BONE MARROW TRANSPLANTATION WITH T LYMPHOCYTE DEPLETED AND STEM CEL ENRICHED BONE MARROW GRAFTS

Experiments in rhesus monkeys

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BONE MARROW TRANSPLANTATION WITH T LYMPHOCYTE DEPLETED AND STEM CELL ENRICHED BONE MARROW GRAFTS

Experiments in rhesus monkeys

BEENMERGTRANSPLANTATIES NA STAMCELVERRIJKING EN T LYMFOCYTEN DEPLETIE

Experimenten in rhesusapen

PROEFSCHRIFT

ter verkrijging van de graad van Doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. dr. A.H.G. Rinnooy Kan
en volgens besluit van het College van Dekanen
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W.R. GERRITSEN

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hij toonde me wat doorzettingsvermogen is en
hoe verwoestend kanker kan zijn.

Aan mijn ouders
Aan Simonetta

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

1.1 BONE MARROW TRANSPLANTATION

Peripheral blood cells have vital and well known functions. Their relatively short lifespan leads to a high production rate of approximately 10^{11} new blood cells daily in man. This process is called haemopoiesis and the main site for the production of blood cells is the bone marrow. Bone marrow is located inside all bones, particularly in the pelvis, the vertebrae, ribs and bones of the extremities. The common ancestor cell for all haemopoietic differentiation pathways is the pluripotent haemopoietic stem cell. This cell maintains the whole process of haemopoiesis by its virtue of self-replication and differentiation. In the differentiation pathway, stem cells differentiate to committed progenitor cells, which are restricted for their development to a specific cell lineage, such as the myeloid, lymphoid, erythroid, thrombocytic and macrophage differentiation series. Committed cells will gradually loose their proliferative capacity and mature into functional end cells, such as leukocytes, erythrocytes, thrombocytes and macrophages. These mature cells leave the bone marrow to circulate through blood vessels and to exert their specific functions, e.g. O_2 and CO_2 transport (erythrocytes), clotting of blood (platelets), and defense against infections (B- and T lymphocytes, granulocytes and macrophages) in various tissues.

Most of the current knowledge of haemopoietic stem cells has been obtained from observations on **radiation chimaeras** (Van Bekkum & de Vries, 1967). After total body irradiation, haemopoiesis is arrested as well as the ability to mount an immunological attack against foreign antigens.

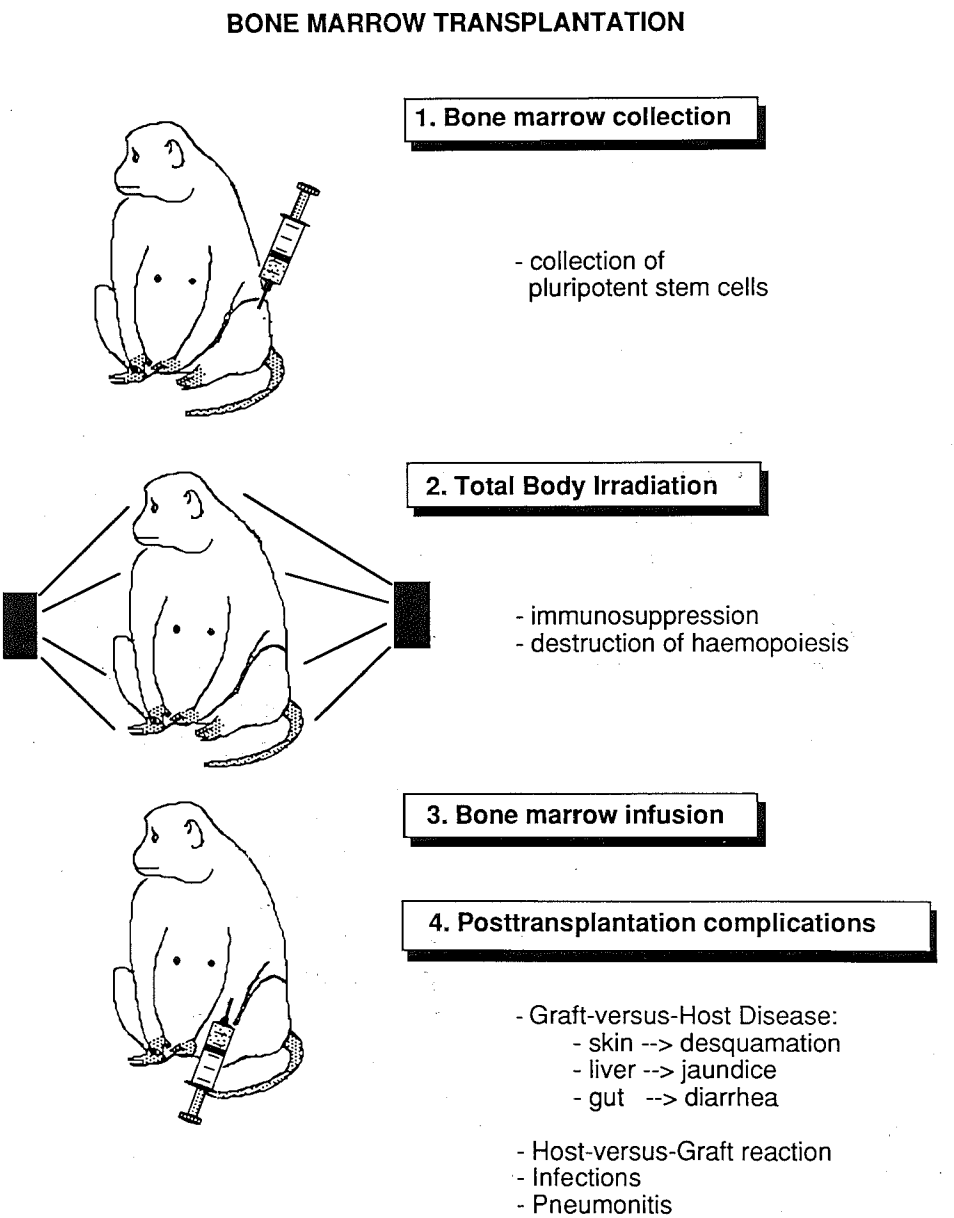

Figure 1.1 **Bone marrow transplantation**

The subsequent intravenous administration of a relative small number of bone marrow cells restores all haematological functions. The term 'radiation chimaera' was introduced when it was detected in a recipient of a bone marrow graft that all haemopoietic cells of such a bone marrow recipient were of donor origin (Ford et al., 1956; Vos et al., 1956; Nowell et al., 1956).

These early observations have led to clinical bone marrow transplantation (BMT) as a treatment modality for damaged or insufficiently functioning haemopoiesis. A bone marrow transplantation (Figure 1.1) starts with preparation of a recipient for grafting. An unprepared recipient, which is not immunodeficient, will oppose engraftment of allogeneic bone marrow cells by immunological reactions. For immunosuppression and elimination of the recipient's own haemopoiesis, total body irradiation and/or high dose chemotherapy is applied. After such a conditioning, a bone marrow graft is injected to provide the recipient with a new, permanently functioning haemopoietic system. For the restoration of the haemopoietic system haemopoietic stem cells are indispensable since these are the only cells which have the ability of self-replication. Dependent on the relationship between donor and recipient a bone marrow graft is designated as: autologous, when the marrow is taken from the same individual; syngeneic, when host and donor are genetically identical, which means that the marrow is derived either from an identical twin (outbred species) or from an animal of the same inbred strain; allogeneic, when host and donor are genetically different but still belong to the same species; and xenogeneic, when host and donor belong to different species.

After transplantation, donor cells may mount an immunological reaction against host tissues dependent on the genetic disparity between host and donor. The result of this graft-versus-Host (GvH) reaction is a disease with high morbidity and mortality. Despite these complications, BMT is an appropriate treatment for a variety of diseases. The updated results of many bone marrow transplantation centers, as reported by the European and International Bone Marrow Transplantation Registry, are presented in Table 1.1. The probability of survival in patients with acute myeloid leukemia in first remission is 50%. Transplants for patients with chronic granulocytic leukemia yielded the best results when undertaken in chronic phase with a long term survival of approximately 60%, while transplants in blast crisis resulted in a 20% survival rate. BMT for nonmalignant disease, such as severe combined immunodeficiencies, aplastic

anemia and thalassemia major, gave encouraging results of 50 - 80% long-term survival rates. In the next paragraphs, the current state of research for haemopoietic stem cells, engraftment and GvH disease is discussed in more detail.

Table 1.1

Current status of survival and relapse after allogeneic bone marrow transplantation.

Type and stage of disease	PROBABILITY OF Survival	Relapse	Reference
ALL, 1st remission	40-55%	20-30%	a
ALL, $\geq$ 2 remission	20-35%	50-60%	a
ANL, 1st remission	50%	20-25%	b
ANL, > 2 remission	25-35%	50-70%	b
CGL, chronic phase	50-60%	10-20%	c
CGL, acc. phase	20-30%	55-80%	c
non malignant diseases:			
SCID	60-70%		d
Aplastic anemia	70-80%		e
Thalassemia major	80%	30%	f

ALL= Acute Lymphoblastic Leukemia; ANL= Acute Nonlymphoblastic Leukemia; CGL= Chronic Granolocytic Leukemia; SCID= Severe Combined Immunodeficiencies
a: (Ringden for EBMT, 1987; Gratwohl for EBMT, 1988; Champlin for IBMTR, 1987), b: (Ringden for EBMT, 1987, ; Gratwohl for EBMT, 1988; Champlin for IBMTR, 1987; Weisdorf et al., 1988), c: (Ringden for EBMT, 1987; Gratwohl for EBMT, 1988; Storb et al., 1987), d: (O'Reilly et al., 1987; Fisher et al., 1986), e: (Bacigalupo for EBMT, 1988; Storb et al., 1987), f: (Lucarelli et al., 1985)

1.2 PLURIPOTENT HAEMOPOIETIC STEM CELLS

Haemopoiesis is a complex process, since it encompasses both the multiplication of all types of precursor cells as well as the differentiation from haemopoietic stem cells to functional peripheral blood cells. The complexity is due to the wide

range of functions, which are exerted by peripheral blood cells, such as clotting, transport of O_2, phagocytosis and defense against infections. If this whole ingenious process has been torn down by irradiation or cytotoxic drugs, transplantation of a relatively small number of stem cells will result in repopulation of the whole haemopoietic system. The transplanted bone marrow cells give progeny to a great variety of functional end cells, which include not only blood cells but also macrophages in tissue such as lung, liver, peritoneal cavity, epidermis, spleen and thymus (reviewed by Wagemaker, 1985; Figure 1.2). Mast cells (Kitamura, 1977) and osteoclasts (Scheven et al., 1986) are also descendents of haemopoietic stem cells and, under special circumstances, macrophages in the brain are of donor origin (Hoogerbrugge et al., 1988).

In search of a cell which is responsible for the maintenance of haemopoiesis, mice appeared to be very valuable.

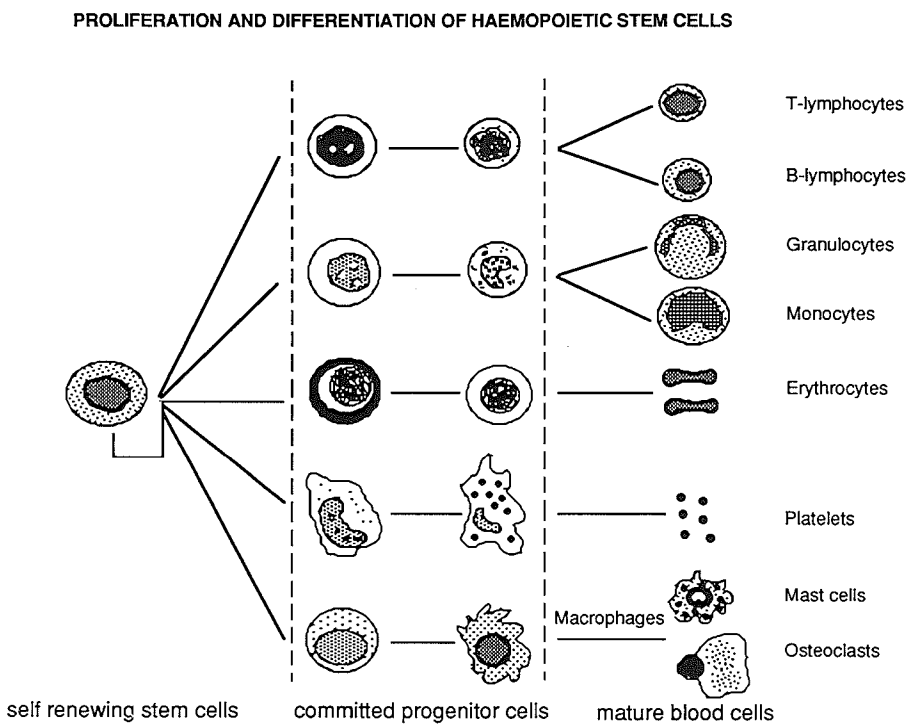

Figure 1.2 **Differentiation of haemopoietic stem cells.**

Starting about seven days after transplantation macroscopically visible nodules, which contain haemopoietic cells, become visible on the spleen of lethally irradiated recipients. The number of spleen colonies appeared to be linearly related to the number of transplanted cells (Till and McCulloch, 1961). Transplantation experiments with bone marrow cells with unique chromosome markers demonstrated that cells present in an individual spleen colony carried the same unique marker, suggesting that spleen colonies were derived from one single cell (Becker et al., 1963; Wu et al., 1967). The properties of extensive self-replication and pluripotentiality were demonstrated by the development of a complete haemopoietic system after transplantation of a single spleen nodule into a lethally irradiated recipient (Trentin and Fahlberg, 1963). Because of these observation, murine pluripotent stem cells can be assayed by their ability to form colonies in a spleen of a lethally irradiated recipient mouse and are referred to as colony-forming unit/spleen (CFU-S). Although recent observations suggest that not every spleen colony forming cell represents a pluripotent haemopoietic stem cell (Magli et al., 1982; Baines et al., 1983; Harris et al., 1984; Visser et al., 1984), this assay is still of considerable value for experimental haematology.

An equivalent of the spleen colony assay for stem cells is not available in outbred species such as humans and rhesus monkeys for obvious reasons. The development of clonal cultures of haemopoietic cells in semisolid culture medium enabled the investigation of haemopoietic differentiation. In those cultures, individual progenitor cells of a particular haemopoietic lineage are able to proliferate and generate a clone of maturating progeny cells that remain in physical proximity to each other and are identifiable as a colony. For the main haemopoietic lineages such assays have been developed (Metcalf, 1984). Most of these in vitro assays represent committed progenitor cells. The assay for multilineage progenitor cells (CFU-GEMM) has some characteristics of haemopoietic stem cells including differentiation along multiple cell lineages (Fauser and Messner, 1979), the capacity for self-renewal (Ash et al., 1981) and originating from a single progenitor (Powell et al., 1984). In humans, autologous bone marrow transplantation after in vitro purging with 4-hydroperoxy-cyclophosphamide has demonstrated that this colony assay failed to predict the marrow reconstituting ability. In spite of a drastic reduction in colony counts by this drug (Rowley et al., 1985; Kaizer et al., 1985), the in vitro treated marrow was

still capable to repopulate autologous recipients conditioned with lethal chemo/radiotherapy (Yeager et al., 1986).

Another approach for the study of haemopoietic cell differentiation is identification of the pluripotent haemopoietic stem cell by surface markers. Especially in mice, several investigators have been successful in the purification of CFU-S on the basis of their physical and surface properties. Rat (Castagnola et al., 1981; Goldschneider et al., 1980) and mouse (Visser et al., 1984, 1988; Spooncer et al., 1985; Bauman et al., 1986; Spangrude, 1988; de Vries, 1988) haemopoietic stem cells have been purified to almost complete purity. Although the haemopoietic stem cells of rats, mice, rhesus monkeys, and man can be distinguished from other cells on the basis of ultrastructural characteristics (Van Bekkum et al., 1971; Moore et al., 1972; Dicke et al., 1973; Van Bekkum et al., 1979), stem cell specific surface antigens have not been identified yet. Class I and/or class II antigens of the major histocompatibility complex are present on the cell surface of stem cells in mice (Visser et al., 1984; Van den Engh et al., 1978), dogs (Szer et al., 1985) and of very primitive progenitor cells (CFU-GEMM) in rhesus monkeys (Winton et al., 1985) and humans (Fitchen et al., 1982). Studies on the purification of human stem cells are hampered by the lack of a reliable assay for haemopoietic stem cells.

1.3 ENGRAFTMENT

The successful engraftment of a bone marrow graft requires proper preparation of a recipient by myeloablative total body irradiation and/or chemotherapy. The objectives of conditioning of an allogeneic recipient are a) the creation of space in bone marrow to allow homing and proliferation of stem cells , b) the suppression of immunological reactions of the recipient directed against histocompatibility antigens present on donor haemopoietic cells in case of allogeneic transplants, and c) in case of malignancies, eradication of tumor cells (Vriesendorp, 1985).

After transplantation of bone marrow cells into untreated, syngeneic mice only a very small proportion (2-16 %) of the cells are of donor origin (Micklem et al., 1968; Takada et al., 1971; Saxe et al., 1984). The concept that a lack of space was responsible for these observations, was supported by the observation that transplantation of normal, syngeneic bone marrow into alpha-thallassemic mice resulted in a sustained conversion to normal red cell phenotype when

recipients were prepared by a sublethal dose of total body irradiation of 2-3 Gy (Barker et al., 1986; Wagemaker, 1986).

The immunological reaction of the host upon the injection of foreign bone marrow cells is dependent on several variables (see Table 1.2). The role of these variables has been extensively described by Vriesendorp (1985) and will be discussed briefly here.

The differences in major and minor histocompatibility antigens between donor and recipient are important factors determining whether a bone marrow graft will be accepted by the host. The influence of genetic disparity between donor and recipient on engraftment is well illustrated by the numbers of cells required in various donor-recipient combinations. Extensive studies in mice have demonstrated that 20-30 times more cells are required for 50% radioprotection (LD_{50}) after supralethal TBI in allogeneic marrow transplantations in comparison to syngeneic transplantations (van Bekkum and de Vries, 1967). For rhesus monkeys 10^7 autologous bone marrow cells/kg body weight are sufficient to protect lethally irradiated recipients, while 3-4 x 10^8 bone marrow cells/kg body weight from an unrelated, mismatched donor are required for 100% radioprotection (Vriesendorp, 1985; personal observations). A similar difference between cell dose required after either autologous or mismatched bone marrow transplantation has been reported for dogs (Vriesendorp, 1985). Another indication for the role of MHC-barriers on engraftment is the frequency of engraftment in different genetic donor-recipient combinations using identical conditioning regimens and cell numbers.

Table 1.2

Factors which determine the severity of a Host-versus-Graft Reaction.

Histoincompatibility between donor and recipient
Conditioning of the recipient
Route of administration
Cellular composition of a bone marrow graft (stem cells/lymphocytes)
Prior sensitization of recipient
Decontamination of the gastrointestinal tract of the recipient
Sex of the donor

Experiments in outbred species such as dogs (Vriesendorp et al., 1982; Deeg et al.,1982), monkeys (Wagemaker et al., 1981) and humans (Powles et al., 1983; Hansen et al., 1986; Anasetti et al., 1989) have shown that transplantation across MHC-barriers are associated with a higher frequency of engraftment failures.

A host-versus-graft reaction normally prevents the engraftment of foreign bone marrow, unless the recipient's competence for an immunological attack is abrogated by an intensive immunosuppressive treatment prior to the transplantation. Total body irradiation, busulphan and cyclophospamide are mostly applied for this purpose. As a single agent, total body irradiation is superior to busulphan or cyclophosphamide in rhesus monkeys as well as in dogs. This effect was observed in matched and mismatched donor-recipient combinations. (van Bekkum et al., 1970; Vriesendorp, 1985).

The cellular composition of a bone marrow graft has a major influence on the outcome of a bone marrow transplantation. The number of pluripotent haemopoietic stem cells determines the repopulating ability of a bone marrow graft. Experiments with highly purified stem cells (Visser et al., 1984; Bauman et al., 1986; Spangrude et al., 1988; de Vries, 1988) have shown that pluripotent stem cells are primarily responsible for sustained engraftment. Donor lymphocytes induce an acute GvH reaction in case of allogeneic bone marrow transplantations. Since the recipient's immunocompetent cells are among the targets of the GvH reaction, donor lymphocytes can abrogate residual immunological resistance against donor cells.

Pluripotent stem cells are not only present in bone marrow, but can also be found in tissues such as fetal liver, spleen and peripheral blood. In mouse, the highest concentration of stem cells is found in bone marrow which is matched only by the fetal liver. In comparison to bone marrow, the proportion of stem cells is 100 times less in peripheral blood and 20 times less in spleen (Wagemaker, 1985).

The presence of pluripotent stem cells in the peripheral blood (Calvo et al., 1976; Storb et al., 1977; Reiffers et al., 1986) indicate that they, apparently, can leave the bone marrow, enter the circulatory system and remigrate to the bone marrow. This migration pattern permits bone marrow transplantation by administration of haemopoietic cells intravenously. This route of administration was proven to be superior to a subcutaneous, intraperitoneal and intra-arterial route (reviewed by Vriesendorp, 1985).

Blood transfusions given prior to bone marrow transplantation can induce sensitization of recipients and can interfere with engraftment as was first demonstrated in rhesus monkeys (van Putten et al., 1967). Subsequent studies in dogs have revealed that donor blood has a more pronounced influence on engraftment than third-party blood transfusions. Whole blood transfusions and donor skin epithelial cells strongly sensitized recipients resulting in engraftment failures in all cases. Sensitization and subsequent engraftment failure could be prevented by using leukocyte-poor platelet and leukocyte-poor red blood cell transfusions (Storb et al., 1979). The problem of sensitization is especially encountered in patients with severe aplastic anemia, who have received multiple blood transfusions. In these patients either multiple platelet or red blood cell transfusions before transplantation were associated with a higher incidence of engraftment failures (Storb et al., 1983). Exposure of blood transfusions to ultraviolet irradiation abolished their ability to sensitize recipients and engraftment failures were no longer observed in dogs (Deeg et al.,1985).

Decontamination of the gastrointestinal tract is employed to decrease the incidence and severity of GvHD (see section 1.5.1), but it also hampers engraftment. This has been clearly demonstrated in dogs. In haplomismatched donor-recipient combinations, a conditioning regimen of 2 x 6.0 Gy resulted in successful engraftment in 5/5 recipients not subjected to gastrointestinal decontamination. When the gastrointestinal tract of the recipients was selectively decontaminated, an identical conditioning regimen and the same number of bone marrow cells resulted in sustained engraftment in only 1/4 of the recipients (Vriesendorp et al., 1981). In rhesus monkeys complete decontamination of the gastrointestinal tract was accompanied by a delayed regeneration of leukocytes. Take failures occurred in a total of 4 out of 25 recipients. This is not statistically significant different from the 23% take failures which were observed in a comparable group of conventional recipients (Heidt, 1989).

1.4 GRAFT-VERSUS-HOST DISEASE

Until recently the most serious complication after transplantation of allogeneic bone marrow was GvHD and its associated infections. Two forms of GvHD have been identified: acute and chronic.

Acute GvHD is a process that develops early after transplantation and it manifests itself by lesions in skin, liver, gut and lymph nodes resulting in a variety of symptoms such as a generalized maculopapular rash, hepatitis, diarrhea, and a delayed reconstitution of haemopoietic and lymphoid function (Thomas, 1975: Zurcher 1985). In the period of early investigations into the nature of GvHD, discussions were held whether the severe atrophy of lymphatic tissue in animals suffering from "secondary disease" was due to a host-versus-graft immunological reaction (Makinodan, 1957) or to a graft-versus-host reaction. Circumstantial evidence for a graft-versus-host reaction was that the transfer of allogeneic lymphoid cells into an immunological nonresistent recipient caused a syndrome very similar to the secondary disease observed after allogeneic bone marrow (Billingham and Brent 1957; van Bekkum et al., 1957; Santos et al., 1958; Nowell et al., 1959; Vos et al., 1959; Trentin, 1959). These observations led to several experiments in which lymph node cells were added to bone marrow cells and grafted in lethally irradiated recipients. These bone marrow grafts caused severe disease and mortality in the recipients. The classical experiment, demonstrating the nature of the graft-versus-host reaction, is that transplantation of a fixed number of bone marrow and a graded number of lymph node cells resulted in a linear-log relationship between mortality and the number of lymphoid cells grafted (van Bekkum, 1964). Subsequent experiments have revealed the factors which influence the induction of acute GvHD:

- the cellular composition of the haemopoietic graft, especially the proportion T lymphocytes and stem cells
- the number of cells transplanted
- the immunogenetic disparity between donor and recipient
- the composition of the microflora of the recipient

For a detailed discussion of the influence of these individual factors, the reader is referred to a recent review (Van Bekkum, 1985).

After the classical experiments in mice which demonstrated that the number of lymphocytes added to a bone marrow graft is related to the severity of an acute GvH reaction, the causative role of lymphocytes in the induction of graft-versus-host disease was established and confirmed in other animal models. In dogs, transplantation of bone marrow from DLA-identical siblings will produce signs of acute GvHD in a minority of the recipients, since in this species bone

marrow contains a low proportion of lymphocytes. When donor lymph node cells are added to the DLA-matched bone marrow graft, the severity and incidence of GvHD accordingly increases. According to the observation that the incidence and severity of acute GvHD is dependent on the number of lymphocytes present in a bone marrow graft, a high incidence of early mortality due to severe acute GvHD is observed after allogeneic bone marrow transplantations in rhesus monkeys. In this species bone marrow contains approximately 20% T lymphocytes and induces severe acute GvHD in the majority of the recipients (7/8) of related, RhLA-identical donors (Wagemaker et al., 1982). Similar to the bone marrow of rhesus monkeys that of humans contains a high proportion of lymphocytes and subsequently the incidence of acute GvHD is approximately 50% in patients who receive a bone marrow graft of an HLA-identical sibling and of whom half will die (Thomas, 1975). Although the proportion of lymphocytes is almost identical in humans and rhesus monkeys, the reported incidence of acute GvHD is not identical. This is attributed to the fact that the rhesus monkeys received no additional treatment for the prevention of GvHD while human patients in general receive prophylactic agents such as methotrexate.

Differences in loci of the major histocompatibility complex (MHC) as well as in loci outside MHC also play an important role in the induction of GvHD. The influence of loci outside the MHC is illustrated by the well known occurrence of GvHD in of 50% of the patients grafted with bone marrow from an MHC-identical sibling. The incidence of severe GvHD is related to the degree of immunogenetic disparity between donor and recipient as was shown in several outbred species such as rhesus monkeys (Wagemaker et al., 1982), dogs (Storb et al., 1985) and humans (Hansen et al., 1986). The risk of severe acute GvHD (grade II to IV) appeared to be 40% for recipients of HLA-identical bone marrow graft, while the risk of acute GvHD was 68%, 75% and 92% when donor and recipient were incompatible for one, two or three loci of the MHC respectively (Hansen, 1986). The role of the different loci of the major histocompatibility complex on the induction of acute GvHD will be further discussed in section 1.7 (mismatched bone marrow transplantation).

The influence of the composition of the microflora of the recipient has been extensively studied in murine models for bone marrow transplantation. Infusion of 10^7 bone marrow cells from H-2 incompatible donors into lethally irradiated mice results in a late onset type GvHD. In this delayed type of GvHD, symptoms of the disease become manifest three weeks after transplantation and a majority

of the recipients will die (van Bekkum and de Vries, 1967). Mortality due to delayed GvHD can successfully be prevented when the recipients are germfree mice (Jones et al., 1971; van Bekkum et al., 1974; Truitt, 1978) or when the gastrointestinal tract of conventional mice has been decontaminated by means of nonabsorbable antibiotics prior to transplantation (Heit et al., 1973; Truitt, 1978; van Bekkum et al., 1974). An acute type of GvHD can be induced in mice by supplementing bone marrow cells with 10^7 spleen cells resulting in the deaths of the recipients within three weeks. Decontamination of the gastrointestinal tract delayed the onset of acute GvHD and mortality but did not prevent it (van Bekkum et al., 1974). The significance of gastrointestinal decontamination for outbred species will be discussed in detail in the next paragraph dealing with methods to prevent acute GvHD.

Because of the overriding influence of acute GvHD on the outcome of an allogeneic BMT, much research effort was put into the treatment of GvHD. In view of the immunologic etiology of GvHD, attempts to prevent it were focused either on the elimination of the immunocompetent donor T lymphocytes from bone marrow grafts or on the administration of immunosuppressive agents started before acute GvHD becomes apparent.

1.5 PREVENTION OF ACUTE GRAFT VERSUS HOST DISEASE

1.5.1 IMMUNOSUPPRESSION AFTER TRANSPLANTATION

Based on results obtained in experiments with mice (Uphoff, 1958) and especially with dogs (Thomas, 1962; Storb et al., 1970; Storb et al., 1970a), methotrexate (MTX) has been widely employed as prophylaxis for acute GvHD in patients (Thomas, 1975). Despite the use of HLA-matched siblings as donors and MTX for GvHD prophylaxis, the incidence of acute GvHD was still 30-70% and it was fatal in approximately one-half of the patients with GvHD (Thomas, 1975; Bortin et al., 1981; Storb et al., 1983; Weiden et al., 1979). The value of MTX as GvHD prophylaxis has been challenged recently. One group reported that four courses of MTX were as effective as seventeen standard courses of MTX for prophylaxis of acute and chronic GvHD (Smith et al., 1985). More intriguing is a report that no difference in the incidence of acute GvHD was

found between patients receiving standard MTX prophylaxis and patients who received no prophylaxis at all (Lazarus et al., 1984). Marrow toxicity of MTX resulted in a delayed engraftment in comparison to a control group. A controlled trial will be necessary to resolve this controversy.

The administration of antithymocyte globulin was quite effective in monkeys (Van Bekkum et al., 1972) and dogs (Storb et al., 1973). In contrast to these preclinical experiments, two randomized trials in human patients did not demonstrate any influence of antithymocyte globulin on the incidence of GvHD (Weiden et al., 1979a; Doney et al. 1981). However, when antithymocyte globulin was given in addition to MTX and prednisone, the incidence of GvHD was 21% in comparison to 48% in the group who received MTX alone (Ramsay et al., 1982).

