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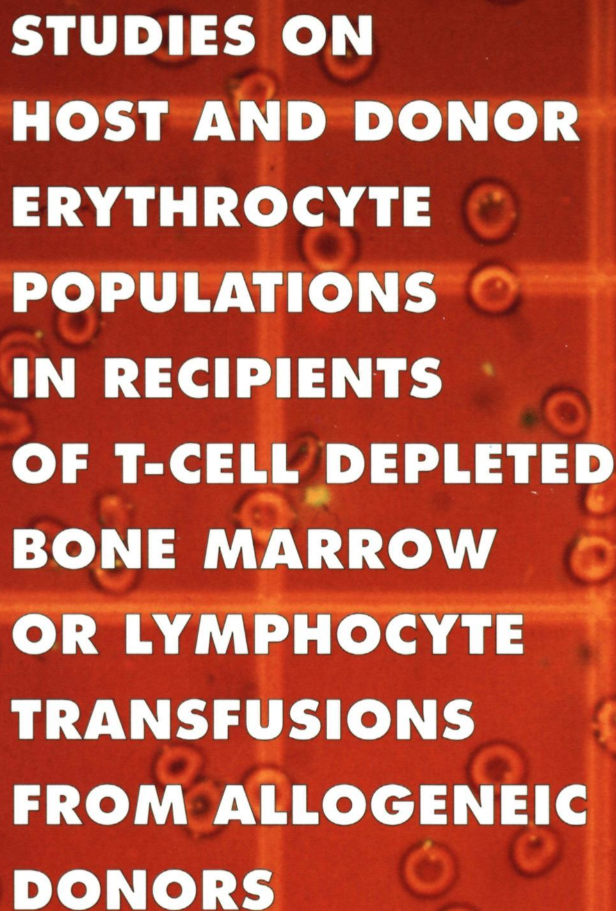

STUDIES ON HOST AND DONOR ERYTHROCYTE POPULATIONS IN RECIPIENTS OF T-CELL DEPLETED BONE MARROW OR LYMPHOCYTE TRANSFUSIONS FROM ALLOGENEIC DONORS

BRIGIT BÄR

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Cover illustration photograph of marker antigen positive erythrocytes, intensively labelled with fluorescent microspheres, among marker antigen negative erythrocytes
Photograph kindly provided by dr A J M de Man

STUDIES ON HOST AND DONOR ERYTHROCYTE POPULATIONS IN RECIPIENTS OF T-CELL DEPLETED BONE MARROW OR LYMPHOCYTE TRANSFUSIONS FROM ALLOGENEIC DONORS

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Drukkerij Benda, Nijmegen, 1996

Aan Rob
en mijn familie

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Chapter 1.

Introduction

Allogeneic bone marrow transplantation and T-cell depletion

Allogeneic bone marrow transplantation (BMT) is the treatment of choice for various, malignant and nonmalignant hematological diseases.[1-8]

During and after successful allogeneic BMT both the hematopoietic and the immune system of the recipient are gradually replaced by the lymphohematopoietic system of the marrow donor. Usually complete donor chimerism has been observed after allogeneic BMT, i.e. all evaluated lymphohematopoietic cells are of donor origin. The coexistence of donor and recipient lymphohematopoietic cells post-BMT has been defined as mixed chimerism.[9]

Success of allogeneic BMT is often limited by post-transplant complications such as graft-versus-host disease (GVHD), graft rejection or leukemic relapse. The major problem after allogeneic BMT is GVHD that is associated with a high incidence of fatal infections due to prolonged duration of a severely compromised immune state. GVHD is mainly caused by the donor T-lymphocytes present in the marrow inoculum. Effective in vitro depletion of T-lymphocytes, achieved by immunological or physical separation techniques, clearly reduces the incidence and severity of GVHD.[10-15] The incidence of moderate to severe acute GVHD in recipients of an unmanipulated HLA matched sibling donor graft varies from 40 to 50% compared to 10 to 15% after transplantation with a T-cell depleted graft.[16-19] T-cell depletion, however, is associated with an increased risk of graft failure and of leukemic relapse, especially in patients with chronic myeloid leukemia (CML).[12,14,15,18,20-24]

Relapse rate after allogeneic bone marrow transplantation; influence of T-cell depletion[12,15,18,20-28]

	No T-cell depletion range %†	T-cell depletion range %†	Nijmegen %‡
AML CR1 *	12-20	30-40	39
ALL CR1-2	6-28	29-40	32
CML CP1	6-25	25-65	39

* AML CR1 acute myeloid leukemia in first complete remission,
ALL CR1-2 acute lymphoblastic leukemia in first and second complete remission,
CML CP1 chronic myeloid leukemia in first chronic phase

† Numbers are reported probabilities of relapse ≥ 2 years after BMT.

‡ Numbers are probabilities of relapse 3 years after BMT.

Moreover, incidences of mixed chimerism, varying from 51% to 100% have been reported after T-cell depletion [29-33] This is clearly higher compared to the incidence of mixed chimerism after BMT with non-T-cell depleted grafts [29,34,35] Studies have demonstrated that mixed chimerism, even with high percentages of autologous cells, does not necessarily predict relapse [33,34,36,40]

Since 1981 more than 400 patients, treated at the University Hospital Nijmegen, have received allogeneic bone marrow partially depleted of T-lymphocytes to prevent GVHD T-cell depletion was performed by density gradient centrifugation followed by counterflow centrifugation using a single chamber rotor or a four-chamber rotor [38,39] To attain a fixed number of 1 0x10^6 T-cells /kg patient body weight in the graft, lymphocyte fractions were added to the maximally depleted graft fraction From 1986 onwards the standard conditioning regimen, consisting of cyclophosphamide and fractionated total body irradiation was intensified by adding anthracyclines in order to reduce relapse rate In the patients treated with anthracyclines the probability of relapse was significantly lower compared to the historic control group Transplant related mortality did not increase by the addition of anthracyclines, resulting in a significantly higher probability of disease free survival in this study group [40-42]

Donor lymphocyte transfusions for treatment of relapse after BMT

CML is a malignant stem cell disorder characterized cytogenetically by the Philadelphia (Ph) chromosome a specific translocation of the Abelson (ABL) oncogene on chromosome 9 to the breakpoint cluster region (BCR) on chromosome 22 At the molecular level this translocation can be detected by polymerase chain reaction (PCR) of BCR-ABL fusion molecules, after isolation of cellular RNA a reverse transcriptase reaction (RT) is performed followed by amplification by PCR After Southern transfer the PCR product is then analyzed by hybridization using a radioactively labeled c-ABL oligonucleotide

As mentioned above, T-cell depletion of the graft increases the risk of relapse, especially in CML patients [21] Part of the reduced antileukemic activity of T-cell depleted grafts can be explained by the reduced incidence of GVHD In CML patients an additional antileukemic effect mediated by T-lymphocytes, independent of GVHD has been suggested [21] Relapse rates in patients transplanted for CML in first chronic phase of 40-60% have been reported after T-cell depletion compared to 10-20% after BMT with unmanipulated grafts [15,18,43]

One of the treatment options for CML patients relapsed after BMT is Interferon alpha (IFNα),

which can achieve a cytogenetic remission in a minority of the patients While IFNα may prolong survival, it is not a curative treatment [44,45] Second BMT with unmanipulated marrow from the original donor is a potentially curative therapeutic option but is associated with a high risk of treatment related mortality [46,47]

In 1990 the first three patients were reported who were successfully treated for relapse after BMT with leukocyte infusions from the original marrow donor All 3 patients achieved complete hematologic and cytogenetic remission [48] Two years later Cullis et al described 2 patients with cytogenetic relapse of CML after BMT, who achieved cytogenetic and subsequently molecular remission of CML after donor leukocyte infusions [49] Recently various studies describing successful treatment of CML patients relapsed after BMT with donor leukocyte transfusions have been published [50-54] In a review of the results reported by 27 transplant centers in the European Group for Blood and Marrow Transplantation (EBMT) 54 out of 75 evaluable CML patients (73%) achieved complete hematologic and cytogenetic remission with this immune treatment [55] Especially in patients with relapse of CML after BMT, donor lymphocytes are capable of exerting a graft versus leukemia (GVL) effect. In the EBMT study a close association between the development of GVHD and/or myelosuppression after donor leukocyte infusions and the GVL effect was found The response rate was 91% in CML patients developing GVHD and/or myelosuppression Conversely, 45% of the patients without GVHD and/or myelosuppression responded favorably to this treatment.

Allogeneic BMT across the ABO barrier

Major ABO incompatible BMT is the situation in which the recipient has the potential to express anti-A and/or anti-B antibodies against donor AB antigens Major ABO incompatibility between recipient and donor occurs in about 10-15% of HLA matched allogeneic bone marrow transplantations Major ABO incompatibility is not an obstacle to successful outcome after BMT No increased risk of graft rejection, GVHD or mortality related to ABO incompatibility have been observed [56,57] Major ABO incompatibility carries the risk of an acute hemolytic transfusion reaction by anti-A or anti-B antibodies of recipient origin at the time of infusion of the donor marrow This can be prevented by reduction of the antibody titers in the recipient or by removal of erythrocytes from the graft [58-63] Immunohematologic complications like delayed hemolytic anemia, delayed onset of erythropoiesis and pure red cell aplasia (PRCA) have been reported after major ABO incompatible BMT [64-69] Delay in erythropoiesis after major ABO incompatible BMT can be explained by the interaction of anti-A or anti-B antibodies with donor erythroid precursors

expressing the A and/or B antigens [70]

In minor ABO incompatibility the donor has the potential to express anti-A and/or anti-B against AB antigens on recipient erythrocytes Several studies have reported the early appearance (1 to 6 weeks) after BMT of antirecipient antibody sometimes causing manifest hemolysis [69 71 73] This phenomenon has been described after minor ABO solid organ transplantation as well and is explained by the adoptive transfer of donor lymphocytes in the marrow or solid organ graft [74-77] The disappearance of this donor antirecipient antibody from 3 months after BMT onward suggests the induction of tolerance for the recipient A and/or B antigens which are widely distributed also in non-hematopoietic tissues [56 71 72] An alternative explanation could be the absorption of antibody, produced by donor lymphocytes, by recipient tissues expressing the A and/or B antigens

Techniques to evaluate hematopoietic chimerism after BMT

To distinguish donor and recipient lymphohematopoietic cells after allogeneic BMT several techniques can be used such as cytogenetic analysis, determination of DNA restriction fragment length polymorphisms (RFLP), polymerase chain reaction (PCR)-based determination of variable numbers of tandem repeats (VNTR) or PCR amplification of microsatellites, analysis of immunoglobulin allotypes, in situ hybridization (ISH) in case of sex mismatched transplant situation or red blood cell antigens [35 78-88]

In this thesis a newly developed sensitive technique to determine red blood cell populations has been used to study the course of recipient erythrocytes after BMT and after donor lymphocyte infusions and to assess the incidence of mixed chimerism from 6 months after BMT onwards We used this technique to monitor donor erythrocyte engraftment especially in the case of major ABO incompatibility between recipient and donor

Analysis of host and donor erythrocyte populations early after BMT requires an adapted transfusion policy [88] Blood samples for red cell phenotyping are drawn from all potential BMT candidates, preferentially before any erythrocyte transfusion is given As soon as the marrow donor is known, patient and donor are phenotyped for the following red cell antigens ABO, CcDEe, MNSs, P1, Kk, Kp^a Kp^b, Fy^a Fy^b, Jk^a Jk^b, Lu^a Lu^b Afterwards marker antigens, i e antigens present in the donor and absent in the host and vice versa, are determined and erythrocytes of blood group O lacking the marker antigens are transfused Both recipient and donor markers, recipient marker only, donor marker only or no markers were available in about 60, 20, 10 and 10 percent of the BMT patients respectively

Initially differential agglutination techniques were used to measure host and donor erythrocyte populations Although the results were informative, limited sensitivity and reproducibility were obvious disadvantages of these semiquantitative techniques [88] Since 1986 a fluorescent microsphere method gradually replaced the agglutination techniques [89] In this assay erythrocytes are first incubated with an IgG antibody directed against the marker under investigation After several washings, anti-human IgG coated fluorescent microspheres are added Contact between red blood cells and microspheres is enhanced by centrifugation Marker antigen positive erythrocytes are labeled intensively with fluorescent microspheres and can be visualized and counted under a fluorescence microscope The sensitivity level of this assay is one positive cell per 10000 negative cells This technique evaluates only the erythroid lineage and this may not reflect the hematopoietic chimerism of the other cell lineages Another disadvantage is the relatively long life-span of mature erythrocytes, which may mask rapid processes like graft rejection

Conventional cytogenetic analysis can only be performed on metaphase cells, which limits the number of available cells The sensitivity level of cytogenetic analysis for the detection of a minor cell population is dependent on the number of cells studied, e g examining 32 cells allows the detection of a level of 9% mosaicism with 95% confidence [90] Bone marrow cells are harvested directly and/or after a 24-hour culture without mitogens Metaphase cells in the bone marrow predominantly belong to the myeloid and erythroid lineage Cytogenetic analysis of peripheral blood is performed after culturing of whole blood in the presence of phytohemagglutinin resulting in a proliferation of T-lymphocytes If patient and donor are of different sex, identification of the sex chromosomes can be applied In case of sex match, the applicability of cytogenetic analysis in studying chimerism is dependent on heteromorphisms of the autosomal chromosomes between recipient and donor These can be found in 20-25% of the donor/recipient sibling pairs using different banding techniques An advantage of chromosome studies after BMT is the possibility to look for cytogenetic abnormalities, which were present in the malignant clone of the recipient at diagnosis before BMT

In case of a sex difference fluorescence in situ hybridization can be applied to discriminate between patient and donor cells [86] This technique is performed on interphase cells which allows the examination of up to 400 nucleated cells A signal that discriminates one to five cells out of 400 cells is considered background This limits the sensitivity of this technique to a level of one percent

Following digestion with restriction endonucleases, variations in DNA sequences among individuals result in DNA fragments of differing lengths known as RFLPs. Subsequently these fragments are separated according to their molecular weight by electrophoresis and transferred to a membrane (Southern blotting). Hybridization with radiolabeled probes is then performed to visualize the fragments.[79-81] Patients and sibling donors are virtually always distinguishable using a panel of restriction endonucleases and probes. The sensitivity of this assay is 5-10%.

A more sensitive molecular method to study mixed chimerism is PCR amplification of regions with a variable number of tandem repeats (VNTR) or microsatellites. The human genome contains hypervariable regions, which are highly polymorphic due to variation in the number of tandemly repeated sequences in different alleles. Microsatellites are short, interspersed repetitive DNA sequences, which are highly polymorphic. Studying multiple VNTR loci or microsatellites, discrimination between patient and sibling donor is almost always possible. The reported sensitivity level of these techniques varies from 0.1-5% depending partly on the number of cells studied.[83,84] DNA based techniques are applicable to all nucleated cells. This gives the opportunity, after isolation of subsets of blood cells, to study mixed chimerism in these cell populations simultaneously.[91,92]

Outline of investigation

Chapter 2 contains observations on donor erythrocyte repopulation measured with the fluorescent microsphere method from 0.5 to 6 months after BMT. The course of autologous erythrocytes was studied in this time period and compared between patients who relapsed or remained in remission during follow up. In a previous study the fluorescent microsphere method has been applied to study erythrocyte survival in orthopaedic patients. These patients received one erythrocyte transfusion positive for an antigen that was negative in the patient. The transfused erythrocytes were detectable for a period of maximally 150 days.[93] Extrapolating this 150 days survival to the course of recipient erythrocytes after BMT, mixed erythrocyte chimerism was defined from 6 months after BMT onward. In patients transplanted between 1981 and 1988 the incidence of mixed erythrocyte chimerism was studied at various time points from 6 months onward. The follow up of mixed red cell chimeras was compared between patients with and without relapse more than 6 months after BMT.

