G. (1977) Conditions controlling the proliferation of hemopoietic stem cells in vitro. J. Cell Physiol. 91, 335.

Dexter, T.M., Moore, M.A.S. & Sheridan, A.P.C. (1979a) Maintenance of hemopoietic stem cells and production of differentiated progeny in allogeneic and semiallogeneic bone marrow chimeras in vitro. J. Exp. Med. 145, 1612.

Dexter, T.M., Wright, P., Krizsa, F. & Lajtha, L.G. (1979b) Regulation of hemopoietic stem cell proliferation in long term bone marrow cultures. Biomedicine 27, 344.

Dexter, T.M. & Testa, N.G. (1980) In vitro methods in haemopoiesis and lymphopoiesis. J. Immunol. Methods 38, 177.

Doenhoff, M.J., Davies, A.J.S., Leuchars, F. & Wallis, V. (1970) The thymus and circulating lymphocytes of mice. Proc. R. Soc. (London) Ser. B. Biol. Sci. 176, 69.

Doenhoff, M.J. & Davies, A.J.S. (1971) Reconsitution of the T cell pool after irradiation of mice. Cell. Immunol. 2, 82.

Doherty, P.C. & Bennink, J.R. (1979) Vaccinia-specific cytotoxic T cell responses in the context of H-2 antigens not encountered in the thymus may reflect abberant recognition of a virus complex. J. Exp. Med. 149, 150.

Douglas, T.C. (1972) Occurrence of Thêta-like antigens in rats. J. Exp. Med. 136, 1054.

Droege, W., Zucker, R. & Jauke, U. (1974) Cellullar composition of the mouse thymus. Cell. Immunol. 12, 173.

Droege, W. (1976) "Early T cells " and "late T cells", suggestive evidence for two cell lineages with separate developmental pathways. Eur. J. Immunol. 6, 763.

Droege, W. & Zucker, R. (1975) Lymphocyte subpopulations in the thymus. Transpl. Rev. 25, 3.

Dukor, P., Miller, J.A.F.P., House, W. & Allmann, V. (1965) Regeneration of thymus grafts. I. Histological and cytological aspects. Transplantation 3, 639.

Duplan, J.F. (1970) Radiosensitivity of the hemopoietic colony-forming cells of the mouse. In: Haemopoietic cellular proliferation. F. Strohman, jr. (ed.), New York, 189.

El Arini, M.O., & Osoba, D. (1973) Differentiation of thymus-derived cells from precursors in mouse bone marrow. J. Exp. Med. 137, 821.

Eliason, J.F., Testa, N.G. & Dexter, T.M. (1977) Erythropoietin-stimulated erythropoiesis in long-term bone marrow culture. Nature 281, 382.

Everett, N.B. & Tyler, R.W. (1969) Radioautographic studies of the stemcell in the thymus of the irradiated rat. Cell Tissue Kinet. 2, 347.

Fathman, C.G., Small, M., Herzenberg, L. & Weissman, I.L. (1975) Thymus cell maturation. II. Differentiation of their "Mature" subclasses in vivo. Cell. Immunol. 15, 109.

Fauser, A.A. & Messner, H.A. (1979) Indentification of megakaryocytes, macrophages, and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. Blood 53, 1023.

Fink, P.J. & Bevan, M.J. (1981) Influence of H-2 antigen expression on killer T cell specificity differentiation and induction. Proc. Natl. Acad. Sci. USA 78, 6401.

Ford, C.E., Hamerton, J.L., Barnes, D.W.H. & Loutit, J.F. (1956) Cytological identification of radiation chimaeras. Nature 177, 452.

Ford, C.E. & Micklem, H.S. (1963) The thymus and lymph nodes in radiation chimaeras. Lancet 7277, 359.

Ford, C.E., Micklem, H.S., Evans, E.P., Gray, J.G. & Ogden, D.A. (1966) The inflow of bone marrow cells to the thymus. Studies with part-body irradiated mice injected with chromosome marked bone marrow and subjected to antigenic stimulation. Ann. N.Y. Acad. Sci. 129, 283.

Goldschneider, I., Shortman, K., McPhee, D., Linthicum, S., Mitchell, G., Battaye, F. & Boilum, F.J. (1982) Identification of subsets of proliferating low Thy-1 cells in thymus cortex and medulla. Cell. Immunol. 69, 59.

Golub, E.S. (1971) Brain associated theta antigens: Reactivity of rabbit-anti-mouse brain with mouse lymphoid cells. Cell. Immunol. 2, 253.

Golub, E.S. (1972) Brain associated stem cell antigen: An antigen shared by brain and hemopoietic cells. J. Exp. Med. 136, 369.

Good, R.A., Dalmasso, A.P., Martinez, C., Archer, O.K., Pierce, J.C. & Papermaster, B.W. (1962) The role of the thymus in development of immune capacity in rabbits and mice. J. Exp. Med. 116, 733.

Goodman, J,W. & Shinpock, S.G. (1968) Influence of thymus cells on erythropoiesis of parental marrow in irradiated hybrid mice. Proc. Soc. Exp. Biol. Med. 139, 417.

Goodman, J.W., Shinpock, S.G. & Basford, H.L. (1979) Thymic involvement in control of bone marrow growth. Use of T cell depleted hybrid mice. Exp. Hematol. 17, 17.

Gorcinsky, R.M., Miller, R.G. & Phillips, R.A. (1970) Homogeneity of antibody-producing cells as analysed by their buoyant density in gradients of ficoll. Immunology 19, 817.

Gorcinsky, R.M., & McRae, S. (1977) Differentiation of functionally active mouse T lymphocytes from functionally inactive bone marrow precursors. I. Kinetics of recovery of T cell function in lethally irradiated bone marrow reconstituted thymectimized and non-thymectimized mice. Immunology 33, 697.

Gottschalk, A. & Drzhiek, R. (1972) Neuraminidase as a tool for structural analysis. In: Glycoproteins, their composition structure and function. A. Gottschalk, (ed.), Elsevier, Amsterdam.

Greenberger, J.S. (1978) Sensitivity of corticosteroid-dependent insulin resistant lipogenesis in marrow preadipocytes of obese-diabetic (db/db) mice. Nature 275, 752.

Haar, J.L. & Loor, F. (1981) Selective migration of "null" cells towards a thymus factor in vitro. Thymus 3, 187.

Haaijman, J.J., Micklem, S.H., Ledbetter, J.A., Dangl, J.L., Herzenberg, L.A. & Herzenberg, L.A. (1981) T cell ontogeny: organ location of maturing populations (defined by surface antigen markers) is similar in neonates and adults. J. Exp. Med. 153, 605.

Haines, K.A. & Siskind, G.W. (1980) Ontogeny of T cell function. I. Acquisition of helper cell activity by the thymus. J. Immunol. 124, 1878.

Hannig, K. (1969) The application of free-flow electrophoresis to the separation of macromolecules and particles of biological importance. In: Modern separation methods of macromolecules and particles. Th. Gerritsen, (ed.), John Wiley and Sons, Inc., New York, Vol.I, 45.

Hannig, K., Wirth, H., Meyer, B.H. & Zeiller, K. (1975) Free flow electrophoresis. I. Theoretical and experimental investigations of the influence of mechanical and electrokinetic variables on the efficiency of the method. Hoppe Seylers, Z. Physiol. Chem. 356, 1209.

Harris, A.W. (1977) Lymphoid properties expressed by cultured mouse T and B tumour cells: Fc receptor and cellular sensitivity to hydrocortisone and thymidine. In: Protides of the Biological Fluids, 25th Colloquium, H. Peeters, (ed.), Pergamon Press, Oxford, 25, 601.

