CHIMERISM IN RECIPIENTS OF LYMPHOCYTE DEPLETED BONE MARROW GRAFTS

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The cover illustration shows the Chimera of Arezzo
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Een wetenschappelijke proeve op het gebied van de
Medische Wetenschappen

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen
volgens het besluit van het College van Decanen
in het openbaar te verdedigen op maandag 1 maart 1993
des namiddags te 1.30 uur precies

door

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geboren op 13 september 1951
te Geleen

Nijmegen, 1993
Drukkerij Benda, Nijmegen

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CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Schattenberg, Antonius, Victor, Martinus, Bernardus

Chimerism in recipients of lymphocyte depleted bone marrow grafts / Antonius, Victor, Martinus, Bernardus Schattenberg
-[S.l.: s.n.]
Thesis Nijmegen.-With ref.
ISBN 90-9005862-1
Subject headings: bone marrow grafts

Aan Grace, Nadine en Sophie

CONTENTS

CHAPTER 1

INTRODUCTION

Allogeneic bone marrow transplantation has evolved from an experimental treatment to a worldwide accepted method to treat a number of severe disorders like leukemia, myelodysplastic syndrome, bone marrow failure, immune deficiency, storage diseases, and hemoglobinopathies.[1] This evolution is described in chapter 2 of this thesis. Worldwide, more than 40,000 patients have received a bone marrow graft from a donor. In 1993 the 250th allogeneic bone marrow transplantation will be performed at the Bone Marrow Transplantation Unit of the University Hospital Nijmegen. Results of the first eighty consecutive patients transplanted in this center for acute leukemia and chronic myelogenous leukemia are given in chapter 3. Special emphasis is put on chimerism and chimerism is the main theme of the present thesis.

The term chimera refers to a monster in greek mythology. This monster, the "Chimera", resembled a lion in the forepart, a goat in the middle and a dragon behind.[2,3] She terrorized Asia Minor and was ultimately killed by Bellerophon, hero of Corinth.[4,5] In the scientific literature, chimerism has been defined as an organism whose cells have been derived from two or more distinct zygotic lineages.[6] In bone marrow transplantation, a chimera is the recipient of a successfully engrafted marrow. Although the term chimera refers to an organism and not to a tissue, the term chimera has been more specifically defined on the base of the origin of the lympho-hematopoietic cells. If all lympho-hematopoietic cells are of donor origin, the chimera is a complete donor chimera and the recipient has a complete donor chimera. If one or more lympho-hematopoietic cell lineages in the blood and/or the bone marrow are of recipient origin and one or more other cell lineages are of donor origin, the chimera is a mixed chimera and the recipient has a mixed chimera (or incomplete chimera). However, the term incomplete chimera is confusing since it suggests incomplete in the sense of inadequate hematopoiesis.

Mixed chimeras are more frequent in recipients of T cell depleted grafts than in patients transplanted with unmanipulated bone marrow.[7-11] Persistence or endogenous repopulation of host-

type lympho-hematopoietic cells after allogeneic transplantation for leukemia have been considered a poor prognostic sign, indicating manifest or imminent relapse.[12-14] In chapter 4 we show that mixed hematopoietic chimerism after allogeneic transplantation with lymphocyte depleted grafts using counterflow centrifugation, is not associated with a higher incidence of relapse. Furthermore, we demonstrate that chimerism may differ between the various subsets of cells analyzed. A comparison of chimerism of red cells, granulocytes, T lymphocytes, and bone marrow cells is made in chapter 5 and results of cytogenetic analysis of lymphocytes and bone marrow cells are given in chapter 6.

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CHAPTER 2

HISTORICAL OVERVIEW ON BONE MARROW TRANSPLANTATION

THE FIRST HISTORICAL ATTEMPTS AT BONE MARROW TRANSPLANTATION

One of the first observations on bone marrow transplantation was made by Neuhof in 1923. He observed that marrow transplanted along with autologous* bone degenerated in the first week and was replaced by hematopoietic cells which were probably not derived from persisting portions of the original graft but from adjacent marrow. In 1930, Loeb briefly referred to necrosis of bone marrow and replacement by fibrous tissue after simultaneous transplantation with autologous, allogeneic, or xenogeneic bone.[2]

In 1937, a 19-year-old woman suffering from aplastic anemia was treated by Osgood and colleagues.[3] She was given pentose nucleotide, liver extract, and 43 units of blood within a period of 52 days. Ultimately, "when all hope from other methods of therapy seemed gone" she received intravenously 18 ml of sternal marrow together with 575 ml blood from her blood group-identical brother. Five days later she died. At necropsy occasional islets of regenerating marrow were found and the authors did not exclude that these had been derived from the injected marrow cells.

Morrison and Samwick wondered if an unknown maturing factor present in normal bone marrow lacked in aplastic anemia and leukemia analogous to the intrinsic factor as the erythrocyte maturing factor in pernicious anemia.[4] In 1940 they treated a 42-year-old man suffering from idiopathic aplastic anemia with pentose nucleotide, liver extract and transfusions of whole blood followed by intrasternal injection of bone marrow. A total dose of 8 ml, which was obtained from the patient's brother, was given on two consecutive days. On the third day, 5 ml bone marrow from another

*In this overview autologous refers to transplantation within the same individual. Syngeneic (isologous) refers to transplantation within an inbred strain or between identical twins (genotypically identical individuals). Allogeneic (homologous) refers to transplantation between inbred strains or between not genotypically identical individuals of the same species. Xenogeneic (heterologous) refers to transplantation between different species.

donor was injected intrasternally. Within two days after the last injection, platelets began to rise followed by an increase in white and red blood cells and normalization of differential counts, and a marked increase of hematopoietic elements in the bone marrow. The patient recovered and the authors concluded that a unknown factor present in normal bone marrow, might have stimulated maturation of hematopoietic constituents already present in the diseased marrow.

In 1950, Rekers *et al* reported on transplantation of allogeneic bone marrow in dogs after exposure to 350 röntgen* of body irradiation.[5] The bone marrow suspension had been administered intramedullary, intravenously, in the spleen or in the liver. However, all transplants failed to grow. Jacobson *et al* showed that mice exposed to a lethal dosis of total body irradiation of 1,025 röntgen did not suffer from anemia and had only a transient leuko- and thrombocytopenia if the surgically mobilized spleen had been covered by lead during irradiation.[6,7] Immediate extirpation of the shielded spleen within 5 minutes after irradiation caused death in all mice but postponed extirpation of the shielded spleen 1 to 6 hours after irradiation had no deleterious effect on recovery. From these experiments the authors concluded that a non-cellular substance produced by the shielded spleen was responsible for recovery from radiation injury. This has been questioned by Lorenz *et al* who believed that not a humoral but a cellular factor was responsible for survival.[8] They injected mice intravenously or intraperitoneally with syngeneic marrow after irradiation with 900 röntgen. Twenty-eight out of 37 (76%) mice were alive at 28 days compared to none of 28 irradiated animals which had not received bone marrow. Active areas of hematopoietic tissue were found in mice infused with bone marrow but not the control animals. However, their experiments did not exclude a humoral factor.

Lindsley and colleagues transplanted rats with allogeneic marrow.[9] In rats irradiated with doses in the LD50 range, donor-type red blood cells were found after 2 to 3 weeks but not in rats which were not irradiated. Using mice with a specific marker-

*for clinical purposes, one röntgen may be read as one rem, one rad, or one cGy.

chromosome as bone marrow donor for mice without this marker, Ford *et al* demonstrated dividing cells of donor origin in the bone marrow, the spleen, the lymph nodes, and the thymus of mice after irradiation and intravenous injection of donor spleen suspension.[10] Virtually all host bone marrow had been replaced by cells originating from the donor. In another experiment they found only rat chromosomes in the bone marrow of mice after irradiation and injection with a suspension of rat bone marrow cells. They concluded that infusion of spleen suspension or bone marrow cells exerted its therapeutic effect by a cellular mechanism although they could not exclude the possibility of simultaneous activity of a humoral factor.

After it became evident that hematopoietic tissue could be transferred from one animal to another, but only after pretreatment with sufficient irradiation, possibilities had been created for the treatment of leukemia. In their experiments on mice in 1957, Barnes *et al* showed that a lethal dose of whole body irradiation followed by transplantation of a syngeneic graft could not eradicate all leukemic cells.[11,12] Mice given syngeneic marrow grafts died from relapsed leukemia. However, recipients of an allogeneic graft died from an unknown secondary syndrome with wasting but without signs of leukemia.

In the same year, Thomas and colleagues published on intravenous infusion of bone marrow in six human beings.[13] Donor marrow was obtained from the long bones and ribs of fetal and adult cadavers, or from living donors by aspiration from the ileum or from ribs removed at surgery. Three patients suffered from end-stage hematologic malignancies, two had metastatic carcinomas and one patient was in coma from a massive cerebral hemorrhage. The patients with hematologic malignancies were irradiated with doses varying from 300 röntgen given in three days to 600 röntgen divided over 17 days. The recipients with carcinoma were not pretreated with irradiation but were conditioned with cytostatics. The patient with cerebral hemorrhage was not pretreated at all. The total amount of nucleated bone marrow cells infused varied from 0.6×10^9 to 3.2×10^9 cells. One of the patients treated for carcinoma received bone marrow from two different donors. The

marrow was administered intravenously in one dose or in divided doses at different time intervals. Using differences in blood group antigens a very transient production of red cells of donor origin was suggested in two patients. None of the patients had prolonged or permanent engraftment.

In 1958, Mathé *et al* transplanted five men who had been accidentally exposed to radiation emitted by a nuclear power plant in Belgrade.[14] Estimated radiation exposition varied from 600 to 1,200 rem. Twenty-seven to 36 days after the exposure the victims received 8.5 to 14 x 10^9 nucleated bone marrow cells from unrelated donors. One patient died, the others recovered. Based on their studies on blood group antigens the authors postulated that donor marrow grafts had functioned temporarily until autologous bone marrow recovery.

Ferrebee *et al* claimed that success of grafting depended on several factors.[15] The recipient had to be in general good condition before and after irradiation, the donor marrow had to be genetically related and the amount of nucleated bone marrow cells infused should be considerable. The host immune system had to be impaired further by splenectomy, by the use of adreno-corticotropic hormone (ACTH) and by irradiation. Furthermore, the total dose of body irradiation had to be increased or given during a prolonged period. A male and a female splenectomised healthy dog were irradiated with 100 röntgen four times daily for 3 consecutive days. Antibiotics and ACTH were started on the first day of irradiation. Immediately after the radiation the female dog was given 2 x 10^9 nucleated marrow cells from an unrelated male. White blood cells did not rise and this dog died from sepsis on the 12th day. The male dog received 8.6 x 10^9 nucleated bone marrow cells from a female sibling. Blood counts normalized at 14 days posttransplant and leukocytes showed female forms. ACTH and antibiotics were discontinued. Thereupon the dog developed fever, was "critically ill" and his blood counts dropped. Within a few days after restarting ACTH and antibiotics he recovered completely and mature granulocytes of female type reappeared.

Congdon and Urso observed that the 15-day mortality of mice irradiated with 900 röntgen and transplanted with allogeneic

marrow was comparable with that of syngeneic grafts.[16] Death was due to the irradiation syndrome. A large number of mice injected with allogeneic marrow died during further follow-up and this was caused by a secondary disease process. This process (secondary phenomenon, secondary phase, homologous disease, delayed foreign bone marrow reaction, graft-versus-host reaction or graft-versus-host disease) consisted of emaciation, dermatitis, laesions in the liver and gastrointestinal tract, and atrophy of lymphatic tissues. It was caused by a reaction of the marrow graft against the host. Uphoff demonstrated that the graft-versus-host reaction could be prevented by using fetal hematopoietic donor tissue.[17] Following 800 röntgen total body X irradiation F_1 hybrids receiving parental marrow died with severe diarrhea and extreme emaciation but those given parental-strain fetal hematopoietic tissue did well and were indistinguishable from those receiving syngeneic marrow.

In 1960 Haurani *et al* reported on bone marrow transplantation in patients with acute leukemia.[18] Donor marrow was obtained from ribs resected from unrelated donors. No more than two ribs were resected from a single donor. Three patients in hematological remission at the time of transplantation were pretreated with 50 to 325 röntgen of irradiation and received corticosteroids in an attempt to impair the host immune mechanism. The number of nucleated cells infused ranged from 5.4×10^9 to 7.4×10^9. None of the three recipients showed evidence of engraftment. They survived for 7 to 10 months with relapsed leukemia. Six other patients with overt leukemia were treated with 300 to 350 röntgen and infusion of 4.3×10^9 to 8.6×10^9 bone marrow cells. No evidence of engraftment was found. Five patients died within two months. Cause of death was infection or hemorrhage in four and leukemia in one recipient. The sixth patient died from leukemia at 8 months after transplantation.

BONE MARROW TRANSPLANTATION WITH IDENTICAL TWINS AND GENETICALLY UNSELECTED DONORS

In 1959 Thomas and colleagues described two patients with acute leukemia who received a graft from their identical twins.[19] A two-

year-old girl without signs of remission after treatment with cytostatics and prednisone was given 200 röntgen of total body irradiation followed by 1.3 x 10^9 nucleated donor bone marrow cells. Blood cell counts normalized quickly, and a bone marrow study performed 25 days after irradiation showed complete remission. She relapsed about two months later. Within one month she was retransplanted with 2.3 x 10^9 nucleated bone marrow cells from the same donor after irradiation with 1,140 röntgen. White blood cell counts rose and the bone marrow showed a normal maturation in myeloid and erythroid cells without any evidence of leukemia. Megakaryocytes were not found, and because of failure of the platelet count to recover, she was given another 0.9 x 10^9 nucleated bone marrow cells from the same donor. A repeated bone marrow aspiration 48 days after irradiation showed relapse of leukemia. Their second patient was a 4-year-old girl with relapsed acute leukemia. She was pretreated with 850 röntgen of total body irradiation followed by the infusion of 3.9 x 10^9 nucleated marrow cells. At 13 days after irradiation the bone marrow was normal but 10 weeks later she relapsed. The authors concluded that leukemia could not be eliminated by whole body irradiation followed by infusion of syngeneic marrow and suggested two alternative approaches. Firstly, after lethal total body irradiation allogeneic marrow could eradicate leukemia by an immunologic reaction. However, patients might die from delayed foreign marrow disease. Their second approach was based on observations in animals which showed that the possibility of cure from leukemia was greater when the number of leukemic cells was smaller.[20] They suggested that a patient with a relatively small mass of leukemic cells after treatment with cytostatics might benefit more from irradiation and subsequent infusion of bone marrow cells.

In 1961 Thomas and colleagues described bone marrow transplantation in five patients.[21] Indications for transplantation were untreated, relapsed or resistant acute leukemias. The patients were nursed in isolated rooms. Dose of irradiation varied from 1,460 to 2,016 röntgen and was given continuously at a dose-rate of 0.5 to 2 röntgen per minute. Marrow donors were identical twins in two cases and a mother and sister in one case each. The fifth

patient received liver and spleen suspensions of three different blood group-identical fetuses and bone marrow from his brother. All patients received a second bone marrow infusion after 11 or more days. The total amount of nucleated cells infused varied from 9.6 x 10^9 to 24.0 x 10^9 cells. Within 22 days after irradiation three patients died from infection and one twin marrow recipient died from hepatic failure. At autopsy no evidence of leukemia was found and the recipient of maternal marrow and the twin marrow recipient showed signs of marrow regeneration. The only patient who survived 22 days received a graft from his identical twin brother. Marrow function recovered and he was discharged from the hospital on the 42d postirradiation day. He died from relapsed leukemia 72 days after BMT. In their comment the authors discussed that in one patient take failure may have been related to pretransplant sensitization to foreign cells by blood transfusions and that the transfusion of fresh blood products might have been the cause of graft failure in another patient. In order to avoid the latter complication they proposed to irradiate fresh blood or platelets with 500 to 1,000 röntgen before administration.

In 1963 Mathé *et al* transplanted a 26-year-old man with relapsed acute lymphoblastic leukemia.[22] He was pretreated with azathioprine 300 mg daily for 4 days followed by 400 röntgen of total body irradiation given on each of two consecutive days. A mixture of equal parts of bone marrow from six donors (his father, mother, three brothers and a sister) to a total dose of 58 x 10^9 nucleated cells was given intravenously. Fifteen days after the infusion of bone marrow cells polymorphonuclear cells began to rise. In the mean time marked diarrhea had occurred followed by generalised eruption and desquamation of the skin. The patient had fever. Skin biopsy showed laesions typical of secondary syndrome. Because of hepatic dysfunction corticosteroids were started. Patient recovered completely. Using red cell phenotyping it was shown that the erythrocytes had the phenotype of one of his brothers, with studies of the leukocyte sex chromatine a few female cells were found and immunoglobulin phenotyping showed donor-type globulins (all bone marrow donors had the same immunoglobulin phenotype). At 44 days posttransplant the patient received skin

grafts from all bone marrow donors. The skin graft of the sister donor was lost by accident and all but the skin graft of the brother donor whose red cell phenotype the patient had acquired had been rejected at 60 days. This skin graft remained perfectly intact for more than seven months. Five months after transplantation blood leukocytes from this brother were given intravenously in order to enhance the presumed anti-leukemic effect of the graft against the host. A total number of 163 x 10^9 leukocytes were given at 4 one-week separated occasions. Recurrence of secondary syndrome was suggested and was successfully treated with corticosteroids. About 3 months after the infusion of donor leukocytes, he developed widespread necrotic herpes zoster complicated by meningo-encephalitis. The patient died 20 months posttransplantation. Post-mortem examination was without evidence of leukemia.

This patient was one of the 21 summarized in 1967 by Mathé *et al.*[23] These patients with acute lymphoblastic leukemia in remission or in active phase were irradiated with 793 to 950 rads and most of them received pooled bone marrow from up to 6 different related and unrelated donors. Transplantation of grafts from several donors was based on experiments in mice showing that simultaneous infusion of bone marrow resulted in engraftment of marrow from the genetically most close donor.[24] Five patients died in aplasia, a sixth patient survived aplasia after retransplantation with a graft from his monozygotic twin. Fifteen patients showed engraftment and all suffered from secondary syndrome causing death in 11 patients. Four patients survived the secondary syndrome but died from leukemia.[25]

THE HUMAN LEUKOCYTE ANTIGEN (HLA) SYSTEM

The major breakthrough in the prevention of secondary disease was the discovery of the genetic principle of histocompatibility. In 1953 Snell *et al* demonstrated that susceptibility and resistence to bone marrow transplantation in mice were determined by multiple dominant genes, the histocompatibility genes.[26] One of these dominant genes was called the H-2 locus. Uphoff observed that differences in histocompatibility genotypes at this locus were

responsible for secondary disease.[27] According to Snell et al[28] the human histocompatibility locus was called Hu-1.[29,30] Leukocytes were carrying transplantation antigens[31,32] and these could be demonstrated by the lymphocytotoxicity test[33] and by leukoagglutination.[34] The antibodies used were obtained from multiparous woman[35] or from volunteers specifically immunised against leukocytes. Apart from serologic typing for specific histocompatibility antigens, the degree of compatibility between individuals could be assessed by the one-way mixed leukocyte culture (MLC) test. In the MLC test, donor leukocytes have been treated with mitomycin C which prevents them to proliferate yet allows them to stimulate untreated leukocytes of the recipient.[36] This stimulation can be measured by incorporation of tritiated thymidine. Using these methods the number of leukocyte antigens that could be recognized increased and this resulted in more insight in the very complex system of histocompatibility in man.[29,30,33,37-40] Standardization of different technologies and of nomenclature had been performed from 1964 onward at the International Histocompatibility Workshops. At the third workshop in 1967 the human histocompatibility system was recognized as one major human leukocyte antigen (HLA) complex. The complexity of the HLA system resulted in a very high number of possible phenotypes. Furthermore it was found that recipient and donor combinations, serologically identical for HLA antigens, could be positive in MLC testing, indicating that HLA loci existed which could not be recognized serologically yet.[41] In the following years it became evident that one single region localised on the short arm of the sixth chromosomes determined the transplantation antigens. This region was called the major histocompatibility complex (MHC). The MHC consisted of two groups of genetically separable loci determining the strong transplantation antigens which could be demonstrated serologically using antisera to detect cell-surface specific antigens (the HLA-A, HLA-B and HLA-C antigens) and by a cellular approach such as the reactivity of lymphocytes in the MLC test (the HLA-D antigens).[42-44]

In 1970 Bortin summarized 203 reported human bone marrow transplants performed for aplastic anemia (N=73), leukemia

(N=84), other malignant diseases and miscellaneous conditions (N=31), and immune deficiency diseases (N=15).[45] More than three quarters of these transplantations were performed between 1959 and 1962 and since it had become evident that outcome was very disappointing, the number of reported transplants decreased to 49 between 1962 and 1968. In at least 125 patients (62%) engraftment did not occur and 152 (75%) were dead at the time of the case report. Donor selection was based on the one-way MLC test in only three of the 203 patients and these three received a graft from an HLA-identical sibling. They were transplanted for immune deficiency disease and were the only reported allogeneic bone marrow chimeras who were still alive at 150 to 365 days. Histocompatibility testing between recipient and donor was not performed in 200 transplants and only two of these transplants were successful. In both cases donors were siblings.