Recently, a new and powerful immunosuppressive agent, cyclosporine A (Borel et al., 1977; Tutschka et al., 1979), has been introduced into clinical studies for the prevention of GvHD. Several pilot studies have been carried out showing that it is effective in preventing acute GvHD in humans (Powles et al., 1980; Speck et al., 1981; Hows et al., 1982). Controlled studies in dogs have shown that it is as effective as MTX in preventing GvHD in different genetic combinations (Deeg et al., 1982). The combined use of a short course of MTX and cyclosporine A till day 100 had superior results in DLA-mismatched littermates in comparison to historical controls treated with MTX alone (Deeg et al., 1984). These results in dogs encouraged the Seattle Bone Marrow Team to start several prospective trials (Table 1.3). An early report on the effect of either cyclosporine A or MTX on the incidence of GvHD in patients transplanted for leukemia in relapse, mentioned a superior result obtained when cyclosporine A was administered (Irle et al., 1985). In a recent report on the same patient group no difference between these drugs with regard to their potency to prevent acute GvHD was found (Storb et al., 1988). These results were confirmed in a randomized trial performed in Sweden (Ringden et al., 1986). In patients transplanted for acute nonlymphoblastic leukemia a 33% incidence of acute GvHD was found in the group receiving cyclosporine A, while 56% of the patients, who were given MTX, developed GvHD (Storb et al., 1986). No difference in the incidence of GvHD was observed in patients transplanted for chronic myelocytic leukemia (Storb et al., 1985). In none of the studies a significant difference in survival was observed. The combination of MTX and cyclosporine was very successful in dogs and a recent clinical studies have confirmed the superiority of

the combination over cyclosporine alone (Storb et al., 1987a) or methotrexate alone (Storb et al., 1987a). In these studies a reduction of the incidence of acute GvHD by the combination of MTX and cyclosporine A was associated with a reduction in the incidence of fatal infections and with an increased survival.

Table 1.3

Efficacy of Cyclosporine A alone or in combination with methotrexate to prevent acute GvHD.

disease	N	treatment	probability of acute GvHD		reference
leukemia, relapse	30	MTX	71%		
	26	CyA	45%	p=0.11	Irle, 1985
ANL	36	MTX	56%		
	39	CyA	33%	p=0.07	Deeg, 1985
CML	23	MTX	42%		
	25	CyA	46%	p=0.70	Storb,1985
Aplastic anemia	24	MTX	53%		
	22	MTX+CyA	18%	p=0.012	Storb,1986
ANL or AML	50	CyA	54%		
	43	MTX+CyA	33%	p=0.014	Storb,1986

ANL= acute nonlymphoblastic leukemia; CML= chronic myeloid leukemia;
MTX= methotrexate; CyA= Cyclosporine A.

Antibiotic decontamination of the intestinal tract did significantly reduce mortality due to delayed GvHD (Jones et al., 1971; van Bekkum et al., 1974; Heit, 1973; Truitt, 1978) and mitigated the severity of acute GvHD in mice (van Bekkum et al., 1974). Heidt (1989) investigated the influence of faecal flora of the donor on the induction of lethal GvHD. Lethal GvHD was only observed in a group of "conventional" recipients which received bone marrow cells from SPF

(specified pathogen free) donors. No GvHD was seen when there is identity between the microflora of recipient and donor or when SPF recipients were given bone marrow cells from "conventional" donors. Clinical studies regarding the influence of GID on the incidence and severity of GvHD are contradictory (Heidt et al., 1985; Heidt, 1989; Storb et al., 1983; Buckner et al., 1983; Buckner et al., 1985; Deeg et al., 1985; Skinhøj, 1987). Heidt (1989) reported a beneficial effect of complete GID in children with none of the 11 patients with successful GID developing severe GvHD. This in contrast to 7 out 29 patients in whom complete GID was not achieved. The difference in results could be due to either incomplete GID or to a too short period of decontamination, since in mice an effect of GID on GvHD is only observed when GID is successfully maintained from 10 days before (Truitt, 1979) until 40 days after bone marrow transplantation (van Bekkum et al., 1977).

1.5.2. T LYMPHOCYTE DEPLETION OF BONE MARROW GRAFTS

The most effective method for prevention of acute GvHD is elimination of immunocompetent T lymphocytes from a bone marrow graft. Two decades ago, experiments with mice have already proven that T lymphocyte depletion is an effective method to prevent acute GvHD (Dicke et al., 1968; Rodt et al., 1974). These results were confirmed in outbred species such as dogs (Kolb et al., 1979) and rhesus monkeys (Dicke et al., 1970; Wagemaker et al., 1982). The elimination of T lymphocytes from human bone marrow transplants required the development of methods, which were applicable for treatment of large volumes of human bone marrow. Meanwhile, several methods have been developed and applied for human transplant purposes such as albumin gradient (Dicke, 1970), a combination of density gradient and E-rosette sedimentation (Löwenberg et al., 1986), differential agglutination of mature blood elements with soybean agglutinin and subsequent removal of T lymphocytes from the unagglutinated bone marrow fraction by E-rosette sedimentation with sheep red blood cells (Reisner et al., 1980; Reisner et al., 1981; O'Reilly et al., 1986), complement mediated lysis of T lymphocytes using monoclonal antibodies (Martin et al., 1985; Sondel et al., 1985; Prentice et al., 1986; Racadot et al., 1986; Mitsuyasu et al., 1986; Hale et al., 1986, Herve et al., 1987), anti-T lymphocyte monoclonal antibodies

coupled to the toxin ricin (Vallera et al., 1983; Kersey et al., 1986) and counterflow centrifugation-elutriation (De Witte et al., 1983; De Witte et al., 1984).

Table 1.4

Prevention of acute Graft-versus-Host Disease by T lymphocyte depletion from bone marrow grafts of HLA-identical siblings.

BMT Center	number of patients	methods	take failure	GvHD >= grade II	reference
New York	57	soyb.	4	3/53	O'Reilly,1986
Seattle	20	mca	6	3/14	Martin,1985
Minneapolis	17	mca-ricin	4	4/13	Kersey,1986
Wisconsin	7	mca	0	0/7	Bozdech, 1985
London	21	mca	1	1/20	Prentice,1986
France	57	mca	11	4/52	Racadot,1986
Los Angeles	20	mca	5	3/20	Mitsuyasu,1985
Campath-1*	264	mca	34	34/230	Hale,1986
Nijmegen	14	elutr.	0	2/14	De Witte,1984
Utrecht	7	soyb.	0	2/7	De Gast,1986
Rotterdam	8	albumin	1	2/7	Löwenberg,1986
Rotterdam	17	alb.+ E-r	0	0/17	Hagenbeek,1987
Paris	21	mca	0	3/21	Gluckman,1987
	530		66 (12.5%)	61/464 (13.1%)	

mca = monoclonal antibody and complement;
soyb. = soybean agglutination and E-rosette sedimentation;
elutr. = elutriation centrifugation;
albumin = discontinuous albumin density gradient;
alb+E-r = disc. albumin density grad. + E-rosette sedimentation.
*: review of results in BMT centers which use the monoclonal antibody CAMPATH-1 for T lymphocyte depletion.

So far reports are very encouraging with respect to the incidence of severe, acute GvHD (13%: see Table 1.4). This score surpasses the best results obtained

by immunosuppressive treatment of recipients after transplantation as prophylaxis for GvHD.

Difference between the various methods used for T lymphocyte depletion are not being observed. All methods deplete 1-2 logs T lymphocytes, which is sufficient to prevent acute GvHD in this donor-recipient combination according to our retrospective analysis of the results of transplantations of T lymphocyte depleted bone marrow grafts in different donor-recipient combinations in rhesus monkeys (Wagemaker, 1986a). The frequency of severe GvHD does not seem to be affected by the use of additional immunosuppressive treatment of recipients after transplantation. Only the BMT center in Nijmegen using elutriation centrifugation for T lymphocyte depletion observed severe GvHD when additional immunosuppresion was not administered after transplantation.

The question of how many T lymphocytes are permitted in a bone marrow graft has yet to be answered. In patients, who received a bone marrow graft depleted from T lymphocytes by CAMPATH-1 and autologous complement, the incidence of acute GvHD was significantly correlated with the presence of more than 4 x 10^6 T lymphocytes/kg body weight after purging (Hale et al., 1988). In 24 patients the measured number of T lymphocytes was more than 4 x 10^6 T lymphocytes/kg body weight and 14 (58%) of them suffered from some degree of GvHD compared to 48 (23%) of 211 patients who's bone marrow graft contained less than 4 x 10^6 T lymphocytes/kg body weight. Quantification of clonable T lymphocytes have demonstrated that 1 x 10^5 clonable T lymphocytes/kg body weight correlated with no acute GvHD (Kernan et al.,1986). Atkinson et al (1987) has also found a direct relation between the number of T lymphocytes in a bone marrow graft and the frequency of acute GvHD. On basis of a retrospective analysis of the relationship between the number of T lymphocytes in a bone marrow graft and the incidence of lethal GvHD in different genetic combinations, the maximum number of T lymphocytes allowable in a bone marrow graft without causing lethal GvHD were calculated. For RhLA-identical sibling monkeys the maximum number of T lymphocytes/kg body weight was found to be 2 x 10^6 T lymphocytes. In a bone marrow graft from an unrelated, A/B matched donor 6 x 10^5 T lymphocytes/kg body weight are permitted and in unrelated, totally mismatched donor-recipient combinations only 10^5 T lymphocytes/kg body weight are allowed (Wagemaker, 1986a).

The encouraging results with T lymphocyte depleted bone marrow grafts in HLA-identical siblings were followed by attempts to apply T lymphocyte deple-

tion for mismatched transplantations. The preliminary results of these trials are discussed in section 1.7.

1.5.2.1 ENGRAFTMENT OF T LYMPHOCYTE DEPLETED BONE MARROW GRAFTS

An undesirable consequence of the utilization of T lymphocyte depletion is the increased frequency of take failures if the conditioning of the patients is not adapted. It was clearly and timely demonstrated in canine (Vriesendorp et al., 1981a), rhesus monkeys (Wagemaker et al., 1982) and murine (Soderling et al., 1985) models of mismatched BMT that a take of T lymphocyte depleted allogeneic marrow requires more intense conditioning than T lymphocyte containing grafts. Results with patients confirmed these findings, especially when partially mismatched, related donors were used (Bozdech et al., 1985; O'Reilly et al., 1985). Nonspecific loss of haemopoietic progenitor cells during the T lymphocyte depletion could partially contribute to this effect as was measured by granulocyte/macrophage colony formation (GM-CFU) (Patterson et al., 1986; Blazar et al., 1985; Martin et al., 1985; Herve et al., 1985). However, the reduced incidence of engraftment was still observed after transplantation of a non limiting number of stem cells in animal experiments (Vriesendorp et al., 1981; Wagemaker et al., 1982; Soderling et al., 1985) and no correlation has been found between the number of GM-CFU infused and failure of engraftment after T lymphocyte depletion (Martin et al., 1985; O'Reilly et al., 1985). A more likely explanation is that T lymphocyte depletion of the graft reduces immunosuppression of the recipient by abrogation of the anti-host immunological reactivity of the graft. This concept of **reciprocal interference** (Nakic, 1966; Vriesendorp et al., 1981b) between GvHD and engraftment is endorsed by the fact that additional immunosuppression of recipients promotes engraftment of T lymphocyte depleted grafts in experimental animals (Vriesendorp et al., 1981a; Wagemaker et al., 1982; Soderling et al., 1985) and humans (O'Reilly et al., 1986; Martin et al., 1985; Sondel et al., 1985) .

An alternative explanation for the increased frequency of engraftment failures of T lymphocyte depleted bone marrow graft is that T lymphocytes have an essential function during the engraftment. This hypothesis is based on the knowledge that T lymphocytes can produce several haemopoietic growth factors (Metcalf, 1986). Hence, depletion of T lymphocytes would deprive the graft of

the growth factors essential for proliferation of haemopoietic cells (Sieff et al., 1985; Soderling et al., 1985).

1.5.2.2 T LYMPHOCYTE DEPLETION AND LEUKEMIA RELAPSE

When leukemic cells are among the targets of a GvH reaction, it is expected that a mitigation of the GvH reaction would have consequences for the graft-versus-leukemia (GvL) reaction. This hypothesis is supported by the observation that the incidence of leukemia relapses in patients with acute nonlymphoblastic leukemia is much higher in recipients of identical twin bone marrow grafts (50%) than in recipients of HLA-matched siblings (reviewed by O'Reilly, 1983). Several studies in humans have reported a decreased risk of leukemia recurrence in patients who develop acute or chronic GvHD following transplantation from HLA-identical siblings (Weiden et al., 1979a; Weiden et al., 1981; Bacigalupo et al., 1985). On the basis of these reports one could predict that a reduction in the incidence of GvH will be correlated with an increase in leukemia relapses. Analysis of the results of patients, who received an allogeneic bone marrow transplantation for chronic myeloid leukemia in chronic phase, has indeed demonstrated that T lymphocyte depletion decreases the incidence and severity of acute and chronic GvHD, but this effect was counterbalanced by a significant increase in leukemia relapses (Apperley et al., 1986, 1988; Goldman et al., 1988). The actuarial survival of patients of T lymphocyte depleted or unmodified bone marrow grafts did not differ significantly. For acute leukemia firm conclusions can not be drawn regarding a relationship between T lymphocyte depletion and leukemia relapse. Mitsuyasu et al. (1986) reported a higher number of relapses in the group of patients which received a T lymphocyte depleted bone marrow graft in comparison to controls but the difference was not significant. In other early reports, the incidence of leukemia relapses in good risk patients, who received a T lymphocyte depleted graft, was not significantly different from the incidence of relapse in comparable good risk patients transplanted with conventional grafts (O'Reilly et al.,1986; Prentice et al.,1986).

1.6 CHRONIC GVHD

Chronic GvHD is a separate clinical syndrome affecting between 15 to 40% of the transplanted patients who survive more than 6 month's. Two thirds of the patients with chronic GvHD have preceding acute GvHD but in one-third it develops without preceding clinical signs of acute GvHD. It results in localized or widespread sclerodermatous changes of the skin, skin and joint contractures, xerostomia, xerophthalmia, biliary cirrhosis, malabsorption, and failure to thrive (Shulman et al., 1980; Sullivan et al., 1981). The disease resembles the systemic collagen vascular diseases, especially systemic lupus erythematosus and progressive systemic sclerosis. Predisposing factors for the development of chronic GvHD are moderate to severe acute GvHD, increasing age of the patient and the use of viable donor buffy coat cells in addition to the marrow in case of aplastic anemia (Storb et al., 1983; Ringden et al., 1985). Donor buffy coat cells were added to bone marrow to prevent engraftment failures. The beneficial effect can be attributed to more GvHD in comparison to bone marrow alone. Subsequently more chronic GvHD was seen. Treatment with immunosuppressive drugs, either prednisone alone or in combination with azathioprine, cyclophosphamide, or procarbazine, has been shown to be effective in abrogating the adverse natural course of extensive chronic graft-versus-host disease (Sullivan et al., 1981).

1.7 MISMATCHED BONE MARROW TRANSPLANTATIONS

HLA compatibility between donor and recipient is generally felt to play a critical role in clinical bone marrow transplantation. The incidence of GvHD is still high (50%) when related donors are used who are totally matched for the MHC antigens and methotrexate is applied for GvH prophylaxis. For this reason it is widely held that the probability of achieving a successful transplant is diminished by HLA incompatibility. However, the majority of patients who are candidates for bone marrow transplantation do not have HLA-identical siblings. Therefore, several studies were started to explore the use of related, mismatched donors and unrelated donors.

In rhesus monkeys the effect of donor selection on acute GvHD has been studied extensively. Transplantation of stem cell enriched, T lymphocyte depleted bone marrow grafts in related donor-recipient combinations has

revealed that the incidence of lethal GvHD is low (1/8: median survival > 2 years) in RhLA-identical siblings, and high (4/5: median survival 25 days) when donor and recipient were mismatched for the D/DR loci (and identical for the A/B loci). Experiments with unrelated donors demonstrated that matching for the A and B loci (and mismatching for the D/DR loci) reduced the incidence and severity of GvHD (5/9: median survival 50 days), but the effect of matching became apparent only when stem cell enriched, T lymphocyte depleted bone marrow grafts were employed (Wagemaker et al., 1982, 1986a). The results of matching for the A and B loci became more impressive when the gastrointestinal tract was completely decontaminated. In this situation no lethal GvHD occurred in 4 monkeys (Heidt, 1989).

In the past decade, cautious exploration of the use of family-member donors other than HLA-identical siblings has been started in clinical centres. As mentioned earlier in this chapter, the probability of severe (grade $\geq$ 2), acute GvHD increases when the immunogenetic disparity between donor and recipient increases. In the study of the Seattle Bone Marrow Transplantation Team (Hansen et al., 1986) the risk of acute GvHD was 40% at day 75 after transplantation when donor and recipient were HLA phenotypically identical and reactive in the MLC. The risk of severe, acute GvHD was 68% in case of HLA one locus incompatibility, 75% when donor and recipient were incompatible at two loci and 92% when three HLA loci differed. Incompatibility for class I antigens appeared to have the same effect on GvHD as incompatibility for class II. In this study of the Seattle Bone Marrow Transplantation Team, it appeared that HLA incompatibility can impede the rate of engraftment, increase the risk of rejection and substantially increase the incidence of acute GvHD. An analysis of the survival of 25 patients transplanted in remission with marrow from a donor who was incompatible for one HLA locus, showed no significant difference with a series of recipients with genotypically identical donors (Hansen et al., 1986). Powles et al (1983) described the clinical course of 35 patients, who received a marrow graft from mismatched family donors. Eleven patients (31%) survived the transplantation. A remarkable finding was that 12 patients died from a capillary leakage syndrome, which was associated with pulmonary oedema, convulsions, intravascular haemolysis and renal failure.

The encouraging results on the prevention of acute GvHD by T lymphocyte depletion prompted several centers to investigate whether this approach could reduce the risk of mismatched transplantations (Table 1.5). The longest expe-

rience with mismatched bone marrow transplantation has been gained with the treatment of young patients with severe combined immunodeficiency (SCID). The published results indicate that engraftment failures is a major obstacle. However, the incidence of severe, acute GvHD is low and the probability of survival (60%) does not differ significantly in those patients, who received HLA mismatched, T lymphocyte depleted bone marrow grafts, and patients who's donors were HLA-identical siblings (Fisher et al., 1986a; O'Reilly et al., 1987). Transplantation of HLA-mismatched, not T lymphocyte depleted marrow grafts was associated with a poor survival prognosis of 18% (Fisher et al., 1986a).

Table 1.5

Transplantations of T lymphocyte depleted bone marrow grafts from related, mismatched donors.

Disease	Conditioning	N (number)	Engraftment (number)	GvHD ≥2	Reference
Leukemia	1320 cGy+Cy	5	0		Bozdech,1985
	1320 cGy+Cy+ARA-C	4	4	3	Bozdech,1985
	1320 cGy+Cy+ARA-C+ Csa	10	10	2	Bozdech,1985
Leukemia	1200 cGy+ARA-C Melphalan+ATG	15	14	2	Cahn, 1988
Leukemia	1320-1440 cGy+ARA-C or + Cy	48	32	3	O'Reilly, 1987
Leukemia	1200 cGy+ARA-C+Cy	8	0		Henslee,1987
	1400 cGy+ARA-C+Cy	24	23	9[a]	Henslee,1987

cGy = Total body irradiation dose in centiGray; ARA-C = cytosine arabinoside;
Cy = Cyclophosphamide; Csa = Cyclosporine A
a: acute GvHD more than grade 2

The early reports on patients with leukemia, who received HLA-mismatched, T lymphocyte depleted bone marrow grafts, indicate that the obstacles are the same as in recipients of T lymphocyte depleted, HLA-identical marrow grafts, namely an increased frequency of engraftment failures. Several clinical centers have searched for adequate conditioning regimens which permit sustained engraftment. In New York 34 recipients of HLA-mismatched marrow were conditioned for grafting by hyperfractionated TBI followed by cyclophosphamide or ARA-C and additional pretransplant immunosuppressive drugs (Anti Thymocyte Globulin (ATG) + procarbazine or ARA-C) and 15 rejected their graft. Of these 34 patients, 22 patients received immunosuppressive drugs (cyclosporine A or steroids) for promotion of engraftment, but 8 of these 22 patients still rejected the bone marrow graft. The most successful treatment was the administration of ATG in the early posttransplant period, which resulted in 17 out of 18 durable engraftments (O'Reilly et al., 1987). The other transplantation centers (Wisconsin and Lexington) also found that the problem of engraftment failures could be overcome by additional immunosuppression (Bozdech et al., 1985; Henslee et al., 1987). The incidence of severe acute GvHD ranged from 10% (O'Reilly et al., 1987) to 40% (Henslee et al., 1987). In contrast to SCID patients, survival of leukemia patients with HLA-mismatched donors was inferior to survival of recipients of HLA-identical bone marrow grafts. O'Reilly (1987) reported that the inferior survival was mainly due to more lethal infections in comparison to HLA-matched bone marrow transplantations.

The possibility of using unrelated donors who are phenotypically HLA-identical with the patient is attracting more attention. A few successful transplantations have been reported (Hansen et al., 1980; Hows et al., 1987; Duquesnoy et al., 1983; Liu Yin et al., 1984; McGlave et al., 1987), but it is to early to draw any conclusions. The same holds for the use of unrelated, partially, mismatched donors (Gingrich et al., 1985; McGlave et al., 1987).

1.8 RHESUS MONKEY; AN ANIMAL MODEL FOR EXPERIMENTAL BMT

For more than 25 years Rhesus monkeys (Macaca Mulatta) and Cynomolgous monkeys (Macaca Iris) have been employed for experimental bone marrow

transplantations. The Radiobiological Institute TNO has a long experience of approximately 30 years with bone marrow transplantation in rhesus monkeys (Crouch et al.,1961).

1.8.1 IRRADIATION AND ENGRAFTMENT IN RHESUS MONKEYS

The radiosensitivity of rhesus monkeys has been determined for 300 kV X-rays as well as for fission neutrons (Broerse et al., 1978). Over a range from 450 to 700 cGy (X-ray), there appeared to exist a steep dose-survival curve with 100% 30 day survival after a dose of 450 cGy and no animal surviving a dose of 600 cGy (hvl 2.0 mm Cu; dose rate 25-30 cGy). The LD_{50} of rhesus monkeys has been estimated to be approximately 5.25 Gy. Autologous bone marrow protects up to a dose of 900 cGy. After a total body irradiation of 1000 cGy and more all the animals died due to gastrointestinal toxicity within 17 days. The dose survival curve was even more steeper for neutron irradiation since after 200 cGy all animals survived and none survived a dose of 300 cGy.For neutrons the LD_{50} was estimated to be 250 Gy. For animals treated with autologous bone marrow grafts the 50% survival point was reached after 475 cGy (Broerse et al., 1978).

The number of fresh autologous bone marrow cells required for engraftment is approximately 1 x 10^7/kg body weight (van Putten et al., 1962), while engraftment of allogeneic marrow grafts 2.5-3 x 10^8/kg body weight (van Putten et al., 1962) is required. Transplantation experiments with fetal monkey liver and spleen of 3-month gestation demonstrated that the number of cells obtained from one single donor (2-3 x 10^8/kg body weight) was insufficient for successful engraftment (van Putten et al., 1962).

1.8.2 BONE MARROW COMPOSITION IN RHESUS MONKEYS

With respect to the potency to induce an acute GvH reaction, rhesus monkey bone marrow is identical to human bone marrow. Aspiration marrow contains approximately 23 $\pm$ 2 % T lymphocytes (Wagemaker et al, 1982). In RhLA-identical siblings 2-4 x 10^8 bone marrow cells/kg body weight induced severe, fatal GvHD in 90 % of the recipients with a median survival of 22 days

(Wagemaker et al., 1982). When unrelated donors were used, the median survival time was 12 days, deaths being uniformly due to severe GvHD (Wagemaker et al., 1982).

In search of methods which could be applied to mitigate the incidence and severity of acute GvHD, density gradients were used to separate stem cells from lymphocytes (Dicke, 1970; Moore et al., 1972). Early progenitor cells, which are able to form colonies in the GM-CFU assay, can be recovered from the light density fractions of a discontinuous albumin density gradient. Among these early progenitor cells, haemopoietic stem cells are present as has been proven by the experiments in which transplantation of 2 x 10^6 autologous, low density cells/kg body weight protected a lethally irradiated monkey (Dicke, 1970; Dicke et al., 1973). The phylogenetic relation between man and rhesus monkeys has always been an advantage. Recently, many monoclonal antibodies directed against human lymphocytes (Letvin et al., 1983; Jonker et al., 1983) and myeloid cells (Letvin et al., 1983; Pesando et al., 1985) appeared to crossreact with rhesus monkey cells. So, for a variety of reasons, rhesus monkeys are well suited to test the feasibility of using monoclonal antibodies.

1.8.3 GRAFT VERSUS-HOST DISEASE IN RHESUS MONKEYS

The morbidity and mortality pattern in rhesus monkeys is identical to humans. The clinical symptoms comprise diarrhea and anorexia and may start as early as the end of the first week, and death ensues between day 8 and 17 after transplantation of marrow from unrelated donors. The median survival of rhesus monkeys is 25 days after transplantation of bone marrow from a RhLA-identical sibling. The use of other genetic donor-recipient combinations, i.e. unrelated matched donors and family donors mismatched for 1 haplotype, resulted in a shorter median survival time (Wagemaker et al., 1982). The pathological features of the lesions induced by acute GvHD have been object of intensive study in the Radiobiological Institute TNO and have been described in detail (de Vries et al., 1961; de Vries, 1967; Zurcher, 1985). Similar to humans, the graft versus host reaction damages particularly the epithelium of the gut, the skin and the liver, resulting in clinical symptoms such as diarrhea, anorexia, jaundice, erythaema, and a rapid loss of weight (wasting).

After establishing the pattern of morbidity and mortality due to GvHD, several methods have been tried to mitigate the incidence of acute GvHD in rhesus monkeys (Table 1.6). The early experiments included preirradiation of bone marrow. Experiments in mice had demonstrated that preirradiation might have a favorable influence on the incidence of secondary disease (the early description for GvHD) (Cudkowicz, 1961). A fourfold increase in cell number was given to compensate for nonspecific cell loss after irradiation. Survival and severity of GvHD was not influenced by preirradiation of a bone marrow graft with 250 cGy X-rays. A similar experiment with a fourfold increase in cell number and 400 cGy of X-rays resulted in a longer survival with evidence of temporary and incomplete regeneration of bone marrow (van Putten et al., 1962).

Table 1.6

Mitigation of Acute GvHD in unrelated rhesus monkeys

Method to prevent acute GvHD	number of animals	mean survival (days)	range (days)	reference
controls	25	14	5-25	van Putten,1962
pre-irradiation graft				
250 cGy	1	8		
400 cGy	1	>40[a]		van Putten,1962
storage at 4° C				
0 hours	2	11	9-13	
48-96 hours	4	13	7-16	
120 hours	1	no regeneration		van Putten,1962
Amethopterin	4	19	11-27	Muller-Berat,1966
cyclophosphamide	7	39	23-65	Muller-Berat,1966
Anti-lymphocyte-serum				
horse	15	27	13-41	van Bekkum,1972

a: endogenous regeneration

A simple method of storage of bone marrow cells at + 4° C had been proved to be much more successful in mice (van Bekkum, 1964). This method mitigated the severity of GvHD and did not diminish the radiation protection potency. In unrelated monkeys, this method appeared not successful (see Table 1.6; van Putten et al., 1962). This was the first experiment in which it was concomitantly demonstrated that methods which are effective in decreasing the secondary disease in rodents are not necessarily effective in monkeys due to the difference in the proportion of immunocompetent lymphocytes present in bone marrow. Accordingly it was recommended to carry out preclinical experiments in monkeys before applying methods, developed in mice, in clinical practice.

Amethopterin (Methotrexate) is one of the earliest drugs applied for the treatment of GvHD. Uphoff (1958) was the first to report a beneficial effect of amethopterin in mice. Thomas described a pronounced beneficial effect of post-transplantation administration of this drug in dogs. Fifty percent of a series of 20 dogs survived more than 150 days after allogeneic bone marrow transplantation. Graft rejection occurred in 5 dogs (Thomas et al., 1962). The bone marrow transplantation group in Rijswijk has tried to reproduce these results in rhesus monkeys. Mortality due to acute GvHD was prevented in 3 out of four 4 monkeys, but long time survivors were not observed (Muller-Berat et al., 1966). Cyclophosphamide mitigated more successfully the incidence and severity of GvHD and markedly prolonged survival when it was administered after transplantation (Muller-Berat et al., 1966).

Going along with the ideas that secondary disease was caused by donor lymphocytes, treatment protocols were designed to eliminate lymphocytes. In the earliest experiments anti-lymphocyte sera (ALS) were prepared by immunizing rabbits or horses with thymocytes. Van Bekkum et al (1972) injected monkeys 24 hours before BMT subcutaneously with ALS and investigated the effect on the incidence and severity of GvHD. The ALS produced in rabbits appeared to be toxic not only to lymphocytes but also to other bone marrow cells since only 3 out of 11 monkeys showed engraftment. Pretreatment of the recipient with horse ALS resulted in engraftment in 15 out of 16 animals. Survival ranged from 7-41 days. Severe to very severe signs of GvHD were seen at autopsy in 5 out of 15 monkeys. Mild GvHD was observed in the other 10 monkeys, but none of the animals escaped death from GvHD at a latter stage. Again these results did not confirm the results in mice, where a single administration of ALS before administration fully prevented GvHD (Van Bekkum et al., 1970).

At the same time it was attempted to eliminate donor lymphocytes from a bone marrow graft by density centrifugation using a discontinuous albumin gradient (Dicke, 1970). In the low density fractions the proportion of haemopoietic stem cells is greater than in normal bone marrow. The proportion of lymphocytes in the low density fraction is similar to normal bone marrow. However, the enrichment of stem cells enabled transplantation of approximately 10 times less cells, which means that the absolute number of lymphocytes is accordingly 10 times lower than in an unseparated bone marrow graft. An one log lymphocyte depletion was sufficient to lower the incidence and severity in RhLA-identical siblings and long term survivors were seen. These results were confirmed in human studies, which demonstrated that this method did reduce the incidence and severity of GvHD in HLA-identical siblings (Löwenberg et al., 1986). For a complete prevention of acute GvHD 1-2 log T lymphocytes had to be removed for rhesus monkey bone marrow (Wagemaker et al., 1982) as well as for human bone marrow (Löwenberg et al, 1986; Hagenbeek, 1987).

1.9 OUTLINE OF THIS THESIS

The major complication after allogeneic bone marrow transplantation is the occurrence of acute GvHD. This complication can be successfully prevented when T lymphocytes are depleted from a bone marrow graft. The beneficial effect of prevention of acute GvHD is counterbalanced by an increased frequency of engraftment failures. In view of this problem several experiments were planned to unravel whether the engraftment failures after T lymphocyte depletion were attributable to the loss of the trophic function of T lymphocytes or were attributable to the loss of the immunosuppressive action of donor T-lymphocytes. When the latter option is valid, more immunosuppression of the recipient would result in sustained engraftment of T lymphocyte depleted bone marrow grafts.