Haskill, J.S. & Moore, M.A.S. (1970) Two dimensional separation: comparison of embryonic and adult haemopoietic stem cells. Nature 226, 853.

Haskill, J.S., McNeil, T.A. & Moore, M.A.S. (1970) Density distribution analysis of in vivo and in vitro colony forming cells in bone marrow. J. Cell. Physiol. 75, 167.

Hasthorpe, S. & Harris, W.A. (1979) Growth control of hemopoietic colony-forming cells by thymidine and desoxycytidine. In: Experimental Hematology Today. S.J. Baum & G.D. Ledney, (eds.), Springer-Verlag, New York, 11.

Health, D.S., Axelrad, A.A., McLeod, D.L. & Shreeve, M.M. (1976) Separation of erythropoietin responsive progenitor BFU-E and CFU-E in mouse bone marrow by unit gravity sedimentation. Blood 47, 777.

Heidt, P.J., Vriesendorp, H.M., Zurcher, C., de Groot, C.W. & Klapwijk, W.K. (1981a) Selective gastrointestinal decontamination in dogs undergoing allogeneic bone marrow transplantation. VII. Int.Symposium on Gerontology Tokyo, Japan, 1981, (in press).

Heidt, P.J., Wagemaker, G., Knaan, S. & Van Bekkum, D.W. (1981b) Two distinct types of late onset graft-versus host disease after bone marrow transplantation in lethally irradiated mice. Transplantation 32, 263.

Hoffman, F. & Globerson, A. (1973) Graft versus host response induced in vitro by mouse yolk sac cells. Eur. J. Immunol. 3, 179.

Hooper, A., McDaniel, M.C., Thurman, G.B., Cohen, A.H., Schulof, R.Z. & Goldstein, A. (1975) Purification and properties of bovine thymosin. Ann. N.Y. Acad. Sci. 249, 125.

Hopper, K. & Shortman, K. (1976) The differentiation of T Lymphocytes. III. The behaviour of subpopulations of mouse thymus cells in short-term culture. Cell. Immunol. 27, 256.

Hopper, K., Ryden, A. & Shortman, K. (1978) The differentiation of T lymphocytes. IV. Net increases in low thêta cells, maintenance of biological activity and death of high thêta cells during short-term culture of mouse thymocytes. Exp. Cell Biol. 46, 31.

Hrsăk, I. (1973) Influence of thymus on haemopoiesis in mice. Biomedicine 18, 213.

Horowitz, S.D. & Goldstein, A.L. (1978) The in vitro induction of differentiation of putative human stem cells by thymosin and agents that affect cyclic AMP. Clin. Immunol. Immunopathol. 9, 408.

Hudson, L. & Hay, F.C. (1978) Practical Immunoloy. Blackwell Scientific Publications, Oxford, England.

Hünig, T.R. & Bevan, M.J. (1982) Antigen recognition by cloned cytotoxic T lymphocytes follows rules predicted by the altered self hypothesis. J. Exp. Med. 155, 111.

Huber, B., Cantor, H., Shen, F.W. & Boyse, E.A. (1976) Independent differentiative pathways of Ly1 and Ly23 subclasses of T cells. Experimental production of mice deprived of selected T cell subclasses. J. Exp. Med. 144, 1128.

Isaak, D.D. (1978) Fate of skingrafts from different inbred strains on nude mice bearing allogeneic thymus grafts. Res. J. Reticuloendothel. Soc. 23, 231.

Iscove, N.N. (1978) Erythropoietin independent stimulation of early erythropoiesis in adult marrow cultures by conditioned media from lectin stimulated mouse spleen cells. In: Hemopoietic Cell Differentiation. D.W. Golde, M.J. Cline, D. Metcalf & C.F. Fox, (eds.), Academic Press, New York, 37.

Iscove, N.N., Till, J.E. & McCulloch, E.A. (1972) In vitro tritiated thymidine suicide technique for CFU. In: In vitro culture of hemopoietic cells. D.W. van Bekkum & K.A. Dicke, (eds.), Radiobiological Institute TNO, Rijswijk, The Netherlands, 270.

Ishidate, M. & Metcalf, D. (1963) The pattern of lymphopoiesis in the mouse after cortisone administration or adrenalectomy. Austr. J. Exp. Biol. Med. Sci. 41, 637.

Jacobs, S.W. & Miller, R.G. (1979) Characterization of in vitro T lymphocyte colonies from spleens of nude mice. J. Immunol. 122, 582.

Jacobson, L.O., Marks, E.K., Robson, M.J., Gaston, E.O. & Zirle, R.E. (1949) Effect of spleen protection on mortality following X-irradiation. J. Lab. Clin. Med. 34, 1538.

Jacobsson, H. & Blomgren, H. (1974) Responses of mouse thymic cells to mitogens PHA and ConA. Cell. Immunol. 11, 427.

Jandinski, J., Cantor, H., Tadakuma, T., Peavy, D. & Pierce, C.W. (1976) Separation of helper T cells from suppressor T cells expressing different Ly components. I. Polyclonal activation: Suppressor and helper activities are inherent properties of distinct T cell subclasses. J. Exp. Med. 143, 1382.

Jenkinson, E.J., Owen. J.J.T. & Aspinall, R. (1980) Lymphocyte differentiation and major histocompatibility complex antigen expression in the embryonic thymus. Nature 204, 177.

Jerne, N.K. (1971) The somatic generation of immune recognition. Eur. J. Immunol. 1, 1.

Joel, D.D., Chanana, A.D. & Cronkite, E.P. (1974) Thymus cell migration. Ser. Haemat. 7, 464.

Joel, D.D., Chanana, A.D., Cottier, H., Cronkite, E.P. & Laissue, J.A. (1977) Fate of thymocytes: studies with ^{125}Iododeoxyuridine and ^{3}H-thymidine in mice. Cell Tissue Kinet. 10, 57.

Johnson, G.R. (1980) Colony formation in agar by adult bone marrow multipotential hemopoietic cells. J. Cell. Physiol. 103, 371.

Johnson, G.R. and Metcalf, D. (1977) Pure and mixed erythoid colony formation in vitro stimulated by spleen conditioned medium with no detectable erytropoietin. Proc. Natl. Acad. Sci. USA 74, 3879.

Jordan, R.K., Owen, J.J.T. & Raff, M.C. (1977) Organ culture studies of nude mouse thymus. Eur. J. Immunol. 7, 736.

Jotereau, F.V., Houssaint, E. & Le Douarin, N.M. (1980) Lymphoid stemcell homing to the early primordium of the avian embryo. Eur. J. Immunol. 10, 620.

Kadish, J.L. & Basch, R.S. (1975) Thymic regeneration after irradiation: Evidence for an intrathymic radioresistant T cell precursor. J. Immunol. 114, 452.

Kadish, J.L. & Basch, R.S. (1976) Hemopoietic thymocyte precursors. I. Assay and kinetics of the appearance of progeny. J. Exp. Med. 143, 1082.

Kadish, J.L. & Basch, R.S. (1977) Hemopoietic thymocyte precursors. III. A population of thymocytes with the capacity to return "home" to the thymus. Cell. Immunol. 30, 12.

Kamarck, M.E. & Gottlieb, P.D. (1977) Expression of thymocyte surface alloantigens in the fetal mouse thymus in vivo and in organ culture. J. Immunol. 119, 407.

Kaplan, H.S., Nagareda, C.S. & Brown, M.B. (1954) Endocrine factors and radiation induced lymphoid tumors of mice. Recent Progr. Horm. Res. 10, 293.