Chapter 3 describes the results of a retrospective analysis of 144 patients transplanted in

Nijmegen from 1981 to 1991. In this time period three consecutive conditioning regimens were applied. The influence of the conditioning regimen on the incidence of mixed erythrocyte chimerism was studied. The incidence and severity of both acute and chronic GVHD were compared between the three patient groups. The relation between the incidence of mixed erythrocyte chimerism and the presence of acute and chronic GVHD was addressed.

The significance of mixed erythrocyte chimerism in CML and acute leukemia was studied. This was focussed on a relation between the percentages of autologous erythrocytes from 6 months after BMT onward and the occurrence of relapse.

Studying the course of autologous erythrocytes was one of the ways to monitor the effects of donor leukocyte transfusions, administered to 6 CML patients who relapsed after BMT. This treatment modality and its results are described in Chapter 4. Chimerism was studied in these patients using erythrocyte and cytogenetic markers. Monitoring of residual disease was performed by cytogenetic analysis of the Ph chromosome and by PCR of the BCR-ABL fusion molecules.

In Chapter 5 donor erythrocyte repopulation was analyzed in 30 patients after major ABO incompatible BMT and compared to a control group of 58 patients who received an ABO compatible or minor incompatible graft. Pretransplant anti-donor, anti-A and anti-B antibody titers and the course of these antibodies after BMT were compared between those patients who developed pure red cell aplasia (PRCA) and those with timely recovery of donor erythropoiesis. The incidence of immunohematologic complications in the major ABO incompatible group was compared to the incidence reported in the literature to study a possible effect of the elutriation of the graft.

The course of anti-donor, anti-A and anti-B antibodies after major ABO incompatible BMT was compared between recipients of elutriated and non-elutriated grafts in a study presented in Chapter 6, which was performed at the Johns Hopkins Oncology Center.

In the same study the occurrence of the antirecipient antibody early and late after BMT was analyzed and compared between recipients of minor ABO incompatible elutriated and non-elutriated marrow grafts.

In our experience the microsphere method is a very sensitive and relatively simple technique to monitor recipient and donor erythropoiesis after BMT and after treatment with donor leukocyte transfusions.

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Chapter 2.

Host and donor erythrocyte repopulation patterns after allogeneic bone marrow transplantation analysed with antibody coated fluorescent microspheres

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Summary

Analysis of erythrocyte populations, using red blood cell antigen differences between host and donor as marker, was performed with a sensitive fluorescent microsphere assay to monitor marrow engraftment and mixed red cell chimerism after allogeneic bone marrow transplantation (BMT) An adapted transfusion policy, using marker negative erythrocyte transfusions, was required for this analysis In all patients the marrow graft was depleted of lymphocytes by counterflow centrifugation Thirty-seven patients were evaluable for donor repopulation at one or more points in the first 6 months after BMT At 0 5 month donor erythrocytes were detectable in 19 out of 22 patients

At 6 months donor erythrocytes were detectable in 100% of the evaluable patients In the first 3 months after BMT the average donor erythrocyte repopulation in recipients of major ABO mismatched transplantations was delayed Thirty-eight patients were evaluable for chimerism at 6 months or later after BMT A high incidence of mixed red cell chimerism was observed varying from 50% to 71% at different points of analysis Mixed red cell chimerism with low percentages (<1%) of host cells was not related with relapse, nor did high percentages (>10%) of host cells necessarily indicate relapse

Introduction

Effective T-cell depletion of the marrow graft can prevent acute graft-versus-host disease after BMT [1,2] A high incidence of mixed chimerism has been reported after T-cell depletion in BMT [3,4,5] To distinguish host and donor type lymphopoietic and hematopoietic cells after allogeneic bone marrow transplantation several techniques such as cytogenetic analysis, determination of DNA restriction fragment length polymorphisms (RFLP), analysis of immunoglobulin allotypes or red blood cell antigens, can be used [6,7,8,9,10] We have employed standard differential agglutination techniques to determine red blood cell populations after BMT [11] During an adapted transfusion policy, the results of these techniques were very informative However, with the previously used differential agglutination techniques donor erythrocytes were only detectable in a minority of the patients at 0 5 month after BMT Recently a much more sensitive technique for detection of minor erythrocyte populations, using fluorescent microspheres, has been described [12] This report contains observations on donor erythrocyte repopulation measured with the fluorescent microsphere method from 0 5 to 6 months after BMT

Since all our patients received T-cell depleted donor marrow, we were interested in the incidence of mixed red cell chimerism at 6 months or later after BMT. Moreover we studied a possible correlation between autologous red cell patterns and relapse after BMT.

Materials and Methods

From May 1981 to May 1988 91 patients underwent an allogeneic bone marrow transplantation. In 90 patients the donor was a HLA-A, B and DR identical, mixed lymphocyte culture (MLC) negative sibling. One patient differed from the donor sibling in one A locus but the MLC was negative. The median age of the patients was 31 years (range 16-47). The indications for transplantation in these patients were acute myeloid leukemia, n=24; acute lymphoblastic leukemia, n=28; chronic myeloid leukemia, n=29; myelodysplastic syndrome, n=6; other malignant disease, n=2; and severe aplastic anaemia, n=2.

Transplant procedure.

In all patients the standard conditioning regimen consisted of cyclophosphamide 60 mg/kg on days -6 and -5 and of fractionated total body irradiation of 4.5 Gy/d on days -2 and -1, administered with a 18 MV photon beam linear accelerator. The midline average dose rate was 4.1 ± 0.5 cGy/min in the first 60 transplanted patients and 12.3 ± 3.2 cGy/min in the following 31 patients. Lungs and eyes were shielded using individually adapted lead blocks. The corrected median lung dose was 780 ± 70 cGy and 720 ± 70 cGy for the patients receiving the lower and higher midline average dose rate respectively.

In the last 41 patients anthracyclines, either daunorubicin 26 mg/m^2/d or demethoxydaunorubicin 7 mg/m^2/d, given as a continuous intravenous infusion for 6 days from day -7 to -1, were added to the conditioning regimen.

On day 0 the patients received donor marrow depleted of 98% of lymphocytes. This was achieved by density gradient centrifugation followed by counterflow centrifugation.[13] Less than 10^8 residual erythrocytes were present in the marrow graft.

Immunoprophylaxis.

Of the first 11 patients 9 patients received only methotrexate (MTX) as immunoprophylaxis postgrafting[14] and two patients had no prophylaxis. Subsequent patients were treated with either MTX and cyclosporin A (n=34) or cyclosporin A only (n=46).[15,16]

Erythrocyte markers.

Analysis of host and donor erythrocyte populations early after BMT, using red blood cell antigens, requires an adapted transfusion policy.[11] If a patient is considered a potential BMT candidate a blood sample for red cell phenotyping is frozen, preferentially before any erythrocyte transfusion is given. As soon as the donor is known, a complete red cell phenotyping of patient and donor is performed and differing antigens, i.e. marker antigens, if present, are determined. In Table I the availability of markers in the 91 transplanted patients is shown. We always attempted to give erythrocyte transfusions lacking the marker antigens. If it was impossible to give transfusions negative for both markers, donor marker negative blood was transfused.

In case of blood transfusions unselected for the marker antigens, we assumed these transfusions to be marker positive. Considering a maximum survival of erythrocytes of 150 days, the marker percentages determined the first 150 days after the unselected transfusion were excluded from analysis.

Table I. The availability of erythrocyte markers in 91 BMT patients

	No. of patients	%
Recipient and donor marker	58	64
Only recipient marker	15	16
Only donor marker	8	9
No markers	8	9
Markers unknown	2	2

Analysis of erythrocyte markers.

For the initial BMT proceedings in our institute, differential agglutination techniques were used to measure host and donor red cell populations.[11] The disadvantages of these techniques are a limited sensitivity of about 1% and limited reproducibility because of intertechnologist variation. Since 1986 a fluorescent microsphere method gradually replaced the agglutination techniques.[12] In this assay red blood cells are first incubated with an IgG antibody directed against the marker antigen under investigation. After washing the erythrocytes several times, anti human IgG coated fluorescent microspheres are added. The contact between cells and microspheres is enhanced by centrifugation. Marker antigen positive erythrocytes are labeled intensively with fluorescent microspheres and can be visualized and counted under a fluorescence microscope. The sensitivity

level of this assay is one positive cell per 10000 negative cells. Blood group antigens examined in this study are A1, A2, B, DCcE, Jk^a, Jk^b, Fy^a, Fy^b, K, MSs and Kp^a. Time schedule for analysis was 0.5, 1, 2, 3, 6, 9 and 12 months after BMT and annually thereafter.

Statistical analysis.

Statistical analysis was performed by the Fisher's exact test and the Student's t-test.

Definitions.

Mixed chimerism was defined by the coexistence of host and donor type hematopoietic cells in the recipient at 6 months or later after BMT; complete donor chimerism was defined by the presence of donor type hematopoietic cells only in the recipient from 6 months after BMT onwards.

Results

Donor erythrocyte repopulation

Data on donor erythrocyte repopulation from 0.5 to 6 months after BMT, as measured with the microsphere method, are given in Table II. 37 patients were studied at one or more points after BMT. Patients were considered not to be evaluable mainly because of lack of donor marker or because they received unselected transfusions within 150 days before analysis. If a patient relapsed, data on donor erythrocytes after date of relapse were excluded from analysis.

As shown in Table II donor cells were detectable in 86% of the evaluable patients at 0.5 month after BMT. Data from differential agglutination techniques at 0.5 month after BMT revealed donor erythrocytes only in five out of 24 evaluable patients (data not shown). From 0.5 to 6 months after BMT the percentages of donor red cells increased steadily from a median of 0.04% donor cells at 0.5 month to 88.8% at 6 months (Table II).

Donor erythrocyte repopulation in major ABO mismatched transplantations

At 1, 2 and 3 months after BMT donor erythrocytes were not detectable with the microsphere method in four, one and two patients respectively. All these patients were recipients of major ABO mismatched transplantations. Fig 1 shows the course of donor red cell repopulation of recipients with or without major ABO mismatched transplantations from 1 to 6 months after BMT. Recipients of major ABO mismatched transplantations showed a trend for delayed donor repopulation. This was significant at 3 months ($p=0.03$).

Table II. Donor erythrocyte repopulation 0 5-6 months after BMT analyzed with the microsphere method

Months after BMT	No. of patients	% of patients with donor RBC*	% of donor RBC median/range
0.5	22	86	0.04/0 01-0.66
1	22	82	3.21/0.04-19.7
2	23	96	27 8/0 02-71.9
3	25	92	59 5/0.23-90.1
6	26	100	88 8/1.53-100

* RBC red blood cells

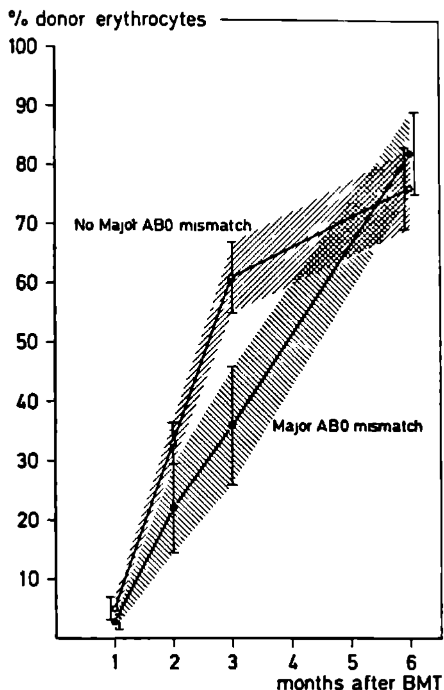

Fig 1 Donor erythrocyte repopulation from 1 to 6 months after BMT in recipients with and without major ABO mismatched transplantations Numbers of patients major ABO/no major ABO, 1 month 13/9, 2 months 11/12, 3 months 11/14, 6 months 9/18 Percentages shown are mean values ± standard error of the mean (SEM)

Autologous erythrocytes in the first 6 months after BMT

The course of autologous erythrocytes in the first 6 months after BMT measured with the microsphere method was studied in 33 patients (Fig 2A and 2B). The other patients were not evaluable mainly because of lack of host marker or because they received erythrocyte transfusions unselected for this marker. Only recently we have started to measure the percentages of host erythrocytes at day 0. These values therefore are only available in eight of the 33 patients. Because of previous transfusions negative for the host marker, values of less than 100% were observed.

In patients with a follow up not complicated by relapse, a gradual decrease of autologous erythrocytes was observed (Fig 2A). Five of the evaluable patients relapsed (Fig 2B). In two of them the autologous red cells initially decreased but increased during follow up resulting in relapse a few months later. In two patients the autologous erythrocytes remained detectable in high percentages or even increased in the first 6 months. Only one patient showed a clear decrease of host erythrocytes shortly before she relapsed.

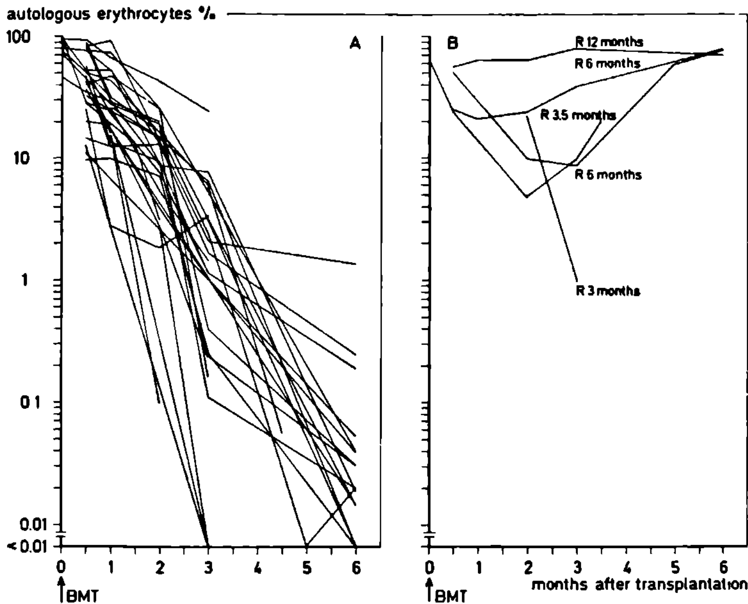

Fig 2. Course of autologous erythrocytes from 0.5 to 6 months after BMT.
(A) no relapse during follow up, (B) relapse during follow up.

Mixed red cell chimerism

Thirty-eight patients were evaluable for chimerism with the microsphere method at 6 months or later after BMT. Results on mixed chimerism are presented in Table III. Patients were excluded from analysis because of death from transplant related complications within the first 6 months, a relapse within the first 6 months, a follow up of less than 6 months or lack of markers.