BONE MARROW TRANSPLANTATION WITH HLA-SELECTED DONORS

In 1968 Bach *et al*[46] and Gatti *et al*[47] described a successful bone marrow transplantation in man using an HLA-identical sibling donor. Identity was based on the lymphocytotoxicity test and MLC test. The patient of Bach *et al* was a 2-year-old boy with the Wiskott-Aldrich syndrome. His lymphocytes responded normally to PHA and lymphocytes of an unrelated donor. Bone marrow donor was his MHC-identical 8-year-old sister. At about 4 weeks posttransplantation bone marrow aspiration showed a cellular marrow with a normal differentiation. Chromosome studies of blood lymphocytes showed 100% donor cells. Follow-up ended 6 weeks after transplantation and no graft-versus-host disease (GVHD) was observed.

The patient of Gatti *et al* was a 5-month-old boy with sex-linked lymphocytopenic immunological deficiency. Lymphocytes of the patient did not respond to PHA and allogeneic cells. Although his lymphocytes had one antigen which was not present on the lymphocytes of his 8-year-old sister, she was considered a MHC-compatible donor since the MLC from donor to patient cells

showed complete absence of response. Without pretreatment 10^9 nucleated bone marrow cells were given intraperitoneally. After nine days a rash developed with biopsy proven findings typical of graft-versus-host disease (GVHD) which subsided spontaneously. Thereafter he had loose stools with small ulcers on proctoscopy and a biopsy showed laesions characteristic of GVHD. From the day of administration of donor cells on, white blood cell count decreased but stabilised at 2 x 10^9/L. Chromosome studies on 25 unstimulated bone marrow cells showed 18 male and 7 female chromosomes. Both humoral and cellular immunological reconstitution occurred. However, the patient became pancytopenic and was successfully retransplanted with 10^9 nucleated bone marrow cells from the same donor given both intraperitoneally and in the right posterior iliac crest resulting in 100% red and white blood cell chimerism.[48]

It had become evident that success of bone marrow transplantation was dependent on the clinical condition of the recipient at transplantation, on histocompatibility matching between recipient and donor, on conditioning regimen, on supportive measures both in the period before and after engraftment and on the treatment of GVHD. This knowledge renewed interest in clinical bone marrow transplantation at the end of the sixties.

In 1971 Thomas *et al* described transplantation in seven patients with malignancies refractory to conventional treatment.[49] Two had advanced acute myeloid leukemia and one had chronic myeloid leukemia in blast crisis. Three patients with acute lymphoblastic leukemia and one with Hodgkin's disease had less advanced disease and were in good clinical condition. The sibling donors were HLA-identical and the one-way mixed leukocyte test was negative in 6 pairs and showed only minimal stimulation in one donor-recipient combination. Five patients were nursed in laminar air flow isolation rooms with sterilisation of the skin, food and gut. They used antibiotic sprays for ears and nose as well as mouth rinses with antibiotics and antifungal drugs. Conditioning consisted of 1,000 rad of total body irradiation at a dose-rate of 5.0 to 5.7 rad/min. In order to prevent rejection and to ameliorate GVHD the patients

received methotrexate 10 mg/m^2 on days 1, 3, 6 and 11 and weekly thereafter. Platelets, buffy coat cells and whole blood cells were irradiated with 1500 rad before infusion. None of the 3 patients treated for advanced leukemia reached white blood cells of > 1.0 x 10^9/L nor suffered from GVHD, but all died within 30 days from renal failure or septicaemia. In the 4 patients treated for less advanced disease white blood cells rose over 1.0 x 10^9/L and all suffered from GVHD. Two died from recurrent leukemia at 85 and 102 days after transplantation, respectively. The patient transplanted for Hodgkin's disease engrafted but died from GVHD at 37 days posttransplant. One patient survived with 100% donor cells and returned to school 200 days after transplantation.

In 1972 Thomas *et al* reported on successful sibling bone marrow transplantation in 2 of 4 patients transplanted for aplastic anemia.[50] They had received multiple blood transfusions and were refractory to conventional treatment. Donors were MHC-identical and sex-mismatched siblings. Pretreatment consisted of cyclophosphamide 50 mg/kg body weight on each of 4 consecutive days according to Santos *et al.*[51] Patients were given one unit of buffy coat cells from the bone marrow donor on the day before cyclophosphamide administration in order to enhance the immunosuppressive effect of cyclophosphamide. Immunoprophylaxis posttransplant consisted of methotrexate. The first patient died from sepsis complicating GVHD. The second patient had no GVHD and at 29 days he had mixed chimerism in PHA-stimulated peripheral blood cells but complete donor chimerism in unstimulated bone marrow cells. In further follow-up cytogenetic analysis of both blood and bone marrow metaphases showed 100% donor cells. The third patient rejected the graft and was retransplanted. He was pretreated with cyclophosphamide 50 mg/kg intravenously on each of 4 consecutive days together with anti-human thymocyte serum but died from widespread Candida infection. The fourth patient had low grade GVHD disease and recovered completely. Repeated cytogenetic analysis showed 100% donor metaphases in both blood and bone marrow cells. Thomas *et al* proposed to transplant patients with aplastic anemia and an HLA-identical sibling early in the course of the disease before major

infections and hemorrhage or sensitisation to transplantation antigens had occurred.

The Seattle transplant center described its experience on 100 patients with end stage acute leukemia and transplanted between 1971 and 1975. Those in good clinical condition had a significantly longer survival time than patients transplanted in poor clinical condition.[52] Prior treatment consisted of several courses of combination chemotherapy, but 19 patients never achieved complete remission and only 10 had < 5% leukemic blasts in the bone marrow at the time of transplantation. Donors were MHC-identical siblings in 99 cases and one MHC-identical cousin who shared the four grandparents with the recipient. In an effort to kill more leukemic cells 1,000 rad total body irradiation in a single dose was preceded by cyclophosphamide 60 mg/kg intravenously on each of two consecutive days with or without the addition of other cytostatic drugs. In 6 cases, cyclophosphamide was omitted. Methotrexate was administered in order to prevent and/or to ameliorate GVHD. Six patients died within 17 days without evidence of engraftment. Ninety-four patients engrafted but one rejected the graft. GVHD grade II or more developed in 50 patients and was treated with one or more courses of anti-thymocyte globulin in 38 cases. Interstitial pneumonitis occurred in 54 patients and was lethal in 34. Both the incidence of interstitial pneumonitis and death from interstitial pneumonitis was higher in patients with acute GVHD ≥ grade II. Thirty-one patients relapsed from leukemia. Due to the large number of deaths, no relation was found between relapse of leukemia and the presence or absence of GVHD. Seventeen patients survived for 330+ to 1654+ (median 892+) days but four of them had relapsed at 210 to 833 days. The actuarial survival curve could be divided into three time intervals: the first 130 days with a high death-rate mainly due to GVHD and infections. A second period between 130 days and two years with less overall mortality and death mainly due to relapses, and a plateau phase from 2 to 4.5 years without deaths, although 4 of 17 patients had a relapse of the leukemia.

The overall results were poor and it was evident that this was due to the poor clinical condition of the patients suffering from end-

stage leukemia. The Seattle group observed a significant improvement in survival in patients transplanted for acute lymphoblastic leukemia in second or subsequent remission compared to those transplanted in relapse.[53] Dinsmore from the group of the Bone Marrow Transplant Center of the Memorial Sloan-Kettering Cancer Center showed that disease-free survival had improved in patients transplanted for acute lymphoblastic leukemia in second remission in comparison with those transplanted in later remission or relapse.[54] The Seattle center reported on good outcome of bone marrow transplantation in patients transplanted for acute myeloid leukemia in first complete remission.[55] The probability of disease-free survival was 63% with 12 of 19 patients living without relapse for more than 471 days after transplantation. These results were comparable with those obtained in Europe.[56] Outcome of bone marrow transplantation in CML patients was disappointing when performed after onset of blast crisis,[57] but improved when patients were transplanted in first chronic phase.[58]

GRAFT-VERSUS-HOST DISEASE (GVHD)

Attempts have been made to reduce the incidence of GVHD. In 1963 Mathé *et al* showed that preservation of hematopoietic cells for 2 hours at 37° C reduced the incidence of secondary syndrome in mice without affecting the restoration capacity of myeloid cells.[59] Van Bekkum demonstrated that mortality due to graft-versus-host reaction was a function of the number of lymph node cells infused. Storage of a mixture of bone marrow cells and lymph node cells at 4° C for 24 to 48 hours had a more profound inactivating effect on the immunologically active cells than on the hematopoietic cells.[60] Epstein *et al* found a reduced severity of the secondary disease when dogs had been treated with methotrexate in the early period of engraftment.[61] Cyclophosphamide or antilymphocytic antisera given to monkeys after bone marrow infusion controlled secondary disease only partially.[62] Harris and Joseph separated bone marrow cells in fractions by differential centrifugation in a density gradient composed of fractions of different concentrations of bovine plasma

albumin.[63] They found the earliest forms of blasts cells, myeloblasts, promyelocytes and proerythroblasts together with lymphocyte-like cells in the upper two layers with the lowest density. In the middle two layers granulocytes and lymphocytes predominated and normoblasts and eosinophils were found in the deepest two layers. The separation of cells had not damaged their viability since rapid recovery was found in animals infused with these recombinated bone marrow fractions. In 1968 Dicke *et al* assessed the repopulation capacity and graft-versus-host activity of the various fractions of mouse spleen cell suspensions after separation on a discontinuous albumin gradient.[64] In comparison to the original spleen cell suspension they observed a ten-fold increase in the concentration of spleen colony forming units and more than a 10-fold decrease of graft-versus-host activity after infusion of the pooled upper layers containing between 30% and 50% of the total number of erythroblasts, myeloblasts and other blast cells. Mice infused with cell fractions from the intermediate layers containing lymphocytes, granulocytes and a small number of blast cells, died from secondary disease. Those infused with fractions from the lower layers containing lymphocytes and granulocytes, died from graft failure. Since the lower layers had the lowest hematopoietic capacity, they concluded that the putative hematopoietic stem cells were not small lymphocyte-like cells.

The clinical manifestations and grading of acute GVHD had been described by Glucksberg *et al.*[65] Acute GVHD typically developed within 3 months after transplantation and was caused by immunocompetent T lymphocytes present in the graft and directed against alloantigens expressed on host cells leading to tissue injury. Acute GVHD was found in 30 to 70% of recipients of an HLA-identical marrow graft and caused death in 20 to 40% of those affected.[66] Shulman and Sullivan summarized the clinical course of chronic GVHD.[67,68] Chronic GVHD developed more than three months after transplantation and resembled an autoimmune disorder with skin pathology resembling scleroderma, abnormal liver tests, ocular and oral symptoms resembling Sjögren's syndrome, pulmonary insufficiency, and manifestations in other organs. Chronic GVHD was observed in 52 of 175 (30%) transplants

and was lethal in 17 (33%) patients.[68] GVHD and associated infections remained the major obstacles to success of bone marrow transplantation.

The Seattle group used methotrexate as standard prophylaxis for acute GVHD but this was only partially effective.[69] Prophylactic administration of anti-thymocyte globulin had no beneficial effect.[70] Established acute GVHD was treated with corticosteroids with or without the addition of anti-thymocyte globulin but was inadequate in many cases.[71,72] Cyclosporine A with its selective inhibitory activity on activated T cells was initially used by Powles *et al* for the treatment of GVHD[73] but later on for prevention of GVHD.[74] Randomised trials comparing methotrexate and cyclosporine A as prophylaxis for GVHD did not show significant differences in the incidence of moderate and severe acute nor chronic GVHD in patients transplanted for acute leukemia[75,76] or chronic myeloid leukemia.[77] When compared to cyclosporine alone, the combination of cyclosporine A and methotrexate decreased significantly the incidence of moderate and severe acute GVHD but not of chronic GVHD in transplants for acute myeloid leukemia in first remission or CML in first chronic phase.[78]

Another approach to prevent GVHD is the in vitro depletion of immunocompetent T lymphocytes from the donor marrow. This can be accomplished by immunological or physical methods.

Immunological methods of T cell depletion. Rodt *et al* incubated donor marrow with anti-human T cell globulin to remove T cells.[79] GVHD grade I-II was observed in only 2 out of 12 engrafted patients. In a pilot study on 10 consecutive patients, Filipovich *et al* showed that histocompatible sibling donor marrow could be safely infused after pretreatment with OKT3, an IgG2a complement-binding mouse monoclonal antibody specifically directed against T lymphocytes.[80] In vitro studies demonstrated that almost all T lymphocytes were coated with OKT-3 and subsequent incubation with neonatal rabbit complement reduced PHA response to 4% of that of untreated bone marrow samples. However, 5 out of 10 patients developed GVHD grade II or more and this was due to the inability of autologous human complement to eliminate OKT-3

coated cells in vivo. Prentice *et al* observed a reduced incidence of severe acute GVHD in recipients of marrow grafts pretreated with OKT3.[81] In vitro lysis with complement was not performed and all patients received methotrexate as graft-versus-host prophylaxis posttransplant. They also showed that incubation of the marrow graft with a combination of two monoclonal antibodies followed by two rounds of lysis with rabbit complement killed > 99% of T cells in all but one graft and prevented significant GVHD.[82] Immunoprophylaxis posttransplant was not given. A mixture of eight monoclonal antibodies providing optimal antibody binding to all T lymphocytes and lysis with two rounds of rabbit complement, was used by Martin *et al* from Seattle resulting in a 2 to 3 logs depletion of T cells in most patients.[83] Prophylaxis after transplantation consisted of cyclosporine A. The incidence and severity of acute GVHD disease had decreased significantly compared with controls. However, T cell depletion was associated with an increased incidence of graft failure.

Other antibody-mediated techniques for in vitro depletion of T lymphocytes were reported such as antibodies coupled to ricin toxin,[84] and antibodies coupled to magnetic microspheres.[85-87]

Physical methods of T cell depletion. Several physical methods have been developed to remove lymphocytes from donor marrow. Bone marrow was fractionated by soybean agglutinin and sheep red blood cells,[88-91] density gradients,[92] and counterflow elutriation.[93]

Reisner *et al* eliminated T cells from the leukocyte-enriched fraction of bone marrow by E-rosetting with sheep red blood cells followed by differential agglutination of residual T lymphocytes in the non-rosetting fraction with soy bean agglutinin, a plant lectin that selectively removes T lymphocytes.[88] In a modification of this method, the leukocyte-enriched fraction was treated first with soybean agglutinin followed by depletion of residual T cells from the unagglutinated fraction by E-rosetting with sheep red blood cells.[89,90] The non E-rosetting cells contained 5% of the nucleated cells of the original marrow, more than 80% of the colony forming cells, and no detectable T lymphocytes. Using this modification O'Reilly *et al* transplanted 22 patients with a graft from an HLA-

identical donor.[91] Conditioning consisted of cyclophosphamide (120 mg/kg) and hyperfractionated total body irradiation to a dose of 13.2 Gy. No additional immunoprophylaxis was given. Twenty patients achieved a durable full engraftment and 16 survived disease-free 3 to 10 months posttransplant. Only one patient suffered from GVHD grade II.

Löwenberg *et al* observed a significantly higher incidence of acute GVHD ≥ grade I in patients receiving marrow grafts depleted of 90% of lymphocytes using discontinuous albumin gradient fractionation when compared to those given transplants depleted of 99% of lymphocytes using E-rosette sedimentation.[92] The authors referred to studies in monkeys where it had been calculated that mortality due to GVHD without additional immunosuppression posttransplantation in MHC-identical transplants, could be prevented fully if not more than 2×10^6 T lymphocytes/kg body weight were given.[94]

Another physical depletion method is counterflow centrifugation (elutriation). Counterflow centrifugation separates cells mainly according to their size.[93,95-98] In summary: bone marrow is filtered through a nylon filter with a pore size of 70 μm. After removal of plasma and fat, a mononuclear cell fraction with a density < 1.073 g/ml is isolated in gradients of Percoll (Percoll, Uppsala, Sweden). Then the low density mononuclear fraction is pumped into the separation chamber which is placed excentrically in a centrifugation rotor. The separation capacity of the original single chamber rotor (Beckman JE-6) has been enhanced by the development of a multichamber counterflow centrifugation system with four separation chambers reducing the total time of the elutriation procedure to about 3 hours.[99] The transparant chambers (International Medical BV, Zutphen, The Netherlands) have been placed in a rotor (type Mark-1, Dijkstra Vereenigde BV, Amsterdam, The Netherlands) that has been installed in a modification (Curame-3000) of the Varifuge-RF centrifuge (Heraeus Separationstechnic, Ostenrode Germany). The particles in the separation chamber are subjected to two opposing forces: the centrifugal force depending upon the rotor speed and the centripetal force caused by the continuous fluid stream pumped

into the chamber with a direction towards the rotor axis. At equillibrium the cells are positioned according to their size with the larger cells in the more centrifugal position and the smaller cells in the more centripetal direction. By increasing the centripetal force and/or decreasing the centrifugal force this equilibrium shifts towards the outlet of the chamber and the smaller cells are the first to leave the chamber. The rotor outlets are monitored for cell number using a Ortec-541 ratemeter (EG7G Ortec, Oak Ridge, Tennessee, USA) and for cell size using a multichannel analyser ND 600 (Nuclear Data Incorporated, Schaumberg, Illinois, USA). The cells are collected in several fractions and of each fraction the numbers of nucleated cells are measured. Of each fraction the number of T lymphocytes are counted using a Coulter Epics Elite (Coulter Corporation, Hialeah, Fl, USA) after binding to CD3-FITC monoclonal antibody (Dakopatts, Glostrup, Danmark). The marrow that is ultimately given to the recipient consists of the larger cells fractions with an absolute total number of T cells between 0.7 and 1.0 x 10^6/kg body weight of the recipient corresponding to a T cell depletion of about 98%. The first clinical results of bone marrow transplantation using counterflow centrifugation at the University Hospital Nijmegen have been described by De Witte *et al* in 1984.[97] Two of the first six patients died within 22 days due to infections and despite the average high age of 30 years only one of the 4 patients at risk developed acute GVHD grade II. He was one of two patients who did not receive additional immunosuppression.

GVHD AND RELAPSE OF LEUKEMIA

In 1957 Barnes and Loutit showed that mice irradiated with 950 rads followed by transplantation with syngeneic bone marrow usually died after about one month with overt leukemia but those transplanted with allogeneic marrow often survived for longer periods and ultimately died in wasted condition but without signs of leukemia.[12] They considered that an immune reaction of the allograft had destroyed residual leukemic cells but also acted on normal tissue causing the secondary phenomenon (GVHD). The

influence of GVHD on relapse of leukemia was analysed in 242 transplants for acute leukemia performed between 1970 and 1978.[100] Forty-six patients received a syngeneic graft and 196 were transplanted with bone marrow from an allogeneic donor. When considering type of marrow graft, diagnosis and remission status, multivariate analysis showed that patients having moderate to severe acute GVHD or chronic GVHD had a significant lower relapse-rate than patients with no or mild GVHD. However, the positive effect of GVHD on relapse-rate was counterbalanced by a higher incidence of death due to other causes, especially interstitial pneumonitis. Consequently, patients with GVHD had no better probability of long term survival. In 1981, Weiden *et al* reported that chronic GVHD was associated with a lower relapse-rate and improved survival in recipients of allografts for acute leukemia.[101]

It had become evident that T cell depletion of the marrow graft was associated with a decreased incidence and severity of GVHD. However, recipients of T cell depleted marrow had a higher probability of relapse. In a prospective randomised double-blind trial from the University of California, T cell depletion was performed using monoclonal T cell antibody CT-2, an IgM antibody directed against the E-rosette receptor antigen on T lymphocytes, and rabbit complement.[102] Immunoprophylaxis posttransplant consisted of methotrexate or cyclosporine A. Recipients of T cell depleted grafts had significantly less mortality due to acute GVHD and a significantly lower incidence of chronic GVHD but more relapses resulting in equal survival when compared to those receiving untreated marrow. Compared to historical controls, Apperley *et al* found a significantly reduced incidence of acute GVHD, but a significantly increased incidence of leukemic relapse in 28 recipients of T cell depleted grafts transplanted for chronic myeloid leukemia in chronic phase.[103] Donor bone marrow had been depleted of T cells with the rat-derived monoclonal antibody Campath-1 together with fresh serum from the donor as source of complement. T cell depletion was nearly complete. All but three patients were given cyclosporine A as additional prophylaxis against GVHD posttransplant. In a prospective randomised trial of T cell depletion, Maraninchi *et al* observed a significant reduction in both

acute and chronic GVHD in recipients of T cell depleted grafts when compared to the patients who received unmanipulated marrow.[104] Bone marrow was treated with two different combinations of monoclonal antibodies and complement removing 97 to 99% of T cells. Immunoprophylaxis posttransplant consisted of cyclosporine A. Relapse-rate was significantly higher in recipients of T cell depleted grafts but disease-free survival did not differ between both groups. An average T cell depletion of 94% was achieved in an open pilot study by Hervé *et al* using two different cocktails of pan-T monoclonal antibodies with rabbit complement.[105] A minority of patients received methotrexate posttransplant. The incidence of both acute and chronic GVHD was low but relapse-rate appeared to be relatively high.