Kindred, B. (1976) Lymphocytes which differentiate in an allogenic thymus. I. Responsive MLC determinants and skin grafts from the thymus donor strain. Cell. Immunol. 25, 189.

Kindred, B. (1977) Effect of thymus donor strain on the response of reconstituted nude mice to GLT. Dev. Comp. Immunol. 1, 271.

Kindred, B. (1980) Lymphocytes which differentiate in an allogeneic thymus. III. Parental T cells which differentiate in an allogeneic or semiallogeneic thymus help only B cells of their own genotype. Cell. Immunol. 51, 64.

Kindred, B. (1981) A comparison of H-2 restriction of helper function using T cells from radiation chimeras and nude mice bearing thymus grafts. Cell. Immunol. 63, 228.

Kindred, B. & Loor, F. (1974) Activity of host derived T cells which differentiate in nude mice grafted with co-isogeneic or allogeneic thymuses. J. Exp. Med. 139, 1215.

Kisielow, P., Hirst, J.A., Shiku, H., Beverley, P.C.L., Hoffmann, M.K., Boyse, E.D. & Oettgen, H.F. (1975) Ly antigens as markers for functionally distinct subpopulations of thymus derived lymphocytes of the mouse. Nature 253, 219.

Komuro, K. & Boyse, E.A. (1973a) In vitro demonstration of thymic hormone in the mouse by conversion of precursor cells into lymphocytes. Lancet 1, 740.

Komuro, K. & Boyse, E.A. (1973b) Induction of T lymphocytes in vitro by a product of the thymus. J. Exp. Med. 138, 479.

Komuro, K., Goldstein, G. & Boyse, E.A. (1975) Thymus repopulating capacity of cells that can be induced to differentiate to T cells in vitro. J. Immunol. 115, 195.

Kraal, G., Van der Hilst, B. & Boden, D. (1979) Functional and morphological recovery of the T-cell compartment in lethally irradiated and reconstituted mice. Immunology 37, 195.

Kraal, G. & Boden, D. (1981) The influence of the thymus on precursor T cells. Biomedicine 35, 115.

Kreeb, G. & Zinkernagel, R.M. (1980) Virus specific proliferative T cell response: Parameters and specificity. Cell. Immunol. 53, 267.

Kruisbeek, A.M. (1978) Thymus dependent immune competence; effects of ageing tumour-bearing and thymic humoral funtion. Thesis, University of Utrecht, The Netherlands.

Kruisbeek, A.M., Hodes, R.J. & Singer, A. (1981) Cytotoxic T lymphocyte responses by chimeric thymocytes. Selfrecognition is determined early in T cell development. J. Exp. Med. 153, 13.

Lahiri, S.K. & van Putten, L.M. (1969) Distribution and multiplication of colony forming units from bone marrow and spleen after injection in irradiated mice. Cell Tissue Kinet. 2, 21.

Lajtha, L.G. (1965) Response of bone marrow stem cells to ionizing radiations. In: Current Topics in Radiation Research, Vol.I, M. Ebert & A. Howards, (eds.), North Holland Publishing Co., Amsterdam, 141.

Lajtha, L.G. (1975) Annotation: Haemopoietic stem cells. Br. J. Haematol. 28, 529.

Lassila, O., Eskola, J., Toivanen, P., Martin, C. & Dieterlen-Lièvre, F. (1978) The origin of lymphoid stem cells studied in chick yolk-sac-embryo chimaeras. Nature 272, 353.

Lassila, O., Martin, C., Dieterlen-Lièvre, F., Nurmi, T.E.T., Eskola, J. & Toivanen, P. (1979) Is the yolk-sac the primary origin of lymphoid stem cells? Transpl. Proc. 11, 1085.

Lassila, O., Eskola, J., Toivanen, P. & Dieterlen-Lièvre, F. (1980) Lymphoid stem cells in the intra embryonic mesenchyma of the chicken. Scand. J. Immunol. 11, 445.

Law, L.W., Trainin, H., Levey, R.H. & Barth, W.F. (1964) Humoral thymic factors in mice: Further evidence. Science 143, 1049.

Ledbetter, J.A. & Herzenberg, L.A. (1979) Xenogenic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47, 63.

Ledbetter, J.A., Rouse, R.V., Micklem, H.S. & Herzenberg, L.A. (1980) T cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens. J. Exp. Med. 152, 280.

Le Douarin, M.N. (1977) Ontogeny of primary lymphoid organs. In: B and T cells in immune recognition. F. Loor & G.E. Roelants, (eds.), John Wiley & Sons, London, 1.

Lepault, F., Dardenne, M. & Frindel, E. (1979) Restoration by serum thymic factor of colony-forming unit (CFU-S): entry into DNA synthesis in thymectomized mice after T-dependent antigen treatment. Eur. J. Immunol. 9, 661.

Lepault, F. & Weissman, I.L. (1981) An in vivo assay for thymus-homing bone marrow cells. Nature 293, 151.

Levy, R.H., Trainin, H. & Law, L.W. (1964) Evidence for function of thymic tissue in diffusion chambers implanted in neonatally thymectomised mice. J. Natl. Cancer Inst. 31, 199.

Lewis, J.P. & Trobaugh, F.Jr. (1964) Haematopoietic stem cell. Nature 204, 589.

Loor, F. & Roelants, G.E. (1975) Immunofluoresence studies of possible prethymic T cell differentiation in congenitally athymic nude mice. Ann. N.Y. Acad. Sci. 254, 226.

Loor, F., Amstutz, H., Hägg, L-B., Mayor, K.S. & Roelants, G.E. (1976a) T lineage lymphocytes in nude mice born from homozygous nu/nu parents. Eur. J. Immunol. 6, 663.

Loor, F., Kindred, B. & Hägg, L-B. (1976b) Incomplete restoration of T cell reactivity in nude mice after neonatal allogeneic thymus grafting. Cell. Immunol. 26, 29.

Loor, F. & Hägg. L.B. (1977) The restoration of the T lymphoid system of nude mice: Lower efficiency of nonlymphoide epithelial thymus grafts. Cell. Immunol. 29, 200.

Lord, B.I., Lajtha, L.G. & Gidali, J. (1974) Measurement of the kinetic status of bone marrow precursor cells: Three cautionairy tales. Cell Tissue Kinet. 7, 507.

Lorenz, E., Upphoff, D.E., Reid, T.R. & Shelton, E. (1951) Modification of injury in mice and guinea pigs by bone marrow injection. J. Natl. Cancer Inst. 12, 197.

Löwenberg, B. (1975) Fetal liver cell transplantation. Thesis, Erasmus University Rotterdam, The Netherlands.

Löwenberg, B., Dicke, K.A. & Van Bekkum, D.W. (1975) Quantitative studies on the take of fetal liver hemopoietic transplants. Transpl. Proc. 7, 1965.

Löwenberg, B., De Zeeuw, H.M.C. & Dicke, K.A. (1977) Nature of the delayed graft versus host reactivity of fetal liver cell transplants. J. Nat. Cancer Inst. 58, 959.

Luekenbach, G.A., Kennedy, M.M., Kelly, A. & Mandel, T.E. (1978) Suppression of an in vitro humoral immune response by cultured fetal thymus cells. Eur. J. Immunol. 8, 8.

Magli, M.C., Iscove, N.N. & Odartchenko, N. (1982) Transient nature of early hemopoietic spleen colonies. Nature 295, 527.

Mandel, T.E. & Kennedy, M.M. (1978) The differentiation of murine thymocytes in vivo and in vitro. Immunology 35, 317.