Table III. Red cell chimerism at 6 months or later after BMT analyzed with the microsphere method

Months after BMT	No. of patients	% of patients MC	% of patients CDC[#]	% of autologous RBC[*] in mixed chimeras median/range
6	26	69	31	0.05/0.01-70.8
9	27	63	37	0.07/0.01-81.6
12	27	59	41	0.06/0.01-57.1
24	17	71	29	0.51/0.02-66
>24	8	50	50	5.5/0.03-34.7

[*] RBC: red blood cells
[#] MC mixed chimerism, CDC complete donor chimerism

A high percentage of mixed chimeras was found at all points after BMT. The median percentages of autologous red cells demonstrate that most patients have only small amounts of autologous cells in circulation. Combining the results of the microsphere method at 6, 9 and 12 months 20 patients were mixed chimeras and 16 patients complete donor chimeras. Between these two groups there were no differences in diagnosis (data not shown). In the 14 patients who received anthracyclines in the conditioning regimen and were irradiated with an average dose rate of 12.3 cGy/min, a lower incidence of mixed chimerism (36%) was found than in the 17 patients without anthracyclines during conditioning and an average dose rate of 4.1 cGy/min (70%). This difference, however, was not significant ($p=0.057$). Five patients received anthracyclines and an average dose rate of 4.1 CGy/min. Three of them were mixed chimeras and two patients were complete donor chimeras.

Follow up of the mixed red cell chimeras

Fig 3A and B shows the follow up of 25 patients, being mixed chimeras at first point of analysis,

6 months or later after BMT. Different patterns in the course of autologous red cells can be recognized in 21 patients without relapse (Fig 3A). Ten patients are stable mixed chimeras with low percentages (<1%) of autologous cells at different moments of analysis. Three patients are stable mixed red cell chimeras as well but their percentages of autologous cells are obviously higher (> 10%) and increase in time in two of them. Cytogenetic analysis of peripheral blood and/or bone marrow has confirmed the results of the erythrocyte antigen analysis in these three patients. RFLP analysis has only been performed in one of the three patients but gives similar results. Six patients have converted to complete donor chimeras. Four of them beyond 6 months after BMT, two patients beyond 12 and 24 months respectively. These patients have remained complete donor chimeras during follow up. Finally, in two patients, initially mixed chimeras, autologous erythrocytes were no longer detectable at next point of analysis. However, autologous red cells reappeared in small quantities during further follow up without clinical evidence of relapse.

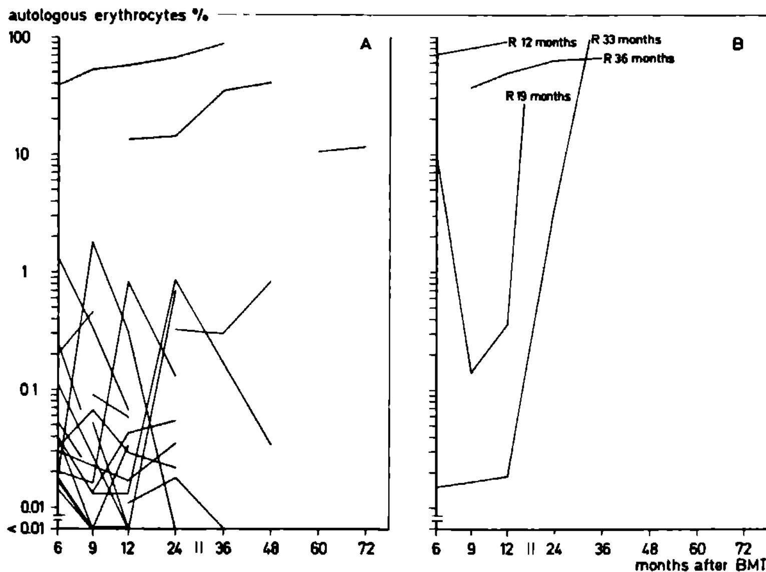

Fig 3 Follow up of patients being mixed red cell chimeras at first point of analysis 6 months or later after BMT (A) No relapse during follow up, (B) Relapse during follow up

Four mixed chimeras relapsed (Fig 3B) Two of them had repeatedly high percentages of autologous red cells, confirmed by cytogenetic analysis of peripheral blood and bone marrow, before relapse In the other two patients the autologous erythrocytes increased markedly in the period before relapse, in one of them after an initial decline

Follow up of the complete donor red cell chimeras

Twelve patients were complete donor chimeras at first point of analysis 6 months or later after BMT One patient, being a complete donor chimera at 6 months, converted to a mixed chimera with 0 02% and 0 01% autologous red cells at 9 and 12 months respectively All other patients who were analyzed 2 to 4 times during follow up (n=7) proved to be stable complete donor chimeras None of the complete donor chimeras was a mixed chimera when bone marrow cells were assessed by cytogenetic analysis (data not shown)

One patient, who was a complete donor chimera at 12 months, relapsed one month later

Discussion

We analyzed erythrocyte populations, using red blood cell antigen differences between host and donor, to study marrow engraftment and mixed red cell chimerism after allogeneic bone marrow transplantation Agglutination techniques and more recently a fluorescent microsphere method were used These assays are simple and results are available within a day The advantages of the microsphere method over the agglutination techniques are, besides accuracy and reproducibility, a higher sensitivity of 0 01% [12] A potential disadvantage of red blood cell antigen analysis is the fact that only one cell lineage is studied Cytogenetic analysis of peripheral blood and/or bone marrow and determination of RFLPs are widely used to distinguish patient and donor lymphopoietic and hematopoietic cells after BMT [6-8,10] The sensitivity of cytogenetic analysis for the demonstration of minor cell populations is limited by the number of metaphases analyzed [17] In the post-transplantation period it may be impossible to obtain sufficient dividing cells RFLP is a highly informative technique with a sensitivity of 1-10% [4,7-9,10] In contrast to cytogenetic analysis it does not depend on dividing cells, but it requires high quality technical staff and equipment and it is also time consuming

Analysis of erythrocyte populations requires an adapted transfusion policy, giving marker negative erythrocyte transfusions This condition was not always fulfilled, especially in the first 25 transplanted patients In case of unselected transfusions we considered the patient not

evaluable in the first 150 days after transfusion. These 150 days originated from an erythrocyte survival study we performed in orthopaedic patients with the fluorescent microsphere method. These patients received one erythrocyte transfusion positive for an antigen that was negative in the patient. The transfused erythrocytes were detectable for a period of maximally 150 days after the transfusion.[18] Taking this survival into account we defined mixed chimerism of erythrocyte populations from 6 months after BMT onwards.

With the fluorescent microsphere method we were able to detect donor erythrocytes in 86% of the evaluable patients at 0.5 month after BMT. None of the recipients of major ABO mismatched transplantations underwent plasma exchange or immunoadsorption. As pointed out by Sniecinski et al[19] and Reviron et al[20] we found that major ABO mismatched transplantations showed a trend for delayed donor repopulation and increased red cell transfusion requirements (data not shown). However, in contrast to Sniecinski et al[19], all patients with major ABO mismatched transplantations in our study repopulated well at 6 months after BMT.

A gradual decrease of autologous erythrocytes in the first 6 months after BMT was seen in all patients whose follow up was uncomplicated by relapse. A progressive increase of autologous red cells within the first 6 months was associated with relapse. Yam et al[7] observed this same pattern in two patients, using RFLP of peripheral blood cells. Knowlton et al[10] described two patients with reappearance of peripheral autologous cells in increasing percentages. Only one of them has relapsed. One of our patients relapsed while host red cells decreased. This might suggest a clonal expansion of a more committed stem cell not involving the erythroid lineage in this particular patient.

A high incidence of mixed red cell chimerism was found in this study. This is certainly a consequence of T-cell depletion as has also been described by Bretagne et al[4], De Witte et al[3] and Ferrara et al.[5] Because of their lower number in the graft, mature T-lymphocytes are less capable of destroying host lympho-hematopoietic cells, which have survived the conditioning regimen. The low incidence of graft-versus-host disease, thanks to T-cell depletion,[1,2,15] may amongst others be responsible for a higher incidence of autologous reconstitution. Another important reason for the high number of mixed chimeras is the 0.01% sensitivity of the fluorescent microsphere method.

No clinical evidence for leukemic recurrence was found in stable mixed chimeras with low percentages (<1 %) of autologous cells (n=10) or mixed chimeras, who converted to complete donor chimeras (n=6). Reappearance of small numbers of autologous cells in one patient who was initially a complete donor chimera, and in two patients who had been mixed chimeras earlier in their posttransplantation course but subsequently lost the host cells, was not associated with

leukemic relapse as well. It is important to stress that mixed chimerism with less than 1% autologous cells can not be detected with cytogenetic analysis and RFLP. Therefore, the risk of relapse after BMT in this group of mixed chimeras can only be evaluated with red cell markers.

A marked increase of autologous cells was associated with relapse in two mixed chimeras. However, mixed chimeras with high (>10), sometimes even gradually increasing, percentages of autologous red cells did not relapse necessarily. In three out of five mixed chimeras with this pattern the results of the red cell marker analysis were confirmed by cytogenetic analysis of bone marrow in one patient and of peripheral blood in two patients. They have not relapsed 36, 48 and 72 months after BMT respectively. Petz et al[21] and Branch et al[22] described similar patients with predominance of host red cells confirmed by cytogenetic analysis without clinical evidence of relapse.

Finally, we found a difference in incidence of mixed chimerism between the patients who received anthracyclines and irradiation at a higher dose rate and the patients conditioned without anthracyclines and irradiated at a lower dose rate, but this difference was not (yet) statistically significant.

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Chapter 3.

Influence of the conditioning regimen on erythrocyte chimerism, graft-versus-host disease and relapse after allogeneic transplantation with lymphocyte depleted marrow

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Summary

Three different conditioning regimens were applied to 144 patients undergoing allogeneic bone marrow transplantation (BMT) with HLA identical sibling marrow, depleted of lymphocytes by counterflow centrifugation. All regimens consisted of cyclophosphamide and fractionated total body irradiation (TBI). In 49 patients treated with regimen A the total TBI dose was 9 Gy. In regimen B the dose rate of TBI was increased and anthracyclines were added (n=65). Thirty patients received regimen C with a total TBI dose of 12 Gy but no anthracyclines.

The different conditioning regimens did not influence the percentage of patients with detectable recipient CFU-GM prior to infusion of donor marrow. The incidences of mixed erythrocyte chimerism at 6 months after BMT were 73, 33 and 20% for regimen A, B and C respectively. The conditioning regimen influenced significantly mixed erythrocyte chimerism from 6 to 24 months after BMT.

Both age and the conditioning regimen influenced significantly the incidence of acute graft-versus-host disease (GVHD) ($p=0.017$ and 0.0001 respectively). Acute GVHD ≥ 1 occurred in 15, 29 and 77% of the patients treated with regimen A, B and C respectively. The incidence of acute and chronic GVHD was significantly higher in complete donor chimeras than in mixed chimeras ($p< 0.001$ and $p<0.01$). The probability of relapse was 43% in 32 and 18% in 43 good risk patients treated with regimen A and B respectively ($p=0.07$). Longer follow up is needed to draw conclusions about relapse in regimen C.

We postulated that a more intensive conditioning regimen resulted in a lower incidence of mixed erythrocyte chimerism, a higher incidence of acute GVHD and a trend to a lower relapse rate.

Introduction

Allogeneic BMT with marrow from a HLA identical sibling has proven to be an effective treatment option for patients with acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and chronic myeloid leukemia (CML) in chronic phase.[1-5] However, moderate to severe acute GVHD occurs in approximately 45% of recipients of HLA identical marrow grafts despite posttransplant immunosuppressive therapy and it is an important cause of morbidity and mortality after BMT.[2,3]

T-cell depletion of the allogeneic graft can be accomplished by various methods. Effective methods clearly decreased the incidence of acute GVHD.[6-8] Several studies have shown an

increased relapse rate after T-cell depletion, especially in patients transplanted for CML [3,9-11] Moreover, incidences of mixed chimerism, varying from 51-100%, have been reported after T-cell depletion [12,16] This is clearly higher compared with the incidence of mixed chimerism after BMT with non T-cell depleted grafts [17]

Intensification of the conditioning regimen could be an option to eradicate more, both normal and leukemic, hematopoietic recipient cells [18,19] In our center all patients have received marrow grafts depleted of lymphocytes by counterflow centrifugation [20] This study describes three different conditioning regimens and their impact on the incidence of mixed erythrocyte chimerism A sensitive fluorescent microsphere assay was used to detect recipient and donor red cells [21] In addition we compared the incidence of acute and chronic GVHD, the number of remaining colony forming units granulocyte/monocyte (CFU-GM) on the day of BMT and the probability of relapse in good risk patients

Materials and methods

From May 1981 till January 1991 157 allogeneic bone marrow transplantations were performed with marrow from a HLA-identical sibling donor Retransplant, transplant for nonmalignant diseases and transplant with donors other than HLA-compatible siblings were excluded from analysis A total of 144 out of 157 transplantations were preceded by one of three following conditioning regimens All three conditioning regimens consisted of cyclophosphamide 60 mg/kg/day (days -6 and -5) and fractionated TBI (days -2 and -1) administered by a 18 MV photon beam accelerator (Saturne, CGR, Buc, France) Lungs and eyes were always shielded using individually adapted lead blocks In regimen A, applied in 49 patients from May 1981 till June 1986 TBI was administered in a dose of 4 5 Gy/day with a midline average dose rate of 4 1 ± 0 5 cGy/min The corrected mean total lung dose was 7 9 ± 1 3 Gy Regimen B was used in 65 patients from October 1986 till January 1990 The TBI dose in this regimen was 4 5 Gy/day with a midline average dose rate of 12 3 ± 2 0 cGy/min The corrected mean total lung dose was 7 1 ± 0 8 Gy In addition anthracyclines, either daunorubicin 156 mg/m² or demethoxydaunorubicin 42 mg/m², were administered as a continuous intravenous infusion for 3 to 6 days on days -7 to -2 Initially regimen C was confined to HLA mismatched transplants but since 1990 it was used as the standard conditioning regimen consisting of cyclophosphamide as described above and TBI in a dose of 6 Gy/day with a midline average dose rate of 12 3 ± 2 0 cGy/min The corrected mean total lung dose was 7 3 ± 1 1 Gy In addition to lung and eye shielding the kidneys were shielded

in these patients using individually adapted lead blocks A total number of 30 patients with a HLA identical donor was treated with this regimen

On day 0 patients received donor marrow depleted of 98% of lymphocytes by density gradient centrifugation followed by counterflow centrifugation [20]

Patient and donor characteristics

In order to compare the three conditioning regimens only the 144 patients with a HLA ABDr identical, mixed lymphocyte culture (MLC) negative sibling donor were evaluated The mean age of these patients treated with regimen A, B or C was 28 9, 32 2 and 35 7 years respectively Indications for transplantation per conditioning regimen are given in Table I

Immunoprophylaxis

Of the first 11 BMT patients nine patients received only methotrexate (MTX) according to the Seattle regimen[22] and two patients had no immunoprophylaxis The next 34 patients were treated with the combination of cyclosporin A (CsA) and MTX CsA (3 mg/kg/day) was given by continuous intravenous (iv) infusion from day -1 to day 28, followed by CsA orally (9 mg/kg/day), with a gradual tapering off after 6 weeks and discontinuation after 12 weeks MTX was given as weekly iv injections from week 5 till week 16 after BMT in a maximal weekly dose of 10 mg/m^2 [23] All other patients received only CsA in a schedule as described above

Erythrocyte markers

The procedure followed to select erythrocyte markers and the adapted transfusion policy have been described before [24] Of the 144 evaluable patients both a recipient and donor marker were present in 83 cases (58%), only a recipient marker in 36 cases (25%), only a donor marker in 15 cases (10%) and no markers or unknown markers because of previous erythrocyte transfusions in 10 cases (7%) In case of an erythrocyte transfusion unselected for the marker antigens we assumed this transfusion to be marker positive Considering a maximum survival time of erythrocytes of 150 days the marker percentages determined the first 150 days after the unselected transfusion were excluded from analysis [25] In this study measurement of host and donor red cell populations after BMT was performed by a fluorescent microsphere method [21] In this assay red blood cells are first incubated with an IgG antibody directed against the marker antigen under investigation After washing the erythrocytes, fluorescent microspheres coated with anti-human IgG are added followed by centrifugation Marker positive erythrocytes which are intensively labeled with fluorescent microspheres can be visualized and counted under a

fluorescence microscope. The sensitivity level of this assay is one positive cell per 10000 negative cells (0.01%). Blood group antigens examined were A1, A2, B, CcDE, K, MSs, Jk^a Jk^b, Fy^a Fy^b and Kp^a. Time points of analysis were 6, 9 and 12 months after BMT and at least once yearly thereafter.