The incidence of GVHD and the probability of relapse in 80 patients transplanted for leukemia with lymphocyte depleted grafts using counterflow centrifugation are described in chapter 3 of this thesis. The low incidence of GVHD was associated with a relatively high probability of relapse. Preliminary data suggested that intensification of the conditioning regimen with anthracyclines reduced relapse of leukemia.[106] A more recent analysis showed a probability of relapse of 18% in patients conditioned with anthracyclines which was significantly lower than the probability of 44% in an earlier cohort of 35 comparable patients treated with the standard conditioning.[107]

BONE MARROW TRANSPLANTATION WITH MATCHED UNRELATED DONORS

A new development in allogeneic bone marrow transplantation is the creation of national and international bone marrow donor registries. Since about 60% of patients lack an appropriate family donor, that means an HLA genotypically identical sibling or a family member incompatible at only one locus, voluntary bone marrow donors registries have been founded. The chance of finding an HLA-A, -B, and -DR identical donor for an individual patient depends on the size of the donor pool and the recipient's HLA phenotype. It can be calculated that the number of possible HLA phenotypes is

$4x10^{18}$, but some phenotypes are much more frequent than others in a given population.[108] For example, 17% of North American whites with a common phenotype have a 90% probability of finding an HLA-A, -B, and -DR matched donor in a registry of 50,000 white donors but this is less than 10% for the 48% of potential recipients with unusual haplotypes.[109] The first bone marrow transplantation with an HLA-A, -B identical, MLC negative unrelated donor was performed in 1973 by Speck *et al.*[110] Indication for transplantation was severe aplastic anemia. The patient died two months after transplantation without signs of engraftment.

More than twenty registries are operating worldwide and donor searches are being performed by computerised systems. At present, reports on unrelated bone marrow transplantation in acute leukemia, myelodysplastic syndromes, and severe aplastic anemia are limited. McGlave *et al* reported on unrelated bone marrow transplantation in 102 patients transplanted for CML in first chronic phase (CP1) or more advanced disease.[111] In 44 cases of HLA-A, -B, and -DR identical, MLC nonreactive (relative respons index < 20%) transplants, actuarial survival was 46 ± 20% at 2.5 years. This was 27 ± 14% in recipients of less matched unrelated donors. Until now, 33 patients have been transplanted with a matched unrelated donor in the Netherlands.[112] Disease-free survival at 2 years postgrafting was 43% in 18 patients transplanted for good risk disease. It may be expected that outcome of unrelated transplantation will improve with better matching and transplantation in an earlier phase of the disease.

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CHAPTER 3

ALLOGENEIC BONE MARROW TRANSPLANTATION FOR LEUKEMIA WITH MARROW GRAFTS DEPLETED OF LYMPHOCYTES BY COUNTERFLOW CENTRIFUGATION

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Published in Blood 1990, 75: 1356-1363

SUMMARY

Eighty consecutive patients were transplanted with human leukocyte antigen (HLA)-identical sibling marrow for acute myelogenous leukemia (AML, N = 29), acute lymphoid leukemia (ALL, N = 23), or chronic myelogenous leukemia (CML, N = 28). Donor marrow was depleted of lymphocytes using counterflow centrifugation. Median age of the recipients was 31 years. Pretransplant conditioning consisted of cyclophosphamide and fractionated total body irradiation (TBI) with a low (4.1 ± 0.3 cGy/min) or high (13.1 ± 1.6 cGy/min) midline average dose-rate. In 43 patients, cytosine-arabinoside or anthracyclines were added to the conditioning regimen. Immunoprophylaxis posttransplant consisted of methotrexate (MTX) alone, cyclosporine A (CsA) in combination with MTX, or CsA alone; two patients received no immunoprophylaxis at all. Graft failure occurred in 4 of 77 evaluable patients (5%). The probability of acute graft-versus-host disease (GVHD) ≥ grade 2 at day 100 after transplantation was 15%. The projected 3-year estimate of extensive chronic GVHD was 12%. Only 3 patients died of cytomegalovirus-interstitial pneumonitis. The projected 3-year probability of relapse was 30% (95% confidence interval [CI], range 8% to 53%) in transplants for AML in first complete remission (CR1), 35% (95% CI, 1% to 69%) after transplantation for ALL in CR1, and 38% (95% CI, 2% to 74%) after transplantation for CML in first chronic phase (CP1). The projected 3-year probability of leukemia-free survival (LFS) was 56% (95% CI, 35% to 77%) after transplantation for AML-CR1, 42% (95% CI, 16% to 69%) in patients transplanted for ALL-CR1, and 49% (95% CI, 18% to 80%) after transplantation for CML-CP1. After transplantation for AML-CR1, ALL-CR1, or CML-CP1, the median follow-up time for leukemia-free survivors was 31+, 30+, and 21+ months, respectively. Probabilities of relapse, survival and LFS in AML-CR1 and ALL-CR1 transplants were comparable with those reported in recipients of untreated grafts. In patients transplanted for CML-CP1, probability of relapse was higher and probability of LFS was lower than in recipients of untreated grafts. In transplants for leukemia in CR1 and CP1, preparative regimen and

immunoprophylaxis posttransplant were not associated significantly with the probability of acute GVHD ≥ grade 2, extensive chronic GVHD, relapse, survival, or LFS. In bone marrow transplantation for leukemia, counterflow centrifugation is a useful technique for the prevention of GVHD. In transplants for leukemia in CR1 and CP1 receiving T cell depleted grafts using counterflow centrifugation, probability of survival and LFS can compete with that reported in studies from the literature on recipients of untreated marrow. Further efforts should be made to reduce relapse-rate, particularly in CML transplants.

INTRODUCTION

Human leukocyte antigen (HLA)-identical sibling bone marrow transplantation (BMT) has become the treatment of choice for acute and chronic leukemias.[1-7] One of the major obstacles for successful outcome is acute graft-versus-host disease (GVHD). It occurs in 30% to 70% of HLA-matched recipients and contributes to death in 20% to 40% of those affected.[2] GVHD, on the other hand, reduces the risk of leukemic relapse.[8] The incidence and severity of acute GVHD can be reduced by removal of immunocompetent T lymphocytes from the graft using different techniques.[9] T cell depletion, however, is associated with increased incidence of graft rejection and leukemic recurrence.[10,11]

In this article, we report on the outcome of 80 consecutive transplants for acute and chronic leukemia. All patients received marrow from HLA-identical siblings after lymphocyte depletion using counterflow centrifugation.[12] Results are compared with the outcome of BMT with unmanipulated marrow and otherwise T cell depleted grafts as reported in the literature.

MATERIALS AND METHODS

Patient and donor characteristics. From May 1981 through September 1988, 80 consecutive patients (42 males and 38 females) were transplanted for acute myelogenous leukemia (AML), acute lymphoid leukemia (ALL), or chronic myelogenous leukemia

(CML). Marrow donors were HLA-identical, mixed lymphocyte culture negative siblings (53 males and 27 females). Syngeneic transplants were excluded. Median age of the recipients and donors was 31 (range 13 to 47) and 32 (range 13 to 51) years, respectively. Informed consent was obtained from all recipients and donors or their guardians. Donor marrow was depleted of 98% of lymphocytes by counterflow centrifugation using a single chamber rotor (Beckman Instruments Inc, Palo Alto, CA, USA)[13] or a four-chamber rotor (Dijkstra Vereenigde BV, Amsterdam, The Netherlands).[14] The absolute number of T cells infused ranged from 0.1 to 3.2 (median 0.7) x 10^6/kg body weight.

Indications for transplantation. The indications for transplantation were as follows: AML in 29 patients (26 in first complete remission [CR1]), ALL in 23 patients (17 in CR1), and CML in 28 patients (19 in first chronic phase [CP1]).

Conditioning regimen. Standard conditioning regimen consisted of cyclophosphamide 60 mg/kg body weight/d (days -6 and -5). In order to reduce relapse-rate, cytosine-arabinoside 3 g/m^2/d (days -4 and -3) was added to the standard conditioning regimen in six patients and anthracyclines were added in 36 cases (daunorubicin 26 mg/m^2/d or demethoxydaunorubicin 7 mg/m^2/d by continuous intravenous (IV) infusion from days -7 to -2, inclusive). All patients received total body irradiation (TBI) in two equal fractions of 450 cGy each on days -2 and -1 using a 18 MV photon beam linear accelerator (Saturne, CGR, Buc, France) with a midline average dose-rate of 4.1 ± 0.3 cGy/min in 50 patients, and 13.1 ± 1.6 cGy/min in 30 patients. Lungs and eyes were shielded using individually adapted lead blocks. The corrected mean total lung dose was 790 ± 130 and 710 ± 80 cGy for the patients with the lower and higher midline average dose-rate, respectively. Donor marrow was infused 24 hours after completion of TBI.

Immunoprophylaxis. Immunoprophylaxis posttransplant was omitted in 2 patients. Since GVHD grade 3 occurred in both patients, nine recipients received methotrexate (MTX) according

to the Seattle regimen.[15] As soon as cyclosporine A (CsA) became available, CsA was added: 30 consecutive patients received both CsA and MTX. CsA (3 mg/kg body weight/d) was given by continuous IV infusion on days -1 to +28, followed by CsA (9 mg/kg body weight/d) orally, with a gradual tapering off after 6 weeks and discontinuation after 12 weeks. Weekly IV injections of MTX were given from week 5 onward, starting with 2.5 mg/m^2 and weekly increments of 2.5 mg/m^2 until a dose of 10 mg/m^2 was reached. MTX treatment was discontinued in week 16 after BMT. Since MTX delayed engraftment, it was omitted and the next 39 consecutive recipients received CsA alone, 3 mg/kg body weight/d by continuous IV infusion from days -1 to +14, followed by 2 mg/kg body weight/d continuous IV on to day +21. From day +21 onward, CsA was given orally in a dose of 6 mg/kg body weight/d to 12 weeks after BMT, followed by a gradual tapering off and discontinuation after 16 weeks postgrafting. All patients were managed in single rooms with filtered air under positive pressure throughout the transplantation period inside the hospital, and all received oral selective gut decontamination, as well as co-trimoxazole for *Pneumocystis carinii* prophylaxis and oral acyclovir for prophylaxis of herpes infection.

GVHD. The clinical manifestations of acute GVHD were graded 1 through 4 according to the criteria described by Glucksberg *et al.*[16] Chronic GVHD was classified as limited or extensive as described by Shulman *et al.*[17]

Engraftment. The first day of engraftment was the first of 3 consecutive days with peripheral white blood cell (WBC) counts of $\geq 1.0 \times 10^9$/L. Engraftment was proven by the presence of donor-type erythrocytes; from 6 months after BMT onward sustained engraftment was also documented by cytogenetic analysis and by studies of restriction fragment length polymorphisms (RFLPs).[18-20]

Statistics. Analyses were performed using the Kaplan-Meier product-limit method. Actuarial curves were calculated for acute GVHD $\geq$ grade 2, extensive chronic GVHD, relapse, survival and

leukemia-free survival (LFS). The effect of diagnosis on the curves was compared using the log rank test and Wilcoxon test. *P* values < 0.05 were considered significant. For a particular diagnosis, effect of pretransplant conditioning and immunoprophylaxis posttransplant on the curves could not be studied in multivariate analysis: the number of patients in the categories was too small. For all diagnoses in CR1 and CP1 together, however, effect of preparative regimen and immunoprophylaxis posttransplant on the curves for acute GVHD ≥ grade 2, extensive chronic GVHD, relapse, survival and LFS was studied in univariate and multivariate analysis. Variables in pretransplant conditioning and immunoprophylaxis posttransplant, evaluable in more than 10 patients, were examined. These variables were the lower or higher midline average dose-rate of TBI, conditioning with or without anthracyclines, and immunoprophylaxis with MTX + CsA or CsA alone. Association of these variables with the incidence of mixed chimerism was studied in univariate and multivariate analysis using the Chi-square test. Follow-up ends on date of evaluation (February 1, 1989).

RESULTS

Primary engraftment. Three patients died within 22 days after transplantation and are not evaluable for engraftment. Two patients died at 37 and 44 days without proof of engraftment. Seventy-five patients showed WBC engraftment at 7 to 47 (median 19) days after BMT.

Rejection of the marrow graft. Two of the seventy-five patients showing engraftment rejected their graft at 2 and 6 months after transplantation. Since the first patient, transplanted for AML-CR1, had no marker specific for donor-type red blood cells, red cell phenotyping could not be used for the demonstration of donor-type erythrocytes; cytogenetic analysis or studies with RFLPs were not performed. The patient died at day 103 after BMT. In the second recipient, transplanted for CML-CP1, donor-type red blood cells had disappeared at 6 months after BMT and could not be demonstrated at several occasions until end of evaluation, 4 years

after BMT. Cytogenetic analysis of bone marrow revealed host-type cells only in determinations from 6 to 48 months after BMT onward, but repeatedly, 4% of peripheral blood lymphocytes were still of donor-type. Using RFLPs, persisting mixed chimerism of peripheral blood lymhocytes was confirmed on several occasions. Despite the reappearance of the Philadelphia chromosome, the patient never showed hematological evidence of relapse.

Graft failure. Graft failure, defined as failure of primary engraftment and early or late rejection of the graft, occurred in 4 of 77 patients (5%).

GVHD. Eleven of seventy-five patients at risk had acute GVHD ≥ grade 2 (seven recipients had grade 2, three patients grade 3, and one recipient had grade 4). Among them were the 2 patients who did not receive immunoprophylaxis posttransplant. The probability of acute GVHD ≥ grade 2 at day 100 after BMT was 15% (95% confidence interval [CI], range 7% to 23%). Sixteen of sixty-three evaluable patients developed chronic GVHD with an extensive manifestation in seven. The 3-year probability of extensive GVHD was 12% (95% CI, 4% to 21%). For acute and extensive chronic GVHD, no significant differences were found in patients transplanted for AML-CR1, ALL-CR1, or CML-CP1. In two cases, extensive chronic GVHD was preceded by acute GVHD ≥ grade 2.

Causes of death. Thirty-five of the eighty patients (44%) died at 8 to 1,360 (median 114) days after BMT. The principal causes of death are shown in Table 1. Three patients died from cytomegalovirus-interstitial pneumonitis.[21]

Relapse. Seven patients transplanted for AML (N = 29) relapsed at 3 to 15 (median 6) months after BMT. After transplantation for ALL (N = 23), five patients relapsed at 1 to 32 (median 5) months after BMT. Seven patients relapsed after transplantation for CML (N = 28): relapse occurred at 6 to 32 (median 19) months after BMT. The projected 3-year probability of relapse for the different diagnoses and for the patients transplanted in CR1 or CP1 is shown

Table 1. Principal causes of death

Causes	N
Relapse	14
Aspergillosis	5
Pneumonia of unknown origin	3
CMV-IP*	3
Pneumocystis carinii pneumonia	1
Candidiasis	2
ARDS#	1
Bacterial sepsis	1
Capillary leakage	1
Renal failue	1
VOD$	1
Acute GVHD	1
Graft-rejection	1
Total	35

*Cytomegalovirus-interstitial pneumonitis

#Adult respiratory distress syndrome

$Veno-occlusive disease

in Table 2. Actuarial relapse curves for AML-CR1, ALL-CR1, and CML-CP1 are depicted in Fig 1. No significant differences were found for the probability of relapse in patients transplanted for AML-CR1, ALL-CR1, or CML-CP1.

Probability of survival. Sixteen patients transplanted for AML survived for 4+ to 84+ (median 31+) months; 12 transplants for ALL survived for 7+ to 61+ (median 26+) months; and 17 patients transplanted for CML survived for 14+ to 59+ (median 23+) months after BMT. The projected 3-year probability of survival for the different diagnoses and for the patients transplanted in CR1 or CP1 is shown in Table 2; survival curves for AML-CR1, ALL-CR1, and CML-CP1 are given in Fig 2. No significant differences were found

Table 2. Projected 3-year probability of relapse, survival, and LFS in patients transplanted for AML, ALL, and CML

Indication/N	% Probability (range)*		
	Relapse	Survival	LFS
AML			
all cases/29	36 (14-59)	49 (28-69)	50 (31-70)
CR1/26	30 (8-53)	55 (34-77)	56 (35-77)
ALL			
all cases/23	36 (7-64)	45 (21-69)	47 (23-71)
CR1/17	35 (1-69)	41 (14-67)	42 (16-69)
CML			
all cases/28	57 (24-90)	45 (17-73)	32 (6-58)
CP1/19	38 (2-74)	79 (61-97)	49 (18-80)

*95% confidence interval

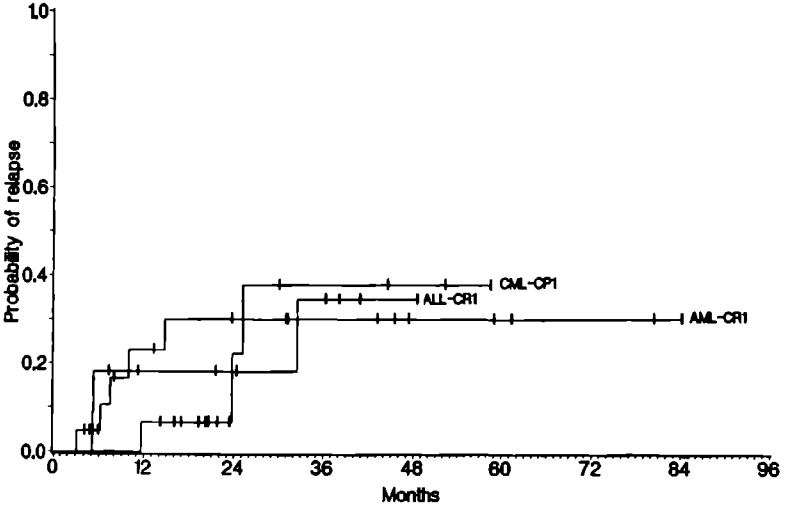

Fig 1. Probability of relapse in patients transplanted for AML-CR1, ALL-CR1, and CML-CP1. Tick marks in the relapse curves denote leukemia-free survivors

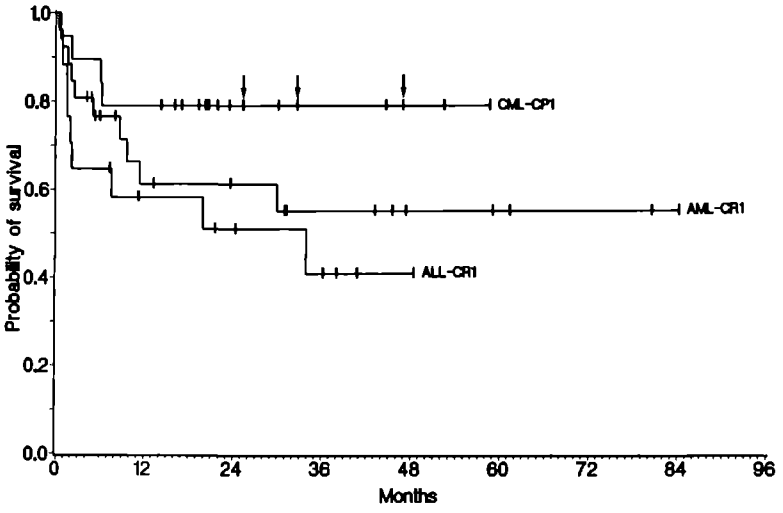

Fig 2. Probability of survival in transplants for AML-CR1, ALL-CR1, and CML-CP1. Tick marks in the survival curves denote patients who are alive. Arrows identify the three CML patients who are alive with a relapse after BMT

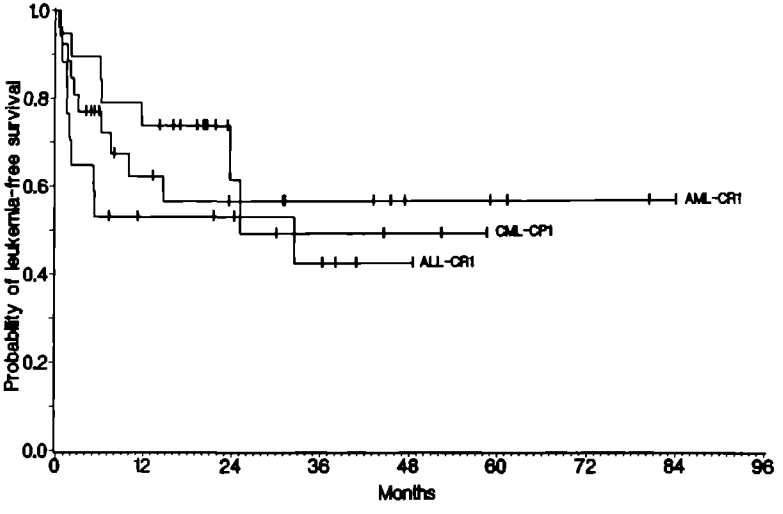

Fig 3. Probability of leukemia-free survival in patients transplanted for AML-CR1, ALL-CR1, and CML-CP1. Tick marks in the curves indicate leukemia-free survivors

for the probability of survival in patients transplanted for AML-CR1, ALL-CR1, or CML-CP1.