With regard to T lymphocyte depletion, it was attempted to positively select for stem cells and concomitantly negatively select for T lymphocytes. Stem cells are the essential cells in a bone marrow graft since they are responsible for sustained engraftment. A positive selection of these cells would yield an uniform method for preparation of a bone marrow graft rich in stem cells and fully depleted of T lymphocytes. A second approach was to investigate whether deple-

tion of a subpopulation of T lymphocytes (helper/inducer or suppressor/cytotoxic T lymphocytes) was sufficient to prevent acute GvHD.

A general introduction to bone marrow transplantation is described in **chapter I.** Materials and methods are described **chapter II.**

To assess the influence T lymphocyte depletion on the repopulating ability of stem cells, an in vivo assay had to be developed, since in outbred species a reliable assay to predict the repopulating ability of a bone marrow graft was not available. The development of the assay was based on the assumption that the regeneration rate of peripheral blood cells after autologous bone marrow transplantation is a function of the number of stem cells present in marrow graft. The relationship between regeneration patterns of peripheral blood cells and graded numbers of autologous bone marrow is described in **chapter 3.** After establishing this in vivo assay, T lymphocyte depleted autologous bone marrow grafts were transplanted. As a control on the predictive value of the assay, Dr-positive cells were depleted from autologous grafts assuming that this would drastically reduce the repopulating ability of a bone marrow graft. This assumption was based on the fact that the Dr antigen is present on stem cells in several species.

In **chapter 4,** an attempt is made to purify the hemopoietic stem cell of the rhesus monkey. A positive selection of stem cells of rhesus monkeys was made using a discontinuous albumin density gradient and a positive selection of Dr-positive cells by flow cytometry. Ultimately the capacity of purified cells to repopulate an irradiated recipient was investigated.

In **chapter 5,** the promotion of engraftment of T lymphocyte depleted, allogeneic bone marrow grafts has been studied. It was attempted to quantify the additional immunosuppression required to obtain engraftment of T lymphocyte depleted grafts.

In **chapter 6,** the efficacy of monoclonal antibodies, directed against either subpopulations of T lymphocytes or against all lymphocytes, to prevent acute GvHD was studied.

A general discussion on these different investigations is given in **chapter 7.**

CHAPTER II

MATERIALS AND METHODS

2.1 ANIMALS

Young male or female rhesus monkeys (Macaca Mulatta), bred at the Primate Center TNO, were used. The monkeys weighed 2.5 to 4 kilogram and were 3 to 4 years of age at the time of transplantation.

2.2 ANIMAL CARE

2.2.1 ISOLATION PROCEDURE

Before transplantation, the physical condition of the animals was inspected and blood samples were taken for hematological and biochemical examination. The faecal flora was investigated on the presence of flagellates. One week before transplantation, animals were transferred to either isolations rooms or laminar air flow units. Transfer of infections was prevented by caging the monkeys separately. To prevent contamination from external sources, barrier nursing and disinfection of materials, introduced into the room, was carried out routinely. The animals were placed in a restraint chair for physical examination and handling such as administration of medicines at least once per day. The animals were not anesthetized for this procedure. Daily blood samples were taken to measure repopulation of the haemopoietic system and two to three times a week for biochemical tests. The sensitivity pattern of faecal flora was determined twice weekly. On the basis of the antibiograms of the faecal flora nonabsorbable antibiotics were given.

2.2.2 FOOD AND DRINKING

Monkeys were fed fresh fruits, vegetables and cooked rice. When an animal did not eat spontaneously, it was fed by stomach tube. A catheter was passed through the nostrils into the stomach. Through the stomach tube the monkeys were administered daily ± 240 ml of a mixture consisting of 120 ml Nutrison (Nutricia, Holland), 80 ml glucose 10% and 40 ml Carvan Cevitam (a baby fruit syrup; Zwaardemaker, Holland). This liquid diet was composed on basis of an estimated energy need of 80-90 kcal/kg body weight. Drinking water was acidified to pH 3 to limit microbial growth.

2.2.3 ANTIBIOTICS

For the prevention of infections caused by Pseudomonas aeruginosa, the nonabsorbable antibiotic Polymyxine B® (10 mg/kg/day; Pfizer) was given to the animals from four days prior to transplantation until hematological recovery. Nystatine® (2 x 200.000 IE/day; Sanofi Labaz) was given together with Polymyxine B to prevent fungal infections. For the elimination of intestinal flagellates, the animals received metronidazole (250 mg/day per os: Flagyl®, Specia/May & Baker) for four days prior to transplantation. When after transplantation the leukocyte counts dropped below 1 x 10^9/l, the antibiotic regimen was extended with the systemic antibiotics tobramycine (Obracin®, Lilly): 3-5 mg/kg/ day intramuscular (i.m.) and cephamandol (Mandol®, Lilly): 100 mg/kg/ day i.m.). Changes in the antibiotic regimen occurred on the basis of the sensitivity pattern of faecal flora and cultures of blood samples. Other antibiotics, which were given regularly, were; ticarcilline (100 mg/ kg/day i.m.: Ticarpen®, Beecham), amukacinesulphate (5 mg/kg/day i.m.:Amukin®, Bristol), rifamycine (15-20 mg/kg per os: Rifadin®, Gist-brocades) and Vancomycin® (20-25 mg/kg intravenous, Lilly).

2.2.4 SUPPORTIVE CARE

Irradiated whole blood or red blood cell concentrates were given when the hematocrit dropped below 20%. Irradiated platelet transfusion were ad-

ministered when thrombocyte counts fell below 40 x 10^{12}/l or when thrombopenic purpura were observed. Balanced fluids (1:1 glucose 5% and Ringer's lactate) were given intravenously or subcutaneously when signs of dehydration were observed.

2.3. PREPARATION OF CELL SUSPENSIONS

2.3.1 BONE MARROW CELL SUSPENSION

Bone marrow cells were obtained either by aspiration from femoral and/or humoral shafts as was described previously (Van Bekkum, 1967) or the donor was sacrificed and bones of the vertebral column, sacroiliac bones, femora, humeri, sternum, ribs and scapula were pressed in a tissue press. Cells were collected in phosphate buffered salt (PBS) or in Hank's balanced salt solution (HBSS) which contained 100 IE heparin/ml (Tromboliquine®; Organon Teknika, Holland) and deoxyribonuclease I (DNAse; Behring Diagnostics, La Jolla, CA). A mononuclear cell suspension was prepared by centrifugation through a ficoll-hypaque solution(LSM®, density of 1.077 g/ml; Bionetics, Kensington, UK) for 30 min at 1600 rpm (TJ-6 Beckman centrifuge; Beckman-RIIC, Scotland). After centrifugation, the mononuclear cells were recovered from the interface. Thereafter, the cell suspension was filtered through a nylon gauze to obtain a monocellular cell suspension.

2.3.2 DISCONTINUOUS ALBUMIN DENSITY CENTRIFUGATION

A discontinuous albumin density gradient, designed by Dicke and van Bekkum (1970), was employed to obtain cell suspensions enriched for stem cells. Albumin powder (fraction V: Sigma Chemical Co., St Louis, MO) was dissolved in Tris buffer (pH 7.2, 230 mOsm/l) to a concentration of 35% (w/w) . The optimal osmolarity of the albumin solution was tested in pilot experiments in which the distribution of GM-CFU's was measured over a gradient. After establishing the optimal osmolarity, a 35% stock solution was prepared. This 35% solution was diluted with different volumes of sodium chloride-sodium phosphate buffer (NaCl-Phosphate buffer; 0.154 M NaCl; 0.01 M Na phosphate buffer; pH 7.2;

299-300 mOsm/l) for the preparation of the desired albumin solutions, namely 17%, 19%, 20%, 21%, 23% and 25%. The final albumin concentration was checked by an Abbe refractometer (Zeiss), as previously described by Dicke (1970).

Mononuclear cells were suspended in 17% BSA and layered on top of a gradient consisting of layers with different density (17% -25%). The tube was centrifuged for 30 min at 10^0 C at 2200 rpm (Beckman centrifuge). After centrifugation, distinct layers were visible at the density interfaces in the gradient. Cells were collected from the interfaces and washed with PBS.

2.3.3 CELL COUNTING

Nucleated cells were counted either in a haemocytometer in Turk solution (0.01% crystal violet and 1% acetic acid in saline) or by an automated cell counter (ELZONE; Particle Data Inc, Elmhurst or BAKER; Baker Instruments, Bethlehem, NY). Both automated cell counters were calibrated for counting cells of rhesus monkeys by values obtained in haemocytometers. The number of erythrocytes and leukocytes in the peripheral blood of the monkeys were counted by either a particle analyzer of ELZONE (Particle Data Inc) or of BAKER (Baker Instruments). The leukocyte counts were corrected for erythroblasts. Thrombocytes were counted by a thrombocyte counter from BAKER. Reticulocytes were counted after staining slides with brilliant cresylblue (3.3% (v/v) in ethanol). Thereafter the percentage of reticulocytes were determined after counting at least 1000 erythrocytes.

2.4 MONOCLONAL ANTIBODIES

For incubation with monoclonal antibodies (MCA), cells were pelleted and MCA's were added to the pellet. The cells were mixed with antibody solution and incubated for 30 min at 4^0 C. After washing with PBS, either goat-anti-mouse(GAM), conjugated to fluorescein isothiocyanate (FITC) (Nordic, Holland) (diluted 1:80 with PBS containing 20% monkey serum) or goat- anti-rat, conjugated to FITC, (GARA/FITC: diluted 1:30; Nordic, Holland) was added to the cells labelled with MCA. The incubation time was again 30 min at 4^0 C.

2.4.1 ANTI T LYMPHOCYTE ANTIBODIES

The OKT antibodies were purchased from Ortho Diagnostics (Raritan,NJ). Per 10^6 pelleted cells 0.02 ml MCA was added. From the Leu antibodies (Becton Dickinson, Sunnyvale, CA) 0.01 ml was added to 10^6 pelleted cells. From B9-pool (CD8; 0.05 mg/ml : provided by Dr G. Mawas, Marseille) 0.02 ml was added to 10^6 cells. WT 1 (CD7; 0.005 mg/ml; Tax et al., 1984) was a gift from Dr W. Tax (St Radboud Hospital, Nijmegen, Holland;) and used in concentration of 0.02 ml for 10^6 cells.

Table 2.1

Anti T lymphocyte antibodies and their reactivity pattern with human lymphocytes.

CD	MCA	reactivity pattern	references
CD1	Leu1	T lymphocytes	Hansen, 1984
CD2	Leu5	E-rosette receptor	Hansen, 1984
CD4	OKT4	helper/inducer	Hansen, 1984
	OKT4a	T lymphocytes	
	Leu3a		
CD8	OKT8	cytotoxic/suppressor	Hansen, 1984
	Leu2	T lymphocytes	
	B9-pool		
CD7	WT 1	T lymphocytes	Tax, 1984
---	CAMPATH-1	T and B-lymphocyte	Hale,1983

CD= cluster defined nomenclature as proposed by the international work-shop on leukocyte antigens (Bernard, 1984). MCA= monoclonal antibody.

CAMPATH-1 (Hale et al, 1983) was provided by Dr G. Hale and Dr H. Waldmann (University of Cambridge, Cambridge, UK) and 0.1 ml CAMPATH-1 (0.1 mg/ml) was added to 10^6 cells. These antibodies were selected on the basis

of their reactivity pattern with human lymphocytes (Table 2.1) and their cross-reactivity with rhesus monkey lymphocytes.

2.4.2 ANTIBODIES DIRECTED AGAINST MYELOID CELLS

For detection of DR-positive cells, bone marrow cells were incubated with either the anti-DR antibody OKIa1 (Ortho Diagnostics), or GM11 (supernatant: Jonker et al. (1984)), or anti HLA-DR (Becton Dickinson), or YCL 6.8 (0.05 mg/ml), or YAML 555.6 (0.01 mg/ml: both provided by Dr G. Hale and Dr H. Waldmann). These antibodies recognize an antigen with a molecular weight of 28-34 kdalton and were used in a concentration of 1 ml for 5 x 10^7 cells.

For the staining of progenitor cells the MCA's OKT10 (Ortho Diagnostics: Reinherz et al., 1980) or GM 1 (production by Jonker M and Van Meurs G) were applied. OKT 10 reacts with human thymocytes and early progenitor cells (Crawford et al., 1980, Sieff et al., 1982). GM 1 reacts with human and rhesus monkey granulocytes, lymphocytes and early progenitor cells (Jonker, personal communication). The antibodies MY 4 and Mo1 (gift from Coulter Immunology) react in humans with mature and immature cells from the granulocyte/monocyte lineage, but not with cells which give progeny to GM-CFU (Griffin et al., 1983) and it cross-reacts with rhesus monkey bone marrow cells (Letvin et al., 1983).

2.4.3 COMPLEMENT LYSIS

After incubation with the MCAs, at the concentrations described above, complement was added without further washing. For CAMPATH-1 sera of two monkeys were pooled and added at a final concentration of 20% (v/v). For the other MCAs rabbit serum (Stichting Bloedonderzoek, Wageningen, the Netherlands) was used as a source of complement. The final complement concentrations for WT 1, OKT4+4a, B9-pool and the cocktail of anti DR antibodies were respectively 30%, 30%, 50% and 50% (v/v). The cells were incubated with complement for 1 hour at 37^0 C. DNAse (Behring Diagnostics) was added to prevent clumping.

2.5 E-ROSETTE SEDIMENTATION

Sheep red blood cells (SRBC) less than 2 weeks old were treated with a 140 mM 2-aminoethylisothiouronium bromide (AET: Sigma, St. Louis, MO) solution (pH 9.0) at 37^{o} C for 15 min and washed 4-5 times in isotonic saline (Madsen et al., 1980). A 10% solution of AET SRBC in PBS with 40% heat inactivated (56^{0} C, 30 min) foetal calf serum (FCS) was prepared. An equal volume of cold 10% AET SRBC was added to a cell suspension of 30 x 10^{6}/ml. This mixture was spun down slowly (800 rpm, TJ-6 Beckman centrifuge; Beckamn-RIIC, Scotland) for 5 minutes to purchase optimal rosette formation of sheep erythrocytes and T lymphocytes (E-rosette forming cells: E-RFC). After gently resuspending the mixture, it was layered on a ficoll-hypaque gradient and centrifuged for 20 min at a speed of 2000 rpm (TJ-6 Beckman centrifuge). E-rosette forming T lymphocytes are spun down the gradient and recovered from the bottom. The E-RF cells at the bottom of the gradient are T lymphocytes. A pure T lymphocyte population could be obtained when the sheep red blood cells were lysed with a hypotonic shock.

2.6 COLONY FORMATION IN VITRO (GM-CFU)

Bone marrow progenitor cells can give rise to clusters of cells consisting of different cell lineages depending on the stimulating factors present in the medium. For our experiments the granulocyte/macrophage colony assay (GM-CFU) according to Pike and Robinson (1970) was applied. Bone marrow cells were cultured using the double layer agar technique with human peripheral blood leukocytes as feeder layer and an overlayer consisting of haemopoietic cells. Feeder layers were prepared by suspending 10^{6} peripheral blood leucocytes in 1 ml of a medium consisting of 30% (v/v) Dulbecco's medium (800 mOsm), 20% (v/v) FCS and 50% (v/v) of 1% agar at 37^{0} C. For each experiments two sets of feeder layers were prepared with leukocytes from two individuals. The haemopoietic cells for the overlayer were resuspended in 1 ml medium in which agar is substituted for distilled water and 1 ml medium as described above. From this mixture 0.2 ml was placed on a feeder layer.

Colonies, defined as cell aggregates of more than 50 cells, were counted at a 25 fold magnification using an inverted microscope(Zeiss). Colonies were scored 7-14 days after initiation of the cultures.

2.7 PROLIFERATION OF T LYMPHOCYTES IN VITRO

Peripheral blood lymphocytes were separated by density centrifugation using ficoll-hypaque (LSM®, density 1.077 g/l; Bionetics, Keningston, UK). Lymphocytes at a concentration of 2 x 10^6 cells/ml were cultured in RPMI 1640 medium containing 20% (v/v) heat inactivated monkey serum, 2 mM L-glutamine and 100 µg/ml streptomycin in microculture plates at 37^0 C in 5% CO_2 for 3-4 days. Stimulation was performed with phytohaemagglutinin (PHA, 1 mg/ml; Borroughs-Wellcome). All cells were cultured in triplicate. In all experiments controls were included where PHA was omitted as stimulator. Twelve hours before the end of the culture period, 1 µCi of [^{14}C]-thymidine was added to each well. At the end of the culture period, the cells were harvested with a multiple automatic sample harvester and the uptake of [^{14}C]-thymidine was measured in a liquid scintillation counter.

2.8 BONE MARROW TRANSPLANTATION PROCEDURE

2.8.1 TOTAL BODY IRRADIATION

Recipients were prepared for grafting by total body irradiation (TBI) delivered by a single or two opposing beams from a Philips-Müller X-ray machine. The monkeys were placed in a cylindric polycarbonate cage which rotated slowly (3 times per minute) around its vertical axis during irradiation. The animals were not anesthetized during irradiation. The one or two X-ray tubes operated at a tube voltage of 300 kV and a current of 10 mA. The half-value layer thickness was 3 mmCu. The focus-skin distance 80 cm and the average dose rate over the animal was 20-22 cGy/min (Broerse et al., 1978).

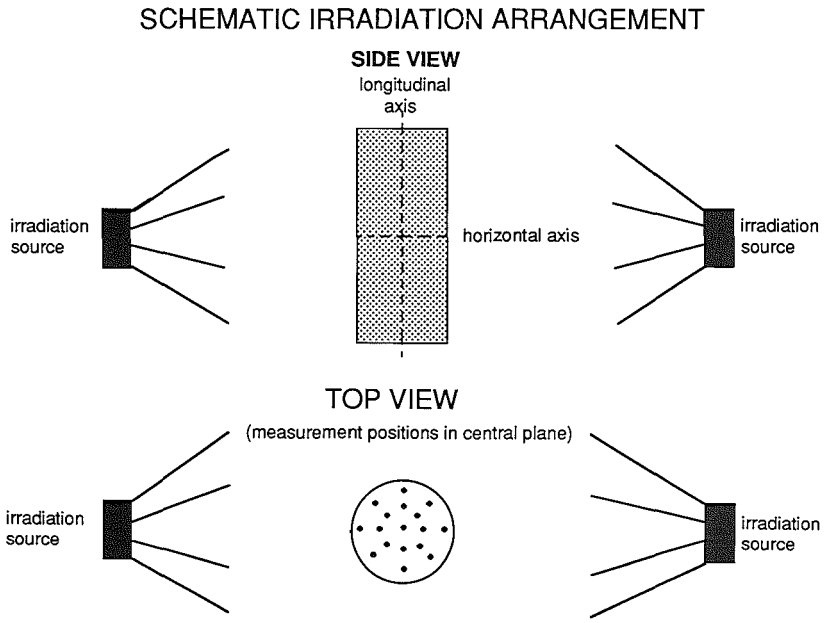

Figure 2.1. **Irradiation arrangement for exposing the cylindric water phantoms to 300 kV X-rays.**

On basis of dosimetry measurements in dogs phantoms (Zoetelief, 1984) it was decided to change the irradiation protocol from radiation with one beam to two opposing beams. Dose measurements were made with an ionization chamber at different positions inside cylindrical monkey phantoms (Figure 2.1). Measurements, done by Dr Zoetelief from the Radiobiological Institute TNO, showed that a more homogeneous dose distribution is obtained by bilateral irradiation (see figure 2.2).

During evaluation of the experimental data it was found that several monkeys which were irradiated with two opposing beams died due to irradiation pneumonitis, while this complication was hardly ever observed in the period when one radiation beam was used. Recent evaluation of the irradiation protocols from 1961-1986 for total body irradiation have demonstrated that replacement of irradiation filters, different assessment of correction factors for weight of the

animal and different assessment of the diameter of an animal and different phantoms were the main cause of errors in the calculated irradiation dose in lungs. Recent simulation of the different irradiation protocols over the last ten years have demonstrated that the actual dose with one single beam was 6-9% lower than the reported dose. The actual dose delivered by two opposing beams appeared to be 10-14% higher than reported. Lung tissue was simulated by dose measurements in cork. The absorbed dose in lungs was 9-13% higher for unilateral irradiation and 22-25% higher for bilateral irradiation in comparison to the reported doses earlier (data obtained by Dr Zoetelief). The dosimetry measurements had their consequences for the experiments performed. For that reason the planned dose of irradiation and the actual dose (according to the new dosimetry measurements) are shown in Table 2.2 for each monkey used for experiments described in this thesis.

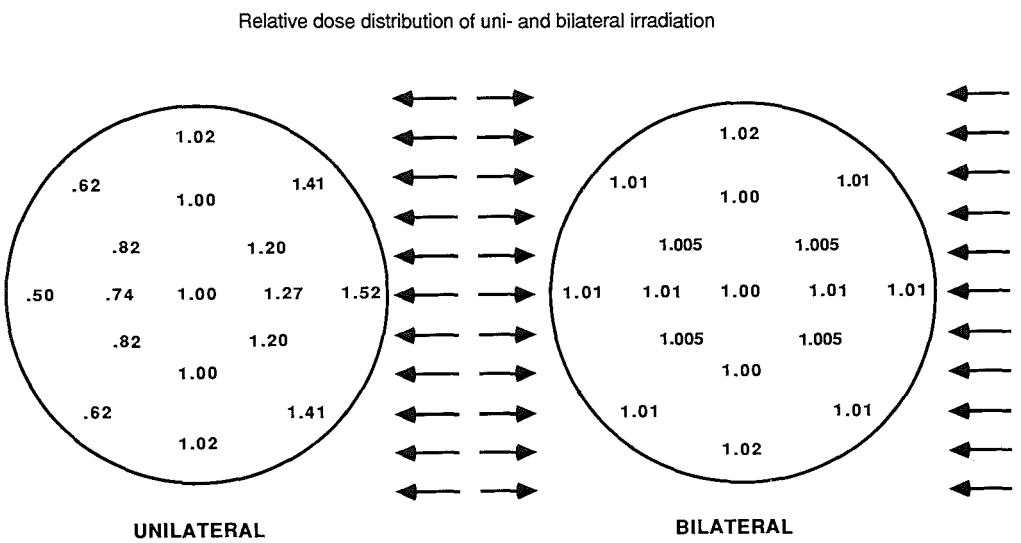

Figure 2.2 **Relative dose distributions for uni- and bilateral irradiation with 300 kV X-rays of a cylindric monkey phantom with its center at 90 cm from the focus or foci. Given are the values in the central circular plane (data obtained by Dr. J. Zoetelief).**

Table 2.2

Total body irradiation of rhesus monkeys.

UMN	BONE MARROW GRAFT		RADIATION DOSE		
	number of cells x 10^6	treatment	planned (Gy)	actual (Gy)	actual lung dose (Gy)
		AUTOLOGOUS			
1 ST	100	----	8.5	9.65	10.55
1 HH	30	----	8.5	9.85	10.60
3425	10	----	8.0	7.50	8.20
3745	10	----	8.0	7.50	8.20
3282	10	----	8.0	7.50	8.20
1 HM	3	----	8.5	9.80	10.60
1 HW	1	----	8.5	9.85	10.60
1 HK	----		8.5	9.80	10.60
1 JB	----		8.5	9.60	10.55
1 GB	100	CAMPATH-1	8.5	9.20	10.40
1 DX	100	CAMPATH-1	8.5	9.80	10.60
1 SN	10	OKT4	8.5	9.80	10.60
1 MY	10	WT-1	8.5	9.65	10.55
1 GA	100	B9-pool	8.5	9.35	10.45
1 FO	10	B9-pool	8.5	9.20	10.40
1 LZ	100	anti-DR	8.5	9.60	10.55
1 SS	100	anti-DR	8.5	9.85	10.40
1 NN	100	YAML + GARA/FITC	8.5	9.50	10.50
1 LP	3	gradient	8.5	9.40	10.45
1 LL	1.5	gradient + FLS	8.5	9.30	10.45
1 LG	3	gradient + CAMPATH-1	8.5	9.45	10.50
1 RP	0.5	stem cells	8.5	9.70	10.65
1 TO	0.7	stem cells	8.5	9.55	10.55
1 OT	0.5	stem cells	8.5	9.50	10.50
1 OW	0.6	DR-negative	8.5	9.40	10.45

1 HJ	50	unmodified	2 x 6.0	2 x 5.7	2 x 6.2
1 FB	50	unmodified	2 x 6.0	2 x 5.6	2 x 6.4
1 AT	9	gradient + E-rosette sed	2 x 6.0	2 x 5.6	2 x 6.0
2 AW	9	gradient + E-rosette sed	2 x 6.0	2 x 5.6	2 x 6.1
		ALLOGENEIC			
1 EA	2.5	gradient + E-rosette sed	2 x 4.5	2 x 4.2	2 x 4.6
1 DV	2.5	gradient + E-rosette sed	2 x 5.0	2 x 4.2	2 x 5.3
1 CK	2.5	gradient + E-rosette sed	2 x 5.0	2 x 4.7	2 x 5.2
1 BA	2.5	gradient + E-rosette sed	2 x 5.5	2 x 5.2	2 x 5.8
1 FE	2.5	gradient + E-rosette sed	2 x 5.5	2 x 5.2	2 x 5.6
1 EP	2.5	gradient + E-rosette sed	2 x 6.0	2 x 5.6	2 x 6.1
1 CC	2.5	gradient + E-rosette sed	2 x 6.5	2 x 6.1	2 x 6.8
1 EG	2.5	gradient + E-rosette sed	2 x 6.5	2 x 6.1	2 x 6.8
1 EC	400	CAMPATH-1	2 x 6.0	2 x 6.5	2 x 7.3
1 JR	400	CAMPATH-1	2 x 6.0	2 x 6.9	2 x 7.5
1 CI	400	CAMPATH-1	2 x 6.0	2 x 6.7	2 x 7.4
1 FN	400	CAMPATH-1	2 x 6.0	2 x 6.7	2 x 7.4
1 LJ	400	CAMPATH-1	2 x 6.0	2 x 6.9	2 x 7.5
1 HC	400	CAMPATH-1	2 x 6.0	2 x 6.6	2 x 7.4
1SK	400	OKT-4+4a	2 x 6.0	2 x 6.7	2 x 7.4
1 LU	400	OKT-4+4a	2 x 6.0	2 x 6.7	2 x 7.4
1 BX	400	B9-pool	2 x 6.0	2 x 6.5	2 x 7.4
1 GF	400	B9-pool	2 x 6.0	2 x 6.8	2 x 7.4
1 SP	400	WT-1	2 x 6.0	2 x 6.8	2 x 7.4
1 LB	400	WT-1	2 x 6.0	2 x 6.9	2 x 7.5

UMN: unique monkey number, FLS: Forward light scatter

2.8.2 AUTOLOGOUS BONE MARROW TRANSPLANTATION

The monkeys were anesthetized with ketalar (5-10 mg/kg body weight: Vetalar®, Parke-Davis) and Vetranquil® (0.3 ml/10 kg body weight). Under general anesthesia bone marrow was aspirated from the femora. After aspiration a mononuclear cell suspension was prepared by density centrifugation using ficoll-

hypaque (see section 2.3.1). Thereafter, either bone marrow cells were cryopreserved as described by Schaefer et al. (1972) or the graft was manipulated in vitro and reinfused in a peripheral vein of the recipient afterwards. The recipients were prepared for transplantation by 9.2-9.7 Gy TBI, two opposing beams (Figure 2.3).

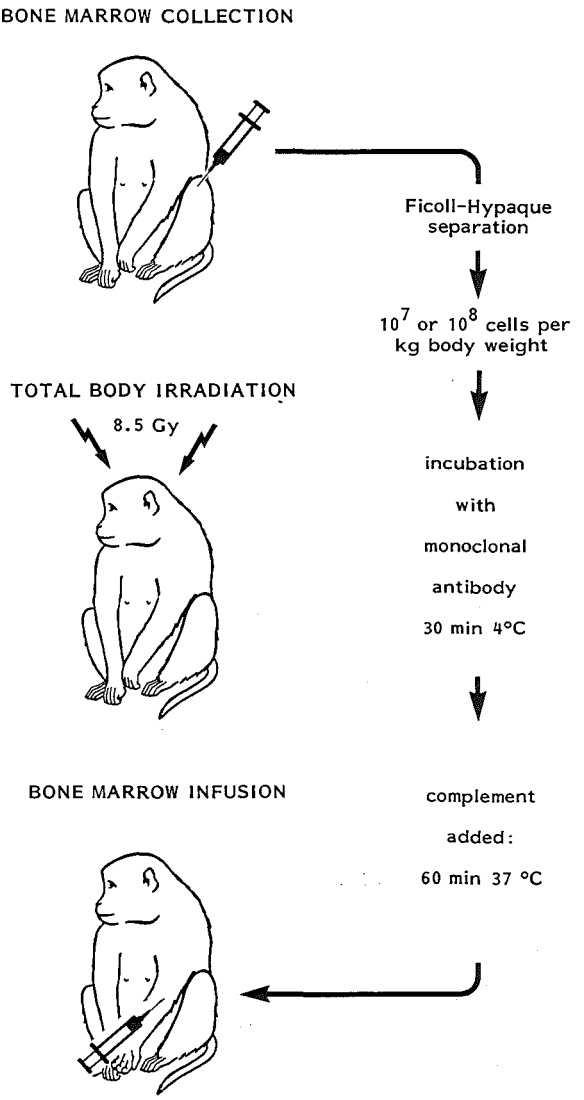

Figure 2.3 **Autologous bone marrow transplantation**

2.8.3 ALLOGENEIC BONE MARROW TRANSPLANTATION

Recipients of allogeneic bone marrow transplants were prepared by fractionated TBI. The irradiation was given in two doses with 24 hours interval. The bone marrow graft was administered intravenously 24 hours after irradiation.

2.9 FLOW CYTOMETRY

A flow cytometer is a machine that measures parameters of cells which are made to flow in suspension through an intense light source. When cells pases through a laser beam, optical signals are generated which contain biochemical or morphological information about the cells. These optical signals are detected and translated in electric signals by photomultipliers. Light scatter, generated when a cell flow through the laser beam, is detected in 0^0 and 90^0 direction and gives information about the size (0^0 scatter = forward light scatter (FLS)) and shape (90^0 scatter = perpendicular light scatter (PLS)). Using laser light with a defined wave length enables the detection of fluorescence particles present on a cell. The signals from the photomutipliers are either linearly or logarithmic amplified.

Except for analysis, flow cytometers are extremely useful because of the opportunity to sort cells according to defined parameters. For sorting, the liquid jet is vibrated with the help of a piezo-electric crystal at a frequency such that droplets break from the flow stream containing individual cells. With "windows" the upper and lower limits for the optical parameters are set. A sort logic circuitry compares the signals of each cell with the preset "windows". By giving droplets different electric charges, the droplets can be deflected when they pass through a constant transverse electric field. The deflected droplets are sorted into glass tubes.