Table I. Clinical details on patients per conditioning regimen

Conditioning regimen	A	B	C
No. of evaluable patients	49	65	30
mean age	28.8	32.2	35.7
(range)	(15-53)	(16-46)	(13-47)
Indications for BMT			
AML CR1	15	12	4
AML CR>1	1	1	
ALL CR1	10	15	5
ALL CR>1	1	4	1
CML CP1	7	16	1
CML AP	6	1	2
MDS	1	5	5
LyLy CR	2	3	1
NHL		2	4
MM		2	5
Others[#]	6	4	
Good risk patients[##]	32/10	43/6	12/4
No. total / relapsed			
Follow-up (months)[*]	74	33	13
Median (range)	(0.25-118)	(1-58)	(1-18)
Time to relapse (months)[@]	11	11	8.5
Median (range)	(5-55)	(3-24)	(6-16)

AML = acute myeloid leukemia; ALL = acute lymphoblastic leukemia, CML = chronic myeloid leukemia, MDS = myelodysplastic syndrome, LyLy = Lymphoblastic lymphoma, NHL = non Hodgkin lymphoma, MM = multiple myeloma, CR = complete remission, CP = chronic phase, AP = accelerated phase

[#] Mainly advanced stage leukemia.

[##] Good risk patients = AML CR1, ALL CR1, CML CP1.

[*] Follow-up for non relapsed good risk patients ends at day of death or day of evaluation.

[@] For good risk patients

CFU-GM assay

At day 0 a bone marrow sample was taken from all patients to assess the number of residual host CFU-GM after marrow ablative therapy. This assay was performed as previously described.[26]

Definitions

Taking into account a 150-day survival time of recipient erythrocytes generated before BMT, mixed chimerism was defined by the coexistence of host and donor erythrocytes in the recipient at 6 months or later after BMT. Complete donor chimerism was defined by the presence of donor type erythrocytes only in the recipient from 6 months after BMT onwards. Patients were considered to be evaluable for acute GVHD if they survived at least 20 days after BMT. The clinical manifestations of acute GVHD occurring till day 100 posttransplant were classified according to the criteria described by Glucksberg et al.[27] Acute GVHD $\geq$ I was treated by adding corticosteroids to CsA prophylaxis. Patients were evaluable for the presence or absence of chronic GVHD if they survived at least 100 days after BMT.

Statistical analysis

The probability of relapse was calculated according to the Kaplan-Meier method from the day of BMT. Follow up ends on the day of evaluation (December 1. 1991). Statistical comparisons between curves were made using the log-rank test. Analysis of variance was done by ANOVA. Multivariate analysis was performed using logistic regression. Frequencies were compared using the Fisher's exact test or the χ^2 test.

Results

Patient characteristics

Age distribution was significantly different between the three conditioning regimens (ANOVA, p=0.008). The age of the patients at the time of BMT increased during the years (Table I).

Graft characteristics

The absolute number of T-cells in the marrow graft was significantly different between the patients treated with regimen A, B and C (ANOVA, p=0.0001) (Table II). The patients in group B especially received more T-cells but compared to transplantation with unseparated marrow grafts the numbers of T-cells were very low in all 144 patients.

Engraftment

Five out of 144 patients died within 20 days after BMT and are not evaluable for engraftment. All remaining patients engrafted. One patient, treated with regimen B, rejected the marrow graft after initial engraftment 2 months after BMT.

Table II. Incidence of GVHD per conditioning regimen

Conditioning regimen	A	B	C	
T-cells (Mean ± SEM) (10^6/kg body weight)	0.66 ± 0.08	1.42 ± 0.08	0.89 ± 0.06	
Acute GVHD:	n (%)	n (%)	n (%)	
Grade 0	39 (85)	45 (71)	7 (23)	
I	2 (4)	7 (12)	19 (63)	p<0.001
II	1 (2)	10 (15)	2 (7)	
III/IV	4 (9)	1 (2)	2 (7)	
Chronic GVHD:	n (%)	n (%)	n (%)	
Absent	27 (77)	30 (57)	13 (54)	p=0.096
Present	8 (23)	23 (43)	11 (46)	

Incidence of mixed erythrocyte chimerism

For each of the three conditioning regimens the total numbers of patients evaluable for mixed erythrocyte chimerism at 6 months or later after BMT are given in Table III. Patients were excluded from analysis because of transplant related mortality, relapse before or at time point of analysis or because a recipient marker was lacking. Initially we did not use the fluorescent microsphere method as routinely as we did during the last 5 years and this explains the lower number of observations in patients transplanted with regimen A at 6 and 9 months after BMT.

During the years, initially using regimen A, subsequently regimen B and finally regimen C, the incidence of mixed red cell chimerism has decreased. The conditioning regimen influenced significantly the occurrence of mixed erythrocyte chimerism at 6, 9, 12 and 24 months after BMT (χ^2 test, p<0.02 at all time points). The majority of mixed chimeras in regimen A and B showed only small percentages of circulating autologous erythrocytes; 0.22 /0.011-81.6 and 0.034 /0.01-14.9 (median/range) for regimen A and B respectively from 6 till 24 months after BMT. For regimen C the number of observations is too low to draw conclusions.

Combining the results of the three regimens 27 patients were mixed and 47 complete donor chimeras from 6 till 12 months after BMT. We performed multivariate analysis to examine the influence of the conditioning regimen, age and the number of T-cells in the graft on the occurrence of mixed erythrocyte chimerism in this time period. The presence of autologous red cells was influenced significantly by the pretransplant treatment regimen (p=0.0004). Neither age (p=0.8) nor the number of T-cells in the graft (p=0.07) had a significant influence on the incidence of mixed erythrocyte chimerism.

Table III. Incidence of mixed erythrocyte chimerism per conditioning regimen at 6 months or later after BMT.

Time	Regimen A		Regimen B		Regimen C	
(months)	No.#	%MC*	No.	%MC	No.	%MC
6	11	73	36	33	15	20
9	12	83	33	36	14	21
12	15	73	32	28	13	30
24	15	73	31	13		

Total number of evaluable patients per time point
* % MC= percentage of mixed erythrocyte chimeras

The significance of mixed erythrocyte chimerism in CML and acute leukemia

When the data of the three conditioning regimens were pooled 35 patients had an allogeneic transplantation for CML either in chronic phase (n=26) or in accelerated phase (n=9). Twenty-two of these 35 patients had a recipient marker and no signs of clinical or cytogenetic relapse within the first 6 months after BMT. Twelve out of these 22 patients showed neither hematological relapse nor cytogenetic abnormalities during further follow up. Ten out of 22 patients developed a cytogenetic relapse at 6 to 60 months after BMT. In four of them hematological relapse converged with cytogenetic relapse. Hematological relapse followed the recurrence of the Ph chromosome after 6 and 12 months in two patients. The other four patients repeatedly have had Ph chromosome positive metaphases in the bone marrow without signs of hematological relapse with a follow up of 8 to 32 months after the first reappearance of the Ph chromosome after BMT. Figure 1A and 1B show the course of autologous erythrocytes in the relapse and no relapse group respectively. Nine out of 10 patients in the relapse group had high (>10%) or increasing percentages of autologous cells at the time of relapse. In one patient, with a cytogenetic relapse

at 12 months, the percentages of autologous erythrocytes decreased from 1.33 to 0.34 and 0.068% at 6, 9 and 12 months after BMT. One year later hematological relapse manifested itself with a percentage of autologous cells of 96.5%. In the no relapse group six patients had no detectable autologous erythrocytes at 3 to 6 time points of analysis. Five patients did have variably detectable autologous cells but only in percentages ≤ 0.5%. The last patient had 0 014% recipient red cells at 6 months but no detectable recipient red cells from 9 to 48 months after BMT.

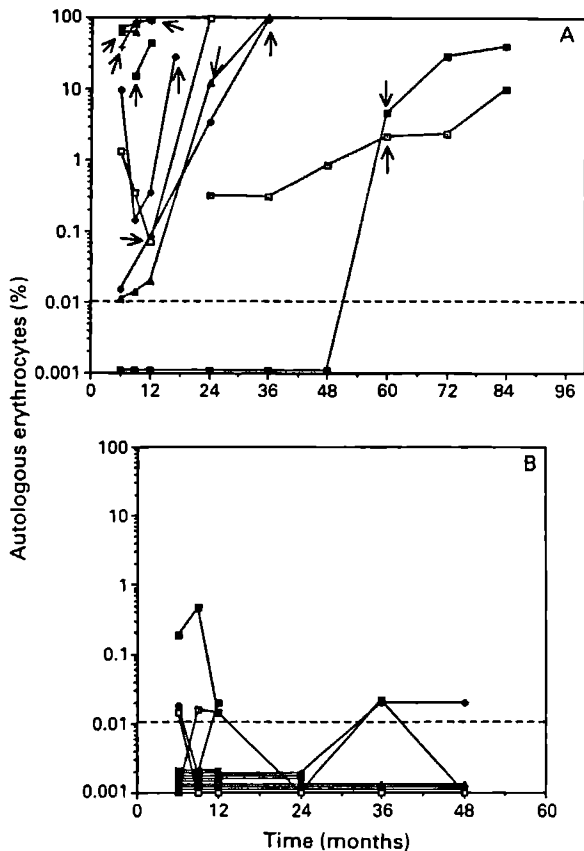

Fig 1. Course of autologous erythrocytes in CML patients from 6 months after BMT onward.
(A) Cytogenetic relapse during follow up indicated by arrow (n=10).
(B) No cytogenetic relapse during follow up (n=12).

Forty-two out of 69 patients transplanted for AML or ALL had a recipient marker and no recurrent disease within the first 6 months after BMT The majority of patients (n=33) who did not relapse during further follow up, were either complete donor chimeras or stable mixed chimeras with percentages of autologous erythrocytes below 1% However, two patients transplanted for AML in CR1, have had repeatedly percentages of recipient red cells varying from 5 to 20% without evidence of relapse during a follow up of more than 9 years Moreover two patients in continuous complete remission, transplanted for ALL in CR1, have shown increasing percentages of autologous red cells up to 88 and 45 % with a follow up of 75 and 95 months respectively Nine out of 42 patients relapsed at 6 to 36 months after BMT In six of these cases (four AML in CR1, one ALL in CR1 and one ALL in CR3) no autologous erythrocytes were detectable at the time of relapse In two patients, both ALL in CR1, relapse was preceded by increasing percentages of autologous cells up to 70% One patient who relapsed at 9 months, had 3 5 and 4 3% recipient erythrocytes at 6 and 7 months after BMT

Graft-versus-host disease

The incidence and the severity of acute GVHD could be assessed in 139 out of 144 patients conditioned with regimen A, B or C (Table II) Forty-eight patients developed acute GVHD grade I or more (34%), 28 patients had grade I and 13 patients grade II GVHD

Looking at each of the regimens the incidence of acute GVHD grade I or more was 15, 29 and 77 % for regimen A, B and C respectively (p<0 001) Although the incidence of acute GVHD in patients treated with regimen C was higher, 19 out of 23 patients had grade I, two patients grade II and two patients grade III/IV GVHD Multivariate analysis showed a significant influence of both age (p=0 017) and the conditioning regimen (p=0 0001) on the incidence of acute GVHD The number of T-cells in the graft had no significant influence on the occurrence of acute GVHD in multivariate analysis (p=0 2)

The majority of patients treated with regimen A received both MTX and CsA as immunoprophylactic treatment in contrast to patients conditioned with regimen B who received only CsA However, the incidence of acute GVHD grade I or more was not significantly different between these two patient groups (p=0 08) All patients treated with regimen B and C received CsA only as immunoprophylaxis, nevertheless the incidence of acute GVHD was significantly higher in the latter group (p<0 001)

The presence or absence of chronic GVHD could be evaluated in 112 patients The overall incidence of chronic GVHD was 38% and the incidence per regimen 23, 43 and 46% for regimen A, B and C respectively (p=0 095) (Table II) In the majority of cases chronic GVHD was limited

to the skin and of mild to moderate severity No significant influence of age, the conditioning regimen or the number of T-cells in the graft on the incidence of chronic GVHD could be detected by multivariate analysis

We assessed the relation between GVHD and mixed chimerism The results of the three conditioning regimens were combined Twenty-seven patients were mixed chimeras and 47 patients were complete donor chimeras from 6 till 12 months after BMT The incidence of both acute and chronic GVHD was significantly higher in the group of complete donor than in the group of mixed chimeras (Fisher's exact test, $p<0$ 001 and $p<0$ 01 respectively) (Table IV)

Table IV Relation between GVHD and mixed chimerism

Acute GVHD Grade	MC	CDC	
0	25	24	p<0 001
≥I	2	23	
Chronic GVHD			
Absent	24	25	p<0 01
Present	3	22	

MC= mixed chimera, CDC= complete donor chimera

CFU-GM assay results

We assessed routinely the number of recipient CFU-GM just prior to bone marrow infusion to evaluate the efficacy of the conditioning regimen to eradicate bone marrow progenitors CFU-GM assay results at day 0 were available in 45 out of 49, 56 out of 65 and in all 30 patients treated with conditioning regimen A, B and C respectively Data of the remaining patients are lacking due to technical reasons Table V gives the results of the CFU-GM assay at day 0 for each of the conditioning regimens Only a minority of the patients appeared to have residual CFU-GM, irrespective of the conditioning regimen used (χ^2 test, p=0 4) When patients transplanted for AML, ALL and CML were analyzed according to disease category no significant influence of the conditioning regimen on the result of the CFU-GM assay was demonstrable Thirty-five out of 131 patients with a result of the CFU-GM assay at day 0 relapsed Ten out of these 35 patients had demonstrable CFU-GM at day 0, which was not significantly different from the 21 out of 96 non-relapsed patients with a positive result of the CFU-GM assay

Table V. Results of the CFU-GM assay at day 0

CFU-GM	Conditioning regimen A	B	C
Negative, n (%)	35 (78)	45 (80)	20 (67)
Positive, n (%)	10 (22)	11 (20)	10 (33)
No of clusters + colonies median/range*	3/1-21	2/1-15	2/1-59

* In patients with positive CFU-GM.

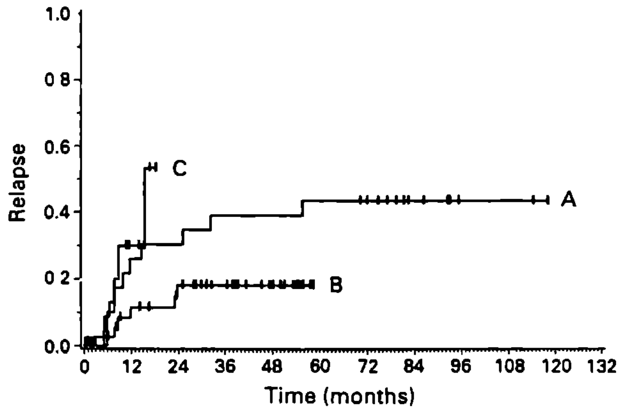

Fig 2. Probability of relapse in good risk patients per conditioning regimen.