Probability of LFS. All 12 patients who relapsed after transplantation for AML or ALL died at 33 to 464 (median 49) and 8 to 446 (median 43) days after relapse occurred, respectively. Four of seven patients with a relapse after transplantation for CML, died at 103 to 591 (median 306) days after diagnosis of relapse. Three are alive at 49 to 663 (median 638) days after relapse occurred. The projected 3-year probability of LFS for the different diagnoses and for the patients transplanted in CR1 or CP1 is shown in Table 2. Curves of LFS after transplantation for AML-CR1, ALL-CR1, and CML-CP1 are depicted in Fig 3. No significant differences were found for the probability of LFS in patients transplanted for AML-CR1, ALL-CR1 or CML-CP1.

Hematopoietic chimerism. In the present study, hematopoietic chimerism was assessed at 6 months after BMT using red blood cell phenotyping, cytogenetic analysis, and RFLP studies as described

Table 3. Number of mixed chimeras and complete donor chimeras at 6 months posttransplant. Number and occurrence of relapses and follow-up of patients in continuous complete remission

	Mixed chimera	Complete donor chimera
No patients (%)	31 (61)	20 (39)
Relapses (%)	5 (16)	6 (30)
Occurrence of relapse range (median)*	12-32 (24)	6-32 (9)
Follow-up patients in CCR# range (median)*	6-61 (24)	6-84 (20)

*Indicates in months

#Continuous complete remission

previously.[20] Fifty-one patients with a follow-up of 6 months or more did not relapse within 6 months after transplantation. At 6 months after BMT, 31 (61%) had mixed chimeras and 20 (39%) had complete donor chimeras (Table 3). Follow-up of the 31 mixed chimeras was 6+ to 61+ (median 24+) months; 5 (16%) relapsed at 12 to 32 (median 24) months after BMT. Follow-up of the 20

Table 4. Variables examined for association with the probability of acute GVHD ≥ grade 2 and extensive chronic GVHD in univariate analysis of patients transplanted for leukemia in CR1 and CP1

	% Probability (Range)*	
Variables	Acute GVHD ≥ grade 2	Extensive Chronic GVHD
TBI		
LDR #(N=37)	12 (1-24)	12 (0-24)
HDR $(N=25)	24 (7-41)	21 (2-40)
Anthracyclines		
Without (N=32)	14 (1-27)	3 (0-27)
With (N=30)	21 (6-35)	18 (2-34)
Immunoprophylaxis		
MTX plus CsA (N=21)	10 (0-24)	11 (0-26)
CsA alone (N=31)	20 (6-34)	17 (1-33)

No significant associations were found. Probability for acute GVHD ≥ grade 2 was calculated at 100 days, for extensive chronic GVHD projected at 3 years after BMT (corrected for patients at risk)

*95% confidence interval

#TBI with the lower midline average dose-rate

$TBI with the higher midline average dose-rate

complete donor chimeras was 6+ to 84+ (median 20+) months; six (30%) relapsed at 6 to 32 (median 9) months posttransplant. Similar to our previous analysis on mixed chimerism, no

relationship between mixed chimerism and relapse after BMT was observed.[20] In univariate and multivariate analysis, pretransplant conditioning and immunoprophylaxis posttransplant were not associated significantly with the incidences of mixed chimerism (data not shown).

Variables examined for association with acute GVHD ≥ grade 2, extensive chronic GVHD, relapse, survival and LFS in patients transplanted for leukemia in CR1 and CP1. Although not significant in univariate analysis, the probabilities of GVHD ≥ grade 2, extensive chronic GVHD, survival and LFS were higher in patients irradiated with the higher midline average dose-rate, in patients prepared with anthracyclines and in patients who received CsA alone as immunoprophylaxis posttransplant (tables 4 and 5). In multivariate

Table 5. Variables examined for association with the projected 3-year probability of relapse, survival and LFS in univariate analysis of patients transplanted for leukemia in CR1 or CP1

	% Probability (Range)*		
Variables	Relapse	Survival	LFS
TBI			
LDR #(N=37)	35 (17-54)	51 (34-67)	45 (29-62)
HDR $(N=25)	36 (0-88)	78 (61-95)	52 (9-96)
Anthracyclines			
Without (N=32)	39 (19-59)	50 (32-67)	44 (27-61)
With (N=30)	20 (0-49)	75 (59-91)	63 (37-89)
Immunoprophylaxis			
MTX plus CsA (N=21)	33 (12-55)	62 (41-83)	57 (36-78)
CsA alone (N=31)	24 (0-53)	76 (61-92)	60 (34-86)

No significant associations were found

*95% confidence interval

#TBI with the lower midline average dose-rate

$TBI with the higher midline average dose-rate

analysis, the lowest probabilities of relapse and the highest probabilities of acute GVHD ≥ grade 2, extensive chronic GVHD, survival, and LFS were found in patients conditioned with both the higher midline average dose-rate and anthracyclines and who received CsA alone as immunoprophylaxis posttransplant (data not shown). Differences, however, were not significant.

DISCUSSION

Acute GVHD is one of the major complications of allogeneic bone marrow transplantation. The incidence and severity of acute GVHD is decreased when the graft is depleted of lymphocytes before BMT. Several techniques have been developed for ex vivo elimination of T lymphocytes. These include antibody-mediated elimination,[10,22-30] and physical methods[31,32] like counterflow centrifugation.[12] In the present study, the probability of acute GVHD ≥ grade 2 at day 100 after BMT was 15%. This compares favorably with the (actuarial) incidence of 39% to 82% in recipients of HLA-identical, unmanipulated grafts.[22-25,27,31] In studies on HLA-identical, T cell depleted transplants, (actuarial) incidence of acute GVHD ≥ grade 2 varied from 0 to 50%.[22-29,31,32] The patients in the different studies, however, are not comparable with regard to risk factors for acute GVHD such as pretransplant diagnosis,[33] older age of the recipient,[26,33,34] sex-mismatch,[33,34] and immunoprophylaxis posttransplant.[34] Furthermore, in recipients of T cell depleted grafts, the number of T cells infused can be factors of risk.[26,32,35-39]

In the present study, the 2-year probability of chronic GVHD and its extensive manifestation was 25% and 12%, respectively. Chronic GVHD occurred in 39 to 62% of recipients of HLA-identical, unmanipulated grafts,[22,25,27] with an extensive manifestation in 35 to 54% of cases.[23,24] In HLA-identical, T cell depleted transplants, these percentages varied from 0 to 30%.[22,25-28,31,32] and from 0 to 11%, respectively.[23-25,28,31,32]

Lymphocyte depletion of the marrow graft is associated with a higher incidence of graft failure: graft failure occurred in 0 to 35% of HLA-identical, T cell depleted transplants[10,22,23,25-29,31,32,40] and in 0 to 1% of recipients of HLA-identical, unmanipulated

marrow.[10,22,23,25,27] In recipients of T cell depleted grafts, an immunologic advantage is created for the host resulting in a higher risk of graft failure. In order to prevent this, additional pretransplant immunosuppression of the host is required. This can be achieved by in vivo application of monoclonal antibodies directed against conditioning regimen surviving host-immunocompetent cells,[41] by application of a higher total dose of fractionated TBI or by TBI in a single dose.[10,40] Certain donor derived T cells or their products can facilitate engraftment by suppressing residual host cells and/or by promoting hematopoietic stem cell growth. This can be accomplished by infusion of irradiated donor buffy coat after BMT.[42] In the present study, graft failure occurred in 5% of patients. Two mechanisms may be responsible for this low incidence: the number of T cells left in the graft and the relatively high midline average dose-rate in 50 patients.

Lymphocyte depletion of the marrow graft is associated with a higher relapse-rate: after transplantation for leukemia in CR1 or CP1, probability of relapse varied from 17% to 60% in recipients of HLA-identical, T cell depleted grafts[22,27,43-45] and from 7 to 35% in HLA-identical, unmanipulated transplants.[22,27,43-45] In the present study, the projected 3-year probability of relapse after transplantation for AML-CR1, ALL-CR1, or CML-CP1 was 30%, 35%, and 38%, respectively. Differences in relapse-rate between transplants with and without GVHD, suggest that GVHD is associated with an antileukemic effect.[1,2,8,9,11,43,46] Butturini and Gale[46] reported on a novel antileukemic mechanism after transplantation for CML in chronic phase: recipients of T cell depleted, HLA-identical transplants had a higher incidence of relapse than syngeneic transplants, suggesting that the removal of immune cells, probably T cells, is responsible for a higher relapse-rate. Slavin *et al*[47] observed a severe suppression of the generation of interleukin-2-induced cytotoxic lymphocytes against natural killer (NK)-resistant and NK-sensitive target cells after pretreatment of human bone marrow cells with Campath-1M. An analysis of the International Bone Marrow Transplant Registry (IBMTR)[48] showed that recipients of T cell depleted marrow without GVHD had a 2-year probability of relapse of 54%, which was

higher than the 22% observed in patients receiving unmanipulated grafts without GVHD. Simultaneously with T cell depletion, cells with antileukemic activity or producing antileukemic factors may be eliminated.

Attempts to decrease leukemic relapse after T cell depletion include intensification of the pretransplant regimen,[49] infusion of irradiated donor buffy coat after BMT,[42] omission of immunosuppressive drugs posttransplant, and the enhancement of donor- or host-derived NK and lymphokine-activated killer (LAK) function.[50] In our patients, preliminary data suggest that intensification of the conditioning regimen with anthracyclines may reduce leukemia relapse.[51]

In transplants for leukemia in CR1 or CP1, the probability of LFS varied from 29% to 74% in recipients of HLA-identical, unmanipulated grafts [7,27,45,52-56] and from 37% to 58% after transplantation with HLA-identical, T cell depleted grafts.[27,44,45] In a recent study from the IBMTR on HLA-identical bone marrow transplantation,[57] 5-year probability of LFS after BMT for AML-CR1, ALL-CR1, or CML-CP1 was 48%, 43% and 41%, respectively. The majority of these patients received unmanipulated marrow. In the present study, the 3-year projected LFS for AML-CR1, ALL-CR1, or CML-CP1 was 56%, 42% and 49%, respectively. Median follow-up time for leukemia-free survivors after transplantation for AML-CR1, ALL-CR1 and CML-CP1 was 31+, 30+ and 21+ months, respectively.

In comparison with studies from the literature on recipients of untreated marrow, patients receiving T cell depleted grafts using counterflow centrifugation have a lower incidence and severity of GVHD. The lower GVHD-related mortality is counterbalanced by an increase of graft failure and relapse. In the present study, the probability of survival and LFS after transplantation for leukemia in CR1 and CP1 can compete with that reported in studies from the literature on recipients of untreated grafts. Further efforts should be made to reduce relapse-rate, particularly in CML transplants.

ACKNOWLEDGMENTS

We thank the nursing staff of the Division of Hematology for unremitting care and Dr. D.Smeets (Department of Cytogenetics, University Hospital Nijmegen) for cytogenetic analysis.

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CHAPTER 4

MIXED HEMATOPOIETIC CHIMERISM AFTER ALLOGENEIC TRANSPLANTATION WITH LYMPHOCYTE DEPLETED BONE MARROW IS NOT ASSOCIATED WITH A HIGHER INCIDENCE OF RELAPSE

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Published in Blood 1989, 73: 1367-1372

SUMMARY

Using red cell phenotyping, cytogenetic analysis of blood lymphocytes, chromosome studies of bone marrow cells, and restriction fragment length polymorphisms (RFLPs) studies of peripheral blood cells, we demonstrated a high number of mixed chimeras after allogeneic bone marrow transplantation (BMT). Donor marrow from HLA-A, -B, and -DR identical, mixed lymphocyte culture (MLC) negative siblings was depleted of 98% of lymphocytes using counterflow centrifugation. Thirty-two of 48 recipients (67%) appeared to be mixed chimeras at 6 months after transplantation. The high number of mixed chimeras is probably a result of lymphocyte depletion of the marrow graft and the high sensitivity of red cell phenotyping for the demonstration of minor cell populations (at levels as low as 0.01%). The probability of relapse-free survival from 6 months to 4 years after BMT was 85% for the mixed chimeras and 65% for the complete donor chimeras. We conclude that in this study, mixed chimerism is not associated with a higher incidence of relapse.

INTRODUCTION

Persistence or endogenous repopulation of host-type lympho-hematopoietic cells after allogeneic bone marrow transplantation (BMT) for leukemia has been considered a poor prognostic sign, indicating manifest or imminent relapse.[1-3] Other studies, however, have shown that mixed lympho-hematopoietic chimerism is not necessarily an indicator of an unfavorable prognosis.[4-12] Even the persistence or reappearence of the Philadelphia (Ph^1) chromosome in patients transplanted for chronic myelogenous leukemia (CML) is not always associated with relapse.[9,13-18]

Using red cell phenotyping, cytogenetic analysis and restriction fragment length polymorphisms (RFLPs) studies, we found a high number of mixed chimeras and demonstrated that mixed lympho-hematopoietic chimerism is not an indicator of imminent relapse in patients transplanted with bone marrow, depleted of lymphocytes by counterflow centrifugation.

MATERIALS AND METHODS

Patient characteristics. Eighty-four consecutive patients were transplanted with lymphocyte-depleted bone marrow from a HLA-A, -B, and -DR identical, MLC negative sibling. Informed consent was obtained from all recipients and donors or their guardians. Twenty-six died within 6 months after transplantation due to transplant-related complications. These 26 patients were excluded from further analysis. Ten additional patients were excluded since they relapsed within 6 months after BMT. The median age of the remaining 48 patients (22 males, 26 females) included was 32 (range, 16 to 47) years.

Indications for transplantation. The indications for transplantation were: acute nonlymphoblastic leukemia (ANLL) in 14 patients (all in first complete remission [CR_1]), acute lymphoblastic leukemia (ALL) in nine patients (seven in CR_1 and two in second complete remission [CR_2]), CML in 19 patients (15 in first chronic phase [CP_1] and four in accelerated phase [AP]), myelodysplastic syndrome (MDS) in three patients (one refractory anemia [RA], one RA with an excess of blasts [RAEB], and 1 RAEB in transformation), severe aplastic anemia (SAA) in one patient, and two cases with lymphoblastic lymphoma (LL) in CR_1.

Conditioning. The standard transplant conditioning regimen consisted of cyclophosphamide 60 mg/kg body weight/d (days -6 and -5). In 22 patients anthracyclines were added to the conditioning regimen: daunorubicin 26 mg/m^2/d or demethoxydaunorubicin 7 mg/m^2/d by continuous intravenous (IV) infusion from day -7 to -2 inclusive. All patients received fractionated total body irradiation (TBI) in two equal fractions of 450 cGy each on two consecutive days (days -2 and -1) using a 18 MV photon beam linear accelerator (Saturne, CGR, Buc, France) with a midline average dose-rate of 4.1 ± 0.5 cGy/min in 31 patients, and 13.0 ± 2.0 cGy/min in 17. Lungs and eyes were shielded using individually adapted lead blocks. The mean corrected total lung-dose was 780 ± 70 cGy and 701 ± 71 cGy for

the patients with the lower and higher midline average dose-rate, respectively.

Donor marrow. Donor marrow was depleted of 98% of lymphocytes by counterflow centrifugation as described by De Witte *et al.*[19] Virtually no residual erythrocytes (< 10^8) were present in the final product. Bone marrow was infused 24 hours after completion of TBI.

Immunoprophylaxis. Immunoprophylaxis postgrafting was omitted in one patient, two patients received methotrexate (MTX) according to the Seattle regimen,[20] 20 patients received cyclosporine A (CsA) and weekly IV injections of MTX as previously described.[19] Twenty-five patients received CsA only, 3 mg/kg body weight/d by continuous IV infusion on days -1 to +14, followed by 2 mg/kg body weight/d continuous IV on to day +21, then replaced by oral administration in a dose of 6 mg/kg body weight/d on to 12 weeks after BMT, followed by a gradual tapering off with 50 mg every week.

Graft-versus-host disease. The criteria for acute graft-versus-host disease (GVHD) described by Glucksberg *et al*[21] were used for grading severity from grade I to IV.

Red cell phenotyping. Red cell phenotyping was performed as described previously.[22,23] In summary: at diagnosis a sample of patient's erythrocytes was stored in frozen state and once the donor was known, patient and donor were phenotyped for a number of red cell antigens. An antigen present on patient and absent on donor erythrocytes was chosen as marker for patient erythrocytes. An antigen present on donor and absent on patient erythrocytes was selected as donor marker. Blood transfusions were given using donor blood lacking the marker antigens. Blood samples were investigated 0.5, 1, 2, 3, 6, 9, and 12 months after BMT and annually thereafter. Depending on the technique used, minor subpopulations of erythrocytes can be detected at levels of 0.01%.

Cytogenetic analysis. Chromosome studies were performed on peripheral blood cells cultured according to Arakaki and Sparkes.[24] Patient and donor cells were analyzed before BMT and from the recipient 6 and 12 months after transplantation and once a year thereafter. Preparations were G-banded,[25] Q-banded,[26] and C-banded.[27] When the recipient and donor were of different sex, identification of the Y chromosome was used. The number of metaphases analyzed varied from one to 100 (median 25). The sensitivity of this method for the demonstration of minor cell populations depends on the number of metaphases analyzed; analysis of 25 metaphases excludes 12% mixed chimerism with 95% confidence.[28]

Chromosome studies were performed on unstimulated bone marrow samples from the recipient before BMT, 6 and 12 months after transplantation, and annually thereafter. The number of cells analyzed varied from two to 50 (median 19). Analysis of 19 metaphases excludes 15% mixed chimerism with 95% confidence.[28]

Analysis with RFLPs. Blood samples were drawn from the patient and donor before BMT and from the recipient 6 and 12 months after transplant and annually thereafter. Nucleated cells were separated by Ficoll-Isopaque centrifugation. High molecular DNA was extracted by standard techniques. Aliquots of 10 µg of purified DNA were digested to completion with *Eco*RI or *Taq*I (Boehringer Mannheim GmbH, Mannheim, FRG) using the conditions recommended by the manufacturer. Completion of digestion was monitored by agarose gel electrophoresis after staining with ethidiumbromide. Digested samples were fractionated by electrophoresis in 0.6% or 0.8% agarose gels and transferred to Biotrace RP filters (Gelman Sciences Inc, Ann Arbor, MI, USA) or Genescreen filters (Du Pont, Boston, MA, USA) by the method of Southern.[29] Hybridisation was carried out using the appropriate probes, radiolabeled by oligonucleotide priming.[30] The probes used were pAW 101,[31] pDP 34,[32] and pYNZ 2, pYNZ 22.1, pYNZ 23.[33] Hybridisation was visualised by exposing the membrane for a variable period of time at -70° C on Kodak X-Omat AR (Eastman

Kodak Company, Rochester, NY, USA) using intensifying screens. Restriction endonucleases and probes were selected to be informative for patient and donor cells. The sensitivity of RFLPs analysis for the demonstration of minor cell populations depends on the probes used and is in our hands about 10% of total DNA.

Definitions. Mixed chimerism was defined by the presence of both host- and donor-type hematopoietic cells in the recipient from 6 months after BMT onward; complete donor chimerism was defined by the presence of donor-type blood cells only in the recipient from 6 months after transplant onward; full host repopulation was defined by the presence of host-type hematopoietic cells only in the recipient from 6 months after BMT onward. In patients who relapsed, follow-up is given as the number of months between transplantation and relapse. In patients without relapse, follow-up ends on date of evaluation (July 1, 1988).