2.9.1 FLOW CYTOMETERS

Two flow cytometers were used for immunofluorescence analysis and cell sorting; a modified FACS II (Fluorescence Activated Cell Sorter; Becton Dickinson) (figure 2.4) or a home build dual laser flow cytometer (RELACS; design: G. van den Engh and W. Stokdijk) (figure 2.5). With the modified FACS it was possible to measure three parameters. The principles of the RELACS have been described previously (Trask, 1985).

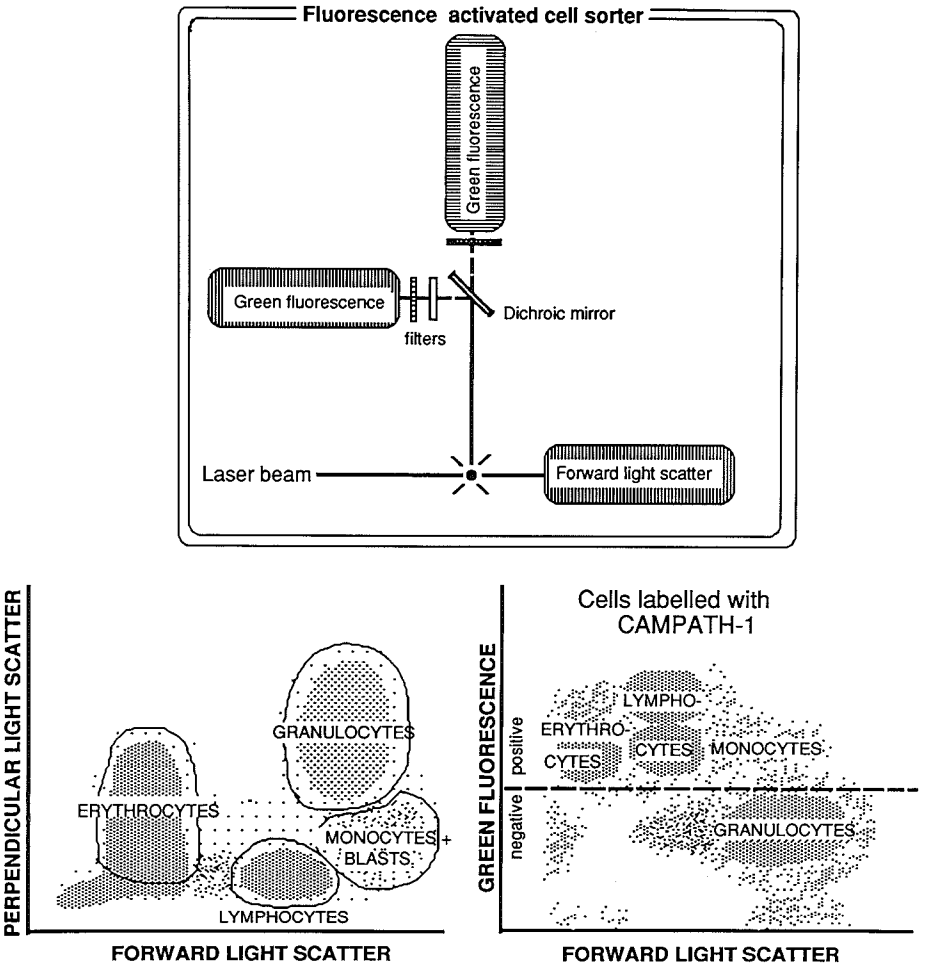

Figure 2.4 **Fluorescence activated cell sorter and dot plots**

Briefly, it is a dual laser flow cytometer with the equipment for multiple parameter signal processing. The amplified signals from the photomultipliers are processed by a "Peak sense and hold" circuitry which determines the the peak of the resultant pulse signals and hold this value temporarily. The peak values are digitized in an analog-to-digital converter. The digital signals are processed for presentation on a two-dimensional oscilloscope, which enables the characterization

of cells by two optical signals, and the parameters can stored in a computer. The novelty of the machine is the speed of signal processing with a very short "dead" time (5 μs) between the detection of the optical signals of a cell and the complete storage of the signals in list data acquisition mode in a Hewlett- Packard 200 computer. The list mode enables analysis afterwards with a software package, which included data display in histograms, 2 or 3 dimensional display and window setting, and was developed in our institution by Drs R.R. Jonker.

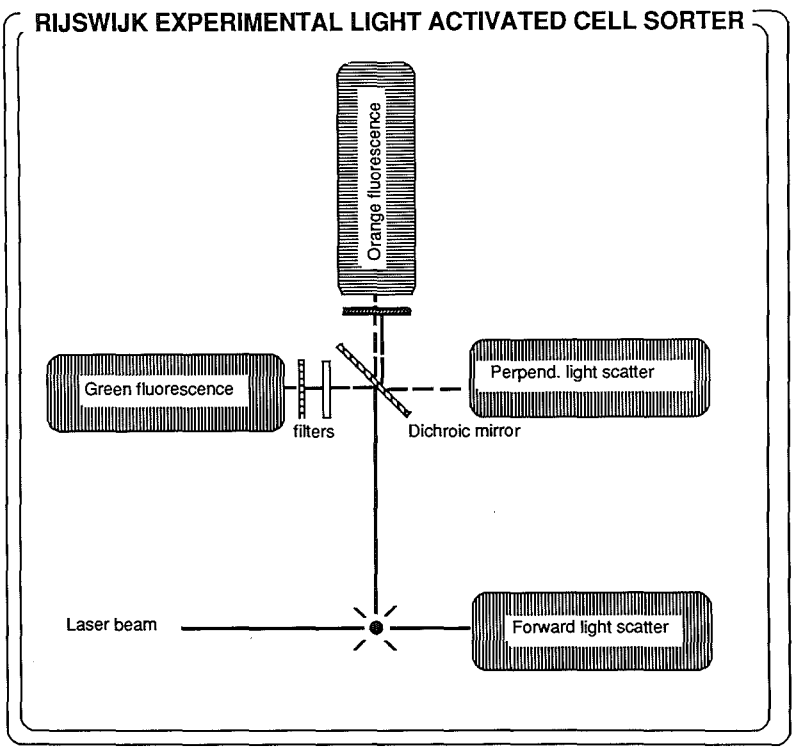

Figure 2.4 **Rijswijk experimental light activated cell sorter**

2.9.2 FLUORESCENT SIGNALS

Besides the forward light scatter (FLS) signals as a measure for the size of the particles, and the perpendicular light scatter (PLS), which is indicative for the shape of a particle, fluorescent signals were used for the characterization of

cells. The fluorescent signals could be generated by excitation of fluorochromes with light of a specific wavelength. The fluorochromes were directly or indirectly coupled to MCA's, which reacted with cells. The fluorochromes fluorescein isothiocyanate (FITC) and phycoerythrin (PE) were excitated with a laser at 488 nm (0.4-0.5 W). The emitted FITC fluorescence was measured through a combination of a broad band multicavity interference filter (520-550 nm: Pomfret Research Optics Inc, Stanford, CT) and a 530 nm cut-off filter (Ditric Optics, Hudson, MA) by an S-11 type photomultiplier (Thorn EMI, Middlesex, U.K.) and PE through a 577 nm cut-off filter (Corion) by a S-20 type photomultiplier (Thorn EMI) or a Hamamatsu multiplier R1477 (Hamamatsu, Japan). Since there is a overlap in the emission spectra of PE and FITC a comparison network (Loken et al., 1977) was used to subtract the FITC emission from the PE emission in the region where the PE fluorescence was measured. After the comparison network the signals were logarithmic amplified. Propidium iodide was used to stain dead cells and measured through a 620 nm cut-off filter (Ditric Optics) by an S-20 type photomultiplier.

2.10 NECROPSY AND HISTOPATHOLOGICAL EXAMINATION

Necropsies and histopathological examinations were routinely done to determine the cause of death and in case of allogeneic transplantations to grade the severity of GvHD according to the criteria of the International Bone Marrow Transplant Registry (1975). Tissue samples were fixed in 4% buffered formaldehyde, trimmed, embedded in paraffin and stained with hematoxylin-phloxine-saffran.

2.11 ENGRAFTMENT

Between day 21 and day 28 after transplantation peripheral blood cells were collected and lymphocytes separated for tissue typing using serologically defined determinants as described previously by Balner et al. (1981).

2.12 STATISTICS

Mean, standard deviation and standard error of mean was calculated according to the standard formula (Colton, 1974). For statistical analysis of the relationship

between regeneration rate of peripheral blood cells and the number of bone marrow cells grafted/kg body weight (see chapter III), a linear regression analysis was used. The regression line was fitted according to the formula $y=a+b\log(x)$. The Student's T test was used for the calculation of the 95% confidence limits of the line and for the calculation the significance of the hypothesis that the line did differ from 0. Since single monkeys were used to measure an effect of in vitro manipulation of autologous bone marrow grafts, the 95% confidence limits were calculated on a predicted individual y value.

CHAPTER III

ASSAY FOR THE REPOPULATING CAPACITY OF AUTOLOGOUS BONE MARROW GRAFTS IN RHESUS MONKEYS

3.1 INTRODUCTION

Pluripotent haemopoietic stem cells are characterized by their virtue of self-replication and differentiation into all blood cell lineages. Because of these properties transplanted stem cells are capable to protect a lethally irradiated recipient. A prerequisite for any in vitro manipulation of a bone marrow graft is that it does not adversely affect pluripotent haemopoietic stem cells.

One of the objectives of this study was to eliminate subpopulations of T lymphocytes from bone marrow grafts by complement fixing monoclonal antibodies in order to prevent GvHD. The toxicity for repopulating stem cells of such a pretreatment had to be investigated. Bone marrow cultures, such as the GM-CFU assay, were thought to provide a good indication for toxicity to stem cells, since any toxicity for pluripotent stem cells will affect the number of descendents. However, a discrepancy has been observed between the results obtained in bone marrow cultures and the repopulating capacity of bone marrow cells when bone marrow cells were incubated with 4-hydroperoxycyclophosphamide. Although severe toxicity was detected in vitro, the bone marrow cells remained their potential to protect a recipient from death due to a lethal dose of chemo- and radiotherapy (Rowley et al., 1985; Kaizer et al., 1985; Yeager et al., 1986). Therefore, it was decided not to rely solely on results of bone marrow cultures but to develop an in vivo assay for reconstitution capacity of bone marrow grafts. The in vivo assay developed was based on the assumption that the rate of recovery of certain peripheral blood cells after an autologous bone marrow transplantation is a function of the number of haemopoietic stem cells

grafted. Therefore, graded numbers of autologous bone marrow cells were transplanted into monkeys, which had received a high dose of whole body irradiation. Peripheral blood cells were counted daily until the values had normalized or until 35 days after grafting. Although after autologous bone marrow transplantation, direct parameters such as MHC-antigens are not available to identify graft derived cells, autologous bone marrow transplantations were preferred to allogeneic transplantations because interfering processes such as GvHD and host-versus-graft reaction are avoided.

The validity of the assay was tested by grafting autologous bone marrow cells from which pluripotent haemopoietic stem cells were eliminated by incubations with anti-DR antibodies. It has been established that the DR-antigen is present on early, pluripotent haemopoietic stem cells of humans (Fitchen et al., 1982) and non-human primates (Winton et al., 1985).In dogs the DR-antigen is present on haemopoietic stem cells (Szer et al., 1985) and the homologue I-E antigen is also present on murine stem cells (Fitchen et al., 1981).

After establishing the validity of the assay, the regeneration pattern of autologous bone marrow grafts following complement mediated lysis of T lymphocyte subpopulations was compared to the regeneration pattern of unmodified bone marrow grafts.

3.2 AUTOLOGOUS REPOPULATION ASSAY

To assess the relation between regeneration rate of peripheral blood cells and cell numbers grafted, irradiated (7.5-9.8 Gy total body irradiation; X-rays) monkeys were given graded numbers of cryopreserved, autologous bone marrow cells over a range of 10^6 to 10^8 cells/kg body weight. For each data point one monkey was used, except for three monkeys at the dose of 10^7/kg body weight. Peripheral blood cell counts were determined daily after transplantation; the day of transplantation was designated as day 0. The reconstitution of the peripheral blood cells is illustrated in the figures 3.1 and 3.2. The leukocyte counts dropped rapidly after total body irradiation and, depending on graft size, the counts remained low for at least two weeks. After the administration of 10^8 cells/kg body weight, leukocyte counts reached the value of 10^9/l after 15 days and subsequently leukocyte counts normalized within a few days. The regeneration rates of leukocytes were slightly slower after the cell doses of 3 and 1 x 10^7 cells/kg body weight.

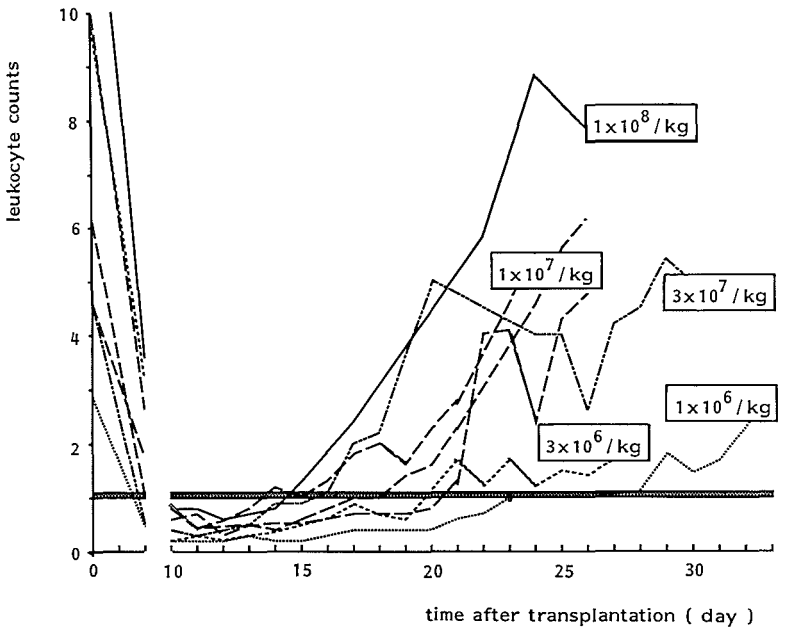

Figure 3.1. **Regeneration of leukocytes after transplantation of graded doses of autologous bone marrow cells.**

After a cell dose of either 1 or 3 x 10^6 cells/kg body weight, leukocyte counts reached the value of 10^9/l after 23 and 20 days respectively and the subsequent rise of leukocytes toward normal values was slower in comparison to higher cell doses. The regeneration pattern of reticulocytes showed a similar pattern as for the leukocytes with early regeneration after high cell doses and a slower regeneration pattern after the cell doses of 1 and 3 x 10^6 cells/kg body weight. The regeneration rate of the autologous grafts was expressed as the time at which the leukocyte counts or reticulocytes definitely exceeded a certain value. For both leukocytes and reticulocytes regression lines were calculated between the graded cell doses and regeneration time. A semilogarithmic relationship between cell dose and regeneration time appeared to exist for different points at the regeneration curves of leukocytes and reticulocytes (Table 3.1).

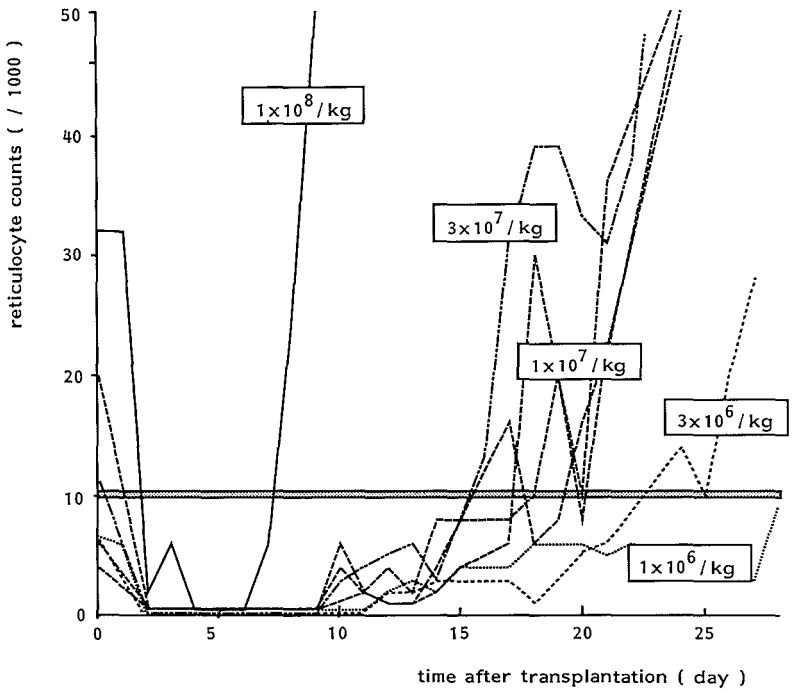

Figure 3.2 **Regeneration of reticulocytes after transplantation of graded doses of autologous bone marrow cells.**

Two variables determine the usefulness of a regression line for calibration purposes; whether the slope of the line significantly differs from zero and whether the distribution of the points around the line is small. The distribution of the points is expressed in the standard deviation and the 95% confidence limits. If the regression line is to be employed either to deduce the number of bone marrow stem cells in a graft of unknown composition or to detect toxicity, narrow confidence limits are required. This requirement was met for the regression lines when reticulocyte counts exceed 1% or 2%. For the assessment of toxicity and enrichment of haemopoietic stem cells the regression line based on reticulocyte counts $\geq$ 1% was applied. Leukocyte counts did not meet the requirements stated above, since the variance of the data points is large. Figure

3.3a + b shows the regression line of reticulocytes and leukocytes with the 95% confidence limits. The differential leukocyte counts gave comparable results for each of the subtypes of white blood cells but this did not improve the limits of the relationship, probably because of inaccuracy of the counting method. Thrombocyte counts could not be used due to the platelet transfusion policy. Platelet transfusions were administered when platelet counts dropped below 40 x 10^9 cells/l.

Table 3.1

Characterization of regression lines calculated on the basis of cell dose grafted and regeneration time of peripheral blood cells.

	Y^a =	r^b	P-value[c]	st. dev.[d]
leukocytes $\geq$ 1 x 10^9/l	26 - 4 x log(x)	.819	.02	1.976
leukocytes $\geq$ 2 x 10^9/l	41 - 9 x log(x)	.915	.004	2.808
leukocytes $\geq$ 3 x 10^9/l	45 - 10 x log(x)	.906	.005	3.32
reticulocytes $\geq$ 0.5 %	35 - 9 x log(x)	.963	.0005	1.83
reticulocytes $\geq$ 1.0 %	39 - 9 x log(x)	.988	.0001	1.05
reticulocytes $\geq$ 2.0 %	45 - 11 x log(x)	.993	.0001	1.02

a: The regression line is characterized by the formula $Y=a+b\log(x)$, where a represents the intercept, b the slope of the line and x the cell number x 10^6/kg body weight.
b: correlationcoefficient
c: significance of slope in comparison to zero
d. standard deviation of points about the fitted line = standard deviation of regression

To test the validity of the obtained relationship between cell dose grafted and regeneration rate, several experiments were performed, which included determination of regeneration rate without transplantation of autologous cells and the regeneration rate of grafts that were depleted of DR-positive cells.

The first experiment involved 2 monkeys that did not receive bone marrow following 9.8 Gy total body irradiation (see Table 2.2). These monkeys showed endogenous regeneration with leukocyte counts reaching levels greater than 10^9/l after 23 and 27 days, while reticulocyte counts were above 1% at day 28 and 31, respectively (Figure 3.4). This is caused by stem cells surviving total body ir-

radiation. When the regression line of reticulocytes is used, the regeneration rate of peripheral blood cells in these two monkeys corresponds with a residual number of stem cells equivalent to an autologous graft of 0.3 - 1 x 10^6 bone marrow cells/kg body weight.

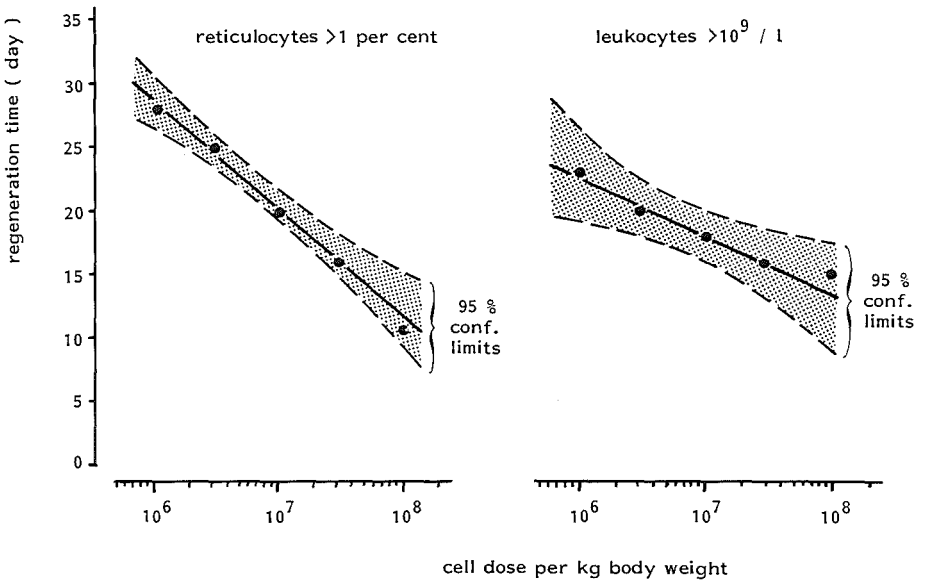

Figure 3.3 **The relationship between bone marrow cell numbers grafted and time of regeneration of leukocytes and reticulocytes.**

3.3 DEPLETION OF DR-POSITIVE CELLS

The experiment designed to determine the regeneration time with stem cell depleted bone marrow grafts involved two monkeys which received autologous bone marrow grafts treated with anti-DR monoclonal antibodies. For the depletion of DR-positive cells several MCA's were selected on basis of their reactivity pattern with rhesus monkey bone marrow cells. Staining bone marrow cells with anti-HLA-DR/PE and either GM-11, YCL 6.8 or OKIa1, which were indirectly labeled with GAM/FITC or GARA/FITC, resulted uniformly in double-stained populations, indicating that the antibodies react with the same cells. Complement- mediated lysis of DR-positive cells was done by the simultaneous

use of four monoclonal antibodies, since only combinations of these antibodies yielded efficient kill of GM-CFU (Table 3.2).

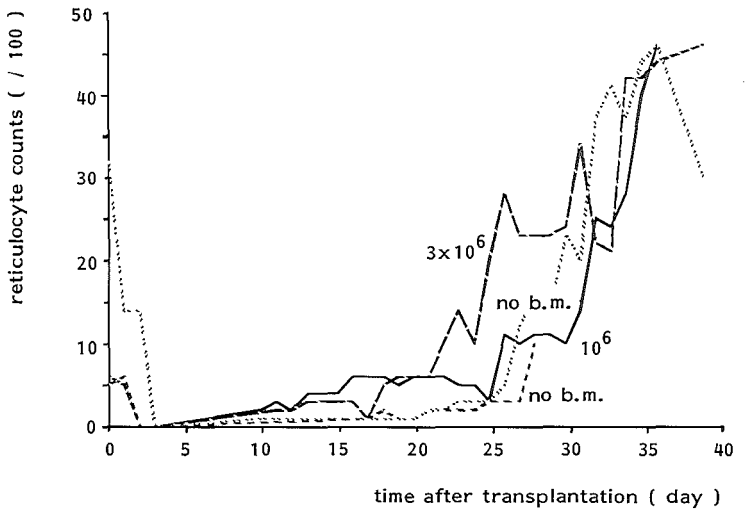

Figure 3.4 **Regeneration of reticulocytes when recipients were given 9.8 Gy TBI and no bone marrow graft.**

The cell dose before antibody treatment was 10^8 viable cells/kg body weight. After the in vitro treatment 20-27% viable cells, 1-2% DR positive cells and 2% GM-CFUs were recovered. The cells left after in vitro treatment were reinfused into the monkey without correction for cell loss. As shown in Table 3.2 the regeneration rate was severely delayed compared to values obtained with 10^8 unmodified cells/kg body weight, with one monkey dying after 22 days without any signs of regeneration. In this monkey histological signs of bone marrow regeneration were not observed. The hematological recovery of the other monkey, which died after 21 days with early signs of regeneration, was estimated to be equivalent to an bone marrow graft of approximately 5 x 10^6 cells/kg body weight, demonstrating that depletion of DR positive cells eliminates at least 95% of the repopulating cells.

Table 3.2

Depletion of Granulocyte Macrophage-CFU (GM-CFU) after treatment with anti-DR antibodies plus complement (N=3).

Monoclonal antibody	GM-CFU/10^5 nucleated cells ± s.d[a]. without complement	with complement	surviving fraction[b] (% ± s.d.)
medium	87 ± 10	86 ± 8	100 ± 9
GM11	86 ± 10	40 ± 5	46 ± 6
YCL 6.8	90 ± 10	36 ± 5	41 ± 6
HLA-DR	91 ± 14	46 ± 6	53 ± 7
OKIa1	74 ± 8	13 ± 2	15 ± 2
all four MCA's	n.d.	5 ± 3	6 ± 3

a : values represent mean ± SD of individual determinations.

b : surviving fraction of GM-CFU after complement lysis

Table 3.3

Toxicity to repopulating capacity of 10^8 autologous bone marrow cells/kg body weight by a preincubation with anti-DR monoclonal antibodies[a] and complement.

	REGENERATION RATE incubated bone marrow	unmodified bone marrow (95% conf. limits)	P -value of difference
Reticulocytes > 1% (day)	21, > 22[b]	8 - 13	< 0.005
Leukocytes > 10^9/l (day)	21, > 22[b]	10 - 18	< 0.01

a : mixture of four anti-DR MCA's GM11, YCL 6.8, anti-HLA-DR, OKIa1

b : died at day 22 after transplantation without evidence of regeneration

3.4 DEPLETION OF T LYMPHOCYTES

CAMPATH-1 plus monkey complement efficiently lysed all T lymphocytes, since no CAMPATH-1 positive cells or T lymphocytes stained with Leu2a and Leu3a could be detected by indirect immunofluorescence analysis after the incubation (see also chapter V). The elimination of the T lymphocyte subpopulations by complement with either OKT4+4a (CD4) or B9-pool (CD8) or WT 1 was also confirmed by immunofluorescence analysis. For the in vivo experiments, the preincubation cell dose was either 10^8 or 10^7 cells/kg body weight . After complement lysis of the T lymphocytes, the remaining cells were reinfused into the monkey.

Table 3.4

Regeneration time of reticulocytes following incubation of autologous rhesus monkey grafts with monoclonal antibodies and complement.

		reticulocytes > 1 %		
monoclonal antibody	cell dose per kg	observed	expected (95% conf. limits)	significance of difference
CAMPATH-1	10^7	19	18 - 21	n.s.
	10^8	10	8 - 13	n.s.
B9	10^7	20	18 - 21	n.s.
	10^8	10	8 - 13	n.s.
OKT4+4a	10^7	19	18 - 21	n.s.
WT-1	10^7	22	18 - 21	$p < 0.05$

n.s.: not significant

Depletion of CAMPATH-1 or B9-pool positive cells did not influence the regeneration of the peripheral blood reticulocytes in 2 monkeys transplanted with either 10^8 cells/kg or 10^7/kg body weight (Tables 3.4). Next, the influence of depleting OKT4+4a or WT 1 positive cells was investigated each in one monkey, each at the same cell dose, i.e. 10^7/kg body weight (Table 3.4). Depletion of OKT4+4a (helper/inducer T lymphocytes) positive cells failed to

exert an influence on the repopulating capacity of the residual bone marrow cells (Tables 3.4). After elimination of WT 1 positive cells the leukocyte regeneration time was within the 95% confidence limits. The reticulocyte regeneration was just outside the 95% confidence limits, but within the 99% confidence limits (the latter are not shown) and, therefore, this difference is probably not of any biological significance.

3.5 DISCUSSION

In the rhesus monkey model the repopulating capacity of a bone marrow graft as measured by the regeneration rate of peripheral blood cells was directly related to the logarithm of the cell number present in an autologous bone marrow graft. The validity of this assay is demonstrated by the highly significant regression obtained and the substantial delay in regeneration time after grafting of bone marrow cells depleted of stem cells by anti-DR antibodies plus complement.

Several investigators (Spitzer et al., 1980; Harada et al., 1983; Meloni et al., 1985) have searched for a relationship between regeneration of peripheral blood cells and size of bone marrow grafts in human patients, but failed to demonstrate such a relationship. The explanation for the discrepancy between rhesus monkey and human results is most likely the small range in cell dose given in the human situation (0.5 - 2 x 10^8 cells/kg body weight) in comparison to the range of autologous bone marrow cells given in this study (10^6-10^8 cells/kg body weight. Besides, all patients have been heavily treated with myelosuppressive drugs prior to bone marrow collection, which could have an detrimental effect on the repopulating capacity of the marrow cells.

The survival of the 9,8 Gy irradiated, nongrafted monkeys is undoubtedly attributable to the profound antimicrobial therapy and the liberal platelet transfusions, which also holds true for the monkeys that received low numbers of bone marrow cells. Without such supportive care, these monkeys would have died prior to haemopoietic regeneration (Van Bekkum, 1967). The delayed reconstitution of the nongrafted monkeys is in accordance with the radiosensitivity of the haemopoietic stem cells. It is estimated that a rhesus monkey has approximately 8.4 x 10^{10} bone marrow cells (Pegg, 1966). The surviving number of stem cells was calculated to be equal to a bone marrow graft consisting of 0.3-1 x 10^6 cells/kg body weight. On the basis of these figures a D_0 of the rhesus monkey stem cells can be calculated using the formula; $S=n \times e^{-D/D0}$,

S: surviving fraction of bone marrow cells, n: a factor two is used because of the shoulder in the cell surviving curve at low doses of TBI, D: irradiation dose. According to this formula a D_0 of 0,8 - 0,9 Gy has been found.

The prolonged delay in regeneration time after depletion of DR positive cells, showed that toxicity to repopulating stem cells could be determined with this indirect assay. The repopulating capacity of the residual cells indicated that approximately a 2 log reduction haemopoietic stem cell number was caused by the anti-Dr antibody treatment. Complement toxicity could not attribute to this result because the same complement concentration was used for depletion of B9 positive cells, which did not cause a detectable significant delay of autologous reconstitution. Apparently, DR-antigens are present on rhesus monkey stem cells, similar as in of other mammalian species (Szer et al.,1985; Fitchen et al., 1981). The antigen is present on early, multipotent haemopoietic cells of humans (Fitchen et al., 1982) and non-human primates (Winton et al., 1985), grown in culture. Results of experiments with long term bone marrow cultures were in contrast with these findings in that colony growth was still observed after depletion of DR positive cells (Moore et al., 1980; Keating et al., 1984). However, this study and two other studies (Greenberger et al., 1985; Falkenburg et al., 1984, 1985) have revealed that the findings of Moore and Keating can be explained by inefficient complement mediated lysis of DR positive cells.