Mathieson, B.J. (1980) Comments on thymocyte and T cell differentiation correlated with function. In: Strategics of immune regulation. E. Sercarz & A.J. Cunningham, (eds.), Academic Press, New York, 465.

Mathieson, B.J., Campbell, P.S., Potter, M. & Asofsky, R. (1978) Expression of Ly-1, Ly-2, Thy-1 and TI differentiation antigens in mouse T cell tumors. J. Exp. Med. 147, 1267.

Mathieson, B.J., Sharrow, S.D., Campbell, P.S. & Asofsky, R. (1979a) An Lyt differentiative thymocyte subpopulation detected by flow cytofluorometry. Nature 277, 478.

Mathieson, B.J., Mage, M., Betel, I. & Sharrow, S.D. (1979b) Cytotoxic lymphocyte precursors: phenotypic analysis and functional activity of peanut lectin fractionated thymocytes. In: Protides of the Biological Fluids. Proc. 27th Colloquium, H. Peeters, (ed.), Pergamon Press, Oxford, 27, 535.

Mathieson, B.J., Sharrow, S.O., Rosenberg, Y. & Hämmerling, L. (1981) Lyt-1$^+$,2,3$^-$ cells appear in the thymus before Lyt-1,2,3$^+$ cells. Nature 289, 179.

Matzinger, P. & Mirkwood, G. (1978) In a full H-2 incompatible chimera T cells of donor origin can respond to minor histocompatible antigens in association with either donor or host H-2 type. J. Exp. Med. 148, 84.

Matzinger, P. & Waterfield, J.D. (1980) Is selftolerance H-2 restricted? Nature 285, 492.

Matzinger, P. (1981) A one receptor view of T-cell behaviour. Nature 292, 497.

McPhee, D., Pyr, J. & Shortman, K. (1979) The differentiation of T lymphocytes. V. Evidence for intrathymic death of most thymocytes. Thymus 1, 151.

Messner, H.A., Linn, B. & Jam, H. (1982) Myeloid and lymphoid components in multilineage hemopoietic colonies. Abstract 18th Alfred Benzon Symposium, Hemopoietic stem cells, characterisation, proliferation and regulation. SV.AA. Killmann, E.P. Cronkite & C.H. Muller-Bérat (eds.), Munksgaard, Copenhagen, 28.

Metcalf, D. (1963) The autonomus behaviour of normal thymus grafts. Austr. J. Exp. Biol. Med. Sci. 41, 437.

Metcalf, D. (1966) The thymus, its role in immune responses, leukemia development and carcinogenesis. In: Recent results in cancer research, Vol.IV. P. Rentchnick, (ed.), Springer, Berlin and New York.

Metcalf, D. (1969) Lymphocyte kinetics in the thymus. In: The lymphocyte in immunobiology and haemopoiesis, J.M. Joffey (ed.), Edward Arnold, London, 333.

Metcalf, D. (1971) Acute antigen induced elevation of serum colony stimulating factors (CSF) levels. Immunology 21, 427.

Metcalf, D., Sparrow, N., Nakamura, K. & Ishidate, M. (1961) The behaviour of the thymus grafts in high and low leukemia strains of mice. Austr. J. Exp. Biol. Med. Sci. 39, 441.

Metcalf, D. & Moore, M.A.S. (1971) Haemopoietic cells: Their origin, migration and differentiation. In: Frontiers of Biology Vol.24. A. Neuberger & E.L. Tatum, (eds.), North-Holland Publ. Co., Amsterdam, 448.

Metcalf, D., Johnson, G.R. & Mandel, T.E. (1979) Colony formation in agar by multipotential haemopoietic cells. J. Cell. Physiol. 98, 401.

Micklem, H.S. & Loutit, J.F. (1966) Tissue grafting and radiation. Academic Press, New York.

Micklem, H.S., Ford, C.E., Evans, E.P. & Ogden, D.A. (1966) Interrelationships of myeloid and lymphoid cells: Studies with chromosome-marked cells transfused into lethally irradiated mice. Proc. R. Soc. (London) Ser. B. Biol. Sci. 165, 78.

Micklem, H.S., Ford, C.E., Evans, E.P. & Ogden, D.A. (1975) Compartments and cell flows within the mouse haemopoietic system. I. Restricted interchange between haemopoietic sites. Cell Tissue Kinet. 8, 219.

Miller, J.F.A.P. (1961) Analysis of the thymus influence in leukemogenesis. Nature 191, 248.

Miller, J.F.A.P. (1962) Role of the thymus in transplantation immunity. Ann. N.Y. Acad. Sci. 99, 340.

Miller, J.F.A.P. (1979) Experimental thymology has come of age. Thymus 1, 3.

Miller, J.F.A.P., Marshall, A.H.E. & White, R.G. (1962) The immunological significance of the thymus. Adv. Immunol. 2, 111.

Miller, J.F.A.P., Doak, S.M.A. & Cross, A.M. (1963) Role of the thymus in recovery of the immune mechanisms in the irradiated adult mouse. Proc. Soc. Exp. Biol. Med. 112, 785.

Miller, J.F.A.P., De Burgh, P.M., Dukor, P., Grant, G., Allman, V. & House, W. (1966) Regeneration of thymus grafts. II. Effects on immunological capacity. Clin. Exp. Immunol. 1, 61.

Miller, J.F.A.P., Mitchell, G.F. & Weiss, N.S. (1967) Cellular basis of the immunological defect in thymectomized mice. Nature 214, 992.

Miller, J.F.A.P. & Osoba, D. (1967) Current concepts of the immunological function of the thymus. Physiol. Rev. 47, 437.

Miller, J.F.A.P. & Mitchell, G.F. (1968) Cell to cell interaction in the immune response. I. Hemolysine-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. J. Exp. Med. 128, 801.

Miller, R.G. & Philips, R.A. (1969) Separation of cells by velocity sedimentation. J. Cell. Physiol. 73, 191.

Miller, R.G., & Phillips, R.A. (1970) Separation of cells by velocity sedimentation. In: The separation of haemopoietic cell suspensions. D.W. Van Bekkum & K.A. Dicke, (eds.), Radiobiological Institute TNO, Rijswijk, The Netherlands, 15.

Mitchison, J.M. (1971) The biology of the cell cycle. Cambridge University Press, London.

Moore, M.A.S. & Owen, J.J.T. (1967a) Stem cell migration in the developing myeloid and lymphoid systems. Lancet 1, 658.

Moore, M.A.S. and Owen, J.J.T. (1967b) Experimental studies on the development of the thymus. J. Exp. Med. 126, 715.

Moore, M.A.S., McNeill, T.A. & Haskill, J.S. (1970) Density distribution analysis of in vivo and in vitro colony forming cells in developing fetal liver. J. Cell. Physiol. 75, 181.

Moore, M.A.S. & Metcalf, D. (1970) Ontogeny of the haemopoietic system. Yolk sac origin in vivo and in vitro colony forming cells in the developing mouse embryo. Br. J. Haematol. 18, 279.

Morrissey, P.J., Kruisbeek, A.M., Sharrow, S.O. & Singer, A. (1982) Tolerance of thymic cytotoxic T lymhpocytes to allogeneic H-2 determinants encountered prethymically: Evidence for expression of anti-H-2 receptors prior to entry into the thymus. Proc. Natl. Acad. Sci., USA 79, 2003.

Mosier, D.E. (1977) Ontogeny of T cell function in the neonatal mouse. In: Development of host defense, M.D. Cooper & D.M. Dayton (eds.), Raven Press, New York, 115.

Mosier, D.E. & Cohen, P.L. (1975) Ontogeny of mouse T lymphocyte function. Fed. Proc. 34, 137.