RESULTS

Red cell phenotyping. Forty-one patients were examined with red cell phenotyping at 6 months after BMT (Table 1). Twenty-one (51%) had mixed chimeras with 0.01% to 50% (median 0.05%) host-type erythrocytes. All remain in CR 9+ to 54+ (median 23+) months after transplant. Five of 18 (28%) complete donor chimeras relapsed, 13 (72%) remained in CR 6+ to 77+ (median 21+) months after transplantation. One of the 2 recipients with only host-type erythrocytes relapsed, the second remains in CR 37+ months after transplantation.

Cytogenetic analysis of blood lymphocytes. Fifteen of 39 recipients (38%) with evaluable cytogenetic data of blood lymphocytes (Table 1) had mixed chimeras with 4% to 73% (median 23%) host-type lymphocytes. Three (20%) relapsed and 12 (80%) remained in CR 12+ to 54+ (median 20+) months after transplant. Twenty-four recipients (62%) were complete donor chimeras. Four (17%) relapsed and 20 (83%) remain in CR 9+ to 77+ (median 28+) months after transplantation.

Table 1. Number of mixed hematopoietic chimeras, complete donor chimeras, and patients with host-type cells only, analyzed at 6 months after transplantation with red cell phenotyping, cytogenetic analysis of peripheral blood lymphocytes, chromosome studies of bone marrow cells, and RFLPs analysis, and number of relapses

	Total number*	Mixed chimera	Complete donor chimera	Full host repopulation
Red cell phenotyping	41 (6)	21 (0)	18 (5)	2 (1)
Cytogenetic analysis of blood lymphocytes	39 (7)	15 (3)	24 (4)	0 (0)
Chromosome studies of bone marrow cells	17 (4)	3 (1)	13 (3)	1 (0)
RFLPs analysis	17 (4)	4 (1)	12 (3)	1 (0)

*Number of patients (number of relapses)

Cytogenetic analysis of bone marrow. Three of 17 patients (18%) with evaluable cytogenetic data of bone marrow cells (Table 1) had mixed chimeras with 9%, 17%, and 33% host-type bone marrow cells, respectively. One relapsed and two remain in CR 14+ and 18+ months after BMT. Thirteen (76%) had donor-type cells only. Three (23%) relapsed and ten (77%) remain in CR 6+ to 45+ (median 15+) months after transplantation. One patient (6%) had host-type metaphases only. He was transplanted for Ph^1 chromosome positive CML. The Ph^1 chromosome reappeared 24 months after transplantation. Until now, 37 months after BMT, he remains clinically and hematologically in CR.

Analysis with RFLPs. Four of 17 patients (23%) with evaluable data of RFLPs (Table 1) had mixed chimeras. Since the autoradiograms were not scanned with a spectrophotometer, we can only speculate about the percentage of host-type cells. One (25%) relapsed, three (75%) remain in CR 30+, 34+ and 40+ months after transplantation. Twelve patients (71%) had complete donor

chimeras. Three (25%) relapsed, nine (75%) remain in CR 25+ to 54+ (median 36+) months after transplantation. One patient (6%) had only host-type cells. He remains in complete clinical and hematological remission.

Concordance of the different methods used. We analyzed the origin of hematopoietic cells at 6 months after BMT with one to four methods: method no. 1 in six patients, 2 in 23 patients, 3 in 14 patients, and 4 in five patients. In patients examined with methods no. 2, 3, or 4, outcome was concordant in 48%, 21% and 40%, respectively. Red cell phenotyping was concordant with cytogenetic studies of blood lymphocytes in 12 of 32 cases (38%; Table 2). When we corrected for the higher sensitivity of red cell phenotyping, concordance was found in 21 of 32 cases (66%; Table 2). Red cell phenotyping was concordant with chromosome studies of bone marrow cells in six of 13 cases (46%). After correction for the higher sensitivity of red cell phenotyping, concordant results were found in 11 of 13 cases (85%). Red cell phenotyping was concordant with RFLPs analysis in eight of 14 cases (57%). When we corrected for the higher sensitivity of red cell phenotyping, concordant results were found in 11 of 14 cases (79%).

Cytogenetic analysis of blood lymphocytes and bone marrow cells were concordant in ten of 15 cases (67%; Table 2). In four of five cases, discordance was due to mixed chimerism found with cytogenetic analysis of blood lymphocytes being complete donor chimeras with chromosome studies of bone marrow cells.

Cytogenetic analysis of blood lymphocytes and studies with RFLPs were concordant in ten of 15 cases (67%). In three of five cases mixed chimerism found with cytogenetic analysis of blood lymphocytes was not confirmed with studies using RFLPs.

Chromosome studies of bone marrow cells and RFLPs analysis of blood cells were concordant in all six cases.

Combination of the different methods. When results of the four different methods were combined, mixed chimerism was observed in 32 of the 48 recipients (67%) at 6 months after transplantation (Table 3). Three mixed chimeras (9%) relapsed 12, 15, and 26

Table 2. Concordance between the various methods used for analysis of the origin of red cells, blood lymphocytes, and bone marrow cells at 6 months after transplantation

	Red cell phenotyping	Cytogenetic analysis of blood lymphocytes*	Chromosome studies of bone marrow cells	RFLPs analysis
Red cell phenotyping	-	12/32 (38%)	6/13 (46%)	8/14 (57%)
Corrected red cell phenotyping#	-	21/32 (66%)	11/13 (85%)	11/14 (79%)
Cytogenetic analysis of blood lymphocytes	12/32 (38%)	-	10/15 (67%)	0/15 (67%)
Chromosome studies of bone marrow cells	6/13 (46%)	10/15 (67%)	-	6/6 (100%)
RFLPs analysis	8/14 (57%)	10/15 (67%)	6/6 (100%)	-

*number of patients with concordant outcome/total number of patients analyzed (percentage)

#corrected for the sensitivity for the demonstration of minor cell populations: 0.01% for red cell phenotyping, 12% for cytogenetic analysis of blood lymphocytes, 15% for chromosome studies of bone marrow cells, and 10% for RFLPs analysis

months after BMT. Twenty-nine (91%) remain in CR 9+ to 54+ (median 23+) months after transplantation. Nine converted to complete donor chimeras 9 to 24 (median 9) months after BMT and are in continuous CR for 9+ to 45+ (median 15+) months. Twenty patients remain mixed chimeras and in CR for 9+ to 54+ (median 24+) months after BMT. Sixteen patients (33%) were complete donor chimeras at 6 months after BMT (Table 3). Four (25%) relapsed 10, 10, 18, and 35 months after transplantation. Twelve patients (75%) remain in CR 6+ to 77+ (median 33+) months after BMT. Seven complete donor chimeras converted to mixed chimeras 9 to 48 (median 12) months after BMT and are in CR for 15+ to 77+ (median 41+) months. Three patients remain

Table 3. Origin of red cells, blood lymphocytes, and bone marrow cells at 6 months posttransplant, measured with the various methods used for analysis* (number and occurrence of relapses and follow-up of patients in continous remission)

	Mixed chimera	Complete donor chimera
No. of patients (%) (N = 48)	32 (67)	16 (33)
Relapse: N (%)	3 (9)	4 (25)
Occurrence of relapse: range (median)#	12-26 (15)	10-35 (14)
Follow-up patients without relapse: range (median)#	9-54+ (23+)	6-77+ (33+)

*Red cell phenotyping, cytogenetic analysis of blood lymphocytes and bone marrow cells, and studies of RFLPs

#Months after transplantation

complete donor chimeras and in CR at 12+, 14+ and 54+ months after transplant, respectively. The follow-up of 2 complete donor chimeras is too short (6 and 7 months, respectively) for further evaluation.

Host- and donor-type hematopoietic cells within 6 months after BMT. Due to the definition of mixed chimerism from 6 months after BMT onward, 36 patients had to be excluded from further analysis. Eighteen have been analyzed using red cell phenotyping: 11 transplant-related deaths and seven patients who relapsed within 6 months after BMT. Eight patients (73%) and four (57%), respectively, had both host- and donor-type red cells before exclusion. The exclusion of these patients from further analysis had no impact on the number of mixed chimeras in this study.

Pretransplant diagnosis and chimeric state at 6 months after BMT. Mixed chimerism was found in seven of 14 patients (50%)

transplanted for ANLL (all in CR1), in seven of nine (78%) transplanted for ALL (five in CR1 and two in CR2), in 12 of 19 patients (63%) transplanted for CML (11 in CP1 and one in AP), and in all patients with a pretransplant diagnosis of MDS, SAA, and LL. The three patients with mixed chimeras who relapsed, were transplanted for AML (one case) and CML in AP (two patients). Pretransplant diagnosis in the four relapsed complete donor chimeras was AML (two patients) and CML (two cases, both in AP).

DISCUSSION

In this study we assessed mixed chimerism from 6 months after BMT onward. Taking into consideration an erythrocyte life-span of 120 days, it is not possible to discriminate between surviving and repopulating host-type red cells within 120 days after transplantation. Although blood samples were investigated 0.5, 1, 2, 3, 6, 9, and 12 months after BMT and annually thereafter, we defined mixed chimerism from 6 months after transplant onward. With red cell phenotyping, minor subpopulations can be detected at levels as low as 0.01%, making this method very sensitive for the demonstration of mixed chimerism. However, only the red cell lineage is evaluated.

Lymphocytes may survive the pretransplant conditioning for months, making discrimination between surviving and repopulating host-type lymphocytes not possible in the early posttransplant period. Donor-type lymphocytes not damaged by the pretransplant conditioning, may be stimulated preferentially in mixed PHA-stimulated cultures,[6] resulting in an overestimation of donor-type lymphocytes. The sensitivity of cytogenetic analysis of blood lymphocytes for the demonstration of mixed chimerism, depends on the number of metaphases examined. To exclude 10% mixed chimerism with 95% confidence evaluation of at least 29 metaphases is necessary.[28] We examined a median number of 25 metaphases, excluding 12% mixed chimerism with 95% confidence.[28] Despite the relatively low median number of metaphases analyzed, we found a high number of mixed chimeras

at 6 months after BMT. We cannot exclude, however, that surviving host-type lymphocytes may have biased these results.

Compared with cytogenetic analysis of blood lymphocytes, chromosome studies of bone marrow cells have less methodological limitations: bone marrow cells are not stimulated, excluding potentially preferential stimulation of donor-type cells. Furthermore, different hematopoietic lineages are examined. A median number of 19 metaphases was analyzed, excluding 15% mixed chimerism with 95% confidence.[28] Using this method a relatively low number of mixed chimeras was found at 6 months after transplant, which to some extent may be due to to the low number of metaphases examined.

With RFLPs analysis the origin of all nucleated blood cells is examined. The number of mixed chimeras at 6 months after BMT may be influenced by surviving lymphocytes.

The origin of hematopoietic cells at 6 months after transplantation was examined with methods no. 1 to 4. Discordance in the outcome of red cell phenotyping and the other methods used was mainly due to the higher number of mixed chimeras found with red cell phenotyping, caused by the higher sensitivity of this method for the demonstration of minor cell populations. Discordance disappeared largely after correction for this higher sensitivity. Remaining discrepancy was caused by mixed chimeras, mainly found with cytogenetic analysis of blood lymphocytes at 6 months after BMT. In further follow-up discordance disappeared partly and was probably due to surviving host-type lymphocytes.

Using red cell phenotyping, Branch *et al* [4] described four patients with mixed chimeras in continuous CR at 375 to 662 days after non T cell depleted BMT for acute leukemia. Two of these four patients were also analyzed using cytogenetic analysis of blood lymphocytes and mixed chimerism was confirmed. All four patients had mixed chimeras according to our definition of mixed chimerism. Singer *et al* [5] described a mixed chimera without evidence of leukemic relapse more than 5 year after transplantation with unmanipulated bone marrow for ALL. At 1,413 days after BMT, mixed chimerism in the myeloid cell lines was

confirmed by the presence of host-type colonies in bone marrow culture studies for granulocyte progenitor cells (CFU-C). Using cytogenetic studies of bone marrow cells and peripheral blood lymphocytes, Lawler *et al* [6] found recipient cells in 12 of 45 patients (27%) who received non T cell depleted grafts from sex mismatched donors. Two remained in continuous CR at 54 and 57 weeks after BMT. According to our definition of mixed chimerism, however, only three of these 12 patients had mixed chimeras and one remained in CR for 54+ months after BMT. With cytogenetic analysis, Schmitz *et al* [7] showed mixed chimerism in four of seven patients with permanent engraftment after non T cell depleted bone marrow transplantation for leukemia. Using our definition of mixed chimerism, however, only one of these four patients had a mixed chimera: in two patients mixed chimerism was not demonstrated from 6 months after BMT onward and the fourth patient relapsed within 6 months after transplantation. Knowlton *et al* [8] described two patients with lympho-hematopoietic chimerism after BMT for hematologic malignancies. Using studies with RFLPs, in one patient host-type cells were found up to 12 months after BMT without evidence of recurrence of leukemic cells. Yam *et al* [9] reported on three stable mixed chimeras in continuous CR at 41, 53, and 58 months after non T cell depleted BMT for acute leukemia. In a prospective study of 19 patients, using cytogenetic studies and/or red cell phenotyping and/or immunoglobulin allotypes analysis, they found two transient mixed hematopoietic chimeras. Both remained in CR. One converted to all donor-type cells within 6 months after BMT; for that reason the patient did not have a mixed chimera according to our definition of mixed chimerism. Two other patients with host- and donor-type cells relapsed within 6 months after transplantation. In our study, we have excluded such patients. Yam *et al* suggested that the prognosis for long-term remission can be good in stable mixed chimeras and in patients who convert to all donor-type hematopoiesis; a progressive increase in the percentage of host-type DNA is associated with relapse. Using RFLPs studies, Bretagne *et al* [10] described mixed chimerism in all of 11 recipients of T cell depleted marrow grafts transplanted for leukemia; five remained in

continuous CR for 9+ to 31+ months after transplantation. Four of these five patients were studied ≥ 6 months after BMT and also had mixed chimeras according to our definition. Petz *et al* [11] observed mixed chimerism in 29 of 172 patients (17%) after non T cell depleted transplantation for hematologic malignancies; 24 (83%) were in continuous CR for up to 116 months after BMT. They used RBC phenotyping with standard techniques, RBC antibody typing (from 6 months after BMT onward), sex chromosomes (usually bone marrow specimens), immunoglobulin allotyping (from ≥ 100 days after the most recent transfusion onward), and RFLPs. In contrast to our study, however, mixed chimerism was defined from 14 days after BMT onward. This may have resulted in a higher number of mixed chimeras due to mature lympho-hematopoietic cells surviving the pretransplant conditioning. In a subsequent analysis, Petz *et al* [12] demonstrated mixed chimerism in 27 of 157 patients (17%), transplanted with non T cell depleted marrow for leukemia and he found a survival in continuous CR of 93% for mixed chimeras, compared with 58% for those with completely allogeneic hematopoiesis ($p < 0.001$). In our study, three of the 32 (9%) mixed chimeras relapsed compared with four of the 16 (25%) complete donor chimeras. Median onset of relapse was 15 months in the mixed chimeras and 14 months in the complete donor chimeras, with an actuarial relapse-rate at 4 year after BMT of 15% in the mixed chimeras and 35% in the complete donor chimeras. Pretransplant diagnosis had no significant impact on chimeric state nor on relapse-rate in mixed chimeras and complete donor chimeras. The percentage of host-type red cells, host-type blood lymphocytes, and host-type bone marrow cells at 6 months after BMT, did not differ between the mixed chimeras who relapsed and who did not.

Several factors may have influenced the high number of mixed chimeras: the lymphocyte depletion of the donor marrow, fractionation of the TBI,[7] the relatively low midline average dose-rate in 31 patients,[4] the immunoprophylaxis used, and the high sensitivity of red cell phenotyping for the demonstration of minor cell populations. Mixed chimerism more than 6 months after transplantation is rare in recipients of nonlymphocyte-depleted

bone marrow transplanted for leukemia. Using cytogenetic analysis, Hill *et al* [34] found mixed chimerism in 56 of 96 recipients (58%) of unmanipulated bone marrow transplanted for SAA. All 96 recipients, however, were not conditioned with TBI. Furthermore, mixed chimerism was defined from 14 days after BMT onward. A high incidence of mixed chimerism was found after transplantation with lymphocyte-depleted marrow grafts. Using cytogenetic studies Bertheas *et al* [35] found mixed chimerism in seven of 14 recipients (50%) of T cell depleted grafts compared with three of 24 recipients (13%) of unmanipulated bone marrow. Using RFLPs, Bretagne *et al* [10] demonstrated mixed chimerism in all of 11 recipients of T cell depleted marrow grafts compared with only one of 9 recipients (11%) of unmanipulated bone marrow. The higher incidence of mixed chimerism in recipients of lymphocyte-depleted bone marrow is probably caused by the removal of mature donor T lymphocytes, leading to a lower immunosuppressive effect on residual host hematopoiesis.[10,35] In the mixed chimeras residual host-type immunocompetent cells could mitigate evolving acute graft-versus-host reaction after engraftment of the donor marrow, resulting in a reduced incidence and severity of acute GVHD. In recipients of unmanipulated bone marrow, persistent mixed chimerism may be evidence of closer tissue matching between recipient and donor, with, consequently, less GVHD.[34] Like others[10,11,34,35] we observed a low incidence of severe acute GVHD. Only five of 84 consecutive patients (6%) developed acute GVHD ≥ grade II. Two of the 48 patients (4%) included in this analysis, had severe acute GVHD ≥ grade II: both were complete donor chimeras at 6 months after BMT. Due to our definition of mixed chimerism from 6 months after transplantation onward and the occurrence of acute GVHD within 3 months after BMT, we cannot indicate if the high incidence of mixed chimerism is the cause or the consequence of the low incidence and severity of acute GVHD.

The number of mixed chimeras did not differ between the two different midline average dose-rates of irradiation nor between patients with CsA and/or MTX as immunoprophylaxis. Furthermore, the addition of anthracyclines to the conditioning

regimen had no impact on the incidence of mixed chimerism (data not shown).

In conclusion, we found a high number of mixed chimeras after lymphocyte-depleted bone marrow transplantation associated with a low incidence of severe acute GVHD. Compared with complete donor chimeras, mixed chimerism was not associated with a higher relapse-rate.

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CHAPTER 5

COMPARISON OF CHIMERISM OF RED CELLS WITH THAT OF GRANULOCYTES, T LYMPHOCYTES, AND BONE MARROW CELLS IN RECIPIENTS OF BONE MARROW GRAFTS DEPLETED OF LYMPHOCYTES USING COUNTERFLOW CENTRIFUGATION

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Published in Leukemia and Lymphoma 1991, 5: 171-177

SUMMARY

We analyzed the origin of red cells (using red cell phenotyping), T lymphocytes (using both cytogenetic analysis and restriction fragment length polymorphisms studies), and of granulocytes and bone marrow cells (using restriction fragment length polymorphisms studies) in 10 consecutive patients. All received bone marrow grafts depleted of lymphocytes using counterflow centrifugation. Analyses were performed on identically timed samples from 6 months after transplantation onward.

After correction for the higher sensitivity of red cell phenotyping, results of red cell phenotyping were concordant with restriction fragment length polymorphisms studies of granulocytes and bone marrow cells in all cases studied. Outcome of cytogenetic analysis and restriction fragment length polymorphisms studies of T lymphocytes were concordant in all 10 cases. Two patients had only mixed chimerism in T lymphocytes but not in red cells nor in granulocytes; in one of these two patients the absence of mixed chimerism was confirmed with restriction fragment length polymorphisms studies of bone marrow cells; bone marrow cells of the second patient were not available for analysis with restriction fragment length polymorphisms, but cytogenetic analysis of his bone marrow cells showed only metaphases of donor-type.

These data show that red cell phenotyping represents the hematopoietic chimeric state of granulocytes and nucleated bone marrow cells. Cytogenetic analysis or restriction fragment length polymorphisms studies of T lymphocytes increases the number of instances of mixed chimerism, but this reflects the higher incidence of mixed chimerism in the clonogenic T cell population. These cells are less sensitive to radiochemotherapy than the hematopoietic stem cells and have retained the capacity for (limited) selfrenewal.