The delayed regeneration observed in our experiments does not conclusively prove that the DR antigen is present on the haemopoietic stem cell of rhesus monkeys, because the possibility remains that accessory cells, essential for repopulating stem cells, were removed. However, Visser et al. (1984) and Spangrude et al. (1988) have demonstrated that highly purified stem cells are able to repopulate a lethally irradiated, syngeneic recipient. The latter results do support the hypothesis that repopulating stem cells can repopulate a recipient without the help of other cells. In chapter IV of this thesis it is demonstrated that highly enriched stem cells are capable to repopulate the haemopoietic system of a recipient.

T lymphocyte depletion from autologous bone marrow grafts with a number of carefully selected monoclonal antibodies did not influence the repopulating capacity of the graft as measured by the reappearance of peripheral blood cells. Most convincing are the results with depletion of CAMPATH-1 positive cells from the bone marrow graft, since allogeneic bone marrow

transplantation experiments (chapter V) have clearly demonstrated the efficient elimination of T lymphocytes by this technique.

The results with T lymphocyte depletion exclude the possibility that either T lymphocytes and stem cells share the antigens detected by the antibodies used in this study, since elimination of T lymphocytes by complement mediated lysis did not reduce the number of stem cells.

The hypothesis that T lymphocytes have a significant trophic function for repopulating stem cells, as proposed by Sieff et al. (1985) and Soderling et al. (1985), is excluded by our observations. The increased frequency of take failures observed after T lymphocyte depletion of allogeneic bone marrow grafts has therefore to be attributed to the diminution of the immunosuppressive action of the GvH reaction exercised by donor T lymphocytes. This conclusion is in complete accordance with the concept of reciprocal interference between GvHD and engraftment (Nakic, 1966; Vriesendorp, 1981). In dogs was demonstrated that transfusion of donor leukocytes or thoracic duct lymphocytes increased the engraftment of histoincompatible bone marrow, but also the incidence and severity of GvHD (Deeg et al., 1979).

CHAPTER IV

ENRICHMENT OF HAEMOPOIETIC STEM CELLS BY SELECTION OF DR POSITIVE CELLS FROM BONE MARROW

4.1 INTRODUCTION

The ablation of the capacity of an autologous bone marrow graft to repopulate an irradiated recipient by depletion of DR-positive cells; as reported in the previous chapter, in principle opens the possibility to sort stem cells by positive selection. From experiments with human bone marrow cells it is known that several cells types have a DR-antigen on their cell surface. Among human bone marrow cells which carry a DR-antigen on their cell membrane are B-lymphocytes, activated T lymphocytes, macrophages, monocytes and myeloid progenitor cells. The DR-antigen has been demonstrated on multipotential progenitor cells that produce colonies consisting of several cell lineages (CFU-GEMM; Fitchen et al., 1982; Ohe et al., 1982) as well as on progenitor cells committed to differentiation along either granulocyte-monocyte, or erythroid, or megakaryocytic-platelet pathway (Kaplan et al., 1978; Winchester et al., 1978; Falkenburg et al., 1985; Linch et al., 1984).

The experiments in this chapter deal with the repopulating capacity of purified stem cells. At the time of these experiments a monoclonal antibody, which exclusively reacts with pluripotent haemopoietic stem cells, was not discovered in any species. Purification of pluripotent stem cells of mice has been achieved with an anti-$H2^k$ monoclonal antibody and sorting in combinations with a variety of other means (Visser et al., 1984).

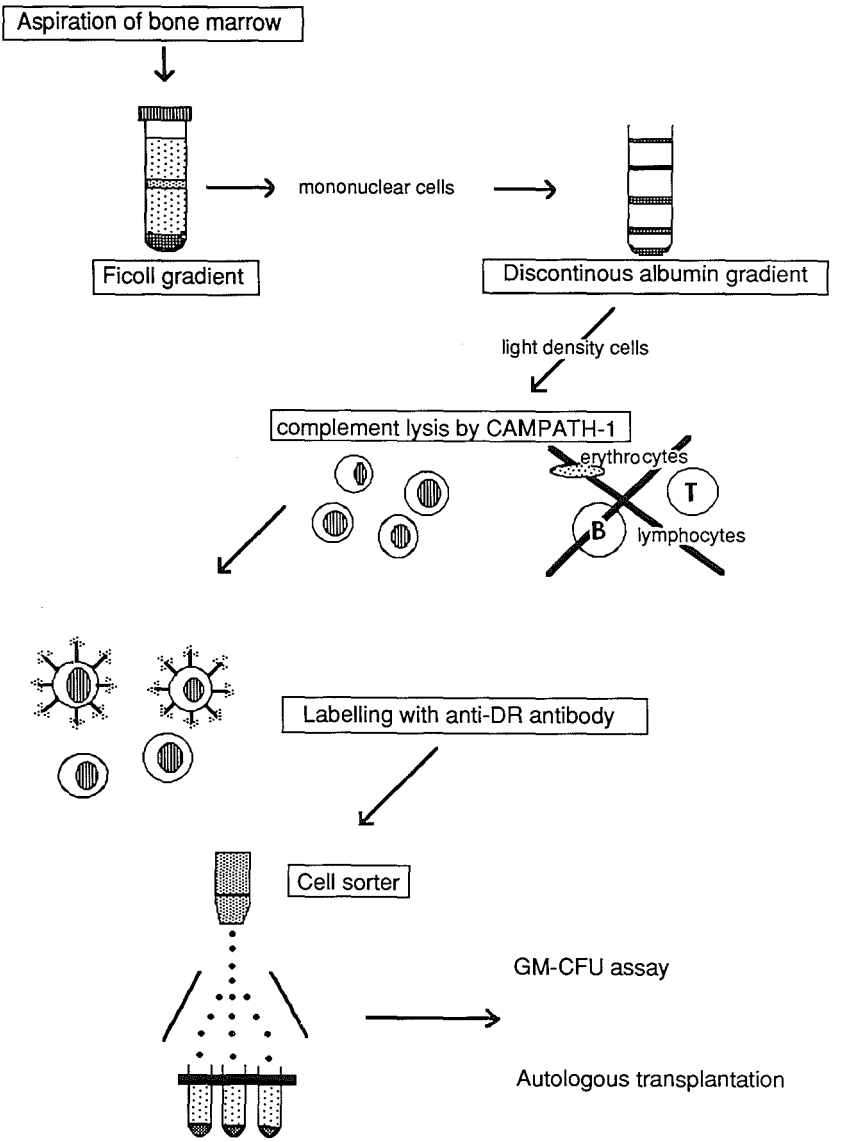

Figure 4.1 **Overview of the methods used for enrichment of haemopoietic progenitor cells**

Using a series of steps analogous to those applied for the purification of murine stem cells, it was attempted to purify pluripotent haemopoietic stem cells of rhesus monkeys using a DR-antibody and a cell sorter. Since it is known that pluripotent haemopoietic stem cells constitute only a small proportion of cells in a bone marrow graft, other purification steps, involving density centrifugation and depletion of T lymphocytes, preceded the isolation of DR-positive cells by the cell sorter.

Before carrying out in vivo experiments to assess the repopulating ability of the purified cells, an in vitro colony assay (GM-CFU) was used to monitor the purification steps. After a reproducible method was developed which yielded highly purified stem cells, such stem cell concentrates were grafted into irradiated, autologous monkey recipient. Using the peripheral blood regeneration assay described in chapter III, a quantitative assessment of enrichment of pluripotent haemopoietic stem cells was made.

4.2 IN VITRO EXPERIMENTS

Figure 4.1 shows the purification procedure, which started with the aspiration of bone marrow from the femoral shafts of the monkeys. Subsequently, a mononuclear cell suspension was prepared by density centrifugation followed by a discontinuous albumin density gradient. After enrichment of pluripotent stem cells, lymphocytes were removed by the antibody CAMPATH-1 plus complement. The last step was sorting DR-positive cells, using a cell sorter. The GM-CFU assay was applied to assess enrichment of pluripotent stem cells.

4.2.1 DENSITY CENTRIFUGATION AND DEPLETION OF LYMPHOCYTES

All experiments were performed with aspiration bone marrow. A ficoll-hypaque (LSM; density 1.077 g/l) density gradient was run to prepare a mononuclear cell suspension and to remove erythrocytes. This was advantageous since an abundant number of erythrocytes disturb a discontinuous albumin density gradient as well as GM-CFU cultures (Dicke 1970). After the ficoll density centrifugation was run, cells were collected from the interface.

Table 4.1

Distribution of nucleated cells and granulocyte/macrophage colonies (GM-CFU) in discontinuous albumin density gradients (N=4).

material	recovery of nucleated cells (%)	recovery of colonies GM-CFU (%)	normalized recovery GM-CFU (%)	enrichment factor GM-CFU
Ficoll	100	100	100	1
albumin gradient				
fraction 19-21%	7 ± 3*	101 ± 46*	72 ± 33	14,1 ± 4,9
fraction 21-22%	16 ± 5	28 ± 13	20 ± 10	1,7 ± 0,5
fraction 22-23%	18 ± 8	5 ± 2	3 ± 1	0,3 ± 0,2
fraction 23-25%	26 ± 2	6 ± 6	4 ± 4	0,2 ± 0,3
bottom	30 ± 10	1 ± 0,2	1 ± 0	0,1 ± 0,02
recovery	98 ± 8	140 ± 40	100 ± 29	

*: mean recovery and standard deviation of mean

In comparison to aspiration bone marrow 41.7 ± 8.7% of the nucleated cells and all GM-CFU's were recovered from the interface of a ficoll gradient (N=24). Since in this chapter recovery and enrichment is expressed in reference to the cell suspension, obtained after ficoll gradient, All enrichment factors should be factors should be multiplied by 2.4 when one wishes to express the enrichment - related to the aspiration bone marrow, i.e. the starting material.

A discontinuous albumin gradient consists of five layers with different density. The distribution of nucleated cells and GM-CFU's over the several layers after density centrifugation are shown in Table 4.1. The highest concentration of progenitor cells is found in the lightest density fraction of a discontinuous albumin gradient. This fraction contains 70% of all GM-CFU's. Since a proportion of all progenitor cells is recovered from this fraction, it was decided to avoid selection in this stage of the purification procedure and to collect all cells from the two top fractions. Eighteen percent of all nucleated cells are

recovered from these two light density fraction and approximately 90% of the GM-CFU's.

After collection of cells form the two light density fractions a cell suspension is obtained in which GM-CFU's are approximately 7 times enriched in comparison to the cell suspension harvested from the ficoll gradient.

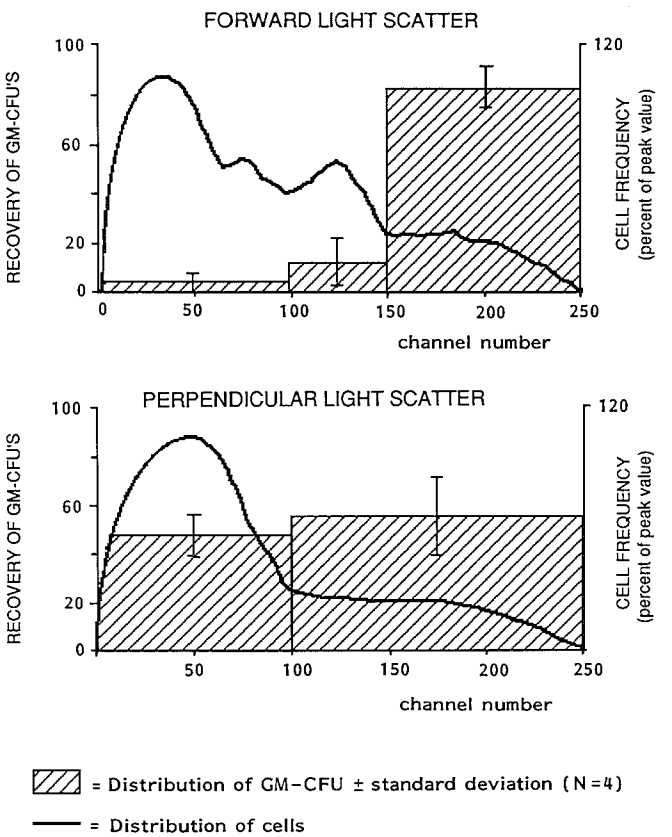

Figure 4.2 **Distribution of GM-CFU's after cell sorting according to their light scatter pattern. Forward light scatter and Perpendicular light scatter**

4.2.2 FORWARD AND PERPENDICULAR LIGHT SCATTER PROPERTIES

Forward light scatter of a laser beam is a function of cell size while perpendicular light scatter is a function of the shape of a cell. Van den Engh et al. (1978) and others (Visser et al., 1984; Bauman et al., 1986) have shown that pluripotent haemopoietic stem cells gave a high forward light scatter signal.Transplantation of cell suspensions obtained by counterflow centrifugation-elutriation has demonstrated that those bone marrow cells, which are capable to repopulate a lethally irradiated recipient, were recovered from the large cell fraction in humans (De Witte et al., 1983) as well as in rhesus monkeys (Monroy et al., 1986).

Cells from the combined two top fractions of a albumin gradient were analyzed for their forward and perpendicular light scatter characteristics. Distribution of cells and GM-CFU according to forward and perpendicular light scatter is depicted in Figure 4.2. In the fraction with a high forward light scatter signal a minority of the nucleated cells was found, but this fraction contained 80% of all the GM-CFU's. GM-CFU's were equally distributed over the different fractions of perpendicular light scatter, indicating that this parameter can not be used for purification.

4.2.3 SURFACE MARKERS OF GM-CFU

With the fluorescence activated cell sorter (FACS) it is possible to isolate cells on the basis of the parameters scatter and fluorescence. For optimal use of the latter parameter several MCA's directed against human myeloid cells were analyzed for their reactivity pattern with GM-CFU's of rhesus monkeys. The antibodies GM1 (Jonker, Primate Center TNO) and OKT10 (Crawford et al., 1981; Sieff et al., 1982) react with the majority of cells which give progeny to colonies consisting of granulocytes and macrophages in humans as well as in rhesus monkeys (see Table 4.2). This means that these antibodies react with either haemopoietic stem cells or with committed precursor cells or with both. Sorting of cells, positive for one of these antibodies, did not result in a marked enrichment of GM-CFU's.

enrichment of GM-CFU. Similar results were obtained with other monoclonal antibodies directed against the DR antigen, such as the antibodies YCL 6.8, YAML 555.6 and HLA-DR.

My4 (Griffin, 1983) and Mo1 (Griffin, 1983: a gift from Coulter Immunology) did not react with myeloid progenitor cells (Table 4.2).

Table 4.2

Surface antigens on progenitor cells in rhesus monkeys.

monoclonal antibody	positive cells	GM-CFU/10^5 negative fraction	positive fraction
none		170 ± 25	
GM-1	50%	32 ± 15 (14%)	195 ± 45 (84%)
GM-11	32%	28 ± 8 (11%)	495 ± 101 (89%)
Mo1	12%	122 ± 31 (95%)	50 ± 36 (5%)
My4	18%	180 ± 29 (93%)	60 ± 29 (7%)
OKT10	67%	32 ± 10 (7%)	228 ± 61 (93%)

The antibodies RFB-1 (a gift form Dr Bodger, New Zealand; Bodger, 1983) and My10 (Strauss, 1987) react with the CD34 antigen present on early progenitor cells in human bone marrow but according to our own determination they react not with rhesus monkey bone marrow cells.

None of the antibodies reacted exclusively with cells which give progeny to GM-CFU's. Therefore, it was decided to purify pluripotent stem cells on the analogy of the purification procedure for mouse stem cells. Mouse stem cells were purified by using a density gradient, a preselection with wheatgerm agglutinin and a final selection on basis of the expression of a class I antigen ($H2^k$). The purification procedure for rhesus monkey stem cells was slightly modified.

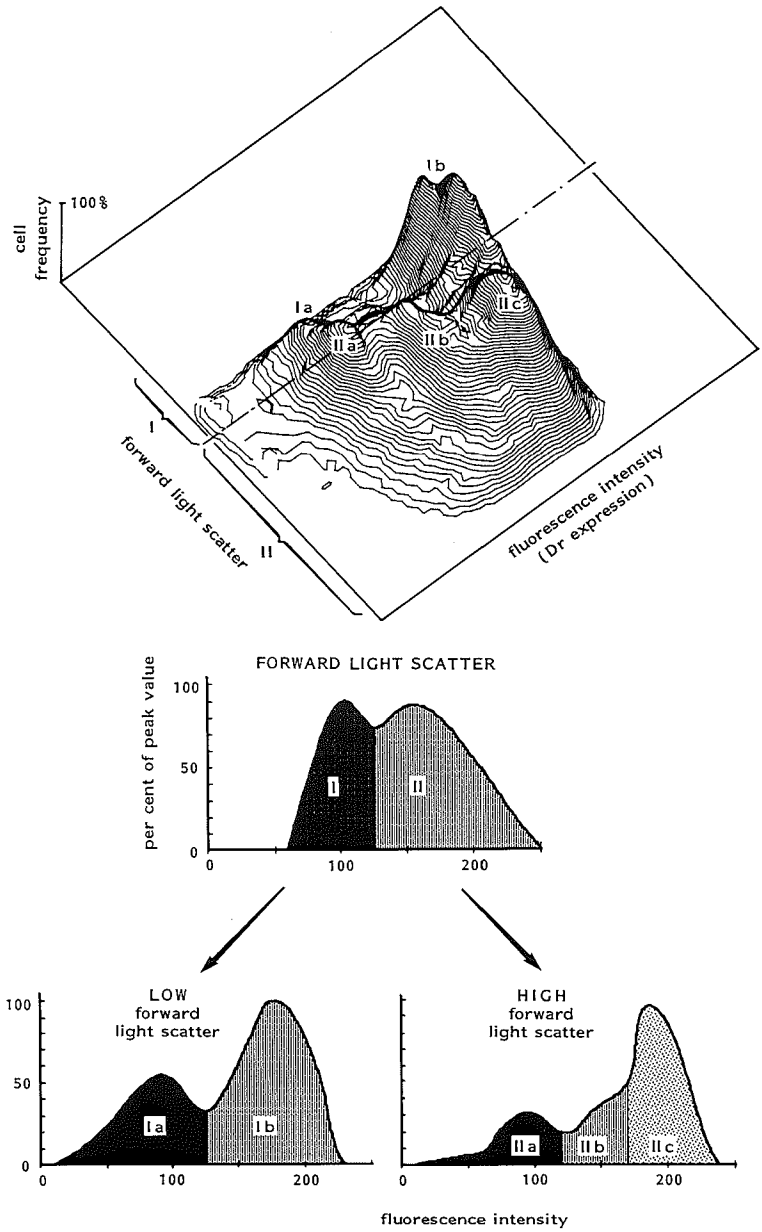

Figure 4.3 **Distribution of cells according to the parameters forward light scatter and fluorescence intensity after labelling with YAML 555.6 (anti-DR) and GARA/FITC.**

After preparation of a stem cell enriched cell suspension using the discontinuous albumin density gradient, lymphocytes were eliminated by a treatment with the antibody CAMPATH-1 plus complement. Subsequently, bone marrow cells were sorted on the expression of the DR-antigen using the antibody YAML 555.6 (Swirsky et al., 1983).

The complement lysis of lymphocytes resulted in a recovery of 11.1% of nucleated cells, 76 ± 9% recovery of GM-CFU's and 12.5-fold enrichment of these progenitor cells in comparison to the suspension harvested from ficoll.

Table 4.3

Distribution of GM-CFU over the parameters forward light scatter and reactivity with the anti-Dr antibody YAML 555.6 (N=4).

Cell fractions	percentage total nucl. cells	GM-CFU/ 10^5	recovery of GM-CFU	enrichment factor
ficoll	100	59 ± 11	100,0 ± 18,6%	1 ± 0,2
CAMPATH-1	12,8	583 ± 145	95,6 ± 27,5%	10 ± 2,4
sort control	12,8	418 ± 63	118,0 ± 16,7%	8 ± 1
Cell sorting:				
FLS Dr-antigen				
low negative (Ia*)	1,2	214 ± 27	4,3 ± 0,6%	3 ± 0,4
low positive (Ib)	3,9	624 ± 30	14,8 ± 1,9%	10 ± 0,5
high negative (IIa)	1,4	225 ± 51	9,0 ± 2,6%	4 ± 0,8
high dull (IIb)	1,4	2224 ± 224	47,3 ± 5,9%	37 ± 4,2
high bright (IIc)	2,7	1772 ± 145	63,5 ± 7,2%	29 ± 2,5
recovery			139,0 ± 18,2%	

abbreviations: FLS= forward light scatter; DR-FITC= fluorescence intensity of antibody YAML and GARA/FITC. *: numbers as shown in Figure 4.3 Mean values of four sort experiments. Enrichment factor is calculated in reference to the ficoll cell suspension.

4.2.4 PURIFICATION BY MULTIPLE PARAMETERS

When the experiments were started to purify haemopoietic stem cells, a cell sorter with three parameters namely forward light scatter, perpendicular light scatter and fluorescence was available. So far, the purification procedure consisted of density centrifugation, collection of light density cells, from a discontinuous albumin density gradient, elimination of lymphocytes by the antibody CAMPATH-1 plus complement, and sorting on basis of the light scatter properties (see also Figure 4.1). The cell sorter offered the opportunity to select cells also on their reactivity pattern with the anti-DR monoclonal antibody YAML 555.6 (Swirsky et al., 1983) and counterstained with GARA/ FITC. The labelled cells with YAML 555.6 were first separated on basis of a low or high forward light scatter signal. Secondly, a further selection was possible on the expression of the antibody YAML 555.6. Among the cells with a low forward light scatter, cells were present which reacted positively or negative with the anti-DR antibody. Cells with a high forward light scatter could be divided in cells reacting negative, positive (dull fluorescence signal) and strong positive (bright fluorescence signal) with the antibody. So, five cell clusters could be distinguished according to the parameters forward light scatter and green fluorescence signal (Figure 4.3). Using electronic sorting windows these cell populations were sorted and the functional properties of these cells were analyzed in the GM-CFU assay. At first, the cells were divided in a population with a low (cell population I) and a high forward light scatter signal (cell population II). Secondly, cells were distinguished according to their reactivity pattern with the anti-DR antibody YAML 555.6. A negative cell population (Ia) and a positive cell population (Ib) was seen in the cell population with a low forward light scatter signal. Three subpopulations could be distinguished in the cell population with a high forward light scatter (IIa: neagtive, IIb: dull fluorescence, IIc: bright fluorescence). Table 4.3 shows the enrichment and distribution of GM-CFU's over these cell clusters defined by forward light scatter and expression of the DR antigen. The highest concentration of GM-CFU's were found in the cell populations characterized by high forward light scatter and a dull (cell population IIb; see Figure 4.3) or bright (cell population IIc; see Figure 4.3) fluorescence signal. A mean 37 and 29-fold enrichment in comparison to the ficoll cell suspension was found respectively. The enrichment in reference to the starting material, i.e. aspiration bone marrow, can be

calculated by multiplying the enrichment factors with a factor 2.4. The result of such a calculation is that GM-CFU's were 89 and 70-fold enriched in the cell populations with a dull (IIb) and bright (IIc) fluorescence signal respectively. These results also indicated that labelling progenitor cells with the antibody YAML 555.6 did not influence the proliferation of these cells in vitro.

4.3 IN VIVO EXPERIMENTS WITH CONCENTRATES OF PLURIPOTENT HAEMOPOETIC STEM CELLS

For assessment of enrichment of pluripotent haemopoietic stem cells, the regeneration assay described in chapter III was used. This autologous transplantation assay is based upon the determinations that the regeneration rate of peripheral blood cells is directly related to the size of autologous bone marrow grafts which reflects the number of pluripotent haemopoietic stem cells in a marrow graft. The purification steps were monitored for the presence of pluripotent haemopoietic stem cells. These experiments were performed to verify whether the GM-CFU's values correlated with the in vivo results.

4.3.1 DISCONTINUOUS ALBUMIN DENSITY GRADIENT

One monkey was transplanted with 3 x 10^6 cells/kg body weight of the top fraction of a discontinuous albumin density gradient. This fraction contained 5.8% of total nucleated cells and 71% (normalized value) of the GM-CFU's were found in this fraction. The enrichment factor for GM-CFU's was calculated to be 8.5 $\pm$ 1.4. After autologous transplantation of these cells, leukocyte counts rose above 10^9/l at day 20 and reticulocyte counts were greater than 1% at day 19. In figure 4.4 it is illustrated in what way these findings were used to calculate the repopulating capacity of the marrow graft. Day 19 of the reticulocytes corresponds with a bone marrow graft of 12.1 x 10^6 unseparated cells /kg body weight. The 95% confidence limits indicate a range of 8.5 to 17.2 x 10^6 cells/kg body weight. From these figures it can be concluded that there is a significant 4-fold (2.7- 5.7) enrichment of pluripotent haemopoietic stem cells.

Leukocytes counts could not be used for the calculation of enrichment because of the low slope of the regression line and the broad 95% confidence range as has been discussed in detail in chapter III.

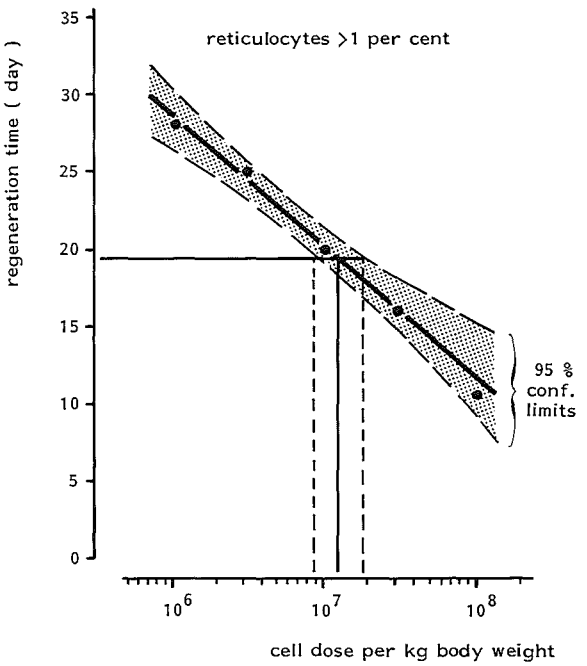

Figure 4.4 **Assessment of enrichment of purified cells using the regeneration assay of reticulocytes.**

4.3.2 ELIMINATION OF LYMPHOCYTES

After a discontinuous albumin density gradient cells from the two top fraction were collected. It was decided to collect cells from the top fraction as well as cells from the second layer since in this way almost all haemopoietic stem cells would be recovered. Lymphocytes were eliminated from this light density cell suspension by complement mediated lysis using the antibody CAMPATH-1. This resulted in a 10-fold enrichment of GM-CFU's with 9.7% of the nucleated cells in comparison to the ficoll cell suspension. After autologous trans-plantation of 1 x 10^7 cells/kg body weight reticulocytes reappeared at day 9, which means that the number of pluripotent haemopoietic stem cells in this graft was equivalent to the numbers present in an unmanipulated bone marrow graft of 1.3 x 10^8 cells/kg body weight (range:0.74-2.37 x 10^8 cells/kg body weight). Since the

expected regeneration of reticulocytes was between day 18 and 21 on basis of the cell number grafted, the observed regeneration at day 9 indicates a significant enrichment of 13 times, which agrees with the enrichment of GM-CFU's.

4.3.3 FORWARD LIGHT SCATTER PROPERTIES OF PLURIPOTENT STEM CELLS

So far, the experiments have demonstrated that both concentration of stem cells by density centrifugation and depletion of lymphocytes are feasible methods to use without loosing the repopulating capability of a bone marrow graft. The next step to isolate cells was to use a light activated cell sorter. The first parameter to be tested was the forward light scatter in view of the results with GM-CFU. Since the optimal sorting speed is approximately 2500 cells per second, it is advisable to preconcentrate the desired cells. On basis of the former experiments the stem cells were concentrated by an albumin density gradient. Cells were collected from the top fraction of the gradient and sorted according to an high forward light scatter signal. After the gradient 3.6% of the cells were recovered and 40% of the GM-CFU's. After sorting 1.3% of the cells were recovered and the enrichment factor on basis of GM-CFU counts was 11.5. Transplantation of 1.5 x 10^6 autologous bone marrow cells/kg body weight, which were isolated on basis of light density and high forward light scatter, resulted in a regeneration of reticulocytes (> 1%) at day 19. This corresponds with a bone marrow graft size of 12.1 x 10^6 /kg and an enrichment factor of 8.1 (range: 5.7-11.5). Although the recovery of stem cells is not optimal in this experiment, the significant enrichment demonstrates that forward light scatter can be used as parameter for isolation of cells with repopulating capacity.

4.3.4 OPSONIZATION OF LABELLED HAEMOPOIETIC STEM CELLS

On basis of previous experiments described above, it would be logical to purify pluripotent stem cells by a combination of density centrifugation, elimination of lymphocytes, forward light scatter. Since elimination of Dr positive cells resulted in markedly delayed regeneration, positive selection of repopulating stem cells would be a logical step in a purification procedure. This latter step

includes that cells, labelled with an antibody, have to be injected into a monkey, which could lead to either complement mediated lysis or antigen modulation or phagocytosis of the labelled cells (van den Engh et al., 1978; Bauman et al., 1985). Incubation of labelled cells with autologous complement did not result in a reduction of the number of GM-CFU's (data not shown). Since it is known that the Dr-antigen has an important function in immunological reactions, antigen modulation could chance the function of the labelled progenitor cells. Therefore, cells collected from the top fractions of a discontinuous albumin gradient were incubated with the antibody YAML and GARA/FITC.