Relapse in good risk patients

In order to compare the probability of relapse between the three different regimens only patients transplanted for AML and ALL in CR1 and CML in chronic phase were included. The numbers of these so-called good risk patients were 32, 43 and 12 for regimens A, B and C. The time to relapse and the follow up for the patients who did not relapse are given in Table I. For CML patients cytogenetic relapse was not considered as a relapse. Although the probability of relapse was decreased in regimen B compared to regimen A (18 versus 43%), this difference was not significant (p=0.07). Because of the short median follow up and the small number of evaluable patients no statements can be made about probability of relapse in group C yet (Figure 2).

Discussion

Several investigators have studied mixed chimerism after BMT with T-cell depleted marrow grafts from a HLA identical sibling. Techniques used were cytogenetic analysis and analysis of restriction fragment length polymorphisms of peripheral blood and bone marrow, mixed agglutination for the red cell lineage, immunoglobulin allotyping for B lymphocytes and more recently in situ hybridization. Mixed chimerism could be detected in all cell lineages. Combining the results of the different techniques the authors found incidences of mixed chimerism varying from 51 to 100%.[12,13,15,16,28] Patients transplanted in our center with marrow grafts depleted of lymphocytes by counterflow centrifugation showed an incidence of mixed chimerism of 67% at 6 months after BMT.[14] This high incidence was explained by the use of a very sensitive fluorescent microsphere assay to detect recipient erythrocytes. Studying mixed erythrocyte chimerism with the microsphere method we found circulating recipient red cells in 59 to 71% of the patients from 6 to 24 months after BMT in contrast to the 36 and 44% found by Schouten et al [13] and Roy et al [16] using agglutination techniques.[24]

In the present study of mixed erythrocyte chimerism we observed a significant influence of the conditioning regimen on the incidence of mixed chimerism from 6 to 24 months after BMT. Intensification of the pretransplant conditioning by adding anthracyclines and increasing the dose rate of TBI caused a decrease of mixed red cell chimerism from 73 to 33% at 6 months after BMT. Toxic effects of the anthracyclines (severe mucositis and prolongation of the neutropenic period by one week compared to regimen A) [29] were the reasons for replacing this regimen. Instead, intensification of the conditioning regimen was achieved by enhancement of the total TBI dose to 12 Gy. This resulted in a 20% incidence of mixed erythrocyte chimerism at 6 months after BMT.

Petz et al [17] observed a lower incidence of mixed chimerism in patients treated with 13 2 Gy fractionated TBI compared with treatment with 10 Gy single dose TBI This difference was not significantly different. Comparing mixed chimeras with complete donor chimeras, Frassoni et al[19] found that the first had received a significantly lower dose of TBI than the latter

An interesting question is the relation between the presence of mixed erythrocyte chimerism and relapse In this study CML patients who never had detectable Ph chromosome positive cells in the bone marrow at 6 months or later after BMT, were either complete donor chimeras or stable mixed chimeras with very low (≤ 0 5%) percentages of recipient red cells High (>10%) or increasing percentages of recipient erythrocytes indicated cytogenetic relapse, which was also observed by Frassoni et al [19] The involvement of the erythroid lineage in CML patients can be explained by the fact that the origin of the malignant clone in CML is at the pluripotent stem cell level The appearance of Ph chromosome positive cells was never transient in our patients in contrast to observations by other authors [19,30] In six out of 10 patients with cytogenetic relapse there was evidence of hematological relapse during follow up

Although in the majority (29 out of 33) of patients transplanted for acute leukemia and remaining in complete remission, either no or low (<1%) percentages of host erythrocytes were detectable during follow up, four patients were observed with a different pattern despite repeatedly high (5-20%) or increasing percentages of autologous erythrocytes no signs of relapse could be detected Other studies confirm that mixed chimerism, even with predominantly host red cells does not necessarily indicate relapse in patients transplanted for acute leukemia [17,31] Like Frassoni et al [19] we also observed patients transplanted for acute leukemia without demonstrable recipient red cells at the time of relapse Whether this has to be attributed to suppressed normal host erythropoiesis or a clonal expansion of a more committed stem cell not involving the erythroid lineage, remains unclear

The incidence of serious GVHD ≥ II was too low to permit an analysis of patients with grade II or more acute GVHD We found a significant influence of the conditioning regimen on the incidence of acute GVHD grade I or more being 15, 29 and 77% for conditioning regimen A, B and C respectively As described by Truitt and Atasoylu [32] in a mouse BMT model, more host T-cells are eliminated by increasing the TBI dose This was associated with increased donor T-cell chimerism and a higher incidence of GVHD Comparing two conditioning regimens with a TBI dose of 12 and 15 75 Gy, Clift et al [33] observed a significantly higher probability of developing grade II or more acute GVHD in the patients receiving the highest TBI dose In that study however, it was not clear whether the higher dose of TBI predisposed to GVHD or whether GVHD was seen more frequently because fewer patients in the 15 75 Gy group received optimal treatment with

MTX and CsA. The significant impact of age on the occurrence of acute GVHD was also observed by Zwaan et al [34]. Gale et al [35], studying risk factors for acute GVHD, found no significant influence of age on the incidence of acute GVHD. Although patients in group B received significantly more T-cells in the graft, the absolute numbers were still very low compared with unseparated marrow grafts. This might be an explanation for the lack of a significant influence of the number of T-cells on the incidence of acute GVHD and mixed chimerism in this study.

No significant influence of the conditioning regimen on the occurrence of chronic GVHD could be observed in our study with an incidence of chronic GVHD of 23, 43 and 46% for regimen A, B and C respectively. This is in contrast to the linear correlation between total dose of TBI and incidence of chronic GVHD found by Frassoni et al.[36] We did find a significantly higher incidence of both acute and chronic GVHD in complete donor chimeras compared with mixed chimeras, suggesting a role for GVHD in eliminating residual host cells. These results are in line with the data of Frassoni et al.[19] The percentage of patients with residual host CFU-GM just prior to bone marrow infusion varied from 20 to 33% amongst the three different regimens. Despite this the incidence of mixed chimerism decreased with intensification of the regimen. This may imply that GVHD which occurred more frequently after the intensified regimens is responsible for the decreased incidence of mixed chimerism rather than the intensity of the regimen. Future strategies should study the possibilities of immunomodulation rather than a mere intensification of the conditioning. Kolb et al [37] were able to reinduce complete hematological and cytogenetic remission in three CML patients, who relapsed after BMT, by transfusion of buffy coat from the original marrow donor. Because of the rather high relapse rate in CML patients, this kind of adoptive immunotherapy might be considered at some time point after BMT.

As pointed out by Apperley et al [30] and Horowitz et al [11] there is a significant influence of the occurrence of acute and/or chronic GVHD on the rate of leukemic relapse in patients receiving T-cell depleted marrow grafts. Comparing patients treated with regimen A and B we observed an increased incidence of chronic GVHD ($p=0.048$) and a trend to a lower relapse rate in the latter group. Intensification of the pretransplant conditioning could also have contributed to this trend. The incidence of acute GVHD increased in patients treated with regimen C, but GVHD was of mild (grade I) severity in the majority of these patients. In view of the increased incidence of acute GVHD and in view of the postulated higher antileukemic activity of regimen C,[33] we anticipate a lower relapse rate. Additional follow up and more patients are needed to assess our expectations.

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Chapter 4.

Donor leukocyte infusions for chronic myeloid leukemia relapsed after allogeneic bone marrow transplantation

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Summary

Purpose

Treatment options for patients with chronic myeloid leukemia (CML) who relapse after allogeneic bone marrow transplantation (BMT) are limited Treatment with lymphocytes from the original marrow donor and the influence on the malignant clone was studied in these patients

Patients and methods

Seven patients with CML that had relapsed after BMT with T-cell depleted grafts were treated Six patients received leukocyte infusions from the original marrow donor One patient received a second BMT with unseparated marrow from the same sibling donor Chimerism was studied using erythrocyte and cytogenetic markers Residual leukemic cells were monitored by cytogenetic analysis of the Philadelphia (Ph) chromosome and by polymerase chain reaction (PCR) of the Breakpoint Cluster Region/Abelson (BCR-ABL) fusion gen

Response

In five patients with hematologic relapse the Ph chromosome disappeared 1 to 3 months after the leukocyte infusions Cytogenetic analysis and in situ hybridization (ISH) showed only donor cells during further follow up Four of five patients became negative for the BCR-ABL translocation by PCR Graft-versus-host desease (GVHD) always preceded response and was severe in two patients One patient with cytogenetic relapse showed no response after leukocyte infusions GVHD after second BMT was of moderate severity One year after second BMT, PCR for the BCR-ABL translocation was negative

Conclusion

Infusion of donor leukocytes is an effective treatment with a low mortality in patients with CML relapsed after BMT with a T-cell depleted graft Longer follow up and more patients will be needed to know whether cure will be permanent

Introduction

CML is a fatal malignant stem cell disorder characterized cytogenetically by the Ph chromosome a specific translocation of the Abelson (ABL) oncogene on chromosome 9 to the Breakpoint Cluster Region (BCR) on chromosome 22

Allogeneic BMT is the only curative option for CML [1] Relapse rates in patients transplanted for CML in first chronic phase vary from 10% to 20% in recipients of a non T-cell depleted graft Higher relapse rates up to 50% to 60% have been observed when T-cell depletion is applied to decrease the incidence of GVHD or when transplantation has been performed in more advanced stages [1,2] Only a minority of patients with relapse CML after BMT achieve a cytogenetic remission after treatment with interferon alfa (INFα) [3] Other treatment possibilities are second BMT [4,5] or, as recently described, infusion of leukocytes from the original marrow donor [6-8]

This report describes the study of seven CML patients who relapsed after BMT with T-cell depleted marrow Six of them were treated with donor leukocyte infusions and one received a second BMT using unmanipulated marrow from the same donor We report on hematologic response and GVHD Mixed chimerism was studied using erythrocyte and cytogenetic markers Monitoring of residual leukemic cells was performed by cytogenetic analysis of the Ph chromosome and by PCR of the BCR-ABL fusion molecules

Patients and methods

Patients

Seven CML patients who relapsed within 6 to 36 months after BMT were treated in an attempt to achieve a second complete remission Patient characteristics are listed in Table I

At the time of BMT, all patients were in first chronic phase of CML except for patient no 91, who was in accelerated phase Patient no 91 received a marrow graft from a one A-locus mismatched, mixed lymphocyte culture (MLC)-negative sibling, all other donors were HLA A/B/Dr identical, MLC-negative siblings The conditioning regimen consisted of cyclophosphamide and total body irradiation (TBI) as previously described [9] All patients received donor marrow depleted of 98% of lymphocytes by density gradient centrifugation followed by counterflow centrifugation [10] Cyclosporin A (CsA) was used as immunoprophylaxis posttransplant in all patients [9] Data on the occurrence of GVHD after BMT are listed in Table I

Table I. Patient characteristics

Patient No.	Date of diagnosis*	Date of BMT*	Age at BMT**	Sex		GVHD	
				Patient	Donor	Acute	Chronic
64	11-85	12-86	31	Male	Male		—
132	5-89	9-89	27	Male	Female	Skin, grade I	Mild
125	8-88	6-89	33	Male	Female		—
51	8-85	5-86	46	Male	Male		—
91	2-83	3-88	25	Female	Male	Skin, grade I	Moderate
166	7-90	10-90	47	Male	Male		—
68	3-84	2-87	35	Female	Female	Skin, grade II	Mild

* Month-year ** Years

Treatment of relapse

Cytogenetic analysis of bone marrow was performed at 6 and 12 months after BMT and yearly thereafter. In five patients (no. 64, 125, 51, 91 and 68), no Ph chromosome positive cells could be demonstrated at one to three time points of analysis after BMT. Subsequently, they became Ph chromosome positive and developed hematologic relapse of CML. Patient no. 132 had Ph chromosome positive cells at 6 and 12 months after BMT with signs of hematologic relapse at the latter time point. Patient no. 166 was Ph chromosome positive at 6 months after BMT, but has shown no signs of hematologic relapse thereafter (Table II).

Table II. Data on relapse and infusion of mononuclear cells

Patient No.	Time to relapse*		Treatment before infusion	Time to infusion*	Nucleated cells ($x10^8$/kg)	Lymphocytes ($x10^8$/kg)	No of infusions
	Cytogenetic	Hematologic					
64	12	24	Busulfan	51	0.34	0.26	4
132	6	12	IFNα	19	0.34	0.25	4
125	24	24	—	25	1.48	1.1	2
51	12	12	Busulfan	63	1.3	0.96	2
			IFNγ	65	2.38	1.78	4
			IFNα				
91	36	36	Busulfan	41	1.65	1.1	2
166	6	–	IFNα	13	1.8	1.27	3
				17	3.4	2 5	8
68	24	24	Busulfan	36	2.7	0.38	Second BMT
			IFNα				

* Months after BMT

Relapse of CML was treated with different drugs (Table II). Patient no. 64 received busulfan in a dose of 2 mg/day, 5 days a week during 30 months. Patient no. 132 was treated with INFα $5x10^6$ U, three times a week for 6 months. Patient no. 51 initially received busulfan 4 mg/day, 5 days a week for 6 months and subsequently interferon gamma (INFγ) 0.5 mg/day, three times a week for 12 months. Treatment consisted of INFα $3x10^6$ U/day during the last 2 years before leukocyte infusions. Patient no. 91 was treated with busulfan 6 mg/day for 1 month. Patient no. 166 received INFα $3x10^6$ U/day, 3 days a week for 6 months. However, 1 week before infusion of donor leukocytes, drug treatment was discontinued in all patients. All patients had Ph chromosome positive cells in the bone marrow immediately before the start of the leukocyte infusions.

Nucleated cells from the donor were obtained by leukapheresis using a blood cell separator: the Baxter CS 3000 (Deerfield Il) in patients no. 64 and 132, and the Fresenius AS 104 (Oberursel, Germany) in the other four patients. Each donor underwent two to eight leukapheresis procedures with a time interval between the sessions of 2 to 5 days. Nucleated cells were administered to the patient within 3 hours after completion of the procedure. Two patients (no 51 and 166) were given 2 courses of leukocytes with an interval between the first and second course of 2 and 4 months, respectively. The total number of infused nucleated cells and lymphocytes, as well as the frequency of infusions, is listed in Table II. In all donors, the infused nucleated cells consisted mainly of lymphocytes (70% to 85%) and monocytes (10% to 15%). Immunophenotypic analysis of the infused cells was performed in three donors (Table III). CD3, CD4, and CD8 were used as markers for T-lymphocytes, T-helper cells, and T-suppressor cells, respectively. Natural killer cells were characterized by CD56 positivity or CD3 negativity/CD16 positivity. CD19 was used as a B-lymphocyte marker. The percentage of T-lymphocytes in the infused cells varied from 69% to 75% among different donors.

Table III. Immunophenotypic analysis of infused nucleated cells (%)

Patient No.	$CD3^+$	$CD4^+$	$CD8^+$	$CD3^-$,$CD16^+$	$CD56^+$	$CD19^+$
125	75	47	38	11	17	10
51*	72	47	28	9	26	14
166†	69	55	21	13	18	10
166*	75	58	21	10	13	11

* Second course of transfusion.
† First course of transfusion.