Mosier, D.E. & Johnson, B.M. (1975) Ontogeny of mouse lymphocyte function. II. Development of the ability to produce antibody is modulated by T lymphocytes. J. Exp. Med. 141, 216.

Muramatsu, S., Monnot, P. & Duplan, J.F. (1976) Competitive proliferation in the haemopoietic tissues of irradiated hybrid mice engrafted with parental bone marrow and spleen. Exp. Hematol. 4, 188.

Nagy, Z.A., Kusnierczyk, P. & Klein, J. (1981) Terminal differentiation of T cells specific for mutant H-2 antigens; conversions of Lyt-1,2 cells into Lyt-2 but not in Lyt-1 cells, in vitro. Eur. J. Immunol. 11, 167.

Niewisch, H., Hajdik, I., Sultaniau, I., Vogel, H. & Matioli, G. (1970) Hemopoietic stemcell distribution in tissues of fetal and newborn mice. J. Cell. Physiol. 76, 107.

Nowell, P.C., Cole, C.J., Habermeyer, G.G. & Roau, P.L. (1956) Growth and continued function of rat marrow cells in X-irradiated mice. Cancer Res. 16, 258.

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

Osmond, D.G. & Nossal, G.J.V. (1974) Differentiation of lymphocytes in mouse bone marrow. II. Kinetics of maturation and renewal of anti-globulin binding cells stained by double labeling. Cell. Immunol. 13, 132.

Osoba, D. & Miller, J.F.A.P. (1963) Evidence for a humoral thymus factor responsible for the maturation of immunological competence. Nature 199, 653.

Osoba, D. & Miller, J.F.A.P. (1964) The lymphoid tissue and immmune responses of neonatally thymectomised mice bearing thymus tissue in millipore diffusion chambers. J. Exp. Med. 119, 177.

Owen, J.J.T. (1977) Ontogenesis of lymphocytes. In: B and T cells in Immune Recognition. F. Loor & Roelants, G.E. (eds.) J. Wiley and Sons, London, 21.

Owen, J.J.T. & Ritter, M.A. (1969) Tissue interaction in the development of thymus lymphocytes. J. Exp. Med. 129, 431.

Owen, J.J.T. & Raff, M.C. (1970) Studies on the differentiation of thymus derived lymphocytes. J. Exp. Med. 132, 1216.

Owen, J.J.T., Andrew, T., Jordan, R.K., McElroy, P., Robinson, J.H., Singh, U. & Wilcox, H. (1977) Factors influencing the primary differentiation of lymphocyte populations. In: Developmental Immunobiology. J. Solomon & J. Horton, (eds.), Elsevier/North Holland, Amsterdam, 131.

Pahwa, R.H., Ikehara, S., Pahwa, S.G. & Good, R.A. (1979) Thymic function in man. Thymus 1, 27.

Papiernik, M. & Bach, J.F. (1979) Postnatal development of T cells. I. Study of the respective contribution of thymic cellular export, thymic humoral function and T cell environment. J. Immunol. 123, 2311.

Papiernik, M. & Nabarra, B. (1981) Thymic reticulum in mice. I. Cellular ultrastructure in vitro and functional role. Thymus 3, 345.

Parker, J.W. & Metcalf, D. (1974) Production of colony stimulating factor in mixed leukocyte culture. Immunology 26, 1039.

Parrot, D.M.V., De Sousa, M.A.B. & East, J. (1966) Thymus dependent areas in the lymphoid organs of neonatally thymectomized mice. J. Exp. Med. 123, 191.

Pfizenmaier, K., Stockinger, H., Röllinghoff, M. & Wagner, H. (1980) Herpes simplex virus specific $H2D^k$ restricted T lymhocytes bear receptors for $H2D^d$ alloantigen. Immunogenetics 11, 169.

Ploemacher, R.E., Brons, N.H.C., De Vreede, E. & Van Soest, P.C. (1981a) Colony formation by bone marrow cells after incubation with neuraminidase. II. Sensitivity of erythroid progenitor cells for burst promoting activity and erythropoietin and restoration of reduced spleen colony formation in mice pretreated with desialated erythrocyte membrane fragments. Exp. Hematol. 9, 156.

Ploemacher, R.E., Brons, N.H.C. & Van Soest, P.C. (1981b) Colony formation of bone marrow cells after incubation with neuraminidase. III. Cell surface repair, homing and growth characteristics of colony forming cells in vivo. Exp. Hematol. 9, 168.

Pritchard, H. & Micklem, H.S. (1973) Hemopoietic stem cells and progenitors of functional T-lymphocytes in the bone marrow of nude mice. Clin. Exp. Immunol. 14, 597.

Pritchard, L.L. & Goodman, J.W. (1978) Dose dependency of the augmentation of hemopoiesis by thymocytes. Exp. Hematol. 6, 161.

Pyke, K.W. & Bach, J.F. (1979) The in vitro migration of murine fetal liver cells to thymic rudiments. Eur. J. Immunol. 9, 317.

Pyke, K.W. & Bach, J.F. (1981) In vitro migration of potential hemopoietic precursors from the murine fetus. Thymus 3, 1.

Raff, M.C. (1971) Surface antigenic markers for distinguishing T and B lymphocytes in mice. Transpl. Rev. 6, 52.

Raff, M.C. (1973) Thêta bearing cells in nude mice. Nature 246, 350.

Reif, E.A. & Allen, J.M.V. (1964) The AKR thymic antigen and its distribution in leukemias and nerve tissues. J. Exp. Med. 120, 413.

Reiman, J. & Burger, H. (1979) In vitro proliferation of hematopoietic cells in the presence of adherent cell layers. I. Culture conditions and strain dependence. Exp. Hematol. 7, 45.

Ritter, M.A. (1971) Functional maturation of lymphocytes within embryonic mouse thymus. Transplantation 12, 279.

Ritter, M.A. (1978) Embryonic mouse thymus development: Stem cell entry and differentiation. Immunology 34, 69.

Roelants, G.E., Loor, F., VonBoehmer, H., Sprent, J., Hägg, L.B., Mayor, K.S. & Ryden, A. (1975) Five types of lymphocytes (Ig-thêta-, Ig-thêta+ weak, Ig-thêta+ strong, Ig+-thêta- and Ig+thêta+) characterised by double immunofluorescence and electrophoretic mobility: Organ distribution in normal and nude mice. Eur. J. Immunol. 5, 127.

Rouse, R.V., Van Ewijk, W., Jones, P.P. & Weissman, I.L. (1979) Expression of MHC antigens by mouse thymic dendritic cells. J. Immunol. 122, 2508.

Russel, J.L. & Van den Engh, G.J. (1979) The expression of histocompatibility-2 antigens on hemopoietic stem cells. Tissue Antigens 13, 45.

Rusthoven, J.J. & Phillips, R.A. (1980) The use of hydroxyurea to study the proliferative state of T cell precursors (CFU-T) in murine bone marrow. Exp. Hematol. 8, 578.

Sato, C. & Sakka, M. (1969) Cell population analysis of radiation damage and recovery of thymus and lymphe nude in mice. Radiation Res. 38, 204.

Scheid, M.P., Boyse, E.A., Carswell, E.A. & Old, R.J. (1972) Serologically demonstrable alloantigens on mouse epidermal cells. J. Exp. Med. 135, 938.

Scheid, M.P., Hoffmann, M.K., Komuro, K., Hämmerling, L., Abbott, J., Boyse, E.A., Cohen, G.H., Hooper, J.A., Schulof, R.S. & Goldstein, A.L. (1973) Differentiation of T cells induced by preparations from thymus and by non-thymic agents. J. Exp. Med. 138, 1027.