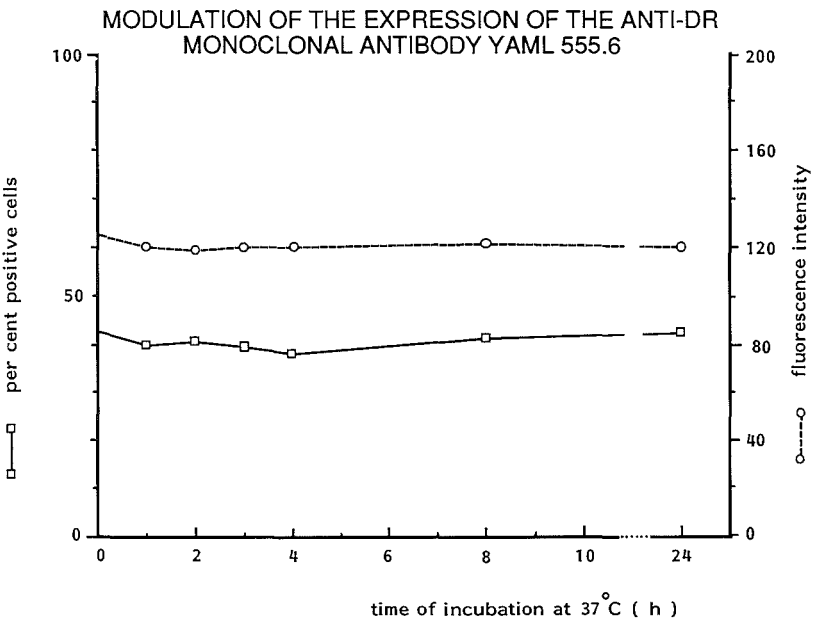

Figure 4.5 **Influence of 37^0 C on the stability of binding of the antibody YAML 555.6 and GARA/FITC at bone marrow cells.**

Thereafter these cells were resuspended in a medium containing 20% autologous serum and placed in an incubator at 37^0 C. At different time intervals the fluorescence intensity and the percentage positive cells were measured to investigate if the antigen-antibody complex with GARA/FITC was modulated. As is shown in Figure 4.5, the number of labelled cells as well as the fluorescence intensity

remained stable during a period of incubation of 24 hours. Autologous bone marrow graft consisting of 10^8 cells/kg body weight was incubated with the monoclonal antibodies YAML and GARA/FITC and injected into a monkey which was prepared by 9.8 Gy total body irradiation (double beam). The regeneration pattern of leukocytes and reticulocytes were identical to that of a monkey which received an untreated bone marrow graft. This result demonstrates that these incubated bone marrow cells were not eliminated, but retained their capacity to repopulate an irradiated recipient monkey.

Table 4.4

Concentration of repopulating pluripotent haemopoietic stem cells (PHSC) calculated from reticulocyte regeneration rate.

FLS	DR	cell dose (x 10^6/kg body weight)	Reticulocyte > 1% expected	Reticulocyte > 1% observed	cell dose fitting to regr. line	PHSC enrichment factor
low	**++ (Ib*)**	0,5	32	23	4,7	9,4
high	**+ (IIb)**	0,7	31	16	24,8	35,4
high	**++ (IIc)**	0,5	32	17	19,5	39,0
all	**- (Ia+IIa)**	0,6	31	>22	<5,9	<9,6

FLS: Forward light scatter, DR: expression of the anti DR antibody YAML 555.6, regr.: regression, PHSC: pluripotent haemopoietic stem cells
*: numbers as shown in Figure 4.3

4.3.5. POSITIVE SELECTION OF HAEMOPOIETIC STEM CELLS

The final step in the purification procedure was isolation of bone marrow cells according to the parameters forward light scatter and reactivity pattern with an indirect labelled anti-Dr antibody, called YAML (see also figure 4.1 and 4.3). Using the modified FACS II, autologous bone marrow cells were sorted and small numbers of cells were injected into an irradiated recipient. On basis of the regeneration rate of reticulocytes, the repopulating capacity of the purified cells

could be calculated (Table 4.4). Transplantation of Dr-negative cells gave no regeneration of peripheral blood cells. The monkey died after 22 days due to sepsis secondary to bone marrow aplasia. At autopsy total bone marrow aplasia was observed.

Cells which were Dr-positive and which gave a low forward light scatter signal, (cell population Ib; see Figure 4.3) had some repopulating ability.

Table 4.5.

Recovery of cells with repopulating ability.

forward light scatter	reactivity with Dr-antibody	% of total nucl. cells	enrich-ment	recovery
low + high	**negative**	2,7%	< 6	13,5%
low	**positive**	1,6%	9,4	15,0%
high	**dull positive**	1,4%	35	49,0%
high	**bright positive**	0,7%	39	27,3%
Total				104,8%

Transplantation of cells with a high forward light scatter and a dull fluorescence signal with the anti-Dr antibody (cell population IIb; see Figure 4.3) resulted in a regeneration of reticulocytes at day 16, which meant a 36.3 (range: 21-64) times enrichment. A graft consisting of cells sorted on basis of a high forward light scatter signal and bright fluorescence signal with the anti-Dr antibody (cell population IIc; see Figure 4.3), showed a different regeneration pattern of reticulocytes. At day 9 reticulocyte counts rose above 1%, but dropped at day 13 and rose again at day 17. This latter day was taken for calculation of the enrichment, since after day 17 the regeneration of reticulocytes showed a normal pattern. The enrichment factor was calculated to be 39.7 (range: 23-70) times. On basis of the percentage of total nucleated cells and the enrichment factors calculated, a recovery can be estimated (Table 4.5). The distribution of stem cells over the different cell clusters is similar to the distribution of progenitor cells with 76% of stem cells characterized by high forward light scatter and positive reaction with the Dr-antibody.

Leukocyte regeneration was comparable to the regeneration rate of reticulocytes in the animals, which received a bone marrow graft consisting of either Dr-negative cells or cells with low forward light scatter signal and Dr-positive signal, while in the other two monkeys, the regeneration rate of leukocytes was slower in comparison to reticulocyte regeneration. However, the number of platelet transfusions required was within normal limits, indicating clearly that the bone marrow graft consisted of multipotent stem cells (Figure 4.6 + 4.7).

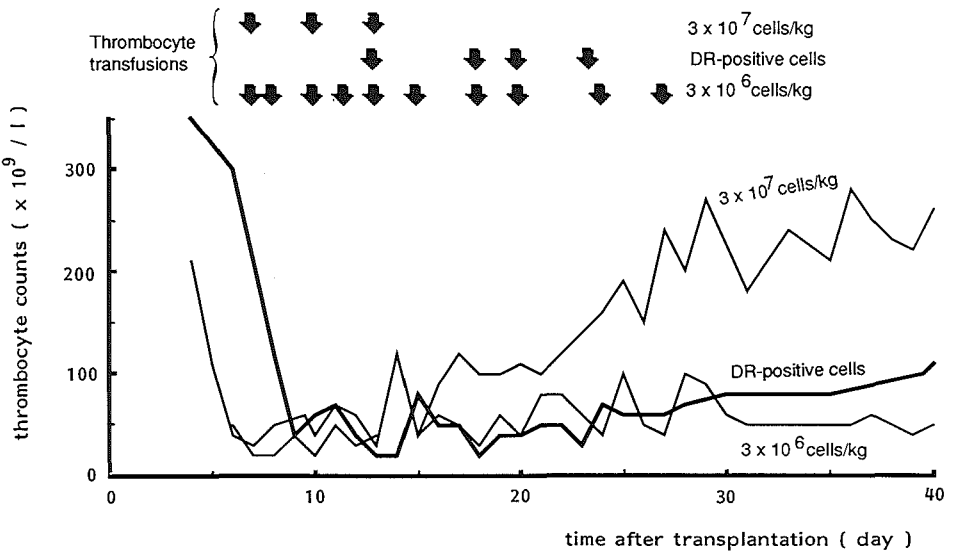

Figure 4.6 **Regeneration of thrombocytes after transplantation of cells selected on the basis of DR expression and forward light scatter. Fraction IIb; high forward light scatter and strong expression of the DR-antigen.**

4.4 ANALYSIS OF GM-CFU BY DOUBLE FLUORESCENCE ANALYSIS

Further enrichment was possible when a new cell sorter was build in our laboratory. This experimental light activated cell sorter was able to identify more

than three parameters and for immunofluorescence analysis a second laser beam was available.

The availability of the fluorescent dye phycoerythrin (PE) allowed further purification since two color immunofluorescence can be obtained using a single wavelength excitation when PE is paired with FITC.As stated before, not only lymphocytes and activated T lymphocytes, but also some macrophages, monocytes and committed progenitor cells have the Dr-antigen on their cell surface. The antibody My4 reacts in humans with monocytes and promonocytes (Griffin et al., 1983). These two cell types also express Dr-antigens on their cell surface. Both the antibody Mol and an anti-Dr antibody recognize monocytes, promonocytes and myeloblasts (not identical to cells which produce granulocytes and monocytes). Besides these cells the antibody Mol is directed against promyelocytes, myelocytes and polymorphs (Griffin et al., 1983). The antibody Mol as well as My4 crossreact with rhesus monkey cells (Letvin et al., 1983) and does not react with cells which produce GM-CFU's (Table 4.2).

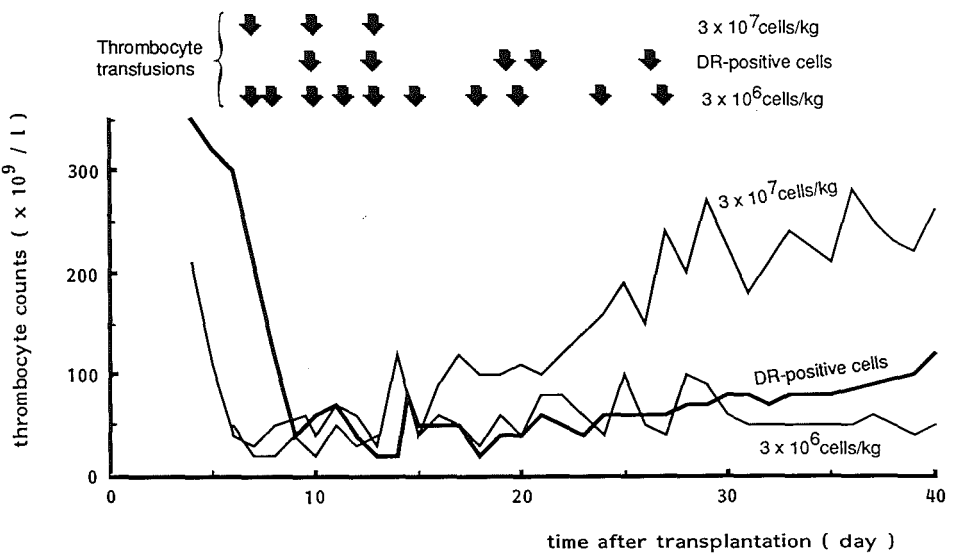

Figure 4.6 **Regeneration of thrombocytes after transplantation of cells selected on the basis of DR expression and forward light scatter. Fraction IIc; high forward light scatter and very strong expression of the DR-antigen.**

A pilot experiment showed that there is a small cell population in rhesus bone marrow which has only the Dr-antigen on their cell surface and not the antibodies CAMPATH-1 and Mol. Selection of those cells which gave a high forward light scatter signal, had the DR-antigen on their cell surface and not the antibodies CAMPATH-1 and Mol lead to further enrichment of GM-CFU (Table 4.6).

Table 4.6

Purification of progenitor cells using a discontinuous albumin density gradient and multiple parameter cell sorter.

cell material		per cent of total nucl cells	GM-CFU/ 10^5	enrichment factor	recovery %
Ficoll		100	15	1	100
albumin gradient top fractions		12	110	7,3	86
high fls/low pls window: DR/PE	CAMPATH-1+Mol/FITC				
pos	**neg**	0,039	2540	169	66
pos	**pos**	0,84	300	20	17
neg	**pos+neg**	2,12	30	2	4
all cells outside this window		8,7	160	10,6	38

total nucl cells: total nucleated cells; DR/PE: anti HLA-Dr directly coupled to phycoerythrin; pos: positive, neg: negative

4.4 DISCUSSION

These experiments were primarily carried out to conduct enrichment procedures to be used for human progenitor cells in a preclinical rhesus monkey model. Therefore, monoclonal antibodies directed against human myeloid progenitor cells were used with rhesus monkey bone marrow. Studies with monoclonal antibodies directed against human granulocytes (Letvin et al., 1983) and lymphocytes (Jonker, 1984, 1986; Hansen, 1984) have demonstrated that these antibodies react with the same antigenic structures in several primates.

The first steps in the purification procedure consisted of density centrifugation. According to the results of Dicke (1970) and Moore et al.(1972), rhesus monkey pluripotent haemopoietic stem cells were recovered from the light density fractions of a discontinuous albumin density gradient as has been demonstrated in the GM-CFU assay as well as in the in vivo experiments. Depletion of lymphocytes had no influence on the colony growth, which is in agreement with results with human bone marrow (Martin et al., 1985; Granger et al., 1982; Berenson et al., 1984). Scatter of light by cells in flow gave a distribution of rhesus bone marrow cells according to their size and shape. The distribution is very similar to human bone marrow cells. GM-CFU's and pluripotent stem cells were recovered from the fractions with a high forward light scatter signal. Experiments with human bone marrow cells using either cell sorters or counterflow elutriation centrifugation did also show that myeloid progenitor cells can be identified by a high forward scatter signal (Beverley t al., 1980; Mouchiroud et al., 1985; Bodger et al., 1983; de Witte et al., 1984).

Since several antibodies directed against human cells crossreact with rhesus monkey cells, they could be used in this preclinical model. Since none of the tested antibodies reacted exclusively with GM-CFU's, the anti-DR antibody YAML 555.6 was applied to purify pluripotent haemopoietic stem cells on the analogy of the purification of mouse stem cells (Visser et al., 1984). Positive selection of haemopoietic stem cells was not accompanied by the problem of opsonization in contrast to the situation in mice (Van de Engh et al., 1983; Bauman et al., 1985). In the murine experiments an increase in antibody concentration was associated with a loss of stem cells. A high concentration of GAM/FITC protected the labelled cells from opsonization in one mouse strain. In the murine experiments, a 50% loss of stem cells was measured when a relatively high concentration of antibody was used. Since in our experiments a

relatively low concentration of antibody was used and a high concentration of conjugate was applied, this could be an explanation for the fact that opsonization was not observed. On the other hand our regeneration assay is not accurately enough to observe a significant reduction of stem cells in the order of 50% or less.

These experiments have demonstrated that the highest concentration of GM-CFU was found in the cell populations characterized by a high forward light scatter signal and a weak (dull fluorescence) or strong (bright fluorescence) expression of the DR antigen. The GM-CFU were 30-37 fold enriched in comparison to the cell suspension harvested from the ficoll gradient. In agreement with the in vitro data pluripotent stem cells were found in the same cell populations. A 40-fold enrichment of pluripotent stem cells was achieved in comparison to the ficoll cell suspension. In comparison to the starting material, i.e. aspiration bone marrow, a 90-fold enrichment was achieved. Dicke et al (1973) determined that the number of pluripotent stem cells in aspiration rhesus monkey bone marrow was approximately 0.5-0.7%. Thus, a cell suspension consisting of pure stem cells would require an enrichment of 143-200 fold. Since in our experiments an enrichment of 90-fold was found, further enrichment is required when a pure stem cell populations is desired.

A pilot experiment has revealed that the antibody Mo1 can be used in combination with the antibody CAMPATH-1 for the identification of committed progenitor cells which have also the DR antigen on their cell surface. More preferable would be antibody which identify early progenitor cells such as the My10 (CD34) in man. Recently, a CD34 antibody has been described which identify early progenitor cells in baboons (Berenson et al., 1988) and rhesus monkeys (Wielenga et al.,1989).

The calculation of enrichment of stem cells on basis of the GM-CFU assay corresponded very well with the results of the in vivo experiments. The feeder layer of leukocytes produce apparently sufficient growth factors for stem cells to differentiate along the granulocyte-macrophage pathway. This observation is important for further experiments because these experiments have proven that enrichment of pluripotent stem cells can be assayed in vitro as good as in vivo.

CHAPTER V

RADIATION DOSE REQUIRED FOR ENGRAFTMENT OF 2 LOG T LYMPHOCYTE DEPLETED BONE MARROW GRAFTS

5.1 INTRODUCTION

The direct relationship between the number of donor T lymphocytes and the incidence of acute Graft-versus-Host Disease (GvHD) was established two decades ago in mice (van Bekkum et al., 1964). Several methods for selective elimination of lymphocytes were developed and applied. All showed that T lymphocyte depletion did indeed prevent acute GvHD. Prevention of acute GvHD could not only be achieved in species with a low proportion of T lymphocytes in bone marrow such as rodents (Dicke et al., 1968; Vallera et al., 1981; Rodt et al., 1974) and dogs (Kolb et al., 1979) but also in rhesus monkeys with a relatively high proportion of T lymphocytes in bone marrow (Wagemaker et al., 1982). It was also established in experimental animals that T lymphocyte depletion of the graft may cause a high incidence of engraftment failures (Vriesendorp et al., 1982; Wagemaker et al., 1982). The logical conclusion of these observations was that donor T lymphocytes exert an immunosuppressive action (the Graft-versus-Host reaction) which suppresses the Host-versus-Graft reaction. Abrogation of the acute GvH reaction by T lymphocyte depletion will require additional suppression of the Host-versus-Graft reaction since the Host-versus-Graft reaction may get stronger. After establishing the reciprocal interference (Vriesendorp et al., 1981) between Graft-versus-Host reaction and Host-versus-Graft reaction, conditioning schemes were developed for the promotion of engraftment of T lymphocyte depleted bone marrow grafts. In our dog and rhesus monkey models for bone marrow transplantation, a high dose of total body irradiation was effective in preventing engraftment failures of T lymphocyte depleted bone marrow grafts.

Unmodified bone marrow grafts from DLA-identical siblings engrafted in 10/10 dogs after conditioning with 7.5 Gy total body irradiation. Transplantation of stem cell enriched T lymphocyte depleted marrow grafts resulted in 4/7 engraftment failures with the same irradiation dose. When the dose of irradiation was increased to either 2 x 4.5 Gy (5/5 dogs) or 2 x 6.0 Gy (7/7 dogs) with a 72 hours interval (Walma et al., 1987) engraftment was uniformly observed. In rhesus monkeys similar results were obtained with T lymphocyte depleted unrelated bone marrow grafts. A single dose of 8.5 Gy total body irradiation resulted in engraftment of 5 out of 18 recipient monkeys. When the conditioning regimen was changed to 2 x 7 Gy (72 hour interval) take failures were no longer observed (Wagemaker et al., 1982).

A disadvantage of higher doses of total body irradiation is the increased toxicity especially to the gastrointestinal tract and the lungs. Previous experiments in dogs and rhesus monkeys did not give exact information on the minimal dose of irradiation required to prevent graft rejection completely when T lymphocyte depleted bone marrow grafts were transplanted. Therefore, graded doses of total body irradiation were administered to rhesus monkeys which received T lymphocyte depleted bone marrow grafts. Since the problem of engraftment failure is especially encountered in mismatched donor-recipient combinations, unrelated, mismatched donors were used in this study.

5.2. ENGRAFTMENT OF 2 LOG T LYMPHOCYTE DEPLETED BONE MARROW GRAFTS

Donor monkeys were sacrificed for these experiments and bone marrow was extruded from the bones by pressure. A buffy-coat cell suspension was prepared by centrifugation. The cell yield from a single donor was 1-2 x 10^{10} cells which is sufficient for 3-4 recipients. Buffy coat cells were layered on top of a discontinuous albumin density gradient. In a discontinuous albumin density gradient T lymphocytes and myeloid progenitor cells are concentrated in different fractions (Figure 5.1). A T lymphocyte depleted, stem cell enriched cell suspension was collected from the two low density fractions in top of a gradient. In these low density fractions the percentage T lymphocytes is still comparable to the concentration of T lymphocytes in the initial cell suspension. However, since stem cell concentration results in an approximately 8-10 fold

lower number of nucleated cells required for sustained engraftment the absolute number of T lymphocytes grafted is correspondingly decreased (Wagemaker et al., 1982). Accordingly, stem cell concentration results in approximately one log T lymphocyte depletion (Table 5.1).

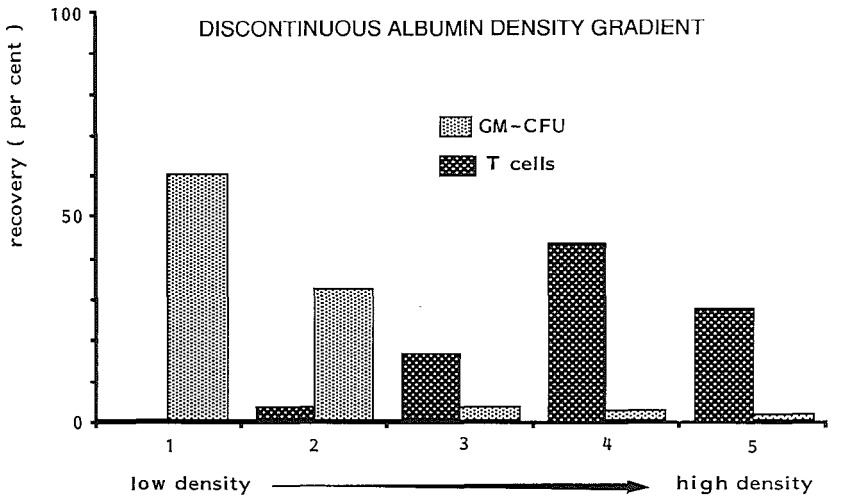

Figure 5.1 **Distribution of GM-CFU and T lymphocytes from Rhesus monkey bone marrow over a discontinuous albumin gradient.**

Table 5.1

Enrichment of GM-CFU by a discontinuous albumin density gradient and depletion of T lymphocytes by E-rosette sedimentation.

bone marrow fraction	enrichment of GM-CFU	graft size in tnc/kg b.w. (x 10^8)	E-RFC (%)	E-RFC/ kg b.w. (x 10^8)	depletion factor
buffy coat	1	4	5	0,2	0
low density fraction	8,4	0,5	5	0,02	10x
E-rosette sedimentation	10,6	0,25	0,20	0,0005	400x

The results of one representative experiment. E-RFC = E-rosette forming cells; tnc = total nucleated cells; kg b.w. = kilogram body weight

Using the property of T lymphocytes to form rosettes with sheep red blood cells, the remaining T lymphocytes were removed from the stem cell fractions by E-rosette sedimentation. After this step a bone marrow graft of 2.5 x 10^7 nucleated cells/kg body weight could be given since the progenitor cells were further enriched. Such a graft contained 0.2-0.5% T lymphocytes (= E-rosette forming cells) corresponding to a 2-3 log depletion of T lymphocytes (Table 5.1) as compared to unmodified bone marrow.

Recipients of T lymphocyte depleted bone marrow grafts were prepared for grafting by fractionated total body irradiation with an interval of 24 hours. Graded doses of total body irradiation were administered in a range of 2 x 4.2 Gy to 2 x 6.1 Gy (Figure 5.2). Up to a dose of 2 x 5.2 Gy engraftment of T lymphocyte depleted bone marrow grafts was not documented.

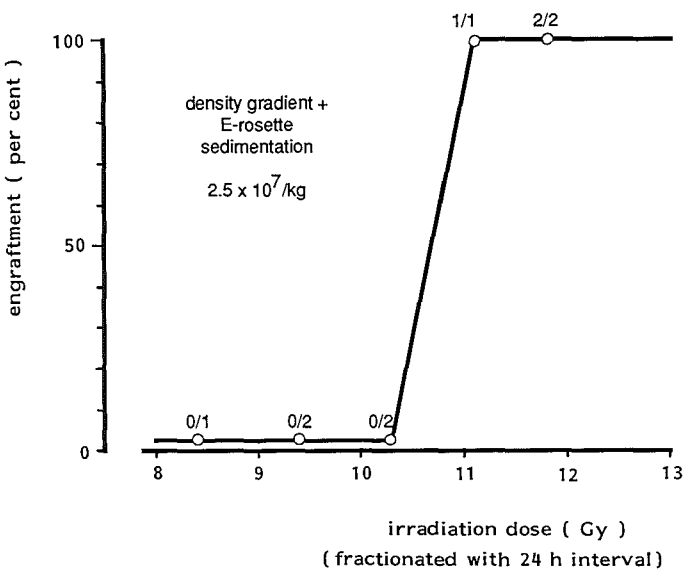

Figure 5.2 **Dose-effect relationship between fractionated total body irradiation (X-rays; 2 fractions with 24 hours interval) and engraftment of 2 log T lymphocyte depleted bone marrow grafts in rhesus monkeys. Unrelated, mismatched donor-recipients combinations.**

An increment of the irradiation dose with 1-2 Gy resulted in sustained engraftment of T lymphocyte depleted bone marrow grafts. These results demonstrate that a steep dose-effect relationship exists between the dose of fractionated total body irradiation (24 hours interval) and engraftment of T lymphocyte depleted bone marrow grafts as illustrated in Figure 5.2.

5.3 LATE REJECTION OF MORE THAN 3 LOG LYMPHOCYTE DEPLETED BONE MARROW GRAFTS

A dose of 2 x 6 Gy total body irradiation was sufficient for engraftment of 2 log T lymphocyte depleted bone marrow grafts. Since earlier experiments have shown that a 2 log T-lymphocyte depletion is not sufficient in preventing completely acute GvHD in unrelated, mismatched donor-recipient combinations, it was investigated whether 2 x 6 Gy total body irradiation would result in engraftment of more than 3 log T-lymphocyte depleted bone marrow grafts.

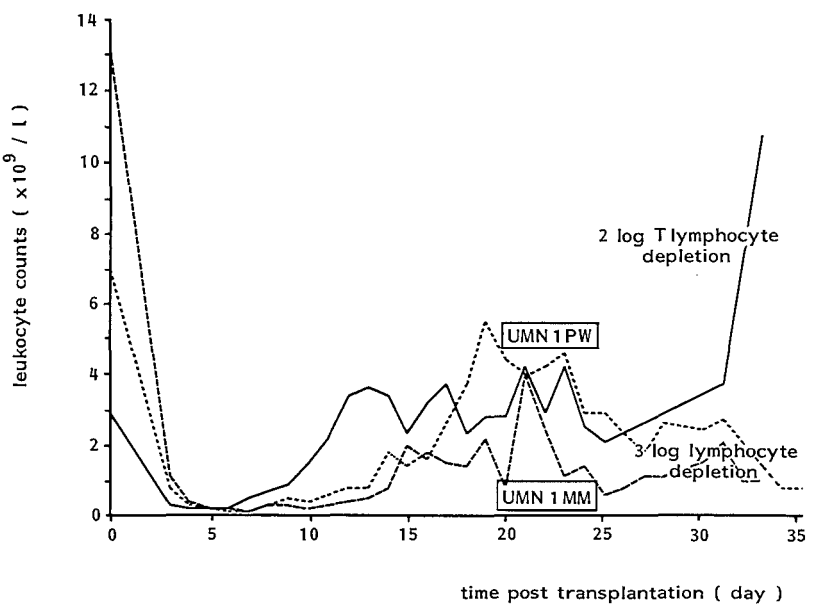

Figure 5.3 **Regeneration of leukocytes after allogeneic bone marrow transplantation with 3 log T lymphocyte depleted bone marrow grafts.**
UMN= unique monkey number

Two rhesus monkeys were conditioned by 2 x 6.0 Gy total body irradiation (24 hours interval; X-rays) and given a bone marrow graft from which T-lymphocytes were depleted by a combination of albumin gradient, E-rosette sedimentation and complement lysis of T-lymphocytes using the antibody CAMPATH-1 plus complement. A discontinuous albumin gradient followed by E-rosette sedimentation deplete 2-3 log T lymphocytes (Table 5.1). Complement lysis of T lymphocytes with CAMPATH-1 plus complement depletes also 2-3 log T lymphocytes. Therefore, it was assumed that these methods would deplete more than 3 log T lymphocytes. After T lymphocyte depletion by these two methods, T lymphocytes were not detected using the E-rosette forming method for enumeration of T lymphocytes. As shown in Figure 5.3 peripheral blood cell counts rose around day 20. After transplantation of a 2 log T lymphocyte depleted bone marrow graft, leukocyte counts continued to rise to normal values. A gradual decrease in leukocyte counts was observed after 25 days posttransplantation when 3 log T lymphocyte depleted bone marrow grafts were administered. Leukocytes of monkey 1MM were of both recipient and donor type at day 25. At day 32 these cells were of recipient type indicating graft rejection had occurred in monkey 1MM. The leukocytes of monkey 1PW were of donor type at day 32, 38 and 42, but at day 45 posttransplantation the cells were both of donor and recipient. Monkey 1 MM was killed after 32 days when it was become transfusion dependent. Monkey 1PW died after 45 days from Cytomegaly virus pneumonitis. The phenomenon of late rejection was not observed when T-lymphocytes were depleted by stem cell enrichment and E-rosette sedimentation alone.

5.4 TOXICITY OF THE CONDITIONING REGIMENS

Autologous bone marrow transplantations were performed to determine the toxicity of the conditioning regimen. The monkeys were prepared for bone marrow grafting by 2 x 5.6 Gy total body irradiation. This was the minimal dose of irradiation required for engraftment of T lymphocyte depleted bone marrow grafts. Four monkeys were given either an unmodified, autologous bone marrow graft, consisting of 5 x 10^7 cells/kg body weight, or a T lymphocyte depleted, stem cell enriched bone marrow graft consisting of 0.9 x 10^7 cells/kg body weight. Concentration factor for GM-CFU was 7.5 and 6.1, which means that

approximately an equal numbers of progenitor cells were present in both unmodified marrow grafts and T lymphocyte depleted marrow grafts.

Table 5.2

Incidence of radiation induced, acute exsudative pneumonitis following total body irradiation and autologous bone marrow transplantation in rhesus monkeys. Monkeys which survived for more than 2 months after transplantation.

UMN	DOSE OF IRRADIATION (Gy) TBI	LUNG DOSE	SURVIVAL TIME (Days)	RADIATION PNEUMONITIS
1 GA	9.35	10.45	80	+
1 LP	9.40	10.45	> 100	-
1 LG	9.45	10.50	87	-
1 NN	9.50	10.50	> 100	-
1 OT	9.50	10.50	> 100	-
1 LQ	9.50	10.55	87	-
1 TO	9.55	10.55	> 100	-
1 MY	9.65	10.55	67	-
1 QT	9.65	10.55	87	-
1 KK	9.70	10.60	60	+
1 SN	9.80	10.60	93	+
1 HH	9.85	10.60	> 100	+
1 HW	9.85	10.70	61	+
1 HJ	2 x 5.6	2 x 6.1	59	+
1 AT	2 x 5.6	2 x 6.1	87	+

TBI: total body irradiation, UMN; unique monkey number

The monkeys survived for 40, 53, 59 and 87 days. At the time of death normal peripheral blood cell counts were documented. At the autopsy whitish nodules were predominantly seen in liver and spleen of monkey 1AT and bacterial cultures revealed that these organs were infiltrated with Yersinia pseudotuber-

culosis. Monkey 2AW suffered from dyspnea and at autopsy pneumocystis carinii were observed in the lungs. The other two monkeys (1FB and 1HJ) lived longer and died from interstitial pneumonitis without positive viral or bacterial cultures.

Since irradiation had most probably induced these lesions in the lungs, it was decided to review all recipients, which were given autologous bone marrow grafts and which survived for more than 60 days, for signs of radiation pneumonitis at autopsy. The results of this survey is shown in Table 5.3. After preparation of recipients with a single beam X-ray signs of exsudative radiation pneumonitis was observed in 5 out of 13 monkeys. The frequency of pneumonitis was 1 out of 9 when monkeys received a total lung dose of 10.45 - 10.55 Gy. When a total lung dose was 10.60 Gy or higher the frequency of pneumonitis was 4 out of 4 monkeys suggesting a steep dose-effect relation between irradiation dose and the induction of radiation induced pneumonitis.