Patient no 68 relapsed 2 years after BMT Treatment consisted of busulfan 4 mg/day for 4 months, followed by INFα $5x10^6$ U/day for 9 months She received a second unseparated marrow graft from the same donor 36 months after the first BMT The conditioning regimen consisted of standard dose busulfan and cyclophosphamide [11]

All patients who were treated with donor leukocyte infusions or second BMT for CML that relapsed after BMT are included in this analysis

Erythrocyte markers

Complete RBC phenotyping of patient and donor was performed before BMT Antigenic differences of patient and donor were used as marker antigens Marker antigens in this study were A1, A2, B, c, E, Fy^a, Fy^b, Jk^b, M, N and S Only erythrocytes lacking these markers were transfused after BMT [12] Measurement of host and donor erythrocyte populations was performed by a fluorescent microsphere method [13] The sensitivity of this assay is one positive cell per 10000 negative cells (0 01%)

Cytogenetic analysis

Bone marrow cells were harvested directly and/or after a 24-hour culture in RPMI 1640 medium without mitogens, and processed for cytogenetic analysis using standard techniques, including GTG banding As a standard, 32 cells were karyotyped and, as a consequence, monitored for the presence of the Ph chromosome In case of a poor mitotic yield, at least 20 cells were karyotyped (range, 20 to 35, mean, 30, allowing the detection of a level of 10% mosaicism with a 95% confidence level) [14] If patient and donor were of different sex, additional identification of the Y chromosome was performed using QFQ banding In situ hybridization (ISH), using a combination of chromosome X- and Y-specific probes (p Bam X5 and DY 21) was performed on 400 bone marrow cells in interphase to discriminate between patient and donor [15] A signal that discriminated one to five cells out of 400 was considered background In case of equal sex, discrimination of patient and donor cells could be made using a chromosome 9 (patients no 64 and 68) or a chromosome 1 (patient no 51) heterochromatin polymorphism in conjunction with C-banding [16] In patient no 166 discrimination of patient and donor cells could not be made by cytogenetic analysis

BCR-ABL breakpoint molecules

To detect BCR-ABL breakpoint molecules in bone marrow, RNA was isolated from 10^6 bone marrow-derived nucleated cells in a modification of the method reported by Chomczynski and

Sacchi [17] One tenth of the RNA amount was used in a reverse transcriptase (RT) reaction using A21 primer (antisense, position 686 to 668[18]) In a final volume of 20 µl, 10 pmoles primer, 200 U Moloney Murine Leukemia virus (MoMulv) RT (Gibco/BRL, Breda, The Netherlands), 12 5 nmol deoxynucleoside triphosphate (dNTP), and 20 U RNAsin (RNASE inhibitor trademark of Promega, Madison, WI) were incubated for 10 minutes at 20°C, followed by a 45 minute incubation at 42°C and denatured at 95°C for 10 minutes After this, two series of PCR were performed In the first series, primers B11 (nucleotide 360 to 382[18]) and A21 were used in a 30 cycle PCR 30 seconds at 95°C, 30 seconds at 72°C, 90 seconds at 58°C For this, 1 5 U Taq polymerase (Gibco/BRL), appropriate PCR buffer, and 10 pmol B11 primer were added to the RT mixture up to a 80 µl reaction volume In the second series, B12 (nucleotide 387 to 408[18]) and A22 (antisense, position 642 to 619[18]) primer were used in a 35 cycle PCR Reaction conditions were as in first series, an additional 30 pmol of both primers, 1 5 U Taq polymerase, 12 5 nmol dNTP, and PCR buffer were added up to a 100 µL reaction volume

To assess the quality of each RNA sample, we used one tenth of the isolation in a RT reaction followed by PCR on β2-microglobulin RNA This reaction was checked by agarose gel electrophoresis During RNA isolation, RNA from 10^6 Jurkat cells was isolated in parallel to check for contamination To prevent contaminations, all solutions used for RT-PCR were aliquoted and checked for contamination Aerosole-resistant pipette tips were used RNA isolations, PCR preparations, and analysis of PCR products were physically separated In each PCR, both positive and negative controls were used

A 15% aliquot of the PCR products was analyzed by hybridization after Southern transfer using a radioactively labelled c-ABL oligonucleotide recognizing the 5' part of exon 2 of the c-ABL gene Bone marrow samples were regarded to be positive for the BCR-ABL breakpoint molecules when a hybridization signal was visible after exposure of the filters, whereas negative controls were negative after exposure of the film and the β_2-microglobulin reaction was positive on agarose gel Samples were regarded to be negative when no signal was visible on the autoradiogram after two rounds of PCR, whereas positive controls and the β_2-microglobulin reaction were positive after one round of PCR This way, one leukemic cell amongst at least 10^5 normal cells could be detected [19]

RESULTS

Hematologic response

Two patients developed severe hypoplasia after infusion of donor leukocytes Patient no 64

had no detectable donor cells pnor to the ınfusıons He became pancytopenıc 5 weeks after the leukocyte ınfusıons sımultaneously to the onset of GVHD The granulocyte count recovered to greater than 0 5x10^9 5 months after ınfusıon and the platelet recovery was only partıal (20x10^9 without platelet support) at the tıme of thıs report The erythrocyte transfusıon requırement has gradually decreased to 2 U per month The second patıent (no 91) developed severe neutropenıa 2 weeks after the leukocyte transfusıons and severe thrombocytopenıa 2 weeks later ın assocıatıon with severe GVHD The hypoplasıa recovered after treatment of GVHD wıth cortıcosteroıds The remaınıng four patıents treated wıth donor leukocytes dıd not develop clınıcally sıgnıfıcant cytopenıa The patıent transplanted wıth unseparated donor marrow after bone marrow ablatıve therapy was granulocytopenıc for 13 days, whıle platelet recovery to more than 50x10^9 occurred after 2 months after BMT

Graft-versus-host dısease

All patıents who responded to the donor leukocyte ınfusıons developed GVHD The clınıcal pıcture of GVHD ın these patıents was more or less ıdentıcal to that of chronıc GVHD, whıch can be observed ın recıpıents of donor marrow 3 months after BMT wıth or wıthout a precedıng phase of acute GVHD Clınıcal manıfestatıons of GVHD ın skın, lıver, and gut, graded accordıng to the cntena publıshed by Glucksberg et al [20] are lısted ın Table IV GVHD was extensıve ın two patıents (no 64 and 91) It consısted of a generalızed exanthema, severe oromucosıtıs, and oesophagıtıs and lıver functıon abnormalıtıes Both patıents showed a partıal response to cortıcosteroıds only and a satısfactory response after addıtıon of CsA However, patıent no 91 dıed suddenly 6 months after leukocyte ınfusıons from sepsıs orıgınatıng from a fulmınant cellulıtıs of her left leg

Table IV Clınıcal manıfestatıon of GVHD

Patıent No	Grade Skın	Lıver	Gut
64	2	2	2
132	2	3	–
125	1	3	–
51	2	1	–
91	3	2	1
166	–	–	–
68	2	–	–

One patıent (no 166) dıd not respond to the donor leukocyte ınfusıons The remaınıng four

patients (including the patient with second BMT) developed clinical signs of GVHD. GVHD was limited to skin and liver in three patients and responded to treatment with corticosteroids only The fourth patient (no. 51) did not respond to the first course of leukocyte infusions, but he developed exanthema, liver function abnormalities, and thrombocytopenia with a nadir of $28x10^9/l$ after the second course. He was treated with corticosteroids and CsA. Cytomegalovirus pneumonitis was diagnosed 10 weeks after the second transfusion period. The pneumonitis responded favourably to gancyclovir.

Erythrocyte markers and erythrocyte transfusions

Recipient erythrocyte markers were available in six of seven patients (Table V). Recipient erythrocytes disappeared completely in three patients. In patient no. 91, the rapid decrease in recipient erythrocytes between 1 week and 1 month after the leukocyte infusions could be attributed to hemolysis. Hemolysis was caused by a donor-derived antibody in a setting of minor ABO incompatibility of patient and donor (patient A, donor O).[21] The percentage of recipient erythrocytes decreased to very low levels (0.02%) in patient no. 64. In patient no. 51, who responded to the second course of infusions, the recipient erythrocyte level is still decreasing. Patient no. 166 showed no response. Donor erythrocyte markers were available in five patients. A complete restoration was observed in three patients. The percentage of donor erythrocytes is slowly increasing in patient no. 64, who is still suffering from moderate GVHD. The fifth patient showed a persistent absence of donor erythropoiesis, probably caused by a major ABO incompatibility of donor and patient (patient O, donor A2).[22]

In situ hybridization and cytogenetic markers

Three patients had donors of opposite sex. All three patients had no recipient cells detectable using ISH 6 months after leukocyte infusions (Table V). Cytogenetic recipient markers were present in six patients. In all six patients, recipient cells were no longer detectable at periods varying from 1 to 6 months after the leukocyte transfusions onward.

Philadelphia chromosome and BCR-ABL breakpoint molecules

Ph chromosome was present in all patients before treatment. It could no longer be detected in the 25 to 32 metaphases analysed in all six responders from 1 to 6 months after treatment onward. Patient no. 166 received a second course of infusions. Follow up in this patient is short (5 months), but there were no signs of cytogenetic response 3 months after leukocyte infusions. The BCR-ABL translocation could be detected in all 6 patients who were treated with donor

leukocytes. Four of six patients became negative in PCR at 3 to 12 months after leukocyte infusions. Patient no 51, who responded cytogenetically to the second course of infusions, still has BCR-ABL breakpoints demonstrable 6 months after the second treatment. Patient no. 166 did not show a molecular response (Table V). Patient no. 68 was PCR negative 12 months after second BMT.

Median follow up after leukocyte infusions is 12 months (range, 5 to 19 months).

Table V. Analysis of mixed chimerism and residual leukemic cells

Patient No	Time related to infusion	Erythrocyte %P	Erythrocyte %D	Cytogenetic analysis P	Cytogenetic analysis D	ISH P	ISH D	BCR-ABL Translocation
64	before	88 6	0	20 (Ph$^+$)	0*			b2a2(+)
	3 months	48 4	0 37	0	32			b2a2(+)
	6 months	0 05	0 47	0	32			b2a2(+)
	12 months	0 02	30	0	32			b2a2 (-)
132	before	48 7	NA	23 (Ph$^+$)	9			b3a2(+)
	2 months			0	32	0	400	
	3 months	1.1	NA	0	32			b3a2(+)
	6 months	0	NA	0	32	0	400	b3a2(-)‡
	12 months	0	NA	0	32	1	399†	b3a2 (-)
125	before	NA	88 6	12 (Ph$^+$)	20	75	325	
	1 week	NA	75 4	7 (Ph$^+$)	25	92	308	b3a2(+)
	1 month	NA	94 7	8 (Ph$^+$)	27			b3a2(+)
	3 months	NA	90 5	0	32	5	395†	b3a2(-)‡
	6 months	NA	99 2	0	32	0	400	b3a2(-)
	12 months	NA	89	0	20	2	398†	b3a2(-)
51	before	100	0	27 (Ph$^+$)	5¶			b2a2(+)
	1 week	100	0	31 (Ph$^+$)	1			b2a2(+)
	1 month	96 7	0 013	24 (Ph$^+$)	8			b2a2(+)
	1 week§	100	0	26 (Ph$^+$)	6			b2a2(+)
	1 month§	100	0	21 (Ph$^+$)	4			b2a2(+)
	3 months§	27 1	0	0	32			b2a2(+)
	6 months§	0 03	0	0	32			b2a2(+)
91	before	82 3	0 18	32 (Ph$^+$)	0	387	13	b2a2(+)
	1 week	92 6	1 74	31 (Ph$^+$)	1			b2a2(+)
	1 month	11 2	1 3	0	32	0	400	b2a2(+)
	3 months	0 01	28 5	0	32	1	399†	b2a2(-)
	6 months	0	95 1	0	32	1	399†	b2a2(-)
166	before	87 5	NA	4Ph+/32¥				b2a2(+)
	1 week	100	NA	2Ph+/20				b2a2(+)
	1 month	95	NA	6Ph+/20				b2a2(+)
	3 months	96 7	NA	14Ph+/20				b2a2(+)
	1 month§	81	NA	15Ph+/20				b2a2(+)
	3 months§	88	NA	12Ph+/20				b2a2(+)
68	6 months	NA	93 4	0	25*			
	12 months	0	100	0	30			(-)

Abbreviations P, patient; D, donor; NA, not available, b, exon of BCR gene, a2, second exon of ABL gene
* Discrimination of patient and donor by variance of chromosome 9 † One of 5 discriminating cells are considered background ‡ First test weakly positive, repeat test negative § After second course of transfusion ¶ Discrimination of patient and donor by variance of chromosome 1 ¥ No discrimination of patient and donor by cytogenetic analysis

DISCUSSION

Relapse rates of 50% and higher have been observed in CML patients after BMT with T-cell depleted grafts compared to 10% to 20% in CML patients transplanted with unseparated marrow [1] Part of the reduced antileukemic activity of T-cell depleted grafts can be explained by a lower incidence of GVHD An additional antileukemic effect mediated by T-lymphocytes independent of GVHD has been suggested to be important for permanent cure of CML patients [2] A seceond BMT with unseparated marrow may achieve long term disease-free survival in patients with relapsed CML, reflecting the antileukemic effect of T-cells in the graft [4,5] This is associated with a high procedure-related mortality [4,5] In this study one patient received a second BMT with unseparated marrow from the same donor 3 years after the first BMT Acute and chronic GVHD after second BMT were of moderate severity and responded well to treatment with corticosteroids only Twenty months after second BMT, she is in hematologic remission with no detectable recipient cells PCR of the BCR-ABL breakpoint molecules was negative 12 months after second BMT

Recently, two case reports have been published on infusion of donor buffy coat in a small number of patients with CML who had relapsed after BMT [6-8] Kolb et al [6] induced cytogenetic remission in three patients with hematologic relapse by treatment with INFα followed by donor leukocyte infusions Two of them developed GVHD Cullis et al [7] described two patients with cytogenetic relapse after BMT Both of them became Ph chromosome negative and subsequently BCR-ABL breakpoint molecule negative after donor leukocyte infusions Both patients developed GVHD Drobyski et al [8] describe one patient with relapsed CML after a second BMT who achieved hematologic, cytogenetic, and molecular remission after leukocyte infusions In this study five of six patients became Ph chromosome negative after donor transfusions In four of five patients, BCR-ABL breakpoint molecules were no longer detectable during further follow up Besides erythrocyte markers, cytogenetic analysis, and ISH, we used PCR on variable number of tandem repeats (VNTR) to evaluate chimerism [23] In the responding patients, recipient cells were no longer detectable using cytogenetic analysis and ISH Recipient erythrocytes disappeared in two and decreased markedly in two patients The results of VNTR were in accordance with the outcome of the other assays (data not shown)

Residual leukemic cells can be detected in CML patients by cytogenetic analysis of the Ph chromosome and by PCR to detect the BCR-ABL breakpoint translocation The latter assay is approximately 1000 times more sensitive than cytogenetic analysis This explains the time difference between attaining a cytogenetic remission and a BCR-ABL breakpoint molecule negative

status

In a study on the role of PCR in predicting relapse after BMT for CML, Hughes et al [24] found that PCR negativity post-BMT was predictive of a low risk of relapse They suggested that PCR positivity up to 6 months after BMT had no adverse prognostic significance, in contrast to PCR positivity after 6 months Extrapolating these findings, we may expect a low relapse rate in five patients in this study who became negative in the PCR of the BCR-ABL fusion gene We recently described a relation between the percentage of recipient RBCs and the cytogenetic status in CML patients after BMT [25] No or low percentages (<0 5%) of recipient RBCs at 6 months or later after BMT were observed in patients with continuous cytogenetic remission In contrast, high (>10%) or increasing percentages of recipient erythrocytes indicated cytogenetic relapse Further follow up will be necessary to prove whether this will also apply to patients after donor leukocyte transfusions

Cytogenetic response was always preceded by manifestations of GVHD in our patients The clinical picture resembled that of chronic GVHD observed 3 months or later after BMT of a first marrow graft The number of lymphocytes transfused was equal to the usual number of lymphocytes in non-T-cell-depleted marrow grafts in two patients (no 64, 132), but three to 10 times higher in the remaining patients We observed no relation between the severity of GVHD and the number of transfused lymphocytes GVHD was of moderate severity and responded well to treatment in three patients (no 132, 125, and 51) GVHD was more severe in patient no 64 and attributed to death in patient no 91 Two patients became pancytopenic after donor leukocyte transfusions, which was associatiated with GVHD with partial recovery in patient no 64 who had no detectable donor cells prior to the infusions Patient no 91 showed complete recovery after corticosteroid treatment This patient died from sepsis 6 months after the leukocyte infusions The mortality rate of this treatment approach appeared to be low, with a high response rate For this reason, donor lymphocyte infusions seem to be the treatment of choice for CML relapsed after BMT The exact number of lymphocytes required for therapeutic effect remains to be determined Patient no 51 is interesting for this point He responded to a second treatment with twice as many lymphocytes compared with the first treatment

We conclude that infusion of donor leukocytes is an effective treatment in CML patients with relapse after BMT with a T-cell depleted graft This treatment is capable of inducing a BCR-ABL breakpoint molecule-negative status Moderate to more severe GVHD always preceded response in these patients Longer follow up and additional patients will be needed to know whether cure will be permanent It will be interesting to test the possibility of separating GVHD from graft-versus-leukemia effect by infusing certain subpopulations of donor lymphocytes, eg, natural-

kıller cells

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Chapter 5.