INTRODUCTION

Despite intensive pretransplant conditioning, autologous peripheral blood and bone marrow cells may persist after allogeneic bone

marrow transplantation (BMT). A recipient with both autologous and donor-type blood cells is defined as a mixed hematopoietic chimera. A high incidence of mixed chimerism has been observed after transplantation with lymphocyte depleted grafts.[1-4] The demonstration of mixed chimerism depends on the sensitivity of the method used to detect this phenomenon; furthermore, different cell populations may also be investigated. Using cytogenetic analysis, Bertheas *et al*[2] found a higher frequency of mixed chimerism in phytohemagglutinin (PHA)-stimulated blood lymphocytes than in (unstimulated) bone marrow cells. With red cell markers, isoenzyme analysis, immunoglobulin allotyping, and chromosome studies, Schouten *et al*[3] demonstrated host-type red cells in 36% of cases, recipient-type myeloid cells in 7% of cases, host-type immunoglobulins in 100% of cases, and recipient-type mitotic cells in 3 of 12 (25%) evaluable patients, respectively. In a previous study using red cell phenotyping, cytogenetic analysis of PHA-stimulated blood lymphocytes, chromosome studies of unstimulated bone marrow cells, and restriction fragment length polymorphisms (RFLPs) of peripheral blood cells, we found mixed chimerism in 51%, 38%, 18%, and 24% of cases, respectively.[4] We confirmed the observation of Bertheas *et al*[2] that mixed chimerism was more frequent in PHA-stimulated blood lymphocytes than in (unstimulated) bone marrow cells. When assessing chimeric state, difference in lifetime of the population of cells under investigation and the sensitivity of the different populations to radiochemotherapy have to be taken into account. All patients had circulating autologous erythrocytes when red cell phenotyping was used to evaluate chimerism during the first three months after transplantation.[5] Taking into consideration an erythrocyte lifetime of 120 days, we defined mixed chimerism from 6 months after transplantation onward.[4] However, lifetime of T lymphocytes may be longer than that of red cells. This may influence the number of mixed chimerism when cytogenetic analysis of PHA-stimulated blood cells is used for assessing chimerism after BMT.

In samples obtained from 10 patients consecutively attending the outpatient clinic, we analyzed the origin of red cells, T lymphocytes, granulocytes and bone marrow cells using red cell

phenotyping, cytogenetic analysis of T lymphocytes and RFLPs studies. Analyses were performed from 6 months after BMT onward on identically timed samples. All patients were transplanted in the same center with grafts depleted of lymphocytes using counterflow centrifugation.

MATERIALS AND METHODS

Patient characteristics. The median age of the ten patients (5 females and 5 males) under investigation was 32 (range 16 to 40) years. Bone marrow donors (5 females and 5 males) were human leukocyte antigen (HLA)-identical, mixed lymphocyte culture (MLC) negative siblings in 9 cases and an HLA-identical, MLC negative cousin in one case. Median age of the donors was 32 (range 18 to 47) years. Informed consent was obtained from all patients or their guardians.

Indications for transplantation. The indications for transplantation were: acute myelogenous leukemia (AML) in 2 patients, acute lymphoblastic leukemia (ALL) or acute undifferentiated leukemia (AUL) in 4 patients, chronic myelogenous leukemia (CML) in 2 patients, and T lymphoblastic lymphoma and nephroblastoma, one case each.

Conditioning. The patients transplanted for leukemia or lymphoma received standard pretransplant conditioning consisting of cyclophosphamide 60 mg/kg body weight/d (days -6 and -5) and fractionated total body irradiation (TBI). TBI was given in equal fractions of 450 cGy each on two consecutive days (day -2 and -1) using an 18 megavolt photon beam linear accelerator (Saturne, CGR, Buc, France). The midline average dose-rate was 4.2 ± 0.1 cGy/min in 5 patients and 13.1 ± 0.9 cGy/min in 4 patients. Lungs and eyes were shielded using individually adapted lead blocks. The mean corrected total lung dose was 782 ± 33 cGy/min and 715 ± 49 cGy/min for the patients with the lower and higher midline average dose-rate, respectively. In 7 patients demethoxy-daunorubicin was added to the conditioning regimen: 42 mg/m^2 by

continouos intravenous (IV) infusion.[6] The patient transplanted for nephroblastoma was conditioned with polychemotherapy consisting of iphosphamide 60 mg/kg body weight/d from day -6 to day -3 inclusive, and melphalan 180 mg/m^2 on day -2.

Donor marrow. Donor marrow was depleted of 98% of lymphocytes using counterflow centrifugation as described previously.[7] Donor marrow was infused 24 hours after completion of TBI; the patient transplanted for nephroblastoma received the graft 48 hours after infusion of melphalan.

Immunoprophylaxis. Two patients received cyclosporine A (CsA) and methotrexate (MTX), in eight recipients immunoprophylaxis consisted of CsA alone as described previously.[8]

Red cell phenotyping. Red cell phenotyping was performed as described earlier.[9,10] In summary: from all potential candidates for BMT, a blood sample was frozen at diagnosis, preferentially before any blood transfusion was given. If the patient had a suitable donor, a complete red cell phenotyping of patient and donor was performed. An antigen present on patient erythrocytes but not on donor red cells, was chosen as a patient marker; an antigen present on donor red cells but not on patient erythrocytes, was selected as a donor marker. From that moment on, only blood transfusions lacking the marker antigens were given. Analyses using the fluorescent microsphere method were performed at 0.5, 1, 2, 3, 6, 9 and 12 months after BMT and annually thereafter. The sensitivity level of this method for the demonstration of minor cell populations is high: one positive cell per 10,000 negative cells (0.01%).

Cytogenetic analysis. Peripheral blood cultures were set up from heparinized whole blood samples in medium 199 (Gibco, Paisley, Scotland, UK) supplemented with 5% (v/v) fetal calf serum (Gibco, Paisley, Scotland, UK) and with the addition of phytohemagglutinin (PHA) (Gibco, Paisley, Scotland, UK) to a final concentration of 26 µg/ml, glutamine (2 mM), heparine (25 U/ml), penicillin (125

U/ml), and streptomycin (125 U/ml). Culture time was 96 hours at 37 °C. Colcemid (Gibco, Paisley, Scotland, UK) to a final concentration of 0.2 μg/ml was added after 94 hours of incubation. Preparations were G-banded,[11] Q-banded,[12] or C-banded.[13] Karyotypes were described according to the International System for Human Cytogenetic Nomenclature.[14] When the recipient and donor were of different sex, identification of the sex chromosomes was used. The sensitivity level of cytogenetic analysis for the demonstration of a minor cell population, depends on the number of metaphases analyzed.[15] In the present study, a median number of 31 metaphases was analyzed (range 12 to 100) excluding 10% mixed chimerism with 95% confidence.

Isolation of subsets of blood cells. Peripheral blood samples were enriched for granulocytes after separation by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) centrifugation (density 1.077 g/ml, 1800 rpm, 18 °C for 20 min) and lysis of the erythrocytes in the pellet with 0.15 M NH_4CL. Purity for granulocytes in the lysed pellets was assessed by morphology of ≥ 200 cells in May-Grünwald and Giemsa (MGG) stained cytospins and varied from 95% to 99% (median 99%). In 5 cases, T lymphocytes were obtained after nylon wool filtration in a modification of the method described by De Pauw *et al.*[16] Purity for lymphocytes was demonstrated by morphology of ≥ 200 cells in MGG stained cytospins and varied from 96% to 98% (median 98%). Purity for T lymphocytes was assessed after incubation with CD2 and CD3 (Ortho Diagnostic Systems Inc, Raritan, NJ, USA) and after counterstaining with goat anti-mouse fluoresceine isothiocyanate (GAM-FITC, American Qualex International, Inc, La Miranda, CA, USA) and was ≥ 93%. Contamination with B lymphocytes was determined after incubation with CD19 and CD20 (Coulter Immunology, Hialeah, FL, USA) and after counterstaining with GAM-FITC and was ≤ 3%. In 5 patients, T lymphocytes were isolated from peripheral blood samples with immunomagnetic beads (Dynabeads M-450 Pan-T (CD2), Dynal A.S., Oslo, Norway) using the conditions recommended by the manufacturer. This positive selection results in a T cell population containing less than 1% B lymphocytes or monocytes.[17]

Preparation of bone marrow samples. Bone marrow samples were filtered through 70 μ nylon filters. After Ficoll-Hypaque centifugation (density 1.085 g/ml, 1800 rpm, 18 °C for 20 min), interphase cells were collected for DNA isolation.

RFLPs studies. DNA was extracted as described by Miller *et al.*[18] Aliquots of 5-10 μg purified DNA were digested to completion with *Taq* I (Boehringer Mannheim, GmbH, Mannheim, FRG) using the conditions recommended by the manufacturer. Completion of digestion was monitored by agarose gel electrophoresis after staining with ethidiumbromide. Digested samples were fractionated by electrophoresis in 0.8% agarose gels and transferred to Genescreen filters (Du Pont, Boston, MA, USA) by the method of Southern.[19] Hybridisation was carried out using a probe, radiolabeled by oligonucleotide priming.[20] The probe consisted of a mixture of the inserts of pYNZ 2, pYNZ 22.2 and pYNH 24[21] in equal proportions. Hybridisation was visualised by exposing the membrane for a variable period of time at -70 °C on Kodak X-Omat AR (Eastman Kodak Company, Rochester, NY, USA) using intensifying screens. The sensitivity of RFLPs for the demonstration of minor cell populations is 10% of total DNA. In mixed chimerism the minor cell population was not quantified.

Definitions. Mixed chimerism was defined by the presence of both host-and donor-type lympho-hematopoietic cells in the recipient from 6 months after BMT onward; complete donor chimerism was defined by the presence of donor-type lympho-hematopoietic cells only in the recipient from 6 months after transplantation onward.

RESULTS

The results of red cell phenotyping, cytogenetic analysis of T lymphocytes, and RFLPs studies of T lymphocytes, granulocytes and bone marrow cells, are shown in the Table.
With red cell phenotyping, four patients (UPN 40, 56, 60, and 92) appeared mixed chimeras at 6 to 36 (median 24) months after

Table. The origin of peripheral blood and bone marrow cells studied with red cell phenotyping, RFLPs studies of granulocytes, bone marrow cells, and T lymphocytes, and cytogenetic analysis of T lymphocytes in 10 patients after bone marrow transplantation with grafts depleted of lymphocytes using counterflow centrifugation

UPN	Diag-nosis	Months after BMT	Red cell pheno-typing	RFLPs studies of			Cytogenetic analysis of T Lympho-cytes
				Granulo-cytes	Bone marrow	T lympho-cytes	
36	CML	48	fhr (100)*	fhr	fhr	fhr	fhr (100)*
40	ALL	36	mc (96)	mc	mc	mc	mc (59)
54	AML	36	cdc (0)	cdc	cdc	cdc	cdc (0)
56	CML	24	mc (1.6)	mc	mc	mc	mc (4)
60	ALL	24	mc (84)	mc	mc	mc	mc (60)
92	NB	6	mc (0.8)	cdc	cdc	cdc	cdc (0)
98	ALL	12	cdc (0)	cdc	cdc	cdc	cdc (0)
99	LL	12	cdc (0)	cdc	cdc	cdc	cdc (0)
103	AML	6	cdc (0)	cdc	cdc	mc	mc (44)
114	AUL	6	cdc (0)	cdc	NA	mc	mc (83)

*in parenthesis the percentage of autologous cells

UPN: unique patient number, BMT: bone marrow transplantation, RCP: red cell phenotyping, RFLPs: restriction fragment length polymorphisms, CML: chronic myelogenous leukemia, ALL: acute lymphoblastic leukemia, AML: acute myelogenous leukemia , NB: nephroblastoma, LL: T lymphoblastic lymphoma, AUL: acute undifferentiated leukemia, fhr: full host repopulation, mc: mixed chimera, cdc: complete donor chimera, NA: not available

BMT. The percentage of autologous red cells varied from 0.8 to 96.

Using RFLPs studies of granulocytes, three recipients (UPN 40, 56, and 60) appeared mixed chimeras at 24 to 36 (median 24) months after BMT.

With RFLPs studies of bone marrow cells, nine patients were analyzed. Bone marrow cells of UPN 114 were not available for

study. Three (UPN 40, 56, and 60) had mixed chimerism at 24 to 36 (median 24) months after BMT.

Using cytogenetic analysis of T lymphocytes, mixed chimerism was observed in five recipients (UPN 40, 56, 60, 103, and 114) at 6 to 36 (median 24) months after transplantation. The percentage of autologous T lymphocytes varied from 4 to 83.

Outcome of RFLPs studies of T lymphocytes were identical to that of cytogenetic analysis of T lymphocytes. Combination of results obtained with red cell phenotyping, cytogenetic analysis of T lymphocytes, and RFLPs studies of T lymphocytes, granulocytes and bone marrow cells, showed mixed chimerism in 6 patients (60%) at 6 to 36 (median 15) months after BMT; mixed chimerism was most frequent in T lymphocytes. In the mixed chimeras, the percentage of autologous cells varied from 0.8 to 96. All remained in CR at 11+ to 52+ (median 30+) months after transplantation. The recipient with only autologous peripheral blood and bone marrow cells remained clinically and hematologically in CR at 56+ months after BMT.

Comparison of the different methods used. Outcome of cytogenetic analysis and RFLPs studies of T lymphocytes were concordant in all patients.

Results of red cell phenotyping and analysis of T lymphocytes were discordant in three patients (UPN 92, 103, and 114); UPN 92 had 0.8% autologous red cells, but no autologous T lymphocytes. This small percentage of autologous red cells is below the sensitivity level of cytogenetic analysis. UPN 103 and 114 had a high percentage of autologous T lymphocytes, but autologous red cells were not found.

Both RFLPs studies of granulocytes and bone marrow cells were performed in 9 patients. Outcome was concordant in all cases.

When we compared outcome of red cell phenotyping with RFLPs studies of granulocytes and bone marrow cells, results were concordant in nine out of 10 and in eight out of 9 cases, respectively. In one patient (UPN 92) mixed chimerism found in red cells was not demonstrated in granulocytes nor in bone marrow cells. The percentage of autologous red cells, however, was 0.8 and

below the sensitivity level of RFLPs studies. After correction for this higher sensitivity, red cell phenotyping was concordant with RFLPs studies of granulocytes and bone marrow cells in all patients studied.

Outcome of analysis of T lymphocytes and RFLPs studies of granulocytes were concordant in 8 of 10 cases. UPN 103 and 114 had mixed chimerism of T lymphocytes but RFLPs of granulocytes showed donor-type cells only.

DISCUSSION

The incidence of mixed chimerism after BMT depends on the sensitivity of the method used, on the population of cells under investigation, and on the number of T lymphocytes in the graft.[1-4] From 6 months after transplantation onward, we assessed the chimeric state of red cells, T lymphocytes, granulocytes and bone marrow cells in 10 consecutive recipients of grafts depleted of lymphocytes using counterflow centrifugation. Red cells were analyzed using red cell phenotyping with a sensitivity level for the demonstration of a minor red cell population of 0.01%; the origin of T lymphocytes was assessed with both cytogenetic analysis and RFLPs with a sensitivity level of about 10%. Granulocytes and nucleated bone marrow cells were investigated using RFLPs studies with a sensitivity level of about 10%. In three patients, red cells as well as T lymphocytes, granulocytes, and bone marrow cells were of donor origin only. One patient (UPN 36) only had autologous red cells, autologous T lymphocytes, autologous granulocytes and autologous nucleated bone marrow cells. Donor-type red cells were demonstrated at 2 and 3 months after transplantation. At 6, 12, 24, 36 and 48 months, red cell phenotyping and cytogenetic analysis of unstimulated bone marrow cells showed erythrocytes and bone marrow cells of recipient origin only. However, using cytogenetic analysis of PHA-stimulated peripheral blood cells, donor-type T lymphocytes persisted in a gradually decreasing percentage and were still detectable at 36 months; they had disappeared at 48 months. In this patient a low number of donor-type bone marrow cells may have persisted but in a percentage below the detection

level of cytogenetic analysis and RFLPs. In that case, however, donor-type red cells had to be demonstrated with the very sensitive technique of red cell phenotyping. It may be concluded that donor-type bone marrow cells have disappeared between 3 and 6 months after transplantation; due to their long lifetime, donor-type T lymphocytes have persisted for more than 30 months after rejection of the graft.

In three of 6 patients with mixed chimerism (UPN 40, 56, and 60), mixed chimerism was demonstrated in red cells as well as in T lymphocytes (both with cytogenetic analysis and RFLPs), in granulocytes, and in bone marrow cells. Three patients had mixed chimerism in one subset of cells only: UPN 92 in red cells and UPN 103 and 114 in T lymphocytes. The percentage of autologous red cells in UPN 92 was 0.8 and below the detection level of the other methods. Since red cell phenotyping was performed at 6 months after transplantation, and taking into consideration an erythrocyte lifetime of 120 days, these autologous red cells must originate from hematopoietic cells surviving pretransplant regimen; due to the relatively low sensitivity level of RFLPs, host-type hematopoietic cells were not demonstrated in bone marrow cells. UPN 103 and 114 had mixed chimerism in T lymphocytes only. Despite the high percentage of autologous T lymphocytes, mixed chimerism was not seen in red cells, granulocytes or bone marrow cells (in UPN 114, bone marrow cells were not available for RFLPs studies, but cytogenetic analysis of 15 metaphases of bone marrow cells showed donor-type cells only). If autologous hematopoietic bone marrow cells were still present but in a percentage below the detection level of RFLPs, autologous red cells should have been found. Since no autologous red cells were demonstrated, the autologous T lymphocytes must originate from clonogeneic T lymphocytes which have survived pretransplant conditioning regimen. This is in agreement with the relatively low sensitivity of T cells to radiation.[22] If these patients do not relapse, T lymphocytes may disappear in further follow-up, assuming limited selfrenewal capacity of clonogenic lymphoid cells.

In conclusion: when assessing chimerism of peripheral blood cells after transplantation with lymphocyte depleted grafts, lifetime

of pretransplant regimen surviving peripheral blood cells has to be taken into account; T lymphocytes which have survived pretransplant conditioning regimen, may increase the number of mixed chimeras. Red cell phenotyping was concordant with RFLPs studies of both granulocytes and bone marrow cells, thus red cell phenotyping may represent chimerism of nucleated bone marrow cells. Studies on mixed chimerism after BMT may be easier to interpret if more precise definitions will be used. Hematopoietic mixed chimerism should be restricted to those mixed chimeras in which the autologous cells clearly originate from autologous hematopoietic stem cells with self-renewal capacity. If mixed chimerism only occurs in the lymphoid population, it may be more appropriate to define this state as mixed lymphoid chimerism.

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CHAPTER 6

COMPARISON OF CHROMOSOME STUDIES ON PHA-STIMULATED BLOOD AND UNSTIMULATED BONE MARROW CELLS IN RECIPIENTS OF LYMPHOCYTE DEPLETED GRAFTS USING COUNTERFLOW CENTRIFUGATION

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Accepted for publication in Bone Marrow Transplantation

SUMMARY

We performed chromosome studies on 121 paired samples of phytohaemagglutinin-stimulated blood and unstimulated bone marrow cells from 57 recipients of lymphocyte depleted grafts using counterflow centrifugation. The paired samples were drawn simultaneously 6 to 108 months after transplantation. The incidence of mixed chimerism was higher in blood than in bone marrow cells, both in patients who relapsed and in patients in continuous complete remission. The higher number of mixed lymphoid chimeras is caused by autologous T lymphocytes which have survived conditioning regimen and/or by donor lymphocytes which persisted after disappearance of the marrow graft.

The type of blood and bone marrow chimerism had no significant impact on the incidence of chronic graft-versus-host disease (GVHD) but the overall incidence of chronic GVHD was too low to allow an accurate assessment.

Cytogenetic analysis is a useful method for assessing chimerism after bone marrow transplantation. Apart from its limited sensitivity for the demonstration of a minor cell population, discrepancies between chromosome studies of blood cells (lymphoid chimerism) and bone marrow cells (myeloid/erythroid chimerism) have to be taken into consideration.

INTRODUCTION

Cytogenetic analysis of blood and bone marrow cells is used to assess chimerism after allogeneic bone marrow transplantation (BMT).[1-19] Both in recipients of untreated grafts and in recipients of T cell depleted marrow the incidence of mixed chimerism was higher in blood than in bone marrow metaphases.[15,16] This discrepancy is caused by the different hematopoietic cell lineages which are analysed with chromosome studies of blood and bone marrow cells, respectively. With chromosome studies of unstimulated bone marrow cells myeloid and erythroid cells are analysed and lymphoid cells are studied with cytogenetic analysis of phytohemagglutinin (PHA)-stimulated blood cells.

In order to assess the degree of discordance between chromosome studies of blood and bone marrow cells in recipients of T cell depleted grafts using counterflow centrifugation, we extended our earlier observations[16] to the analysis of 121 paired blood and bone marrow samples of 57 patients. Chromosomal abnormalities in autologous blood and bone marrow cells and causes of discordant outcome are discussed.