Scheid, M.P., Goldstein, G., Hämmerling, U. & Boyse, E.A. (1975) Lymphocyte differentiation from precursor cells in vitro. Ann. N.Y. Acad. Sci. 249, 531.

Scheid, M.P., Goldstein, G. & Boyse, E.A. (1978) The generation and regulation of lymphocyte populations. Evidence from differentiative induction systems in vitro. J. Exp. Med. 147, 1727.

Schofield, R. (1970) A comparative study of the repopulating potential of grafts from various hemopoietic sources: CFU-repopulation. Cell Tissue Kinet. 3, 119.

Schrader, J.W. & Schrader, S. (1978) In vitro studies on lymphocyte differentiation. I. long term in vitro culture of cells giving rise to functional lymphocytes in irradiated mice. J. Exp. Med. 148, 823.

Scollay, R.G., Kochen, M., Butcher, E. & Weissman, T. (1978) Lyt markers on thymus cell migrants. Nature 276, 79.

Scollay, R.G. & Weissman, I.L. (1980) T cell maturation: thymocyte and thymus migrant subpopulations defined with monoclonal antibodies to the antigens Lyt-1, Lyt-2 and ThB. J. Immunol. 124, 2841.

Scollay, R.G., Jacobs, S., Jerabek, L., Butcher, E. & Weissman, I.L. (1980) T cell maturation: thymocyte and thymus migrant subpopulations defined with monoclonal antibodies to MHC region antigens. J. Immunol. 124, 2845.

Scollay, R.G., Butcher, E.C. & Weissman, I.L. (1981) Thymus cell migration: Quantitative aspects of cellular traffic from the thymus to the periphery in mice. Eur. J. Immunol. 10, 210.

Sharp, J.G. & Thomas, D.B. (1975) Thymic regeneration in lethally X-irradiated mice. Radiat. Res. 64, 293.

Sharp, J.G. & Thomas, D.B. (1977) Origin of the radioresistant precursor cells responsible for the initial phase of thymic regeneration after X-irradiation. Res. J. Reticuloendothel. Soc. 22, 169.

Sharp, J.G. & Watkins, E.B. (1981) Cellular and immunological consequenses of thymic irradiation. In: Immuno-pharmacological effects of radiation therapy, Vol.8. J.B. Dubois, B. Serrose & C. Rosenfeld, (eds.), Raven Press, New York, 137.

Shisa, H., Daculsi, R. & Duplan, J.F. (1977) Production of thymic cells by mouse spleen an bone marrow. Biomedicine 27, 73.

Shortman, K. (1972) Physical procedures for the separation of animal cells. Annu. Rev. Biophys. Bioenerg. 1, 93.

Shortman, K., Byrd, W.J., Cerottini, J.C. & Bruma, K.T. (1973) Characterisation and separation of mouse lymphocyte subpopulations. Cell. Immunol. 6, 25.

Shortman, K. & Jackson, H. (1974) The differentiation of T lymphocytes. I. Proliferation kinetics and interrelationship of subpopulations of mouse thymus cells. Cell. Immunol. 12, 230.

Shortman, K., Von Boehmer, H., Lipp, J. & Hopper, K. (1975) Subpopulations of T lymphocytes: Physical separation, functional specialisation and differentiation pathways of subsets of thymocytes and thymus dependent peripheral lymphocytes. Transpl. Rev. 25, 163.

Shortman, K., Linthicum, D.S., Battye, F.L., Goldschneider, I., Liabeuf, A. Goldstein, P., Clark, E.A. & Lake, P. (1979) Cytotoxic and fluorescent assays for thymocyte subpopulations differing in surface Thy-1 level. Cell. Biophys. 1, 255.

Silini, G., Pons, S. & Pozzi, L.V. (1968) Quantitative histology of spleen colonies in irradiated mice. Br. J. Haematol. 14, 489.

Singer, A.K., Hatchock, K.S. & Hodes, R.J. (1981) Self recognition in allogeneic radiation bone marrow chimaeras. A radiation resistant host element dictates the self specificity and immune response gene phenotype of T-helper cells. J. Exp. Med. 153, 1286.

Singer, A.K., Hatchock, K.S. & Hodes, R.J. (1982) Self recognition in allogeneic thymic chimaeras. Self recognition by T helper cells from thymus engrafted nude mice is restricted to the thymic H-2 haplotype. J. Exp. Med. 155, 339.

Singh, L. & Owen, J.J.T. (1975) Studies on the effect of various agents on the maturation of thymus stem cells. Eur. J. Immunol. 5, 286.

Splinter, Th.A.W. & Reiss, M. (1974) Separation of lymphoid-line cells according to volume and density. Exp. Cell Res. 89, 343.

Stobo, J.D. & Paul, W.L. (1972) Functional heterogeneity of murine lymphoid cells. II. Acquisition of mitogen responsiveness and of thêta antigen during ontogeny of thymocytes and T-cells. Cell. Immunol. 4, 367.

Stutman, O. (1975) Humoral thymic factors influencing postthymic cells. Ann. N.Y. Acad. Sci. 249, 89.

Stutman, O. (1977) Two main features of T cell development: thymus traffic and postthymic maturation. In: Contemporary Topics in Immunobiology, Vol.7. O. Stutman, (ed.), Plenum Press, New York, 1.

Stutman, O. (1978) Intrathymic and extrathymic T cell maturation. Immunol. Rev. 42, 138.

Stutman, O., Yunis, E.J. & Good, R.A. (1969) Carcinogen-induced tumors of the thymus. IV. Humoral influences of normal thymus and funtional thymomas and influences of postthymectomy period on restoration. J. Exp. Med. 130, 809.

Stutman, O., Yunis, E.J. & Good, R.A. (1970a) Studies on thymic functions. I. Cooperative effect of thymic function and lymphohemopoietic cells in restoration of neonatally thymectomized mice. J. Exp. Med. 132, 583.

Stutman, O., Yunis, E.J. & Good, R.A. (1970b) Studies on thymus function. II. Cooperative effect of newborn and embryonic hemopoietic liver cells with thymus function. J. Exp. Med. 132, 601.

Suda, T. & Dexter, T.M. (1981) Effect of hydrocortisone on long-term human marrow cultures. Br. J. Haematol. 48, 661.

Sutherland, D.J.A., McCulloch, J.E. & Till, E.A. (1971) Short term culture of mouse marrow cells separated by velocity sedimentation. Cell Tissue Kinet. 4, 479.

Takada, A., Takada, Y., Kim, U. & Ambrus, J.L. (1971) Bone marrow, spleen and thymus regeneration patterns in mice after whole body irradiation. Radiat. Res. 45, 522.

Takada, A. & Takada, Y. (1972) Proliferation of donor hemopoietic cells in lethally irradiated host mice. Transplantation 13, 276.

Tao, T.W. & Floersheim, G.L. (1976) Reconstitution capacity of bone marrow cells from nude mice in radiation chimearas. Acta Haematol. 55, 332.

Till, J.E. & McCulloch, E.A. (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14, 213.

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

Till, J.E. & McCulloch, E.A. (1980) Hemopoietic stem cell differentiation. Biochem. Biophys. Acta 605, 431.

Ting, R.C. & Law, L.W. (1965) Immunologic competence and induction of neoplasms by polyoma virus. Proc. Soc. Exp. Biol. Med. 119, 823.

Toogood, I.R.G., Dexter, T.M., Allen, T.D., Suda, T. & Ragtha, L.G. (1980) The development of a liquid culture system for the growth of human bone marrow, Leuk. Res. 4, 449.