Erythrocyte repopulation after major ABO incompatible transplantation with lymphocyte-depleted bone marrow

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Summary

Forty-four out of 258 allogeneic BMT were performed across the major ABO barrier Donor erythrocyte repopulation could be evaluated in 30 cases Fifty-eight patients transplanted with an ABO compatible or minor incompatible graft served as the control group All patients received a marrow graft depleted of lymphocytes by counterflow centrifugation Less than 10^8 residual erythrocytes were present in the graft Cyclosporin A was used as immunoprophylaxis after transplantation Erythrocyte repopulation was measured using a fluorescent microsphere method An adapted transfusion policy was applied

Eight out of 30 patients (27%) with major ABO incompatibility had no detectable donor erythrocytes 2 months after BMT Up to 3 months after BMT donor erythrocyte repopulation was significantly delayed in the ABO incompatible group ($p \leq 0\ 03$) Significantly more erythrocyte transfusions were required in the ABO incompatible group ($p<0\ 001$) Six patients with blood group O (20%) developed pure red cell aplasia which resolved in 5 without therapeutic intervention In these six patients anti-A antibody titers were persistently high the first 3 months after BMT This was in contrast with 22 patients with timely recovery of erythropoiesis in whom anti-A and anti-B antibody titers showed a steady decrease after BMT

The incidence of immunohematological complications in these patients who received a lymphocyte depleted major ABO incompatible graft is similar (20%) to the incidence reported in the literature Serious morbidity related to major ABO incompatibility did not occur

Introduction

Major ABO incompatibility between recipient and donor occurs in about 10-15% of HLA matched allogeneic bone marrow transplantations Acute hemolysis by anti-A or anti-B antibodies of recipient origin at the time of infusion of the donor marrow can be prevented by reduction of the antibody titers in the recipient or by removal of erythrocytes from the graft [1 5]
Studies by Bensinger et al [6] and Buckner et al [7] have demonstrated that major ABO incompatibility is no obstacle to successful outcome after BMT No increased risk of graft rejection, graft versus host disease (GVHD) or mortality related to ABO incompatibility has been observed Several reports have described delayed hemolytic anemia, delayed onset of erythropoiesis, pure red cell aplasia (PRCA) and increased posttransplant transfusion requirements after major ABO incom-

patible BMT.[8-13]

In contrast to the previous studies all patients transplanted in our center received marrow grafts depleted of lymphocytes by elutriation. Donor erythropoiesis and transfusion requirements were evaluated in 30 patients who received a major ABO incompatible graft and compared to an ABO compatible or minor ABO incompatible control group of 58 patients. Delayed onset of erythropoiesis occurred in eight patients who received a major ABO incompatible graft. The clinical course after BMT of these patients is described in more detail. The pretransplant titers and the posttransplant course of ABO antibodies in these patients were compared to those of the other 22 patients transplanted with a major ABO incompatible graft.

Subjects and methods

Patient groups

From 1981 to 1994, 285 allogeneic bone marrow transplantations were performed in adults. In 44 cases (15%) major ABO incompatibility existed between patient and donor. In 14 out of 44 patients donor erythrocyte repopulation could not be evaluated for the following reasons; early transplant related mortality within one month after BMT in three, early relapse within 6 months after BMT in five, graft failure and autologous recovery in three and no available data in three patients. The remaining 30 patients were transplanted for acute myeloid leukemia (AML) (n=6), acute lymphoid leukemia (ALL) (n=8), chronic myeloid leukemia (CML) (n=14), severe aplastic anaemia (SAA) (n=1) and eosinophilic syndrome (n=1). Twenty patients with blood group O received either a blood group A graft (n=19) or a blood group B graft (n=1). AB marrow was transplanted to three patients with blood group A and one patient with blood group B. Three patients with blood group A received a blood group B graft and three patients with blood group B received a blood group A graft.

Twenty-six donors were HLA-identical sibling donors and four donors were HLA matched unrelated volunteers (UPN 168, 199, 226 and 281). Conditioning regimens always included cyclophosphamide (Cy) in a total dose of 120 mg/kg. This was followed by total body irradiation (TBI) in two equal fractions with 24 h interval in a total dose of 9 or 12 Gy in 28 patients. Anthracyclines (daunorubicin 156 mg/m^2 or demethoxydaunorubicin 42 mg/m^2) were added in 18 out of 22 patients who received TBI in a dose of 9 Gy. One patient (UPN 281) was conditioned with Cy, TBI (9Gy) and total lymphoid irradiation (TLI) (8Gy). Another patient was treated with busulfan and Cy (UPN 126). In the only patient (UPN 226) transplanted for SAA the conditioning

regimen consisted of Cy and TLI (12Gy) Details on the conditioning regimens have been described earlier [14,15] Plasma exchange or plasma immunoadsorption to reduce anti-A or anti-B antibody titers before BMT were never applied

In the same time period, 241 ABO compatible or minor ABO incompatible transplantations were performed Fifty-eight patients could be used as a control group to study donor erythrocyte repopulation The remaining patients were considered not to be evaluable mainly because of lack of a donor erythrocyte marker (see below) or because they received unselected transfusions not taking into account the donor marker Other reasons were early transplant-related mortality, early relapse or graft failure Indications for transplantation were AML (n=14), ALL (n=15), CML (n=17), low-grade non-Hodgkin lymphoma (n=5), multiple myeloma (n=4), SAA (n=2) and myeloproliferative syndrome (n=1) Fifty-four donors were genotypically HLA-identical sibling donors and four donors were one locus mismatched siblings The conditioning regimens always included Cy (120 mg/kg) Except for one patient who was treated with TLI, all patients received TBI in a total dose of 9 or 12 Gy Anthracyclines were added in 35 out of 39 patients treated with TBI in a dose of 9 Gy

Both in the study and in the control group all marrow grafts were depleted of lymphocytes by density gradient centrifugation followed by counterflow centrifugation [16] Less than 10^8 residual erythrocytes were present in the marrow graft Cyclosporin A (CsA) was used as immunoprophylaxis posttransplant in all patients in a schedule, as previously described [17]

Erythrocyte markers

Analysis of patient and donor erythrocyte populations the first months after BMT required an adapted transfusion policy [18,19] Before BMT, preferentially before any transfusions were given, a sample for complete red cell phenotyping was drawn from the patient After phenotyping of the donor, marker antigens, i e antigens present in the donor and absent in the recipient and vice versa, were determined In case of major ABO incompatibility the A or B antigen was used as a donor marker In the control group donor marker antigens were C, c, D, E, K, M, N, S, s, Fy^a, Fy^b, Jk^a, Jk^b and Kp^a Erythrocyte transfusions of blood group O lacking the marker antigens were administered to all patients in both groups

Measurement of host and donor populations was performed by a fluorescent microsphere method The sensitivity of this assay is one positive cell per 10000 negative cells (0 01%) [20] Donor erythrocyte repopulation was studied at 0 5, 1, 2, 3, and 6 months after BMT

Anti-A and anti-B antibodies

Anti-A and anti-B agglutinin (IgM) titers were determined by incubating a 3% standard A and

B erythrocyte suspension in saline with 2-fold serial dilutions of patient serum at 37°C for 45 min followed by centrifugation and scoring for macroscopic agglutination. Anti-A and anti-B IgG titers were measured by pretreating the patient serum with dithiotreitol at 37°C for 45 min followed by indirect antiglobulin testing with anti IgG serum and scoring for macroscopic agglutination.

Antibody titers were determined before and at least 0.5, 1, 2, 3, and 6 months after BMT.

Statistical analysis

The Fisher's exact test was used to compare clinical variables between groups. The Kruskal-Wallis test was applied to compare percentages of donor erythrocytes at different time points after BMT. Within the study group antibody titers were compared using the Mann-Whitney U test.

Results

Recovery of erythropoiesis in the study and control group

Because of reasons mentioned earlier it was not possible to form a fully matched control group based on matching for indication for BMT, conditioning regimen, age and acute GVHD. Table I shows the comparison of clinical variables between the two groups. No significant differences could be found.

Table II shows the means and standard deviations of percentages of donor erythrocytes at various time points after BMT. Significant delay in donor erythrocyte repopulation in the ABO incompatible patient group compared to the control group could be demonstrated up to 3 months after BMT. The difference between the two groups was no longer significant at 6 months after BMT.

To demonstrate delayed donor erythrocyte repopulation in the major ABO incompatible group we compared the relative number of patients without detectable donor erythrocytes (i.e. <0.01%) between both groups at various time points early after BMT. In the study group 62% and 33% of the evaluable patients had no donor red cells detectable at 0.5 and 1 month after BMT, respectively. In the control group these percentages were 19% and 0% respectively ($p<0.01$).

The median number of erythrocyte transfusions after BMT was 18 (range 6-64) in the ABO incompatible group and 10 (range 2-46) in the control group ($p<0.001$).

Leukocyte and platelet recovery did not differ between the ABO incompatible group and the control group (data not shown).

Table I. Comparison of clinical data between the study and the control group

	major ABO incompatible BMT n = 30	control group n = 58	
Conditioning regimen			
TBI: 9 Gy	22	39	
12 Gy	6	18	NS
anthracyclines (added to TBI 9 Gy)	18	35	NS
Indications for BMT:			
acute leukaemia	14	29	
CML	14	17	
others	2	12	NS
Age (years):			
< 40	21	30	
≥ 40	9	28	NS
Donors.			
histocompatible sibling	26	54	
others	4	4	NS
GVHD (grade):			
0 - I	23	41	
≥ II	7	17	NS

No significant differences of clinical variables between the groups were found (Fisher's exact test).

Table II. Donor erythrocyte repopulation in time

Time (months)	major ABO incompatible BMT % donor erythrocytes			control group % donor erythrocytes			
	n[1]	mean	SD[2]	n[1]	mean	SD[2]	
0.5	26	0.04	0.07	54	0.23	0 56	p<0.01
1	30	1.73	2.66	54	5.26	4.81	p<0.01
2	27	18.02	20.47	50	29.64	14.76	p<0 01
3	25	38 65	32.25	51	55 93	22 02	p=0 03
6	25	73.93	31.29	51	83.93	18 69	p=0.2

[1] number of evaluations [2] standard deviation

Delayed onset of erythropoiesis of at least 2 months after BMT

Eight patients in the ABO incompatible group (recipients blood group O, grafts blood group A) had no detectable donor erythrocytes up to 2 months after BMT (UPN 70, 83, 100, 126, 162,

199, 253, 281). In this same time period percentages of recipient erythrocytes in the six out of eight patients with a recipient marker (UPN 83 and 281 had no recipient marker) were progressively decreasing. In these eight patients leukocyte counts >1.0x10^9/l were reached at a median of 29 days after BMT (ranging from 19 to 35 days). Patient UPN 199 died of cerebral haemorrhage while hypertensive due to CsA intoxication. At the time of death, 2 months after BMT, the platelet count was still below 20x10^9/l. In the remaining patients a platelet concentration of > 20x10^9/l without transfusion support was reached at a median of 21 days with a range of 18 to 116 days. Data on these patients are given in Table III.

Table III. Data on 30 major ABO incompatible patients

UPN	pretransplant anti-A or anti-B titers IgM	IgG	leukocytes[1] >1.0x10^9/l	platelets[1] >20x10^9/l	time to onset of erythropoiesis[2]	erythrocyte transfusions (no.)
delayed onset of erythropoiesis (n=8):						
70	32	512	30	19	5.5	36
83	256	512	33	116	3	42[3]
100	32	128	19	23	3.5	15
126	512	16000	21	18	7	37
162	64	64	30	21	4	29
199	128	1024	35	–	NE	28[3]
253	32	128	28	19	6	49
281	64	2048	22	27	>9	64[3]
median	64	512	29	21	5.5	37
timely onset of erythropoiesis (n = 22):						
median	64	16	20	25	1	16
range	4 - 512	1 - 512	10 - 29	11 - 50	0.5 - 2	6 - 30

[1] days after BMT [2] months after BMT [3] until death
NE = not evaluable, patient died 2 months after BMT

UPN 83 developed grade II acute GVHD of the skin 3 weeks after BMT. Treatment consisted of addition of corticosteroids to the CsA. While tapering off these drugs she developed limited chronic GVHD of the gut. Two months after BMT a bone marrow aspirate showed active myelopoiesis, absent erythropoiesis and no megakaryocytes. Three months after BMT the percentage of donor erythrocytes was 0.22. At that time point erythropoiesis in the marrow was active but the number of megakaryocytes was still low. She remained erythrocyte transfusion

dependent with 0.07% of donor erythrocytes 4 months after BMT. Platelet counts never rose above $50x10^9/l$. She died of CMV pneumonitis 6 months after BMT.

UPN 100 remained transfusion dependent up to 3 months after BMT. During this period there were no signs of acute GVHD. Bone marrow morphology at 1and 3 months showed PRCA. At 3 months after BMT no donor erythrocyte percentage was available. At 3.5 and 4 months after BMT these percentages were 4.2 and 19.2, respectively. He is in remission of ALL with 100% donor erythrocytes and no recipient erythrocytes at 6.5 years after BMT.

UPN 70 had no detectable donor erythrocytes up to 3 months after BMT with the picture of PRCA in the marrow aspirate. The patient did not suffer from acute GVHD. Treatment with corticosteroids was started 4.5 months after BMT in an attempt to stimulate donor erythropoiesis. One month later the percentage of donor erythrocytes was 36 and this gradually increased to 100% afterwards. This patient is in complete hematological, cytogenetic and molecular remission of CML 7 years after BMT.

UPN 126 had no detectable donor red cells up to 6 months after BMT reflected by PRCA in the marrow aspirates performed 1, 3 and 6 months after BMT. Cytogenetic analysis of the bone marrow performed 6 months after BMT showed only cells of donor origin. Neither acute nor chronic GVHD occurred. The last erythrocyte transfusion was required 6.5 months after BMT. Nine months after BMT, 78% donor erythrocytes were present. This patient died of marrow and CNS relapse of AML 10 months after BMT while only donor, but no recipient red cells could be demonstrated.