Table 5.4

Engraftment, severity of Graft-versus-Host Disease and survival of monkeys which received a T lymphocyte depleted bone marrow graft from unrelated, mismatched donors.

UMN	Irradiation dose (Gy)	Engraftment	survival (days)	cause of death	GvHD grading
1 EP	2 x 5.6	+	34	CMV	0
1 CC	2 x 6.1	+	24	sepsis	I
1 EG	2 x 6.1	+	34	CMV	II

UMN: unique monkey number, GvHD: Graft-versus-Host Disease, CMV: Cytomegaly virus

5.5 ENGRAFTMENT AND ACUTE GVHD

Three monkeys were conditioned with either 2 x 5.6 Gy or 2 x 6.1 Gy (unilateral total body irradiation) and received T lymphocyte depleted bone

marrow grafts from unrelated, mismatched donors. They survived for 34, 24 and 34 days respectively. Survival was significantly prolonged in comparison with historical controls. In this donor-recipient combination (unrelated and totally mismatched for the MHC-antigens) survival ranged from 8-17 days (N=7: Wagemaker, 1982). Historical control monkeys, which were given unmodified bone marrow grafts, all died from severe GvHD (grade III), while the recipients of T lymphocyte depleted grafts died from complications secondary to the transplantation procedure such as Cytomegaly virus (CMV) infection and bacterial infections (Table 5.4). Only mild signs of acute GvHD were seen (Table 5.4).

5.6 DISCUSSION

It is estimated that approximately 60-70% of patients who might benefit from a bone marrow transplant will not have an HLA-identical sibling as donor. Therefore it was attempted to use HLA-nonidentical siblings as donors. In humans (Hansen et al., 1986; Powles et al., 1983), dogs (Vriesendorp, 1985) as well as rhesus monkeys (Wagemaker et al., 1982) similar obstacles were observed when HLA-nonidentical siblings were used as donors; HLA incompatibility creates a higher immunological barrier which results in an higher incidence of engraftment failures and an higher incidence of acute GvHD. T lymphocyte depletion as a method to prevent GvHD has dramatically decreased the incidence of acute GvHD to less than 10% in HLA-identical, related donor-recipient combinations (see Table 1.4). On basis of these encouraging results, several transplantation centers have started to apply this method for prevention of GvHD in HLA-nonidentical, related donor-recipients combinations. Engraftment failures occurred in 25-50% of the patients (see Table 1.5).

The aim of the present study was to investigate which additional dose of irradiation is required for sustained engraftment of T lymphocyte depleted bone marrow grafts in unrelated, mismatched donor-recipient combinations. Rhesus monkeys were chosen for these experiments since their bone marrow composition and immunological reactions are very similar to man (van Bekkum, 1978). Using a combination of discontinuous gradient and E-rosette sedimentation, depletion of T lymphocytes was in the order of 2-3 log depletion. In accordance to calculations by Wagemaker (1986) which predicted that a reduction of 3 log T lymphocyte depletion is required to prevent lethal GvHD in unrelated, MHC-

mismatched donor-recipient combinations, mild symptoms of acute GvHD were observed in two monkeys and severe GvHD was seen in one monkey.

The steep dose-effect curve between dose of fractionated irradiation and engraftment indicates that a difference of 1-2 Gray fractionated total body irradiation determines a successful engraftment of T lymphocyte depleted bone marrow grafts. A radiation dose of 2 x 6.0 Gy offered sufficient immunosuppression to permit engraftment of 2 log T lymphocyte depleted bone marrow grafts. In accordance with the concept of reciprocal interference, this radiation dose was insufficient when 3 log T lymphocytes were removed from a bone marrow graft. These results will have important implications for clinical centers. In the first place it provides support to do bone marrow transplantations with HLA-nonidentical siblings since T lymphocyte depleted grafts did engraft and the incidence of acute GvHD was low in these unrelated, mismatched donor-recipient combinations. Secondly, this study strongly indicates that the problem of engraftment failures of T lymphocyte depleted marrow grafts may be solved by a relatively small increment in immunosuppression.

In our study total body irradiation was used for this purpose. A disadvantage of increasing the dose of irradiation is toxicity particularly to the lungs. In our study two out of two evaluable monkeys, transplanted with autologous bone marrow grafts, died due to interstitial pneumonia. From human studies it is known that there exists a marked increase in the incidence of this complication when irradiation doses above 10.5 Gy are administered (Barrett, 1982). Almost all conditioning regimens for human patients lung shielding is applied when the irradiation dose exceeds 8-8.5 Gy. After these experiments a new conditioning protocol with lung shielding was developed for rhesus monkeys.

An alternative approach is the use of immunosuppressive agents in combination with total body irradiation such as ARA-C and cyclosporine A (Bozdech, 1985). This combination promoted the engraftment of T lymphocyte depleted marrow grafts from HLA-nonidentical siblings. Another approach is the use of more selective agents specially cytotoxic for T lymphocytes. In rhesus monkeys (Reisner, 1986) as in humans (Kernan, 1987) residual alloreactive T lymphocytes can be detected after a conditioning regimen consisting of cyclophosphamide and hyperfractionated total body irradiation. Injection of anti-T lymphocyte monoclonal antibodies in recipients prior to a sublethal dose of total body irradiation (6 Gy) prevented rejection of T lymphocyte depleted marrow grafts in 7/7 mice (Cobbold, 1986), while normal recipients all rejected

their bone marrow graft. In human patients similar results were obtained with an antibody against against human leucocyte functional antigen (HLFA-1), which is expressed on T lymphocytes, natural killer cells, leukocytes and macrophages. In 7/7 patients with immunodeficiencies engraftment of T lymphocyte depleted bone marrow grafts from HLA-haploidentical siblings was facilitated by injections of the antibody HLFA-1. In a historical control group engraftment was only documented in 1/7 patients using the same condition regimen containing busulphan without injection of this antibody (Fisher, 1986).

CHAPTER VI

PREVENTION OF ACUTE GRAFT VERSUS-HOST DISEASE BY DEPLETION OF T LYMPHOCYTE SUBPOPULATIONS

6.1 INTRODUCTION

In several species, T lymphocyte depletion is successfully applied for the prevention of acute GvHD. The mature T lymphocyte population can be divided into two subpopulations with distinct functions; suppressor/cytotoxic T lymphocytes, characterized by the CD8 antigen, and helper/inducer T lymphocytes, characterized by the CD4 antigen. The role of these T lymphocyte subpopulations in the induction of acute GvHD has been investigated in rodents. These murine experiments have demonstrated that depletion of helper T lymphocytes could mitigate the severity of GvHD after bone marrow transplantation across major histocompatibility barriers (Mason, 1981; Vallera et al., 1982; Korngold and Sprent, 1985; Sprent et al., 1986; Pietryga et al., 1987). It was decided to deplete either helper/inducer T lymphocytes or cytotoxic/suppressor T lymphocytes or all T lymphocytes from bone marrow grafts and to observe the resulting effect on acute GvHD in monkeys.

Monoclonal antibodies directed against human subpopulations of T lymphocytes were analyzed for their reactivity with rhesus monkey T lymphocytes. Subsequently, the function of the T lymphocyte subpopulations, recognized by the selected antibodies, was studied in vitro. When it was demonstrated that the selected antibodies reacted with only one of the two distinct T lymphocyte subpopulations, these monoclonal antibodies were used to investigate whether depletion of subpopulations of T lymphocytes prevented acute GvHD in mismatched donor-recipient combinations.

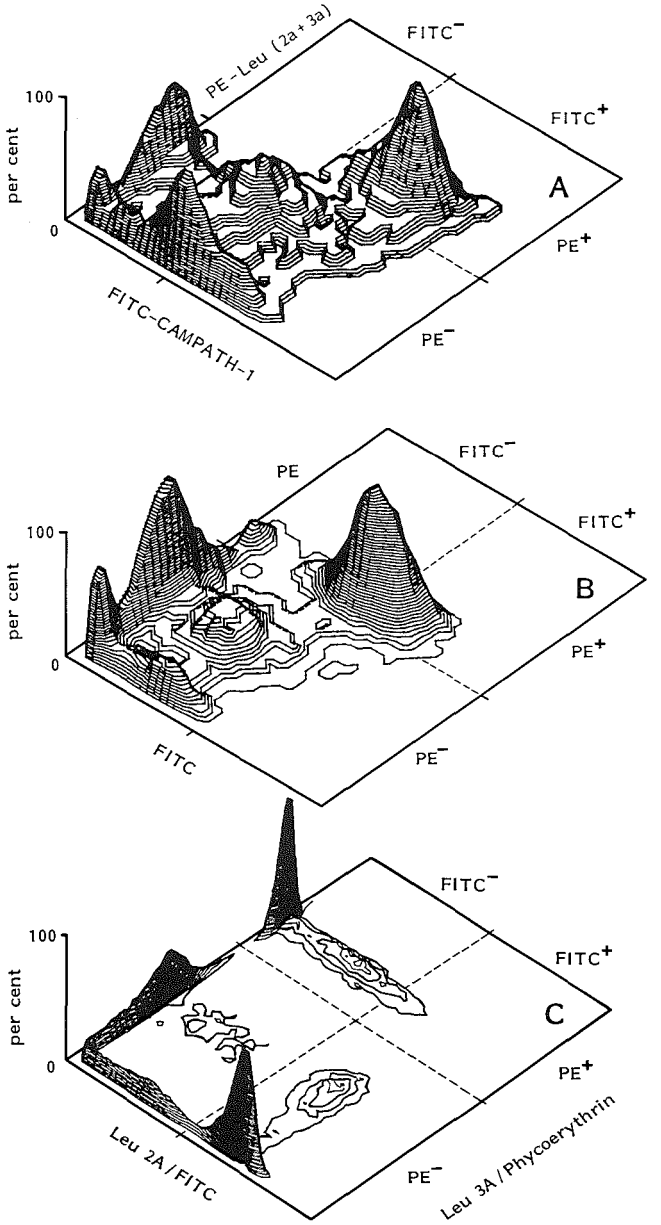

Figure 6.1 **Double fluorescence analysis of rhesus monkey T lymphocytes. (A): CAMPATH-1, (B): WT-1, (C): T lymphocytes**

In chapter V it was demonstrated that 2 x 6 Gy total body irradiation (X-ray; unilateral irradiation; 24 hours interval) would be sufficient to allow engraftment of T lymphocyte depleted bone marrow grafts. For a more homogeneous dose distribution of radiation bilateral irradiation was applied for the experiments in this chapter. Experiments in chapter V have shown that a dose of 2 x 6 Gy is accompanied by lung toxicity. At the time of these experiments, a new total body irradiation setting for total body irradiation including lung shielding would not yet been available for at least half a year. Symptoms of acute GvHD are observed before day 40 in unrelated, mismatched donor-recipient combinations. Dyspnea from lung toxicity is observed after day 40. Therefore, it was anticipated that the effect of depletion of T lymphocyte subpopulations on acute GvHD could be measured when total body irradiation was applied without lung shielding.

6.2 CHARACTERIZATION OF ANTI T LYMPHOCYTE ANTIBODIES IN VITRO

6.2.1 FLUORESCENCE ANALYSIS

The reactivity pattern of rhesus monkey lymphocytes with the antihuman T lymphocyte monoclonal antibodies was determined by use of immunofluorescence with 2 fluorchromes. Leu 2a/PE (CD8) and Leu 3a/PE (CD4) were used as reference antibodies.

Pure T lymphocytes were obtained by E-rosette sedimentation with rhesus monkey peripheral blood cells; a combination of Leu 2a and Leu3a stained 99% of all the T lymphocytes. Incubation of mononuclear peripheral blood cells with Leu2a/FITC and Leu3a/PE resulted in 2 separate subpopulations of approximately equal size with only 1-3% of the cells sharing the markers (Figure 6.1c), demonstrating great similarity between humans (Clark et al., 1986) and rhesus monkeys with respect to the major T lymphocyte subsets. Figure 6.1a demonstrates that CAMPATH-1 reacts with all bone marrow cells positive for Leu2a and/or Leu3a, which represented 68% of the cell populations reacting with CAMPATH-1 (Hale et al.,1983) cells; the remaining 32% were most probably, analogous to human bone marrow, B-lymphocytes and monocytes. Figure 6.1b shows that all bone marrow cells that react with the WT 1 (CD7) antibody shared the T lymphocyte markers Leu2a and/or Leu3a. A small subpopulation of

Leu 2a/Leu3a positive cells was observed that lacked the the WT 1 antigen. Also, these findings are similar to the reactivity pattern of WT 1 with human lymphocytes (Tax et al., 1984). Double fluorescence analysis demonstrated that the B9 monoclonal antibodies and Leu2a recognize the same T lymphocyte subpopulation and that the antibodies OKT4+4a and Leu3a also shared the same subpopulation of T lymphocytes.

6.2.2 FUNCTIONAL CAPACITY OF LYMPHOCYTES IN VITRO AFTER DEPLETION.

The functional capacity of residual T lymphocytes after depletion was studied in vitro. Peripheral blood cells (PBL) was stimulated to proliferate by the mitogen phytoagglutinin (PHA). Since some anti-T lymphocyte antibodies such as OKT3 induce and enhance proliferation of PBL's, it was investigated whether a proliferative response could be attributable to labelling lymphocytes with either B9-pool (a combination of four antibodies directed against cytotoxic/suppressor T lymphocytes) or OKT4+4a (helper/inducer T lymphocytes) or CAMPATH-1 (anti T and B-lymphocytes). The proliferative capacity was compared to proliferation of unlabeled cells. Labelling of PBL's with antibodies, directed against T lymphocyte subpopulations, did not influence their proliferative capacity. The proliferation of lymphocytes with CAMPATH-1, an antibody directed against all lymphocytes, on their cell surface was reduced to $34 \pm 4\%$ in comparison to untreated PBL's.

Depletion of either B9-pool positive lymphocytes or OKT4+4a positive lymphocytes, prior to stimulation with PHA, provides insight in the function of these subpopulations in rhesus monkeys. After complement lysis of labelled cells, residual cells were counted and the same number of viable cells were used as in the control experiments. Complement lysis with the antibody CAMPATH-1 eliminated the proliferative response completely as could be expected. After elimination of B9-positive cells by complement lysis, the residual cells had a similar proliferative response to PHA stimulation as the untreated cells. Depletion of OKT4+4a positive cells did reduce significantly the proliferative capacity of PBL's. These latter results suggest that OKT4+4a positive cells have an helper/inducer function in rhesus monkeys similar to that in humans. A functional assay to investigate the cytotoxic capacity of lymphocytes is not yet available for rhesus monkeys.

Fluorescence analysis of the OKT4 and B9-pool positive cells has shown that these antibodies recognize complementary T lymphocyte subpopulation in rhesus monkeys. The results of fluorescence analysis together with the experiments described above provide sufficient evidence that the antibodies OKT4 and B9-pool recognize the same T lymphocyte subpopulations in man as in the rhesus monkey. Therefore, we used these antibodies to investigate the influence of depletion of T lymphocyte subpopulations on the induction of acute GvHD in rhesus monkeys.

6.3 PREVENTION OF ACUTE GVHD.

Recipients of unrelated, mismatched bone marrow grafts were prepared with 2 x 6.7 Gy total body irradiation (X-ray; bilateral irradiation; 24 hours interval). Bone marrow cells were obtained by aspiration. A mononuclear cell suspension was prepared by ficoll density centrifugation. One monkey received 4 x 10^8 unmodified bone marrow cells/kg body weight. This animal died after 12 days from severe GvHD. This result was in accordance with survival and cause of death of historical controls. These latter animals were prepared with a single dose of 8.5 Gy TBI and all died due to severe GvHD within 17 days after transplantation (median: 12 days; N=7).

Before the in vitro treatment with antibody and complement, the cell number given was 4 x 10^8 bone marrow cells/kg body weight. Cells left after incubation were directly infused into recipients.

Depletion of suppressor/cytotoxic T lymphocytes from a bone marrow graft by the antibodies B9-pool did not eliminate the cells responsible for induction of acute GvHD. Two rhesus monkeys died after 14 and 15 days from severe GvHD (Table 6.1). Elimination of helper/inducer T lymphocytes (OKT4+4a) neither prevented GvHD and both monkeys died due to severe GvHD (Table 6.1). WT 1 is present on most mature T lymphocytes and thymocytes.

After elimination of WT 1 positive cells the residual cells could induce GvHD. Both monkeys died due to kidney failure secondary to shock induced by severe GvHD (Table 6.1). Incubation of a bone marrow graft with CAMPATH-1 plus autologous monkey serum as complement source reduced the number of T lymphocytes from 5-16% E-rosette forming (E-RF) cells prior to treatment to 0.1-0.5% E-RF cells after complement lysis. Using immunofluorescence analysis as shown in figure 6.2 did not detect any T lymphocytes after complement lysis

with CAMPATH-1. The same holds for the antibodies B9-pool, OKT4+4a and WT 1 where after complement lysis no target cells could be detected by immunofluorescence analysis (Figure 6.2).

Table 6.1

Effect of depletion of T lymphocyte subpopulations on the occurrence of acute GvHD in unrelated, mismatched donor-recipient combinations.

pretreatment	number of animals	survival (range in days)	histopathological GvHD grading (0-III)	cause of death
none	7	7-18 (median:12)	II-III	GvHD
OKT4+4a	2	10, 13	II-III	GvHD
B9-pool	2	14, 16	II-III	GvHD
WT 1	2	13, 18	II	GvHD, shock

A total of six monkeys received a bone marrow graft depleted of lymphocytes by CAMPATH-1 and complement (Table 6.2).The monkeys were prepared for grafting by 2 x 6.7 Gy total body irradiation Monkey 1EC died after 19 days due to intussusception of jejunum. This monkey showed no signs of engraftment. In comparison to the other monkeys with documented engraftment the initial cell dose was 2.7 x 10^8 bone marrow cells/kg body weight instead of 4 x 10^8 bone marrow cells/kg body weight. In all the monkeys within 14 days after transplantation the leukocyte counts normalized and engraftment was documented by taken blood samples 3 weeks after transplantation and using serologically defined determinants (Balner et al., 1981). At autopsy of monkey 1 JR a massive infiltration of the gastro-intestinal tract of protozoa, resembling lambliasis, was observed and only mild signs of GvHD.

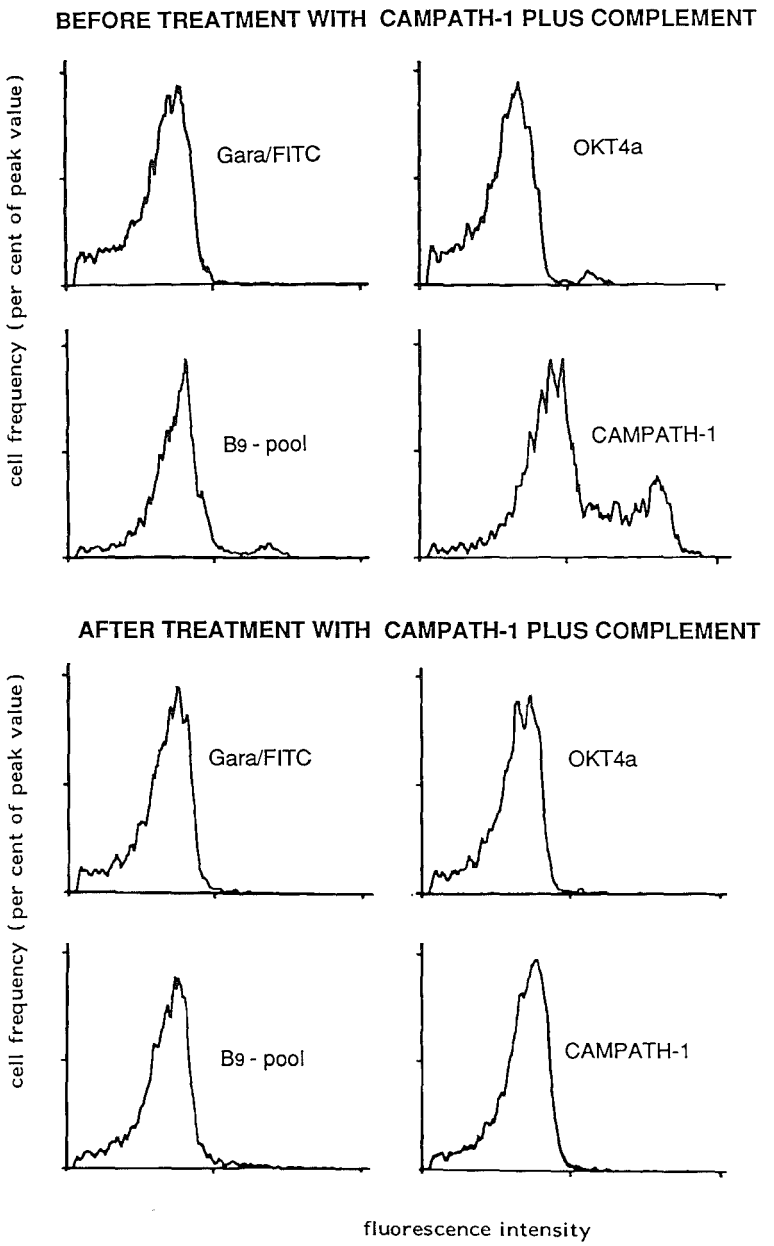

Figure 6.2 **Effectiveness of elimination of T lymphocytes using CAMPATH-1 plus complement. Fluorescence analysis before and after treatment of bone marrow cells.**

Table 6.2

Incidence of acute GvHD in recipients of bone marrow grafts depleted of CAMPATH-1 positive cells.

		GvHD grading			
UMN	survival	skin	liver	GI-tract	cause of death
1EC	19	I	0	0	intussusception jejunum, graft failure
1JR	18	I	0	I	extensive lambliasis in GI-tract
1CI	22	II	I	II	GvHD and sepsis
1FN	27	*	I	I	euthanasia
1LJ	40	0	0	0	radiation pneumonitis
1HC	45	0	0	0	radiation pneumonitis

* extensive necrosis of skin with massive infiltration of granulocytes (see also text)

Monkey 1CI showed grade II GvHD in skin and gastro-intestinal tract and this animal died due to a combination of acute GvHD and sepsis. In the skin of monkey 1FN extensive necrosis with massive infiltration of granulocytes was seen. The latter lesions are not typical for acute GvHD and no signs of GvHD could be observed in other epithelial tissues such as tongue and lip, and neither in liver and gastro-intestinal tract. Therefore, it was concluded that these lesions were not attributable to GvHD. Monkeys 1LJ and 1HC had no signs of GvHD but died after 40 and 45 days due to radiation induced pneumonitis. This diagnosis was made after exclusion of viral infections. Depletion of CAMPATH-1 positive cells result in a very substantial mitigation of the incidence and severity of acute GvHD.

6.4 DISCUSSION

In humans as well as in rodents two distinct T lymphocyte subpopulations, namely helper/inducer T lymphocytes (murine markers: Lyt 1,L3T4; human markers: ea Leu 3, OKT4) and cytotoxic/suppressor T lymphocytes (murine markers: Lyt 2; human markers: ea Leu 2, OKT8, B9-pool) can be dis-

tinguished. After establishing that T lymphocyte depletion by the use of antiserum or monoclonal antibody against Thy 1.2 plus complement successfully prevented GvHD (Ledney and van Bekkum, 1968; Tyan, 1973; Rodt et al., 1974; Müller-Ruchholtz et al., 1979; Korngold and Sprent, 1978 Vallera et al., 1981), the possible role of T lymphocyte subpopulations in induction of GvHD has been investigated. The first study was performed in rats (Mason, 1981). Depletion of OX8 (cytotoxic/suppressor T lymphocytes) donor cells failed to prevent GvHD in a P --> F_1 combination. Removal of helper/inducer T lymphocytes mitigated the incidence of GvHD; the induction of GvHD was not prevented, but survival was prolonged. Successful prevention was obtained after depletion of both subpopulations of T lymphocytes. Similarly, lethal GvHD was observed after depletion of Lyt 2^+ donor cells and transplantations across full H-2 differences in mice (Vallera et al., 1982; Korngold and Sprent, 1985). Results of depletion of Lyt 1^+ donor cells differed in that Vallera et al (1982) reported complete prevention of GvHD while Korngold and Sprent (1985) observed only prolongation of survival. Using a more specific antibody against helper/inducer T lymphocytes (L3T4) for depletion of this subpopulation a prolongation of survival time was observed by both groups of investigators, indicating that helper/inducer cells mediate lethal GVHD (Korngold and Sprent, 1987; Pietryga et al., 1987) when donor and recipient are fully H2-mismatched. Recently, more different donor-recipient combinations were tested and it was found that GvHD could be caused by helper/inducer T lymphocytes as well as suppressor/cytotoxic T lymphocytes (Korngold and Sprent, 1987).

In our rhesus monkey model acute GvHD could not be prevented by depletion of OKT4+4a (CD4; helper/inducer T lymphocytes) positive cells in an unrelated, mismatched donor-recipient combination. Severe GvHD was observed and survival was not prolonged in comparison to untreated historical controls. Depletion of either WT 1 (CD7; majority of mature T lymphocytes) or B9-pool (CD8; suppressor/cytotoxic T lymphocytes) positive cells could neither mitigate the incidence of acute acute GvHD. Only depletion of all lymphocytes by complement lysis of CAMPATH-1 positive cells mitigated the incidence and severity of acute GvHD. The results of CAMPATH-1 were comparable to results with stem cell enrichment by density centrifugation followed by further T lymphocyte depletion using E-rosette sedimentation. In the latter group, the survival of the monkeys ranged from 24 to 86 days (median: 36 days) and acute GvHD > grade II was observed in 2 out of 7 monkeys. Causes of death were viral

(Cytomegaly virus) and bacterial infections (Wagemaker et al., 1982; Wagemaker, 1986). These data are conclusive in that acute GvHD can only be prevented in unrelated, mismatched donor-recipient combinations when both subsets of T lymphocytes are sufficiently eliminated. The results do not confirm the results obtained in some rodent combinations where depletion of T lymphocyte subpopulations could prevent GvHD. The discrepancy can be explained by the patterns of mortality due to GvHD in rhesus monkeys and rodents. Severe GvHD is observed with early mortality (survival < 18 days) in rhesus monkeys when unrelated, mismatched donor-recipients were used. It is possible that in other donor-recipient combinations such as RhLA-identical siblings a mitigation of the incidence of acute GvHD can be observed after depletion of subsets of T lymphocytes. Until more information is available about this subject, our data argue that elimination of all T lymphocytes is necessary for prevention of GvHD prophylaxis, especially when mismatched donor-recipient combinations are used.

CHAPTER VII

GENERAL DISCUSSION

The outcome of a bone marrow transplantation is determined by the functioning of pluripotent haemopoietic stem cells, which sustain engraftment by their capacity of self-replication and differentiation, and T lymphocytes, which are responsible for the induction of the acute Graft-versus-Host reaction. T lymphocytes promote engraftment of an allogeneic bone marrow graft by inducing an antihost immune reactivity, i.e. Graft-versus-Host reaction. These two cell population were the main subject of this thesis (Figure 7.1).

7.1 HAEMOPOIETIC STEM CELLS

7.1.1 HAEMOPOIETIC STEM CELLS AND THE TROPHIC FUNCTION OF T LYMPHOCYTES

The process of proliferation and differentiation to functional end cells, such as leukocytes, thrombocytes and erythrocytes, is controlled by haemopoietic growth factors (Metcalf, 1986; Clark et al., 1987). The growth factors were discovered and defined by their virtue to support haemopoietic colony formation in vitro. Several factors have been purified and produced by recombinant DNA techniques such as the granulocyte-macrophage colony stimulating factor (GM-CSF; Wong et al., 1985), the granulocyte-CSF (G-CSF; Souza et al., 1986), the macrophage-CSF (M-CSF; Kawasaki et al., 1985), the interleukins-1,3,4, and 6 (IL-1; Moore et al., 1987: IL-3; Yang et al., 1986; Dorssers et al., 1987: IL-4; Yokata et al., 1986: IL-6; Ikebuchi et al., 1987) and erythropoietin (Jacobs et al., 1985). Most growth factors are produced by T lymphocytes (Metcalf, 1986; Clark et al., 1987).

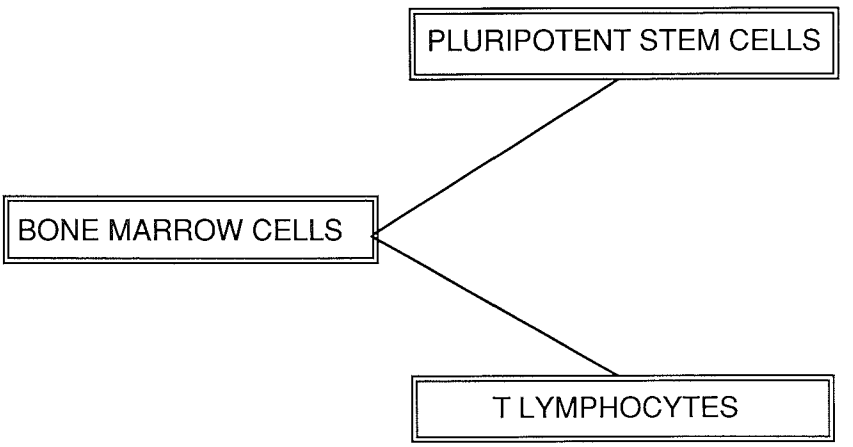

Figure 7.1 **The most essential cells in a bone marrow graft**

Therefore it was suggested that removal of T lymphocytes would deprive a bone marrow graft of the trophic function of lymphocytes and T lymphocyte depletion would result in delayed engraftment (Sieff et al., 1985; Soderling et al., 1985).

In chapter III an in vivo assay was developed to assess quantitatively the influence of in vitro manipulation of bone marrow on the repopulating ability of a bone marrow graft. Indirectly this provided information on the influence on haemopoietic stem cells. Murine experiments have proven that these cells are primarily responsible for the repopulation of an haemopoietic system of a lethally irradiated recipient (Trentin, 1963; Visser et al., 1984; Bauman et al., 1986; Spangrude et al., 1988). Early autologous repopulation was used to measure stem cell toxicity. Autologous transplantation avoided the complicating and unpredictable (i.e. multifactorial) host-versus-graft reactions that occur in an allogeneic setting.

This semiquantitative assay provided information with regard to the influence of T lymphocyte depletion on the regeneration of leukocytes and reticulocytes. T lymphocyte depletion did not significantly reduce the repopulating capacity of an autologous bone marrow graft. This finding means that in rhesus monkey no direct evidence is found for a trophic function of T lymphocytes

during the phase of early repopulation after total body irradiation. This observation is in accordance with the experiments with purified stem cells, which could repopulate a lethally irradiated recipient without help from other cell populations.