Trainin, N. & Resnitzky, P. (1969) Influence of neonatal thymectomy on cloning capacity of bone marrow cells in mice. Nature 221, 1154.

Trainin, N., Small, M. & Kook, A.I. (1977) The role of thymic hormones in regulation of the lymphoid system. In: B and T cells in immune recognition. F. Loor & G.E. Roelants, (eds.), J. Wiley and Sons, London, 83.

Trask, B. & Van den Engh, G. (1980) Antigen expression of CFU-S determined by light activated cell sorting. In: Experimental Hematology Today, S.J. Baum, G.D. Ledney & D.W. Van Bekkum (eds.), S. Karger, Basel, New York, 299.

Trentin, J.J. (1978) Hemopoietic microenvironments. Transpl. Proc. X, 77.

Trentin, J.J. & Fahlberg, W.J. (1963) An experimental model for studies of immunologic competence in irradiated mice repopulated with "clones" of spleen cells. In: Conceptuel advances in Immunology and Oncology, Harper and Row, New York, 66.

Trentin J.J., Wolf, N., Cheng, V., Fahlberg, W., Weiss, D. & Bonhage, R. (1967) Antibody production by mice repopulated with limited numbers of clones of lymphoid cell precursors. J. Immunol. 98, 1326.

Trowel, O.A. (1961) Radiosensitivity of the cortical and medullary lymphocytes in the thymus. Int. J. Radiat. Biol. 4, 163.

Tyan, M.L. (1964) Thymus: Role in maturation of fetal lymphoid precursors. Science 145, 934.

Tyan, M.L. (1968) Studies on the ontogeny of the mouse immune system. I. Cell bound immunity. Immunology 100, 535.

Udupa, K.B. & Reissman, K.R. (1978) Cell kinetics of erythroid colony forming cells (CFU-E) studied by hydroxyurea injection and sedimentation velocity profile. Exp. Hematol. 6, 398.

Upphoff, D.E. (1958) Preclusion of secondary phase of irradiation syndrome by inoculation of immunocompetence of embryonic liver cells in vitro. Proc. Soc. Exp. Biol. Med. 129, 598.

Van Bekkum, D.W. (1967) Immunological and hematological aspects of recovery: Implications for therapeutic use of bone marrow grafts. In: Recovery and Repair Mechanisms in Radiobiology. Brookhaven Symposia in Biology, Upton, Brookhaven National Laboratory 1968. 20, 190.

Van Bekkum, D.W. (1974) Pathways and control of lymphocytopoiesis. In: Leukemia and aplastic anemia. Proc. Conf. on Leukemia and Aplastic Anemia, Naples 1974. Il pensiero scientifico, (ed.) Roma, 31.

Van Bekkum, D.W., Roodenburg, J., Heidt, P.J. & Van der Waaij, D. (1974) Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora. J. Nat. Cancer Inst. 52, 401.

Van Bekkum, D.W. & Schotman, E. (1974) Protection from haemopoietic death by shielding versus grafting of bone marrow. Int. J. Radiat. Biol. 25, 361.

Van den Engh, G.J. (1976) Early events in haemopoietic cell differentiation. Thesis, University of Leiden, The Netherlands.

Van den Engh, G.J. & Golub, E.S. (1974) Antigenic differences between hemopoietic stem cells and myeloid progenitors. J. Exp. Med. 139, 162.

Van den Engh, G., Russel, J. & De Cicco, D. (1978) Surface antigens of hemopoietic stem cells; The expression of Bas, Thy-1 and H-2 antigens on CFU-S. In: Experimental Hematology Today. S.J. Baum & G.D. Ledney, (eds.), Springer-Verlag, New York, 9.

Van der Waaij, D., Berghuis-De Vries, J.M & Ouderkerk-Van der Wees, J.E.C.J. (1971) Anaerobic flora in GI tract has advantage of making animals less susceptible to colonisation with exogenous micro-organisms. J. Hyg. 69, 405.

Van Ewijk, W., Rouse, R.V. & Weissman, I.L. (1980) Distribution of H-2 microenvironments in the mouse thymus. Immuno electronmicroscopic identification of I-a and H-2k bearing cells. J. Histochem. Cytochem. 28, 1089.

Van Ewijk, W., Van Soest, P.L. & Van den Engh, G.J. (1981) Fluorescence analysis and anatomical distribution of mouse T Lymphocyte subsets defined by monoclonal antibodies to the antigens Thy-1, Lyt-1, Lyt-2 and T 200. J. Immunol. 127, 2594.

Van Putten, L.M. (1964) Thymectomy: Effect on secondary disease in radiation chimeras. Science 145, 935.

Van Putten, L.M., Van Bekkum, D.W. & De Vries, M.J. (1968) Transplantation of foetal haemopoietic cells in irradiated rhesus monkeys. In: Radiation and the control of the immune response. Proc. of a panel in Paris, 1967, International Atomic Energy Agency, Vienna, 41.

Vassort, F., Winterholer, M., Frindel, E. & Tubiana, M. (1973) Kinetic parameters of bone marrow stem cells using in vivo suicide by tritiated thymidine or by hydroxyurea. Blood 41, 789.

Viktora, L., Fiala, J. & Hermanová, E. (1978) Transplantation of bone marrow cells to lethally irradiated mice. Scand. J. Haematol. 20, 13.

Visser, J.W.M. & den Engh, G.J., Williams, H. & Mulder, D. (1977) Physical separation of cycling and non-cycling compartments of murine hemopoietic stem cells. In: Experimental Hematology Today. S.J. Baum & G.D. Ledney, (eds.), Springer-Verlag, New York, 21.

Visser, J.W.M., Van den Engh, G.J. & Van Bekkum, D.W. (1980) Light scattering properties of murine hemopoietic cells. Blood Cells 6, 391.

Vogel, H., Hajdik, I., Niewisch, H., Sultamiau, I. & Matioli, G. (1970) Growth kinetics of hemopoietic fetal stem cells. J. Cell. Physiol. 76, 117.

Von Boehmer, H., Shortman, K. & Nossal, G.J.V. (1975) The separation of different cell classes from lymphoid organs. XI. Preparative electrophoretic separation of lymphocyte subpopulations from mouse spleen and thoracic duct lymph. J. Cell. Physiol. 83, 231.

Von Boehmer, H., Haas, W. & Jerne, N.K. (1978) Major histocompatibility complex-linked immune responsiveness is acquired by lymphocytes of low-responder mice differentiating in the thymus of high-responder mice. Proc. Nat. Acad. Sci. USA 75, 2439.

Vos, O., Davids, J.A.G., Weyzen, W.W.H. & Van Bekkum, D.W. (1956) Evidence for the cellular hypothesis in radiation protection by mouse bone marrow cells. Acta Physiol. Pharmacol. Neerl. 4, 482.

Vos, O., Goodman, J.W. & Congdon, C.C. (1960) Donor type lymphatic tissue cells in lethally irradiated mice treated with homologous fetal liver. Transpl. Bull. 7, 408.

Wagemaker, G. (1980) Early erythropoietin independent stage of in vitro erythropoiesis: Relevance to stem cell differentiation. In: Experimental Hematology Today. S.J. Baum, G.D. Ledney & D.W. Van Bekkum, (eds.), S. Karger, Basel, New York, 47.

Wagemaker, G., Ober-Kieftenburg, V.E., Brouwer, A. & Peters-Slough, M.F. (1977) Some characteristics of in vitro erythroid colony and burst-forming units. In: Experimental Hematology Today. S.J. Baum & G.D. Ledney, (eds.), Springer-Verlag, New York, 103.