MATERIALS AND METHODS

Paired blood and bone marrow samples were available in 57 patients (34 males and 23 females) transplanted between May 1982 and September 1989. Bone marrow donors were HLA -A, -B, and -DR identical, mixed lymphocyte culture negative siblings. Median age of the patients was 32 years (range 16-47). The indications for transplantation were acute myeloid leukaemia (AML, 13 patients), acute lymphoblastic leukaemia (ALL, 16 patients), chronic myeloid leukaemia (CML, 20 patients), myelodysplastic syndrome (four patients), and others (four patients). Median age of the marrow donors (39 males and 18 females) was 32 years (range 13-49). Informed consent was obtained from all patients and donors or their guardians.

Transplant procedure. The standard conditioning regimen consisted of cyclophosphamide 60 mg/kg on days -6 and -5, and of fractionated total body irradiation (TBI) in two equal fractions of 4.5 Gy each on days -2 and -1, using an 18 MV photon beam linear accelerator (Saturne, CGR, Buc, France). The midline average dose-rate was 44 ± 3 mGy/min in 28 patients and 133 ± 16 mGy/min in 29 patients. Lungs and eyes were shielded using individually adapted lead blocks. The average lung dose was 7.8 ± 0.4 Gy and 7.0 ± 0.7 Gy for the patients with the lower and higher midline average dose-rate, respectively. In 33 patients anthracyclines were added to the standard regimen, either daunorubicin 156 mg/m^2 or demethoxydaunorubicin 42 mg/m^2 given by continuous intravenous infusion. Twenty-three patients were conditioned with cyclophosphamide and TBI at the lower midline average dose-rate

but without the addition of anthracyclines (conditioning regimen 1) and 28 were conditioned with cyclophosphamide and TBI at the higher midline average dose-rate and with the addition of anthracyclines (conditioning regimen 2). Five patients received cyclophosphamide and TBI at the lower midline average dose-rate and with the addition of anthracyclines, one patient was conditioned with cyclophosphamide and TBI at the higher midline average dose-rate but without the addition of anthracyclines.

On day 0 the patients received donor marrow depleted of lymphocytes using density gradient centrifugation followed by counterflow centrifugation.[20]

Immunoprophylaxis. Immunoprophylaxis postgrafting consisted of methotrexate (MTX) in two patients,[21] both MTX and cyclosporine A (CsA) in 17 patients,[22] and CsA only in 38 patients.[22]

Cytogenetic analysis. Peripheral blood samples from the recipient and the marrow donor were analysed before BMT. Blood and bone marrow samples of the recipient were analysed at 6 and 12 months after transplantation and annually thereafter. Each pair of blood and bone marrow samples was collected on the same day. Peripheral blood cells were cultured from heparinized whole blood samples in RPMI 1640 medium (Gibco, Paisley, Scotland, UK) supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland, UK), glutamine (2 nM), penicillin (125 U/ml) and streptomycin (125 U/ml). After addition of PHA (Gibco, Paisley, Scotland, UK) to a final concentration of 26 µg/ml, blood cells were incubated for 92 hours at 37° C. After 90 hours, colcemid (Gibco, Paisley, Scotland, UK) was added to a final concentration of 0.2 µg/ml. Then the cells were washed and resuspended in 0.075 mol/l KCL for 20 minutes at 37° C. Finally, chromosome slides were prepared after several rounds of fixation in 1:3 glacial acetic acid-methanol solution. Preparations were GTG-banded,[23] QFQ-banded,[24] and/or CBG-banded.[25] In 32 cases the recipient and donor were of different sex and identification of the sex chromosomes was used. Autosomal heteromorphisms on chromosomes 1, 3, 4, 9, 13, 14, 15, 21, and 22 were used to differentiate between donor and recipient in 24

patients with sex-matched donors. In five of these recipient-donor pairs, differentiation was based on heteromorphism on two or more different autosomes. One sex-matched donor had a paracentric inversion of the short arm of chromosome 7 which was used for differentiation. Karyotypes were described according to the standard international nomenclature (ISCN).[26] The mean number of blood metaphases analysed was 26 (range 3-100). This average number allows the detection of a level of 11% mosaicism with 95% confidence.[27]

Bone marrow cells were prepared directly for cytogenetic analysis or cultured for 24 hours in RPMI 1640 medium without PHA stimulation. Further preparation and banding techniques and describing of karyotypes were performed as mentioned above. The mean number of bone marrow cells analysed was 18 (range 5-50). This average number allows the detection of a level of 16% mosaicism with 95% confidence.[27]

Statistical analysis. For statistical analysis the Fisher's exact test was used. Calculations were made using the SAS programme (SAS Institute Inc., Cary, NC, USA). *P* values < 0.05 were considered significant.

Definitions. Chimerism was defined in blood cells (lymphoid chimerism) and bone marrow cells (myeloid/erythroid chimerism). Mixed chimerism was defined as the presence of both host and donor-type metaphases; complete donor chimerism was defined by the presence of 100% donor metaphases; full host repopulation was defined by the presence of 100% autologous metaphases. Relapse was defined as the reappearance of any clinical, cytogenetic or hematological feature of the original disease.

RESULTS

Overall incidence of mixed chimerism in paired blood and bone marrow samples. Mixed chimeras were found in 64 of 121 (53%) blood samples and in 34 of 121 (28%) bone marrow samples ($p < 0.05$, Table 1).

Table 1. Number (%) of mixed chimeras in paired blood and bone marrow samples

	Number (%) of mixed chimeras		
	In blood cells	*p* value	In bone marrow cells
All patients (121 samples)	64 (53)	< 0.05	34 (28)
Patients conditioned with regimen 1 (50 samples)	27 (54)	< 0.05	16 (32)
Patients conditioned with regimen 2 (58 samples)	30 (52)	< 0.05	15 (26)

In patients conditioned with regimen 1, mixed chimeras were found in 27 of 50 (54%) blood samples and in 16 of 50 (32%) bone marrow samples ($p < 0.05$) In the patients conditioned with the intensified regimen (regimen 2) these figures were 30 of 58 (52%) and 15 of 58 (26%) ($p < 0.05$), respectively.

In patients conditioned with the intensified regimen, the incidence of mixed chimeras in blood cells was lower than in patients conditioned with regimen 1 but the difference was not statistically significant. The same was true for the incidence of mixed chimeras in bone marrow cells.

Chromosome studies in blood and bone marrow cells of patients who relapsed. Sixteen of the 57 patients (28%) relapsed. Relapse occurred at 6 to 60 (median 24) months after BMT. Indications for transplantation were CML in 12 cases (eight in first chronic phase and four in accelerated phase), and one case each of AML in first complete remission (CR1), ALL in CR1, ALL in third complete remission (CR3), and refractory anemia with an excess of blasts (RAEB) in CR1. At diagnosis and immediately before conditioning, bone marrow cells of the patients transplanted for CML were Philadelphia (Ph^1) chromosome positive. In these patients relapse occurred with the reappearance of the Ph^1 chromosome in bone

marrow cells and in one patient the Ph^1 chromosome was also found in autologous lymphocytes. Bone marrow cells of the patient transplanted for ALL-CR1 showed polyploidy at diagnosis which had disappeared immediately before conditioning for transplantation. When he relapsed autologous bone marrow cells showed del (13q) and multiple non-clonal chromosomal abnormalities. The other three patients had no chromosomal abnormalities at diagnosis or immediately before conditioning for transplantation. When relapse occurred, the patient transplanted for ALL-CR3 had pseudotriploidy in autologous bone marrow cells and bone marrow cells of the patient transplanted for RAEB-CR1 showed multiple non-clonal chromosomal abnormalities. Bone marrow cells of the patient transplanted for AML-CR1 were not available for cytogenetic analysis after relapse had occurred.

Results of chromosome studies on 38 paired blood and bone marrow samples of the 16 patients who relapsed are presented in two sections: the first section presents chromosome studies on blood and bone marrow cells before relapse occurred and the following section presents chromosome studies on blood and bone marrow cells from occurrence of relapse onward.

Chromosome studies on blood and bone marrow cells of patients in remission with subsequent relapse. Seventeen paired samples were studied before relapse occurred (Table 2). The interval between the last cytogenetic analysis performed in the patient being in remission and the first chromosome study when the patient had an overt relapse, varied from 6 to 18 (median 12) months. Mixed chimeras were found in 10 of 17 blood samples (59%) and in four of 17 bone marrow samples (24%) ($p > 0.05$). In all but three mixed lymphoid chimeras autologous cells had multiple non-clonal but no clonal chromosomal abnormalities. Autologous cells of three of the four bone marrow mixed chimeras had chromosomal abnormalities. These were non-clonal in two cases (ALL and CML), and clonal in the patient transplanted for RAEB: he showed del (12p) in four of eight autologous male bone marrow cells. The 16 female donor cells were without abnormalities.

Complete donor chimeras were found in 7 of 17 blood samples (41%) and in 12 of 17 bone marrow samples (71%) ($p > 0.05$, Table 2).

Table 2. Chimerism in blood and bone marrow (BM) cells of patients in remission with subsequent relapse (Group 1), of patients with relapse (Group 2), and of patients in continuous complete remission (Group 3)

	Number (%) of samples with					
	Mixed chimeras		Complete donor chimeras		Full host repopulation	
	in Blood	in BM	in Blood	in BM	in Blood	in BM
Group 1 (17 samples)	10 (59)	4 (24)	7 (41)	12 (71)	0 (0)	1 (5)
Group 2 (19 samples)	16 (84)#	5 (26)#	2 (11)	0 (0)	1 (5)#	14 (74)#
Group 3 (85 samples)	38 (45)	25 (29)	47 (55)	60 (71)	0 (0)	0 (0)

#$p < 0.05$

Only autologous cells were found in none of 17 blood samples and in one of 17 bone marrow samples (5%) (Table 2). The autologous bone marrow cells showed multiple but non-clonal chromosomal abnormalities.

Chromosome studies on blood and bone marrow cells of patients with relapse. Nineteen paired samples were studied from diagnosis of relapse onward (Table 2). Eighteen were from patients transplanted for CML, one paired sample was from the patient transplanted for RAEB (the patient who relapsed from AML died without further cytogenetic follow-up, and in the two patients with a relapse of ALL only bone marrow cells were analysed). Mixed chimeras were found in 16 of 19 blood samples (84%) and in 5 of

19 bone marrow samples (26%) ($p < 0.05$). Eleven of 16 mixed lymphoid chimeras (69%) had chromosomal abnormalities in autologous cells. These were multiple but non-clonal in 10 cases and clonal in one case showing the Ph^1 chromosome in two autologous cells. The autologous bone marrow cells of the five bone marrow mixed chimeras were without abnormalities in the patient transplanted for RAEB-CR1. The del (12p) which was found in four of 8 autologous bone marrow metaphases of this patient before he relapsed, had disappeared and could not be demonstrated in 12 autologous bone marrow metaphases. The other four patients were transplanted for CML and all had the Ph^1 chromosome in autologous bone marrow cells. In one case the Ph^1 chromosome was associated with a clonal abnormality [t(1;2)] and in another case multiple but non-clonal chromosomal abnormalities were found.

Two of 19 samples (11%) had only donor lymphoid cells. Complete donor chimeras were not observed in any of the bone marrow samples.

One hundred percent autologous cells were found in one of 19 blood samples (5%) and in 14 of 19 bone marrow samples (74%) ($p < 0.05$, Table 2). In the patients with 100% autologous blood cells multiple non-clonal but no clonal chromosomal abnormalities were found. In all cases with 100% autologous bone marrow cells the Ph^1 chromosome had reappeared and in almost all patients the Ph^1 chromosome was found in all autologous metaphases. In all but three cases bone marrow cells with the Ph^1 chromosome showed additional multiple non-clonal and clonal chromosomal abnormalities like t(1;10), t(2;6) together with del (7q), t(11;15), t(1;10), inv (17q), del (1q), del (7q), del (5q), 12q+ and t(12;22).

Chromosome studies in blood and bone marrow cells of patients in continuous complete remission. Forty-one of the 57 recipients (72%) did not relapse. Time interval between the last cytogenetic analysis and the end of follow-up was 2 to 13 (median 8) months. The patients who did not relapse were transplanted for AML in CR1 (13 cases), ALL in CR1 (11 cases), ALL > CR1 (three cases), CML in CP1 (eight patients) and others in six cases. Both at diagnosis and immediately before conditioning for transplantation cytogenetic

analysis of bone marrow cells was performed in 28 patients (68%) transplanted for AML (N=8), ALL (N=9), CML (N=8), and others (N=3). At diagnosis clonal chromosomal abnormalities were found in 17 of 28 patients (61%) transplanted for AML [N=5, three had t(8;21) and two had trisomy 8], ALL [N=4, one had the Ph^1 chromosome, one had t(1;12), one had hyperploidy, and the fourth patient had del (7p), del (9p), and del (12p)], CML [N=7, all were Ph^1 chromosome positive], and one case with refractory anaemia (RA) with del (13q). Immediately before conditioning for transplantation the Ph^1 chromosome was still present in the patients with CML, but chromosomal abnormalities had disappeared in all other patients, except the patient transplanted for RA. This patient was not treated before conditioning.

Follow-up varied from 29 to 116 (median 58) months. The number of paired blood and bone marrow samples analysed was 85 (Table 2). Mixed chimeras were found in 38 of 85 blood samples (45%) and in 25 of 85 bone marrow cells (29%) ($p > 0.05$). In 83% and 67% of the blood and bone marrow mixed chimaeras, respectively, autologous cells had non-clonal chromosomal abnormalities. Autologous bone marrow cells with a clonal chromosomal abnormality were found in only one patient (UPN 4). She was transplanted for t(8;21)-positive AML. At 60 and 72 months she had mixed chimerism in bone marrow cells with autologous cells showing del (13q) and multiple non-clonal abnormalities but no t(8;21). At 84 and 96 months chromosome studies were not performed on bone marrow cells but at 108 months bone marrow cells showed mixed chimerism with autologous cells still showing del (13q) and multiple non-clonal abnormalities but no t(8;21). Until now, more than 108 months after transplantation this patient remains in complete clinical, cytogenetic, and hematological remission of the original disease. Probably, this patient has developed a myelodysplastic syndrome.

Complete donor lymphoid chimeras were found in 47 of 85 cases (55%) and complete donor bone marrow chimaeras in 60 of 85 samples (71%) ($p > 0.05$, Table 2).

None of the blood or bone marrow samples had only autologous cells.

Incidence of GVHD. Since chromosome studies were not performed within 6 months after transplantation we could not assess the impact of chimerism on the incidence of acute GVHD. Eleven of 57 recipients (19%) had chronic GVHD with an onset from 3 to 6 (median 5) months after BMT. Cytogenetic analysis of blood cells at 6 months after BMT was performed in 50 patients. Three of 21 (14%) recipients with mixed lymphoid chimeras had chronic GVHD compared to six of 29 (21%) patients with 100% donor blood cells ($p > 0.05$, Table 3).

Table 3. Chimerism in blood cells of patients with chronic GVHD and of patients without chronic GVHD at 6 months after transplantation

	mixed chimeras (N=21)	*p* value	complete donor chimeras (N=29)
Patients with chronic GVHD	3 (14%)	> 0.05	6 (21%)
Patients without chronic GVHD	18 (86%)	> 0.05	23 (79%)

Chromosome studies of bone marrow cells at 6 months showed mixed chimeras or complete donor chimeras in 32 patients. None of six bone marrow mixed chimeras and 9 of 26 (35%) patients with 100% donor bone marrow cells had chronic GVHD ($p > 0.05$, Table 4).

DISCUSSION

Cytogenetic analysis is a useful method to assess chimerism after allogeneic BMT. It must be emphasized that with chromosome studies of PHA-stimulated blood cells only T lymphocytes are analysed (lymphoid chimerism) and with cytogenetics of unstimulated bone marrow cells myeloid and erythroid cells are studied (myeloid/erythroid chimerism). The difference in cell

Table 4. Chimerism in bone marrow cells of patients with chronic GVHD and of patients without chronic GVHD at 6 months after transplantation

	mixed chimeras (N=6)	*p* value	complete donor chimeras (N=26)
Patients with chronic GVHD	0 (0%)	> 0.05	9 (35%)
Patients without chronic GVHD	6 (100%)	> 0.05	17 (65%)

lineages under investigation may result in discordant outcome of chromosome studies on paired blood and bone marrow cells. Furthermore, the number of metaphases analysed has to be taken into consideration since the sensitivity of cytogenetics for the demonstration of a minor cell population depends on the number of metaphases analysed. We analysed an average number of blood and bone marrow metaphases of 26 and 18, respectively, allowing the detection of a level of 11% and 16% mosaicism with 95% confidence, respectively.[27] This is far below the sensitivity level of red cell phenotyping using the fluorescent microsphere assay[28] and molecular techniques.[29-31] In this study, the significant higher number of mixed lymphoid chimeras was not caused by the higher average number of blood metaphases analysed: when we compared pairs of blood and bone marrow samples in which the number of blood metaphases was equal to or lower than that of bone marrow metaphases the difference between mixed lymphoid and bone marrow chimeras remained significant (17 of 27 (63%) versus seven of 27 (26%), respectively, ($p < 0.05$).

Significant more blood than bone marrow mixed chimeras were found in patients with a relapse. The incidence of mixed chimerism was higher in blood than in bone marrow cells of patients in remission with subsequent relapse but the difference was not significant. The same was true for patients in continuous complete

remission. After adding up the numbers of blood and bone marrow mixed chimeras, respectively, of these two categories of patients, significant more blood than bone marrow mixed chimeras were found (48 of 102 (47%) versus 29 of 102 (28%), $p < 0.05$).
We confirmed the observation of Bertheas *et al.*[15,32] Using cytogenetics, they observed more mixed lymphoid chimeras than bone marrow mixed chimeras, both in recipients of T cell depleted and untreated grafts.

In the first year(s) after BMT the higher number of mixed chimerism in blood cells can be explained by the persistence of autologous T lymphocytes which have survived pretransplant conditioning regimen.[33,34] These T lymphocytes are expected to disappear during longer follow-up.[19] One patient had mixed lymphoid chimerism but only donor bone marrow metaphases at 6, 12 and 24 months. The percentage of autologous lymphoid metaphases was 30, 4, and 4, respectively. At 36 months after BMT only donor metaphases were found in both blood and bone marrow cells.

On the other hand, in patients who relapse or reject the graft circulating donor T lymphocytes may persist after disappearance of the graft.[5,19,35] During longer follow-up host lymphoid cells may reappear causing mixed lymphoid chimerism in patients with only autologous bone marrow cells. Ultimately, donor-type lymphoid cells will disappear leading to 100% autologous cells in both blood and bone marrow. This was observed in a patient transplanted for CML-CP1. Within 6 months after transplantation he rejected the graft and from 6 to 48 months after transplantation cytogenetic analysis of bone marrow cells showed only autologous cells with reappearance of the Ph^1 chromosome. However, at 6, 12, 24 and 36 months blood metaphases showed mixed chimerism with 42%, 5%, 23%, and 4% donor lymphocytes, respectively. Lymphoid cells of donor origin had disappeared completely at 48 months.

The incidence of mixed chimerism in both blood and bone marrow cells did not differ significantly between patients in remission with subsequent relapse and patients in continuous complete remission. In this larger population we thus confirmed

our earlier observations that mixed chimerism is not associated with a higher incidence of relapse.[16]

In most cases autologous lymphoid cells showed multiple non-clonal chromosomal abnormalities probably as a consequence of radiation damage.[5,12,17] Ph^1 chromosome positive autologous blood cells were found in only one case. This patient had a cytogenetic relapse of his CML at three years after BMT. All bone marrow cells were male and Ph^1 chromosome positive. Of the 35 blood cells analysed, two were male and Ph^1 chromosome positive, the remaining 33 cells were Ph^1 negative and from his female donor. Peripheral blood smears showed a low percentage of immature cells (1% metamyelocytes and 4% rods). We cannot exclude that the two autologous Ph^1 positive blood cells were of myeloid origin although the great majority of blood cells after 92 hours of incubation with PHA are T lymphocytes. Although T lymphocytes usually are not involved in the leukemic process[5,36] we confirm the observation of Schmitz *et al* [6] and Alimena *et al* [17] who observed Ph^1 positive cells in PHA stimulated cultures of blood cells. Two of the four patients described by Alimena *et al* [17] were in complete remission with 100% donor bone marrow cells, the other two patients had bone marrow relapse but normal leukocyte numbers and normal differentials. The authors did not rule out a lymphoid origin of these Ph^1 positive blood cells but hypothesized that these cells were morphologically unidentifiable leukemic stem cells recirculating from bone marrow or other sites.

Also autologous bone marrow cells showed multiple non-clonal chromosomal abnormalities in most instances. After relapse had occurred additional clonal abnormalities were found.[5,12,17,19] One patient without cytogenetic or hematological relapse of the original disease, developed a clonal chromosomal abnormality in autologous bone marrow cells at 5 years after transplantation probably as a sign of a myelodysplastic syndrome.