7.1.2 ENRICHMENT OF PLURIPOTENT HAEMOPOIETIC STEM CELLS

Depletion of DR-positive cells from an autologous bone marrow graft ablated the repopulating capacity of such a graft resulting in a severely delayed regeneration of peripheral blood cells. Several monoclonal antibodies were tested for their reactivity with rhesus monkey GM-CFU's, but none of the tested antibodies reacted primarily with GM-CFU's. Therefore, analogous to the results of murine experiments with an antibody against a class I antigen (Visser et al., 1984) it was attempted to enrich haemopoietic stem cells by using an anti-DR antibody (class II) for positive selection of stem cells. The experiments described in chapter IV have revealed that stem cells of rhesus monkeys can be characterized by low density, by a negative reaction with the anti-lymphocyte antibody CAMPATH-1, high forward light scatter and positive reaction with the DR-antigen. An enrichment of approximately 40 times was observed in vivo in comparison to ficoll bone marrow cell suspension and 94 times when compared to the original cell suspension obtained directly after aspiration. Dicke et al (1973) have described the morphological characteristics of the haemopoietic stem cells in rhesus monkeys and estimated that aspiration bone marrow contains approximately 0.7% stem cells. On basis of this value an enrichment of at least 150 times is required to obtained a pure stem cell suspension. Since these experiments were performed in 1985/1986, new monoclonal antibodies have become available. Very promising are the results with the CD34 antibody ICH3 (Watt et al, 1987), which reacts with 5 % of the cells in human as well as rhesus monkey bone marrow. Transplantation of ICH3-positive cells has demonstrated in rhesus monkeys that positive selection of these cells result in a 75 fold (95% confidence limits: 40-120 fold) enrichment (Wielenga et al., 1989). At present no stem cell specific antigens are available and thus further enrichment of rhesus monkey stem cells can be achieved by multiparameter cell separation similar to the purification of mouse haemopoietic stem cells (Visser et al., 1984; Bauman et al., 1986; De Vries et al., 1988; Spangrude et al., 1988).

The availability of highly purified stem cell populations could have important consequences for experimental and clinical bone marrow transplantations. Haemopoietic growth factors, especially GM-CSF and G-CSF, are widely applied to stimulate early regeneration of peripheral blood cells after either bone marrow transplantation (Donahue et al., 1986; Donahue et al., 1988; Mayer et al., 1987; Monroy et al., 1987; Gillio et al., 1987; Nemunaitis et al., 1988) or myeloablative, high dose chemotherapy (Brandt et al., 1988; Morstyn et al., 1988; Socinski et al., 1988, Gabrilove et al., 1988). At present it can not be distinguished whether the more rapid recovery of peripheral blood cells after myeloablative treatment and administration of GM-CSF or G-CSF is due to stimulation of pathway restricted progenitor cells or to direct stimulation of the proliferation and differentiation of haemopoietic stem cells. Purified haemopoietic stem cells can be used to elucidate which growth factors have receptors on stem cells and what the consequence is of stimulation of stem cells with a specific growth factor.

An other application of purified stem cells is the eradication of malignant cells from autologous bone marrow grafts for hematological malignancies and solid tumors. At the moment several methods have been developed to remove tumor cells from bone marrow graft. A disadvantage of this approach is that specific methods are required for each specific tumor. A generally applicable method would be the positive selection of haemopoietic stem cells from tumor cells, contaminating bone marrow as was recommended by van Bekkum in 1986).

7.2 T LYMPHOCYTES

Donor T lymphocytes play an important role in the outcome of allogeneic bone marrow transplantation. They induce the acute Graft-versus-Host reaction. A strong Graft-versus-Host reaction will inhibit the Host-versus-Graft reaction and prevent rejection.

7.2.1 T LYMPHOCYTES AND ENGRAFTMENT

As early as 1957 Van Bekkum et al noticed that host lymphoid cells could reject rat bone marrow cells transplanted into lethally irradiated mice. At that time their interpretation was that rejection was mediated via the production of anti-

bodies against the graft. Later, more evidence for the role of T lymphocytes in the Host-versus-Graft reaction was brought in by the observations that stable mouse-to-rat chimaeras could be obtained when the recipients were conditioned with supralethal TBI and a high dose of anti-rat lymphocyte serum (Bau, 1973).

Donor T lymphocytes induce the Graft-versus-Host reaction and host T lymphocytes are among the cells which are subject of the GvH reaction. Therefore, one of the solutions to reduce the immunological activity of host lymphocytes against an allogeneic bone marrow is to knock out the host T lymphocytes by a GvH reaction induced with donor T lymphocytes. Studies with dogs, where transfusion of donor leukocytes or lymphocytes promoted the engraftment of histoincompatible bone marrow, supported this concept (Deeg et al., 1979). In agreement with the concept of reciprocal interference the success of engraftment was counterbalanced by an increase in lethal GvHD. Irradiated donor leukocytes were not effective (Deeg et al., 1979). So, the outcome of a bone marrow transplantation is the result of competing Host-versus-Graft and Graft-versus-Host Reactions. Depletion of donor T lymphocytes will reduce the incidence of acute GvHD and subsequently a stronger HvG reaction will arise resulting in more rejections in comparison to unmanipulated bone marrow grafts. Additional immunosuppression will be required to suppress the stronger HvG following T lymphocyte depletion.

In chapter V the minimal dose of fractionated total body irradiation was determined for engraftment of T lymphocyte depleted bone marrow grafts. A rather steep dose-effect curve established the relation between graded doses of fractionated TBI (24 hours interval) and engraftment. The difference between sustained engraftment and engraftment failure appeared to be 1-2 Gy fractionated total body irradiation (X-rays).

These data seem to agree very well with observations in mice where titrated doses of spleen cells, syngeneic with host cells, were added to 10^7 allogeneic bone marrow cells and injected into lethally irradiated recipients (van Bekkum, 1988). In this experimental setting mortality is due to graft failure. When no recipient-type spleen cells were added, 90% of the recipients survived. The addition of 10^4 recipient-type spleen cells gave 100% survival, while the addition of 10^5 host spleen cells reduced survival to 25% due to more graft failures. Addition of 10^6 host spleen cells resulted in a poor survival of 10%. These results indicate that the difference between 100% engraftment and 50% engraftment is less than one log immunocompetent cells in mice. Since one log cell kill of lymphocytes

corresponds with 2-3 fold D_0 in mice (Smith, 1963), this would imply that 1.5-2 Gy is sufficient to overcome engraftment failures assuming a D_0 of 70 cGy. Several groups have confirmed this concept in their murine models for engraftment of T lymphocyte depleted bone marrow grafts (Soderling et al., 1985; Schwartz et al., 1987; Ferrara et al., 1988; Gassmann et al., 1988). Additional support for our results in rhesus monkeys has come from clinical studies. Several transplant centers have reported that increasing the dose of fractionated total body irradiation with 2 Gy prevented engraftment failures (Martin et al., 1985; Burnett et al., 1988; O'Reilly et al.,1986; Champlin et al., 1987).

Alternative approaches, which have been investigated in animal models, are the addition of total lymph node irradiation (Blazar et al., 1988), monoclonal antibodies directed against either T lymphocytes (Cobbold et al., 1986) or class II antigens (Deeg et al., 1987), and immunosuppressive 'drugs (Gassmann et al., 1988). Fractionated total lymph node irradiation (2 x 3.3 Gy) in addition to 7.5 Gy total body irradiation resulted in improved survival in mice grafted with allogeneic bone marrow and none of the mice rejected their T lymphocyte depleted grafts in contrast to a 50% rejection rate in recipient mice which were prepared by 7.5 Gy total body irradiation (Blazar, 1988). Cobbold et al (1986) demonstrated the usefulness of monoclonal antibodies against T lymphocytes for the promotion of engraftment of T lymphocyte depleted grafts. A sublethal dose of 6 Gy total body irradiation was not sufficient to prevent rejection in mice. Addition of a monoclonal antibody against T lymphocyte to 6 Gy total body irradiation resulted in 90% chimaerism. Injection of monoclonal antibodies directed against subpopulations of T lymphocytes had no effect on engraftment. Recipient dogs reject bone marrow grafts from unrelated, DLA-nonidentical donors when they are conditioned with 9.2 Gy total body irradiation. Administration of anti-class II monoclonal antibody pregrafting and methotrexate postgrafting proved to result in engraftment in 9/10 dogs. Methotrexate postgrafting alone resulted in engraftment in 6 out 10 recipient dogs. The results of these two groups did not differ significantly (Deeg et al., 1987). Depletion of DR-positive cells ablated the repopulation capacity of bone marrow grafts in dogs (Szer et al., 1986) and rhesus monkeys (this thesis). The use of anti-DR antibodies for conditioning can not be recommended since the risk of jeopardizing haemopoietic reconstitution is great. The immunosuppressive potential of cyclophosphamide, etoposide and cytarabin (ARA-C) was compared in addition of a standard conditioning regimen of 35 mg/kg busulphan in mice.

Cytarabin (dose range 600-1200 mg/kg) and etoposide (dose range 30-60 mg/kg) were significantly inferior to cyclophosphamide (dose range 30-360 mg/kg) in their potential to promote engraftment (Gassmann et al., 1988).

In case of leukemia, a conditioning regimen for T lymphocyte depleted bone marrow grafts has to fulfill two criteria; it has to give extra immunosuppression and it should eliminate residual leukemia cells to prevent the higher increased risk of relapse after T lymphocyte depleted grafts. Assuming that the anti-leukemic effect of GvHD is due to alloreactivity, T lymphocyte depletion will require additional anti-leukemic treatment as part of the conditioning regimen. Schultz et al (1989) calculated that the difference between 90% cure and 40% cure corresponds with a difference of one log leukemic cell kill, which is in turn equivalent to 2-3 D_0. Although exact values for the D_0 are not available, one can assume that it is in the order of the same magnitude for lymphocytes and this will mean that an additional dose of 1-2 Gy total body irradiation will provide the additional one log kill of leukemia cells.

This means that when additional immunosuppression is given in the form of anti-lymphocyte antibodies or total lymph node irradiation, an additional anti-leukemia treatment is required. In view of the experiments of Gassmann (1988) with ARA-C and Etoposide, extra addition of these agents to eliminate more leukemic cells, will require additional immunosuppression in case of T lymphocyte depleted bone marrow grafts. At this moment an increment of 1-2 Gy (single fraction) of total body irradiation is the best option since this approach fulfils both criteria.

7.2.2 T LYMPHOCYTES AND GRAFT-VERSUS-HOST DISEASE

The relation between donor T lymphocytes and acute GvHD has been well established in several species. In chapter VI it was investigated whether anti T lymphocyte monoclonal antibodies plus complement eliminated sufficient T lymphocytes to prevent acute GvHD in a unrelated, mismatched donor-recipient combination. As expected, removal of all detectable T lymphocytes successfully prevented acute GvHD. Depletion of subpopulation of T lymphocytes did not prevented the induction of lethal GvHD in this donor-recipient combination. These data prove that it is hazardous to extrapolate results of murine bone marrow transplantation to outbred species. The morbidity and mortality in man

and monkeys is much higher than in mice due to a higher proportion of lymphocytes in bone marrow. In an unrelated, mismatched donor-recipient combination the severity of acute GvHD is higher than in related, HLA-matched donor-recipient combinations. Subsequently, more efficient T lymphocyte depletion is required to prevent acute GvHD (Wagemaker, 1986). It is therefore not excluded that depletion of subpopulation of T lymphocytes would not result in prevention of GvHD in related, HLA-identical donor-recipient combinations.

Successful prevention of acute GvHD by either albumin density centrifugation followed by E-rosette sedimentation or by CAMPATH-1 plus complement did not result in long term survival due to viral and bacterial infections and late toxicity of irradiation. Late toxicity of irradiation manifested in interstitial pneumonia with evidence of a viral or bacterial infection. After these experiments a new conditioning regimen was prepared where the monkeys were irradiated with 2 x 6 Gy (24 hour interval), but the lungs were shielded after 8.5 Gy. With this new conditioning regimen radiation pneumonitis was no longer observed and recipients of autologous bone marrow grafts became long term survivors (Wielenga: personal communication).

With regard to the influence of T lymphocyte depletion on engraftment and acute GvHD, Vriesendorp et al (1985) draw the attention to the consequences of higher irradiation doses for the incidence of acute GvHD. The incidence of mortality due to either Graft-versus-Host or to Host-versus-Graft reaction has been investigated in dogs. A single dose of 4.25 Gy TBI (300 kV X-ray) resulted in 100% graft rejections in 8 dogs. Graft rejections were not observed after 5 Gy TBI, but 20% of the dogs (N=5) showed signs of GvHD. The incidence of GvHD increased to 40% (N=40) when recipients were prepared for grafting by 7.5 Gy TBI. According to the concept of reciprocal interference between Host-versus-Graft and Graft-versus-Host reactions an optimal conditioning regimen has to be carefully developed.

7.3 THE RHESUS MONKEY AS A PRECLINICAL MODEL FOR BONE MARROW TRANSPLANTATION

For nearly 25 years the rhesus monkey has been employed for investigations on bone marrow transplantations. Since it is felt that the gap between mouse models and clinical bone marrow transplantations is too big, experimental bone marrow transplantations in rhesus monkeys have been initiated. Bone marrow composi-

tion, especially the proportion of lymphocytes, are very similar to humans. Their immunological reactivity has proven to be very similar in various transplantations models such as bone marrow transplantation (see chapter I and chapter V), kidney transplantation (Borleffs, 1983; Jonker et al., 1985; 1984) and liver transplantation (Neuhaus et al., 1985).

The crossreactivity of several monoclonal antibodies, directed against human antigens present on human cells(Jonker, 1984; this thesis), with rhesus monkey cells provide that preclinical experiments can be performed to investigate the usefulness of antihuman monoclonal antibodies in bone marrow transplantation. At present the role of monoclonal antibodies in conditioning of the recipient is subject of study. The human haemopoietic growth factors G-CSF and GM-CSF has shown an enhancement of peripheral blood recovery following bone marrow transplantations in rhesus monkeys (Donahue, 1986; Donahue, 1988; Mayer, 1987; Monroy, 1987; Gillio, 1987). From all the experiments with monkeys it can be concluded that the rhesus monkey provides a near optimal model for preclinical investigations of bone marrow transplantation.

Disadvantages of the monkey model are the susceptibility for bacterial and viral infections, the scarcity of monkeys and the high costs of housing and handling of monkeys. The small family size of rhesus monkeys and slow breeding is a biological limitation for studies with realted donor-recipient combinations. The scarcity of the monkeys is the reason that experiments had to performed with a low number of monkeys. Nevertheless, experiments in a low number of rhesus monkeys has provided crucial information for clinical bone marrow transplantations.

SUMMARY

Bone marrow transplantation is a successful treatment for many lethal diseases of the haematopoietic system such as severe aplastic anemia and leukemia. Patients with inborn errors of the immune system such as severe combined immunodeficiency can be cured by bone marrow transplantation. In a bone marrow graft many different haemopoietic cells are present. Among these cells pluripotent haemopoietic stem cells and T lymphocytes mainly determine the outcome of a transplantation. Haemopoietic stem cells have the ability of self-replication and they can differentiate into one of the cell lineages. Because of their ability of self-replication haemopoietic stem cells are responsible for sustained engraftment. The function of T lymphocytes is to react against foreign antigens. When there is a discrepancy between donor and recipient with regard to the minor or major histocompatibility antigens, mature T lymphocytes of the donor will mount an immunological attack against foreign antigens in the recipient resulting in a graft-versus-host reaction. Depletion of donor T lymphocytes will eliminate those immunocompetent cells which are responsible for the graft-versus-host reaction. A disadvantage of this method for prevention of acute graft-versus-host disease is that it is associated with more engraftment failures.

The main subjects of this thesis was the investigation of the influence of T lymphocyte depletion on engraftment of autologous and allogeneic bone marrow grafts, the influence of depletion of subpopulations of T lymphocytes on the severity of acute GvHD and the enrichment of pluripotent haemopoietic stem cells. The rhesus monkey was used as a preclinical model since its bone marrow composition and their immunological reactions are very similar to those of humans.

The principles of bone marrow transplantation and the role of pluripotent haemopoietic stem cells are described in the first part of chapter I. Most part of chapter I is dealing with engraftment, the nature of graft-versus-host disease and the methods to prevent graft-versus-host disease.

In chapter II is a summary of the materials and methods which are relevant for the studies discussed in the chapters III to VI.

T lymphocytes produce several haemopoietic growth factors. A hypothesis is that depletion of T lymphocyte will result in a deprivation of haemopoietic growth factors essential for the proliferation and differentiation of haemopoietic stem cells. This hypothesis was tested in chapter III. After transplantation of graded number of autologous bone marrow cells, relationship between between the number of cells transplanted and the regeneration rate of leukocytes and reticulocytes was observed. This allows to asssay for stem cells in bone marrow. This in vivo assay enabled the investigation of the influence of depletion of T lymphocytes and DR-positive cells on the regneration of peripheral blood cells. Monoclonal antibodies and complement were used for complement lysis. Depletion of subpopulation of T lymphocytes from an autologous bone marrow graft by the antibodies OKT4+4a (helper/inducer T lymphocytes) and B9-pool (cytotoxic/suppressor T lymphocytes) did not affect the regeneration rate of leukocytes and reticulocytes. Depletion of all lymphocytes, using CAMPATH-1, did neither influence the regeneration of peripheral blood cells. A strong delay in regeneration occurred when all DR-positive cells were removed from a bone marrow graft illustrating that toxicity to stem cells could be assessed with this autologous assay.

Depletion of DR-positive cells eliminated the capacity of a bone marrow graft to repopulate a sublethally irradiated recipient. This result suggested that pluripotent haemopoietic stem cells carry the DR-antigen on their cell surface. In chapter IV, pluripotent stem cells were enriched using a combination of density centrifugation, depletion of T lymphocytes and cell sorting with a fluorescence activated cell sorter. Progenitor cells, measured in the GM-CFU assay, were characterized by light density cells in a discontinuous albumin density gradient, negative for the anti-lymphocyte antibody CAMPATH-1 and they gave a high forward light scatter during the flow through a laser beam of the cell sorter. After labelling bone marrow cells with an anti-DR antibody YAML 555.6, several cell clusters could be distinguished. The highest concentration of GM-CFU's (37 x) were found in the cell populations characterized by a high forward light scatter and positive for the DR-antigen. Autologous transplantation experiments with the enriched fractions did show similar results. Transplantation of cells, selected on basis of high forward light scatter and presence of the DR-antigen on the cell surface, were able to repopulate an irradiated recipient. In

comparison to unseparated bone marrow cells, pluripotent haemopoietic stem cells were 40 x times enriched.

After T lymphocyte depletion a higher incidence of engraftment failures is observed than with T lymphocyte containing bone marrow. Since the experiments in chapter III have demonstrated that T lymphocyte do not have a significant trophic function, the engraftment failures are attributable to the elimination of the immunosuppressive action of donor T lymphocytes on the immunological reactivity of the recipient. Donor T lymphocytes exert an immunosuppressive action since the lymphocytes of the recipient (inducing the host-versus-graft-reaction) are among the targets of the graft-versus-host reaction. Hence, depletion of donor T lymphocytes will require more immunosuppression of the recipient. In chapter V, the minimal dose of fractionated total body irradiation was determined for 2 log T lymphocyte depleted bone marrow grafts. A steep dose-effect relationship appeared to exist between engraftment and total body irradiation. A difference of 1-2 Gy fractionated total body irradiation determines the outcome of a transplantation with 2 log T lymphocyte depleted bone marrow.

In view of the problems with T lymphocyte depleted bone marrow grafts, it was investigated whether depletion of subpopulations of T lymphocytes was sufficient to prevent acute graft-versus-host disease. Murine experiments had suggested that depletion of subpopulations of T lymphocytes successfully prevented graft-versus-host disease. Depletion of subpopulations did not prevent acute graft-versus-host disease in our rhesus monkey model for mismatched bone marrow transplantations (chapter VI). Depletion of all lymphocytes resulted in a successful mitigation of the severity of graft-versus-host disease.

Finally, in chapter VII (General Discussion) the most important results and their possible implications for clinical transplantations has been discussed.

SAMENVATTING

Voor vele dodelijke aandoeningen van het haemopoietische systeem, zoals ernstige aplastische anemie en leukemie, is beenmergtransplantatie een succesvolle behandeling. Patiënten met aangeboren afwijkingen van het immuun systeem zoals 'severe combined immunodeficiency' kunnen genezen worden door een beenmergtransplantatie.

In een beenmergtransplantaat zijn verschillende beenmergcellen aanwezig. Van deze cellen bepalen vooral de T lymfocyten en de haemopoietische stamcellen hoe een beenmergtransplantatie zal verlopen. Haemopoietische stamcellen hebben de mogelijkheid tot zelf-replicatie en de stamcellen kunnen gaan differentiëren tot perifere bloedcellen. Vanwege hun mogelijkheid tot zelf-replicatie zorgen de haemopoietische stamcellen ervoor dat een transplantaat niet uitsterft, maar dat er steeds weer opnieuw cellen aangemaakt kunnen worden. De functie van T lymfocyten is om te reageren op vreemde antigenen. Wanneer er een verschil bestaat tussen donor en gastheer met betrekking tot de transplantatieantigenen, zullen rijpe donor T lymfocyten de gastheer als vreemd herkennen en een immunologische reactie ontketenen tegen de gastheer. Deze reactie wordt de 'Graft-versus-Host' reactie genoemd. Verwijdering van de donor T lymfocyten uit een beenmergtransplantaat betekent dat de cellen verantwoordelijk voor de 'Graft-versus-Host' reactie verwijderd zijn. Een nadeel van methode om 'Graft-versus-Host' reactie te voorkomen is dat het gepaard gaat met een toename in het aantal afstotingsreacties.

De belangrijkste onderwerpen van dit proefschrift zijn het onderzoeken van de invloed van het verwijderen van T lymfocyten op het aanslaan van autoloog of allogeen beenmerg, het onderzoeken van de invloed van het verwijderen van subpopulaties van T lymfocyten op de ernst van de acute 'Graft-versus-Host' reactie en de verrijking van pluripotente haemopoietische stamcellen. De rhesusaap werd gebruikt als preklinisch model omdat rhesusapen wat betreft hun beenmerg samenstelling en hun immunologische reacties heel veel op de mens lijken.

De principes van een beenmergtransplantatie en de rol van de pluripotente stamcellen worden beschreven in het eerste gedeelte van hoofdstuk I. Het

grootste gedeelte van hoofdstuk I gaat over het aanslaan van beenmerg, de oorsprong van de 'Graft-versus-Host' reactie en de methoden die toegepast werden en worden om de 'Graft-versus-Host' reactie te voorkomen.

In hoodstuk II worden de belangrijkste materialen en methoden beschreven die gebruikt zijn voor de experimenten welke beschreven staan in de hoofdstukken III tot en met VI.

T lymfocyten produceren verscheidene haemopoietische groeifactoren. Er is een hypothese dat verwijdering van T lymfocyten tot gevolg zal hebben dat er een tekort aan groeifactoren ontstaat. Groeifactoren zijn essentieel voor de proliferatie en differentiatie van haemopoietische stamcellen en een tekort aan groeifactoren zal daarom leiden tot het niet aanslaan van een beenmergtransplantaat. Deze hypothese werd onderzocht in hoofdstuk III. Na transplantatie van verschillende aantallen autologe beenmergcellen bleek er een directe relatie te bestaan tussen het aantal beenmergcellen wat getransplanteerd was en de regeneratiesnelheid van leukocyten en reticulocyten. Met behulp van dit in vivo assay kon de invloed onderzocht worden van het verwijderen van T lymfocyten en DR positieve cellen op de regeneratiesnelheid van perifere bloed cellen. Monoclonale antistoffen en complement werden gebruikt om cellen te lyseren. Verwijdering van subpopulaties van T lymfocyten door de antistoffen OKT4+4a (helper/inducer T lymfocyten) en B9-pool (cytotoxische/suppressor T lymfocyten) had geen invloed op de regeneratiesnelheid van perifere bloedcellen. Ook verwijdering van alle lymfocyten door het antilichaam CAMPATH-1 liet geen verandering zien in de regeneratie van perifere bloedcellen. Een ernstige vertraging in de regeneratie werd gezien na het verwijderen van alle DR positieve cellen. Dit laatste toonde aan dat met behulp van dit assay toxiciteit gemeten kan worden.

Verwijdering van DR positieve cellen uit een beenmergtransplantaat heeft tot gevolg dat een dergelijk transplantaat niet in staat is om een bestraalde gastheer een nieuw haemopoietisch systeem te geven. Dit doet vermoeden dat pluripotente haemopoietische stamcellen het DR antigeen op hun celoppervlak hebben. In hoofdstuk IV worden haemopoietische stamcellen verrijkt door een combinatie te gebruiken van een dichtheidsgradient, verwijdering van T lymfocyten en een celsorteermachine, een zogenaamde 'fluorescence activated cell sorter'. Voorlopercellen werden gemeten in het GM-CFU assay en bleken gekarakteriseerd te worden door een lage densititeit, ze reageerden niet met het antilichaam CAMPATH-1, en ze gaven een hoog 'forward light scatter' signaal.

Nadat de cellen gelabeld waren met een anti-DR antilichaam, genaamd YAML 555.6, konden verscheidene celclusters onderscheiden worden op de cell sorter. De hoogste concentratie GM-CFU's (37 x) werd gevonden in de celclusters die een hoog 'forward light scatter' signaal gaven en het DR antigeen op hun celoppervlak hadden. Autologe beenmergtransplantaties met cellen, die op deze wijze gezuiverd waren, lieten dezelfde resultaten zien. De cellen die een hoog 'forward light scatter' signaal gaven en positief reageerden met het anti-DR antilichaam, konden een bestraalde ontvanger beschermen. In vergelijking met normaal beenmerg bleken de stamcellen 40 x verrijkt te zijn.

Na het verwijderen van T lymfocyten wordt een hoger aantal afstotingsreacties gezien. Aangezien de experimenten in hoofdstuk III hebben aangetoond dat T lymfocyten geen trofische functie hebben, moeten de afstotingsreacties een gevolg zijn van de verminderde immunosuppressieve werking van donor T lymfocyten. Donor T lymfocyten richten hun immunologische aanval ook op de T lymfocyten van de gastheer. Aangezien de T lymfocyten van de gastheer verantwoordelijk zijn voor de afstotingsreactie zal verwijdering van de donor T lymfocyten tot gevolg hebben dat de gastheer T lymfocyten de overhand krijgen met als gevolg een toename van het aantal afstotingsreacties. Daarom zal meer immunosuppressie voor de gastheer nodig zijn wanneer donor T lymfocyten verwijderd worden. In hoofdstuk V, werd onderzocht hoeveel meer bestraling er nodig was voor het aanslaan van een beenmergtransplantaat waaruit de T lymfocyten 2 log gedepleteerd zijn. Er bleek een stijle dosis-effect relatie te bestaan tussen het aanslaan van beenmerg en de dosis bestraling. Een verschil van 1-2 Gray gefractioneerde lichaamsbestraling bepaalde het verschil tussen het aanslaan of afstoting van beenmerg.

Met het oog op het probleem van het aanslaan van beenmerg waaruit T lymfocyten verwijderd zijn, werd onderzocht of het wellicht voldoende was om een subpopulatie T lymfocyten te verwijderen om de 'Graft-versus-Host' reactie te voorkomen. Experimenten met muizen hadden gesuggereerd dat de 'Graft-versus-Host' reactie voorkomen kon worden op deze manier. Na verwijdering van subpopulaties van T lymfocyten werd nog steeds ernstige 'Graft-versus-Host' ziekte gezien in niet verwante donor-ontvanger combinaties (Hoofdstuk VI). Verwijdering van alle lymfocyten met behulp van het antilichaam CAMPATH-1 verminderde de ernst van de 'Graft-versus-Host' reactie zeer duidelijk.

In hoofdstuk VII (algemene discussie) worden de belangrijkste resultaten en hun betekenis voor de klinische beenmergtransplantaties besproken.

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ABBREVIATIONS

AET	= 2-aminoethylisothiouronium
BMT	= Bone Marrow Transplantation
CD	= Cluster Defined
CFU-S	= Colony Forming Unit/Spleen
CFU-GEMM	= Colony Forming Unit - Granulocyte, Erythrocyte, Macrophage and Megakaryocyte
DNAse	= Deoxyribonuclease
E-RFC	= E-rosette Forming Cells
FACS	= Fluorescence Activated Cell Sorter
FCS	= Fetal Calf Serum
FITC	= Fluorescein Isothiocyanate
FLS	= Forward Light Scatter
GAM	= Goat-anti-Mouse
GARA	= Goat-anti-Rat
GID	= Gastro-Intestinal Decontamination
GM-CFU	= Granulocyte-Macrophage Colony Forming Unit
GVHD	= Graft-versus-Host Disease
GVL	= Graft-versus-Leukemia
Gy	= Gray
LSM	= Lymphocyte Separation Medium
MCA	= Monoclonal Antibody
MHC	= Major Histocompatibility Complex
MTX	= Methotrexate
PBL	= Peripheral Blood Lymphocytes
PBS	= Phosphate Buffered Salt
PE	= Phycoerythrin
PHA	= Phytohaemagglutinin
PLS	= Perpendicular Light Scatter
RELACS	= Rijswijk Experimental Light Activated Cell Sorter
SPF	= Specific Pathogen Free
SRBC	= Sheep Red Blood Cells
TBI	= Total Body Irradiation

CURRICULUM VITAE

Winald R. Gerritsen was born in Wageningen on March 12, 1955. He completed his secondary education (Gymnasium-ß) in 1973. After studying political sciences for one year, he started to study at the Medical School of the Catholic University Nijmegen in 1974. His interest in research was stimulated during the three months which he spent at the Department of Haematology in Nijmegen (supervisors: Prof. dr. D. J. Th. Wagener and Dr. B. de Pauw) and during the three months which he spent at the Department of Internal Medicine of the National Cancer Institute in Amsterdam (supervisors: Dr. G. McVie and Dr. W. ten Bokkel Huinink). After graduation in 1982, he worked at the Primate Center TNO (head: Dr. A.A. van Es) and the Radiobiological Institute TNO (head: Prof. dr. D.W. van Bekkum) from January 1983 till August 1986. In the stimulating environment of both institutes the experiments, described in this thesis, were performed.
In September 1986 he started the education for internist and became a resident in internal medicine at the Majella Hospital in Bussum (head: Dr. D. Maingay). In September 1988 he continued his education at the Department of Internal Medicine of the National Cancer Institute in Amsterdam (head: Dr. R. Somers).
From July 1989 till July 1990 he will investigate the influences of haemopoietic growth factors on haemopoietic stem cells in the laboratory of the Bone Marrow Transplantation Service at the Memorial Sloan-Kettering Cancer Center in New York (head: Prof. dr. R.J. O'Reilly). For the study in New York, he has been awarded an International Research Fellowship of The Fogarty International Center and he is recipient of The Aplastic Anemia Foundation of America's 1989 Postdoctoral Fellowship Award.