Wagemaker, G., Peters, M.F. & Bol, S.J.L. (1979) Induction of erythropoietin responsiveness in vitro by a distinct population of bone marrow cells. Cell Tissue Kinet. 12, 521.

Wagemaker, G. & Visser, T.P. (1980) Erythropoietin-independent regeneration of lymphoid progenitor cells following multiple injections of hydroxyurea. Cell Tissue Kinet. 13, 505.

Wagner, H., Hardt, C., Bartlett, R., Röllinghoff, M. & Pfizenmaier, K. (1980a) Intrathymic differentiation of cytotoxic T lymphocytes (CTL) precursors. I. The CTL immune competence of peanut agglutinin-positive (cortical) and negative (medullary) Lyt-1,2,3, thymocytes. J. Immunol. 125, 2532.

Wagner, H., Röllinghoff, M., Rodt, H. & Thierfelder, S. (1980b) T cell mediated cytotoxic immune responsiveness of chimeric mice bearing thymus graft fully allogeneic to the graft of lymphoid stem cells. Eur. J. Immunol. 10, 521.

Wagner, H., Hardt, C., Stockinger, H., Pfizenmaier, K., Bartlett, R. & Röllinghoff, M. (1981) Impact of thymus on the generation of immunocompetence and diversity of antigen-specific MHC restricted cytotoxic T lymphocyte precursors. Immunol. Rev. 58, 95.

Wallis, V.J., Leuchars, E., Chwalinsky, D. & Davies, A.J.S. (1975) On the sparse seeding of bone marrow and thymus in radiation chimaeras. Transplantation 19, 2.

Warren, L. (1963) Thiobarbituric acid assay of sialic acids. In: Methods in Enzymology, Vol.6. S.P. Colowick & H.O. Kaplan (eds.), Academic Press, New York, 463.

Weissman, I.L. (1973) Thymus cell maturation. Studies of the origin of cortisone resistant thymic lymphocytes. J. Exp. Med. 137, 504.

Weissman, I.L., Small, M., Fathman, C.G. & Herzenberg, L.A. (1975a) Differentiation of thymus cells. Fed. Proc. 34, 141.

Weissman, I.L., Masuda, T., Olive, C. & Friedberg, S.H. (1975b) Differentiation and migration of T lymphocytes. Israel. J. Med. 11, 1267.

Wekerle, H. & Ketelsen, U.W. (1980) Thymus nurse cells: Ia bearing epithelium involved in T lymphocyte differentiation. Nature 283, 402.

Whittle, E.D. (1966) Effect of thymidine on deoxyribonucleiec acid synthesis and cytidine metabolism in rat-thymus cells. Biochem. Biophys. Acta 114, 44.

Williams, A.F. (1977) Differentiation antigens of the lymphocyte cell surface. Mol. Immunol. 6, 83.

Williams, N. & Van den Engh, G. (1975) Separation of subpopulation of in vitro colony forming cells from mouse bone marrow equilibrium density centrifugation. J. Cell. Physiol. 86, 237.

Williams, H. & Pluznik, D. (1978) Differences in the buoyant density characteristics of murine granulocyte-macrophage progenitor cells in the presence of serum and/or hemolysate. Exp. Hematol. 6, 383.

Wilson, D.B., Fischer-Lindahl, K., Wilson, D.M. & Sprent, J. (1979) The generation of killer cells to trinitrophenyl-modified allogeneic targets by lymphocyte populations negatively selected to strong alloantigens. J. Exp. Med. 146, 361.

Worton, R.G., McCulloch, E.A. & Till, J.E. (1969a) Physical separation of hemopoietic stem cells differing in their capacity for self-renewal. J. Exp. Med. 130, 91.

Worton, R.G., McCulloch, E.A. & Till, J.E. (1969b) Physical separation of hemopoietic stem cells from cells forming colonies in culture. J. Cell. Physiol. 74, 171.

Wu, S. (1978) Ontogeny of cell mediated immunity of murine thymocytes and spleen cells. In vitro mixed leukocyte culture and cell mediated lympholysis reaction. Differentiation 11, 169.

Wu, A.M., Till, J.E., Siminovitich, L. & McCulloch, E.A. (1967a) A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. J. Cell. Physiol. 69, 177.

Wu, A.M., Till, J.E., Siminovitich, L. & McCulloch, E.A. (1967b) Cytological evidence for a relationship between normal colony-forming cells and cells of the lymphoid system. J. Exp. Med. 127, 445.

Yen, A., Lambek, C. & Clarkson, B. (1981) Mascaracy of estimations of S-phase fraction by reduction in cloning efficiency with hydroxyurea or tritiated thymidine. Cell Tissue Kinet. 14, 301.

Zeiller, K., Paschen, G., Wagner, G., Liebich, H.A., Holzberg, E. & Hannig, K. (1974) Distinct subpopulations of thymus-dependent lymphocytes. Immunology 26, 995.

Zinkernagel, R.M. (1978) Thymus and lymphohemopoietic cells: Their role in T cell maturation in selection of T cells, H-2 restriction specificity and H-2 linked Ir gene control. Immunol. Rev. 42, 224.

Zinkernagel, R.M. & Doherty, P.C. (1974) Restriction of in vitro T cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature 248, 701.

Zinkernagel, R.M. & Doherty, P.C. (1977) Major transplantation antigens, viruses and specificity of surveillance T cells. Contemp. Top. Immunobiol. 7, 719.

Zinkernagel, R.M., Callahan, G.H., Althage, A., Cooper, J., Streilein, J.W. & Klein, J. (1978a) The lymphoreticular system in triggering virus-plus-self specific cytotoxic cells: Evidence for T Help. J. Exp. Med. 147, 897.

Zinkernagel, R.M., Callahan, G.H., Klein, J. & Demert, G. (1978b) Cytotoxic T cells learn specificity for self H-2 during differentiation in the thymus. Nature 271, 251.

Zinkernagel, R.M., Althage, A., Waterfield, E., Kindred, B., Welsh, R.M., Callahan, G.H. & Pincet, P. (1980) Restriction specificities, alloreactivity and allotolerance expressed by T cells from nude mice reconstituted with H-2 compatible or H-2 incompatible thymus graft. J. Exp. Med. 147, 882.

CURRICULUM VITAE

Na het behalen van het diploma HBS-B in 1964 werd aangevangen met de studie voor het kandidaatsexamen in de scheikunde, natuurkunde, wiskunde en mineralogie aan de Universiteit van Amsterdam. Na het kandidaatsexamen in juni 1968 werd gekozen voor een doctoraal studie met als hoofdvak biochemie, bijvak fysiologie en speciale richting reactie mechanismen in de organische chemie. Na het doctoraalexamen in juni 1973 volgde een tewerkstelling in het klinisch-chemisch laboratorium van het voormalige Weesperplein Ziekenhuis in Amsterdam in het kader van vervangende burgerdienstplicht. In juli 1975 vond tijdelijke aanstelling plaats in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek in het kader van het Zwaartepunt Programma Hemopoietische Regulatie met als standplaats het Radiobiologisch Instituut TNO te Rijswijk onder leiding van Prof.Dr. D.W. van Bekkum. Daar heeft het in dit proefschrift beschreven onderzoek voor het grootste deel plaatsgevonden. In september 1980 vond een aanstelling plaats in dienst van het Instituut voor Experimentele Gerontologie TNO te Rijswijk (Directeur Prof.Dr. C.F. Hollander) met als taakomschrijving het onderzoek naar de rol van T lymfocyten in het verouderende proefdier.