The incidence of chronic GVHD was higher in patients with 100% donor lymphoid cells than in recipients with mixed lymphoid chimerism although the difference was not statistically significant. We thus confirmed the observation of Bertheas *et al.*[33] None of six patients with bone marrow mixed chimeras had

chronic GVHD in contrast to nine of 26 with 100% donor bone marrow cells. Using red blood cell markers and cytogenetic studies of bone marrow cells, Frassoni *et al* found a significant higher score of chronic GVHD in patients with complete chimerism.[18] They suggested that mixed chimerism may be involved in the development of tolerance but on the other hand, they do not rule out that mixed chimerism could be the consequence of a low GVHD activity. In the present study, mixed chimerism was associated with less chronic GVHD but we can only speculate on the impact of blood versus bone marrow mixed chimerism on the occurrence of chronic GVHD.

In conclusion: in patients who relapsed, both before relapse occurred and after relapse had occurred, and in patients who did not relapse, the incidence of mixed chimerism was higher in blood than in bone marrow cells. This is caused by conditioning regimen surviving T lymphocytes and/or by persisting donor lymphocytes after disappearance of the marrow graft. Less mixed chimeras were observed in blood and bone marrow samples of the patients conditioned with the intensified regimen although the difference was not statistically significant. The incidence of chronic GVHD was higher in patients with only donor lymphoid cells although this difference was not statistical significant and the same was true for patients with 100% donor bone marrow cells.

ACKNOWLEDGMENTS

We thank the cytogenetic technicians for performing karyotypic analyses and W.Brouwer, MsC (Department of Radiotherapy, University Hospital Nijmegen) for the calculations on TBI.

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CHAPTER 7

SUMMARY
SAMENVATTING

SUMMARY

In chapter 1 the origin of the term chimera is explained and a definition of chimerism is given.

Chapter 2 describes the history of bone marrow transplantation. In the fifties, it was shown that animals could be protected against otherwise lethal irradiation by covering of the spleen with lead or by infusion of donor bone marrow. It became evident that this effect was not caused by an humoral factor but by colonization of the recipient's bone cavity with hematopoietic cells of the donor marrow. However, success of bone marrow transplantation in leukemia was disappointing. Recipients of genetically identical grafts died from relapsed leukemia. Most recipients of genetically non-identical grafts died ultimately from secondary syndrome (graft-versus-host disease, GVHD), but without signs of leukemia. It was recognized that GVHD was associated with a graft-versus-leukemia effect. The recognition of the major histocompatibility complex in the sixties and seventies contributed greatly to prevention of GVHD and thus to success of bone marrow transplantation. However, GVHD remained one of the major obstacles. T cell depletion of the marrow graft effectively prevented GVHD, but was associated with a higher relapse-rate. Reduction of relapse-rate in recipients of T cell depleted grafts has been achieved by intensification of the conditioning regimen.

Allogeneic bone marrow transplantation has become a worldwide accepted treatment modality for several malignant and nonmalignant diseases. The number of patients that can be cured by allogeneic bone marrow transplantation has increased by the creation of national and international bone marrow donor registries.

Results of the first eighty consecutive patients transplanted for acute leukemia and chronic myelogenous leukemia (CML) at the University Hospital Nijmegen are given in chapter 3. All patients received human leukocyte antigen (HLA)-identical sibling-marrow. In all cases, donor marrow was depleted of lymphocytes using counterflow centrifugation. Median age of the recipients was 31

years. Pretransplant conditioning consisted of cyclophosphamide and fractionated total body irradiation. In 43 patients, cytosine-arabinoside or anthracyclines were added to the conditioning regimen. Graft failure occurred in 4 of 77 evaluable patients (5%). Both the probability of acute GVHD grade 2 or more and the probability of extensive chronic GVHD was low (15% and 12%, respectively). The projected 3-year probability of relapse was 30%, 35%, and 38% after transplantation for acute myeloid leukemia (AML) in first complete remission (CR1), acute lymphoblastic leukemia (ALL) in CR1, and CML in first chronic phase (CP1), respectively. The projected 3-year probability of leukemia-free survival was 56%, 42%, and 49% in patients transplanted for AML in CR1, ALL in CR1, and CML in CP1, respectively. Probabilities of relapse, survival and leukemia-free survival in AML-CR1 and ALL-CR1 were comparable with those reported in the literature on recipients of untreated grafts. In patients transplanted for CML-CP1, probability of relapse was higher and probability of leukemia-free survival was lower than in recipients of untreated grafts. In transplants for leukemia in CR1 and CP1, preparative regimen was not associated significantly with the probability of acute GVHD grade 2 or more, extensive chronic GVHD, relapse, survival, or leukemia-free survival. The high number of mixed chimeras was indicated.

We conclude that in bone marrow transplantation for leukemia, counterflow centrifugation is a useful technique for the prevention of GVHD. In our patients transplanted for leukemia in CR1 and CP1, probability of survival and leukemia-free survival can compete with that reported in studies from the literature on recipients of untreated marrow. Further efforts have to be made to reduce relapse-rate, particularly in CML transplants.

In chapter 4 we report on the high number of mixed chimeras in recipients of marrow grafts depleted of 98% of lymphocytes using counterflow centrifugation. Marrow donors were HLA-A, -B, and -DR identical, mixed lymphocyte culture negative siblings. Methods used for the demonstration of mixed chimeras were red cell phenotyping, cytogenetic analysis of blood lymphocytes,

chromosome studies of bone marrow cells, and restriction fragment length polymorphisms (RFLPs) studies of peripheral blood cells. Thirty-two of 48 recipients (67%) appeared to be mixed chimeras at 6 months after transplantation. The high number of mixed chimeras was probably a result of lymphocyte depletion of the marrow graft and the high sensitivity of red cell phenotyping for the demonstration of minor cell populations (at levels as low as 0.01%). The probability of relapse-free survival from 6 months to 4 years after BMT was 85% for the mixed chimeras and 65% for the complete donor chimeras. We conclude that in this study, mixed chimerism is not associated with a higher incidence of relapse.

In chapter 5 we analyse the origin of red cells (using red cell phenotyping), T lymphocytes (using both cytogenetic analysis and RFLPs studies), and of granulocytes and bone marrow cells (using RFLPs studies) in 10 consecutive patients. All received bone marrow grafts depleted of lymphocytes using counterflow centrifugation. Analyses were performed on identically timed samples from 6 months after transplantation onward. After correction for the higher sensitivity of red cell phenotyping, results of red cell phenotyping were concordant with RFLPs studies of granulocytes and bone marrow cells in all cases studied. Outcome of cytogenetic analysis and RFLPs studies of T lymphocytes were concordant in all 10 cases. Two patients had only mixed chimerism in T lymphocytes but not in red cells nor in granulocytes. In one of these two patients the absence of mixed chimerism was confirmed with RFLPs studies of bone marrow cells, cytogenetic analysis of bone marrow cells of the second patient showed only metaphases of donor-type. These data show that red cell phenotyping represented the hematopoietic chimeric state of granulocytes and nucleated bone marrow cells. Cytogenetic analysis or RFLPs studies of T lymphocytes have increased the number of instances of mixed chimerism, but this reflects the higher incidence of mixed chimerism in the clonogenic T cell population. These cells are less sensitive to radiochemotherapy than the hematopoietic stem cells and have retained the capacity for (limited) selfrenewal.

In chapter 6 we describe chromosome studies on 121 paired samples of phytohemagglutinin-stimulated blood and unstimulated bone marrow cells of 57 recipients of a graft which was lymphocyte depleted using counterflow centrifugation. The paired samples were taken simultaneously 6 to 108 months after transplantation. The incidence of mixed chimerism was significantly higher in blood than in bone marrow cells. The higher number of mixed lymphoid chimeras is caused by autologous T lymphocytes which have survived conditioning regimen and/or by donor T lymphocytes which persisted after disappearance of the marrow graft.

The autologous blood and bone marrow metaphases of the mixed chimeras of the patients in remission showed multiple non-clonal abnormalities probably as a consequence of radiation damage. In almost all patients with relapsed CML the Ph^1 chromosome together with multiple non-clonal and clonal chromosomal abnormalities were observed.

Chimerism had no significant impact on the incidence of chronic GVHD but the overall incidence of chronic GVHD was too low to allow an accurate assessment.

SAMENVATTING

In hoofdstuk 1 definiëren we de term chimerisme. Iemand die met succes getransplanteerd is met beenmerg van een donor bezit in principe cellen van twee verschillende individuen. Naast zijn eigen lichaamscellen heeft hij beenmerg-en bloedcellen van de donor: hij is een chimeer. Ondanks het feit dat de term chimerisme in principe betrekking heeft op een individu en niet op een orgaanweefsel, wordt het begrip chimerisme nader gedefiniëerd naar de oorsprong van de beenmerg- en bloedcellen. Zijn na een beenmergtransplantatie alle bloed- en beenmergcellen afkomstig van de donor, dan is de chimeer compleet en heeft de getransplanteerde een complete donor chimeer. Indien één of meer cellijnen in het bloed en/of het beenmerg afkomstig zijn van de beenmergdonor en één of meer cellijnen in het bloed en/of het beenmerg afkomstig zijn van de getransplanteerde, dan is de chimeer gemengd (mixed) en heeft de patiënt een gemengde chimeer. De benaming chimeer (chimera) is ontleend aan een monsterdier (de "Chimera") uit de griekse mythologie. Het lichaam van dit monster was van voren een leeuw, in het midden een geit (het griekse woord chimera betekent geit) en van achteren een draak.

In hoofdstuk 2 worden de ontwikkelingen beschreven die uiteindelijk geleid hebben tot de mogelijkheid om beenmergtransplantaties te verrichten met behulp van beenmerg van donors. In de jaren vijftig werd aangetoond dat dieren beschermd konden worden tegen de gevolgen van beenmergvernietigende bestraling door de milt tijdens de bestraling met lood af te dekken of door infusie van beenmerg van een donor. Aanvankelijk werd het beschermend effect aan een humorale factor toegeschreven doch later werd duidelijk dat het beenmerg van de donor uitgroeide in de beenmergholte van de ontvanger. De resultaten van beenmergtransplantatie bij proefdieren en mensen met leukemie waren echter teleurstellend. Ontvangers van genetisch identiek beenmerg overleden met een recidief van de leukemie, zij die niet genetisch identiek beenmerg

kregen overleden meestal aan de gevolgen van de "secundaire ziekte", later herkend als de omgekeerde afstotingsreactie of graft-versus-host disease (GVHD). Bij proefdieren en patiënten die langer overleefden doch uiteindelijk overleden met GVHD kon meestal geen leukemie meer aangetoond worden. Blijkbaar ging GVHD gepaard met een anti-leukemisch effect. De kans op GVHD is kleiner naarmate het weefseltype van patiënt en donor méér overeenstemmen. Het weefseltype wordt genetisch bepaald en selectie van beenmergdonors op basis van het weefseltype leverde een grote bijdrage aan de preventie van GVHD en zodoende aan het uiteindelijke slagen van beenmergtransplantaties. Toch bleef GVHD veel morbiditeit en mortaliteit veroorzaken. Verwijdering van de T lymfocyten uit het donor beenmerg veroorzaakte een sterke daling in zowel de incidentie als de ernst van GVHD maar ging gepaard met een toename van het aantal recidieven. Bij deze patiënten kan de kans op recidief verminderd worden door intensivering van de conditionering.

Allogene beenmergtransplantatie is een, niet meer experimentele, behandeling voor een steeds groter aantal maligne en niet maligne ziekten. Door het oprichten van nationale en internationale bestanden van beenmergdonors kunnen steeds meer patiënten genezen worden met behulp van een allogene beenmergtransplantatie.

In hoofdstuk 3 worden de resultaten beschreven van de eerste 80 patiënten die een allogene beenmergtransplantatie ondergingen in het Academisch Ziekenhuis te Nijmegen. Transplantatie werd verricht wegens acute leukemie of chronische myeloide leukemie (CML). De beenmergdonors waren genotypisch identieke broers of zusters. Alle patiënten kregen beenmerg waaruit 98% van de lymfocyten verwijderd was door middel van tegenstroom-centrifugatie (counterflow centrifugation). De mediane leeftijd van de patiënten was 31 jaar. Conditionering geschiedde met cyclofosfamide en gefractioneerde totale lichaamsbestraling. Bovendien kregen 43 patiënten cytosine-arabinoside of anthracyclines toegediend. Het beenmerg sloeg niet aan of werd afgestoten bij 4 van de 77 patiënten (5%) die voor evaluatie in

aanmerking kwamen. De kans op acute GVHD graad II of hoger en ernstige chronische GVHD was laag en bedroeg respectievelijk 15% en 12%. De recidiefkans geprojecteerd op 3 jaar na transplantatie bedroeg respectievelijk 30%, 35% en 38% voor patiënten getransplanteerd voor acute myeloide leukemie (AML) in eerste complete remissie (CR1), acute lymfatische leukemie (ALL) in CR1 en CML in eerste chronische fase (CP1). De kans op overleving zonder leukemie geprojecteerd op 3 jaar na transplantatie bedroeg respectievelijk 56%, 42% en 49% voor patiënten getransplanteerd voor AML-CR1, ALL-CR1 en CML-CP1. In tegenstelling tot patiënten getransplanteerd voor CML-CP1 was de recidiefkans en de kans op overleven zonder leukemie voor patiënten die getransplanteerd werden voor AML-CR1 en ALL-CR1 vergelijkbaar met de resultaten uit de literatuur betreffende beenmergtransplantaties met niet gedepleteerd beenmerg. Voor de in Nijmegen getransplanteerde 80 patiënten hadden de verschillende conditioneringsschema's geen invloed op de kans op acute GVHD graad II of hoger, noch op de kans op ernstige chronische GVHD, noch op de recidiefkans en evenmin op de kans op overleving noch de kans op leukemie-vrije overleving. Gewezen werd op het grote aantal gemengde chimeren.

Geconcludeerd wordt dat tegenstroom centrifugatie de incidentie en de ernst van acute en chronische GVHD vermindert en dat de kans op overleven en op overleven zonder leukemie voor patiënten die getransplanteerd werden voor leukemie in CR1 en CP1 kan wedijveren met de resultaten zoals beschreven in de literatuur die betrekking hebben op patiënten die niet gedepleteerd beenmerg ontvingen.

In hoofdstuk 4 vermelden wij het grote aantal patiënten met gemengd chimerisme na transplantatie met beenmerg waaruit de lymfocyten verwijderd werden met behulp van tegenstroom centrifugatie. De beenmergdonors waren genotypisch identieke broers en zusters. Gemengd chimerisme werd aangetoond met behulp van fenotypering van bloedgroepen, cytogenetisch onderzoek van perifere lymfocyten en beenmergcellen en middels onderzoek van perifere bloedcellen met behulp van restrictiefragmentlengte polymorfisme (RFLP). Zes maanden na de

beenmergtransplantatie bleken 32 van de 48 getransplanteerden (67%) gemengde chimeren te hebben. Blijkbaar werd het grote aantal gemengde chimeren veroorzaakt door T cel depletie van het beenmerg en de grote gevoeligheid van de door ons gebruikte fenotypering van erythrocyten. De gevoeligheid van deze methode is 0,01%, dit wil zeggen dat één positieve cel aangetoond kan worden te midden van 9999 negatieve cellen). De recidief-vrije overleving gerekend vanaf 6 maanden tot 4 jaar na transplantatie was 85% voor de patiënten met gemende chimeren en 65% voor de patiënten die uitsluitend donor cellen hadden.

De conclusie van deze studie is dat gemengd chimerisme niet geassocieerd is met een verhoogde kans op recidief.

In hoofdstuk 5 wordt de origine geanalyseerd van rode bloedcellen met behulp van fenotypering van bloedgroepen en van T lymphocyten middels cytogenetisch onderzoek en RFLP. De oorsprong van granulocyten en beenmergcellen werd bepaald door middel van RFLP. Dit geschiedde bij 10 patiënten die beenmerg ontvingen waaruit 98% van de lymfocyten verwijderd was met behulp van tegenstroom-centrifugatie. De verschillende methoden van onderzoek werden toegepast op bloed- en beenmergmonsters die gerekend vanaf 6 maanden na transplantatie simultaan afgenomen werden. Na correctie voor de grotere gevoeligheid van bloedgroepfenotypering vonden we een volledige overeenkomst tussen de resultaten van fenotypering van rode bloedcellen en van RFLP van granulocyten en beenmergcellen. De resultaten van onderzoek van T lymfocyten met behulp van cytogenetica en RFLP stemden volledig overeen. Twee patiënten hadden uitsluitend gemengde chimeren in hun T lymfocyten maar niet in hun rode bloedcellen of granulocyten. Eén van deze twee patiënten had met RFLP ook geen gemengd chimerisme in de beenmergcellen. Cytogenetisch onderzoek van beenmergcellen van de tweede patiënt liet uitsluitend cellen van donor origine zien. Fenotypering van erythrocyten was voor deze 10 patiënten representief voor het chimerisme van granulocyten en kernhoudende beenmergcellen. Door onderzoek van het chimerisme van T lymfocyten met behulp van cytogenetica en RFLP neemt het aantal patiënten met gemengde chimeren toe

maar dit weerspiegelt het grotere aantal gemengde chimeren in de clonogene T cel populatie. Deze cellen zijn minder gevoelig voor radio-chemotherapie dan de hematopoietische stam cel en behouden hun vermogen tot (beperkte) proliferatie.

In het laatste hoofdstuk, hoofdstuk 6, worden de resultaten beschreven van het cytogenetisch onderzoek van 121 gepaarde, door fytohemagglutinine gestimuleerde bloedcellen en niet gestimuleerde beenmergcellen. Deze bloed- en beenmergmonsters waren afkomstig van 57 patiënten die getransplanteerd werden met beenmerg waaruit 98% van de lymfocyten verwijderd was door middel van tegenstroom centrifugatie. De gepaarde monsters werden tussen 6 en 108 maanden na de beenmergtransplantatie afgenomen. De incidentie van gemengde chimeren was significant hoger in bloed- dan in beenmergcellen. Méér gemengde chimeren werden aangetoond in bloed- dan in beenmergcellen. Het groter aantal gemengde chimeren in bloedcellen werd veroorzaakt door zowel T lymphocyten van patiënt-origine die de conditionering voorafgaand aan de beenmergtransplantatie overleefd hadden als door T lymfocyten van de donor die bleven circuleren na verdwijning van het transplantaat door afstoting of recidief.

De bloed- en beenmergcellen van patiënt origine in de gemengde chimeren van patiënten in remissie vertoonden multipele niet-clonale afwijkingen die het gevolg waren van de bestraling voorafgaande aan de transplantatie. Bijna alle patiënten met een recidief chronische myeloide leukemie na de beenmergtransplantatie, hadden naast het Philadelphia chromosoom multipele niet-clonale en clonale chromosomale afwijkingen. Het soort chimerisme (gemengd of compleet) had geen significante invloed op de incidentie van chronische omgekeerde afstotingsreactie waarbij opgemerkt dient te worden dat de incidentie van chronische omgekeerde afstotingsreactie in de onderzochte populatie laag was.

Ik dank allen die een bijdrage hebben geleverd aan het tot stand komen van dit proefschrift. Met name wil ik noemen Prof. dr. Theo de Witte die mij inspireerde en stimuleerde, drs. Jacqueline Vet die mij in het laboratorium leerde werken en dr. Martin Salden en zijn opvolger dr. Ewald Mensink die mij steeds bleven boeien met hun moleculair biologische technieken. Verder dank ik dr. Hans Wessels, hoofd van het Centraal Hematologisch Laboratorium, dr. Frank Preijers en al hun medewerkers voor hun hulp en begeleiding tijdens en na mijn verblijf in het laboratorium. In Marcel Schellart, verpleegkundig hoofd van de afdeling Bloedziekten dank ik alle verpleegkundigen voor hun toegewijde en professionele zorg voor de patiënten.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 13 september 1951 te Geleen geboren. Aan het Bisschoppelijk College te Sittard behaalde hij in 1970 het diploma Gymnasium-ß. Hij studeerde Geneeskunde aan de Katholieke Universiteit van Nijmegen en werd op 18 november 1978 tot arts bevorderd. In 1979 was hij dienstplichtig eerste luitenant-arts. Van 1980 tot 1985 werd hij opgeleid tot internist in het Sint Joseph Ziekenhuis toentertijd te Eindhoven (opleider destijds dr. P.F.L. Deckers, internist). Na 8 maanden werkzaam te zijn geweest op de afdeling Algemene Interne Geneeskunde van het Academisch Ziekenhuis Sint Radboud te Nijmegen, werkt hij sedert 1 april 1986 op de afdeling Bloedziekten en is sedert 1 augustus 1990 chef de clinique van de beenmergtransplantatie afdeling.

ISBN 90-9005862-1