UPN 162 had PRCA in the marrow aspirate and no detectable donor erythrocytes 3 months after BMT. In these first months he was treated with corticosteroids for acute GVHD grade I limited to the skin. From 4 months onwards the percentage of donor cells started to rise to 100% at 1 year after BMT. Cytogenetic relapse of CML occurred 1 year later.

UPN 253, transplanted for eosinophilic syndrome, had no detectable donor erythrocytes until 6 months after BMT at which time point the percentage was 2 and erythropoiesis could for the first time be observed in the marrow aspirate. Acute GVHD did not occur. He died of Aspergillus sepsis complicating de novo chronic GVHD of the liver 9.5 months after BMT.

In UPN 281, transplanted with an unrelated graft for CML in first accelerated phase, donor erythrocytes were never demonstrable during follow up. She developed acute GVHD grade I limited to the skin which was successfully treated by addition of corticosteroids to the CsA. Because of limited chronic GVHD of the skin and gut, treatment with low-dose CsA was continued. Bone marrow morphology at 6 and 9 months after BMT showed PRCA. Cytogenetic and molecular remission existed at these observation points. In situ hybridization showed only donor cells. Relapse with blast crisis occurred 11 months after BMT. The patient died 1 month later.

All eight patients with delayed onset of erythropoiesis had blood group O in contrast to 12 out of 22 patients in the major ABO incompatible group with timely onset of erythropoiesis (p=0 02)

Course of recipient erythrocytes in the study and control group

The course of recipient erythrocytes after BMT could be studied in 20 patients in the study group and 36 patients in the control group The same rates of decreasing percentages of recipient erythrocytes were observed in the control group and in the study group, both in patients with delayed and in patients with timely recovery of donor erythropoiesis Six months after BMT recipient erythrocytes still were detectable in eight out of 17 patients in the study group and nine out of 32 patients in the control group (p=0 1) At that time point means and standard deviations of percentages of recipient erythrocytes were 0 23 ± 0 2 and 0 48 ± 0 47 for the study and the control group respectively (not significantly different) This data implicates a high percentage of mixed erythrocyte chimerism in both the major ABO incompatible and the control group

Anti-A and anti-B antibody titers in major ABO incompatible BMT

Anti-A and anti-B titers before BMT were compared between the eight patients with delayed onset of donor erythropoiesis and the remaining 22 patients in the major ABO incompatible group (Table III) No significant difference in saline (IgM) titers before BMT could be observed between the two groups However, IgG titers before BMT were significantly different (p<0 01) between the groups This difference still existed after leaving out the IgG titre of 16000 of UPN 126 (p=0 01)

In the 22 patients, mentioned above, a sharp decrease of anti-A and anti-B antibodies IgM as well as IgG, was observed after BMT Three months after BMT IgM and IgG titers were measured in 20 out of 22 patients At that time point the IgM titer was zero in 14 patients and varied from one to eight in the remaining six patients The IgG titer was zero in 11 patients and varied from one to eight in the other nine patients The mean antibody titers (IgM and IgG) of the 22 patients before and in the first 6 months after BMT are shown in Figure 1A and 1B

The course of the anti-A and anti-B antibodies after BMT in the eight patients with delayed onset of donor erythropoiesis also is shown in Figure 1A (IgM) and 1B (IgG) Within the first month after BMT an increase of titers was observed in some patients and stable titers in other patients Only UPN 83 showed a three-step decrease of titers in this time period Three months after BMT all seven patients alive still had detectable antibody titers in a range higher than the other 22 patients in the ABO incompatible group

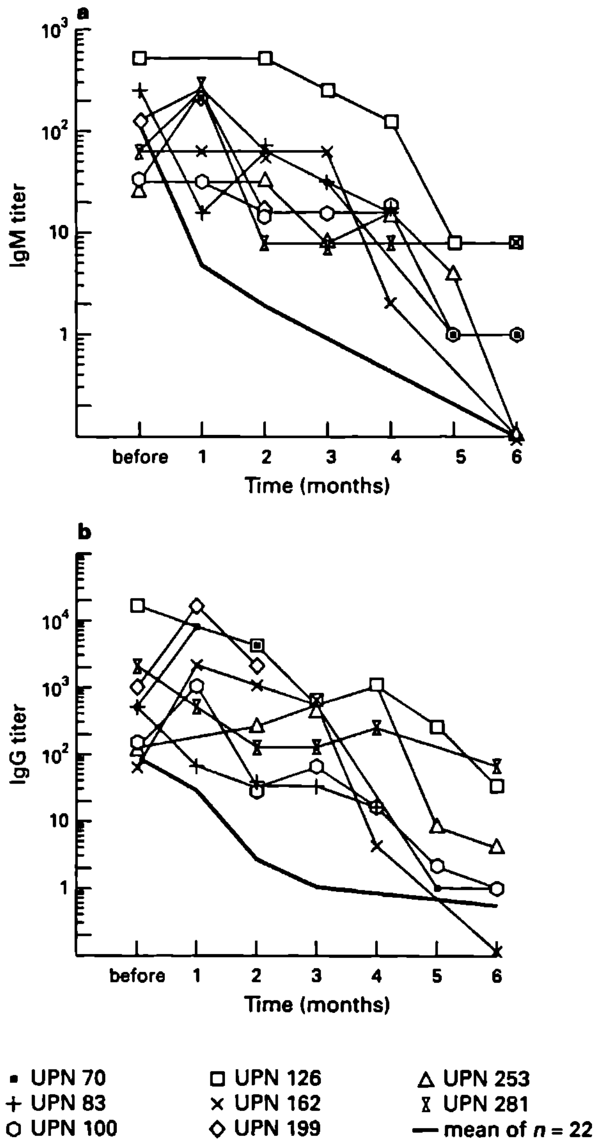

Fig 1 Course of ABO antibody titers IgM (A) and IgG (B) after major ABO incompatible BMT
(A) shows the mean course of anibody titers (IgM) in 22 patients with timely recovery of donor erythropoiesis after major ABO incompatible BMT (bold line), the eight patients with delayed onset of erythropoiesis are shown separately, (B) shows the same course for IgG

Discussion

Delayed onset of donor erythropoiesis and persistence of anti-A or anti-B hemagglutinins are well known phenomena after major ABO incompatible BMT [5 9 10] After red cell depletion of the graft Sniecinski et al [9] observed delayed onset of erythropoiesis >40 days after BMT and persistence of anti-A and anti-B antibodies >120 days in 10 out of 66 (15%) major ABO incompatible transplantations In a later study on major ABO incompatibility these complications occurred in nine out of 58 (16%) patients while the overall incidence of immunohematological complications was 21% Serious associated morbidity was not observed however and erythropoiesis became normal in all but one patient [10] In a study by Gmur et al [11] three out of 15 major ABO incompatible transplants (20%) developed PRCA lasting 5 to 8 months

In our study erythropoiesis was delayed in the 30 patients who received a major ABO incompatible graft compared to an ABO compatible or minor ABO incompatible control group In eight out of 30 patients, donor erythrocytes were still undetectable 2 months after BMT Six out of eight patients developed the clinical picture of PRCA lasting from 3 5 to 9 months after BMT Except for a transient positive direct antiglobulin test in UPN 70 no clinical or laboratory signs of hemolysis were observed at the beginning of erythrocyte production in five of the six PRCA patients In one patient donor erythrocytes were never detectable after BMT UPN 83 had 0 22% of donor erythrocytes 3 months after BMT Major ABO incompatibility may have played a role in the delayed onset of erythropoiesis in this patient but GVHD and recurrent viral infections can not be ruled out as additional causative factors

All patients with delayed onset of donor erythropoiesis were blood group O which was significantly more often than in the major ABO incompatible patients with timely onset of erythropoiesis Most cases of PRCA after BMT reported in the literature involve patients with blood group O and donors with blood group A or B although 2 cases of a recipient with blood group A and a donor with blood group B and vice versa have been described [10 11 21 22]

Twenty-two patients in the major ABO incompatible group had detectable donor erythrocytes within 2 months after BMT Pretransplant IgM anti-A and anti-B antibody titers were identical and IgG titers were significantly lower compared to the eight patients with delayed onset of erythropoiesis Various reports in the literature did not agree on a correlation between pretransplant ABO antibody titers and the number of days posttransplant before onset of erythropoiesis [5 10 11]

In the 22 patients antibody titers decreased immediately after BMT and were undetectable or present in low titers at 3 months after BMT This is in agreement with the data of Gmur et al [11] in 11 patients with timely recovery of erythropoiesis after major ABO incompatible BMT They

described a progressive decrease and titers persistently ≤16 from day 32 onward The course of ABO antibodies after BMT was clearly different in seven out of eight patients in our study with delayed onset of erythropoiesis An early rise or persisting high titers (IgG more than IgM) were observed in this group A correlation between delayed onset of erythropoiesis and posttransplant rise or persistence of high (above 16) antibody titers was also described in recipients of a non-lymphocyte depleted major ABO incompatible graft Only after a substantial reduction in these titers to a level of four to 16 erythrocyte production began [5,10,11] Delay in erythropoiesis can be explained by the interaction of anti-A or anti-B antibodies with donor erythroid precursors expressing the A and/or B antigens [23,24]

A study comparing the course of anti-donor ABO antibodies after BMT between recipients of elutriated and non-elutriated major ABO incompatible grafts was performed by Bar et al [25] Although the number of patients studied was small, no evidence for prolonged persistence of anti-donor antibodies in the elutriated group was found

The incidence of immunohematologic complications and PRCA (20%) in our study is similar to the incidence reported in the literature [10,11] Theoretically one could have expected a higher incidence of immunohematological complications in our patients Firstly, all our patients received red cell depleted grafts instead of lowering pretransplant ABO antibody titers in the recipient In the study by Gmur et al [11] 14 evaluable patients received an ABO incompatible graft Seven patients underwent large volume plasma exchange and seven patients received an erythrocyte depleted graft Timely recovery of erythropoiesis was observed in all but three patients who had received a red cell depleted graft Jin et al [26] however found no difference in erythrocyte transfusion requirements between recipients of a red cell depleted graft and patients who had plasma exchange or immunoadsorption before major ABO incompatible BMT Secondly, all our patients received a lymphocyte depleted graft in contrast to the patients reported in the literature Earlier studies in our patient population showed that lymphocyte depletion of donor marrow resulted in a high incidence of mixed chimerism and persistence of host lymphocytes [27,28] However, cytogenetic evaluation of blood and marrow was usually not performed before 6 months after BMT In the major ABO incompatible patients of the current study cytogenetic analysis of the blood was not performed Cytogenetic analysis of the bone marrow 6 months after BMT showed no difference in mixed chimerism between the group with PRCA and without PRCA (data not shown) This data suggests that mixed chimerism of the bone marrow does not play a major role in the development of PRCA

CsA was given as immunoprophylaxis posttransplant in all patients reported in the literature who developed PRCA after major ABO incompatible BMT [10-12,21,22,29-33] In these studies CsA was administered alone or in combination with prednisone or a short course of methotrexate (MTX)

PRCA was never observed after the use of only MTX to prevent GVHD CsA acts primarily as an immunosuppressive agent on T-lymphocyte proliferation in response to primary antigen stimulation In the case of major ABO incompatibility T-lymphocytes are re-exposed to the stimulating A or B antigens In this setting CsA does not inhibit T-lymphocyte proliferation

All our patients were treated with CsA In one patient with PRCA (UPN 100) erythropoiesis began while CsA was still administered In the other four patients erythropoiesis started 0 5, 1, 2 and 3 months after cessation of CsA according to the treatment protocol In the three patients with PRCA described by Gmur et al [11] erythropoiesis began during CsA treatment in one patient and 10 and 83 days respectively after conclusion of CsA in two patients Volin and Ruutu[21] described one patient recovering from PRCA while still on CsA treatment In the case study by Ohashi et al [29] stopping CsA had no effect on the recovery of PRCA So, in our opinion CsA only plays a role in the possibility to develop PRCA after allogeneic BMT

Treatment with corticosteroids for PRCA was not found to be beneficial [10 29 32] In our study UPN 162, 253 and 281 received corticosteroids for GVHD without apparent effect on erythro- poiesis Corticosteroids were administered to UPN 70 4 5 months after BMT in an attempt to treat PRCA No further erythrocyte transfusions were required One month afterwards 36% erythrocytes of donor origin were present Spontaneous recovery can not be excluded as anti-A antibody titers were clearly decreasing before corticosteroid treatment was started

Treatment successes and failures in PRCA after BMT have been described with plasma exchange [10 21 22 32] and erythropoietin (EPO) [29 30 33] A patient described by Ohashi et al [29] did not respond to EPO alone but erythropoiesis began after the addition of methylprednisolone to EPO Gamma globulins were not found to be beneficial in two case reports [29 33] In two cases of PRCA treatment with anti-lymphocyte globulin resulted in restoration of erythropoiesis [22 31]

Immunohematologic complications did not occur more frequently in recipients of major ABO incompatible marrow grafts, depleted of lymphocytes by counterflow centrifugation, compared to recipients of unmanipulated grafts Serious morbidity usually did not occur and immuno-suppressive intervention was only occasionally necessary

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Chapter 6.

Reconstitution of antibody response after allogeneic bone marrow transplantation: effect of lymphocyte depletion by counterflow centrifugal elutriation on the expression of hemagglutinins

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Summary

The generation of ABO hemagglutinins was used as a model to assess the tempo of reconstitution of antibody responses in recipients of elutriated (CCE) and nonelutriated (nonCCE) HLA matched bone marrow allografts. The study included 29 CCE recipients (10 major, six minor, and one major/minor ABO mismatched, and 12 ABO matched) and 40 nonCCE recipients (14 major, 12 minor, two major/minor, and 12 matched). Plasma volume in the graft and in blood product transfusions was uncorrelated with changes in hemagglutinin titers and therefore was excluded as a significant source of antibody. Removal of graft lymphocytes by CCE did not result in prolongation of host-derived hemagglutinins in recipients of major ABO mismatched grafts. However, CCE resulted in a complete abrogation of the adoptive transfer of donor-derived antibody as detected in recipients of minor ABO mismatched grafts. Despite the absence of adoptively transferred donor immunity in recipients of CCE grafts, they had hemagglutinin levels comparable with those of recipients of nonCCE grafts by 6 months after transplantation. This demonstrates that recipients of elutriated marrow were competent to mount de novo responses at that time. The strong correlation between donor pretransplant hemagglutinin titer and recipient titer one year after bone marrow transplantation in recipients of nonCCE grafts suggests that even late after transplant, antibody remains the product of adaptively transferred memory cells in recipients of grafts containing large numbers of mature lymphocytes.

Introduction

Counterflow Centrifugal Elutriation (CCE) has emerged as an efficient and safe methodology for the removal of lymphocytes from bone marrow allografts.[1-3] Lymphocyte depletion by this method effectively prevents acute graft-versus-host disease (aGVHD) in a lymphocyte dose dependent fashion in patients at high risk for this complication of bone marrow transplantation (BMT).[3] Beyond simple depletion, CCE has proven to be a flexible technique capable of subfractionating lymphocytes into functionally distinct subpopulations.[4,5] This may ultimately open the way for immunologic graft engineering. To realize this potential, it is important to closely document immune reconstitution in recipients of elutriated marrow and assess the impact of this procedure by comparison with patients transplanted with conventional allografts. By evaluating the expression of hemagglutinins we have implemented a simple, biologically meaningful method for the prospective evaluation of the development of T- and B-lymphocyte dependent immunity. Further, the availability of ABO mismatched donor/recipient pairs has enabled us to ascribe the

origin of particular antibody specificities uniquely to donor or recipient, and track the course of their disappearance or emergence as a function of graft type (elutriated or nonelutriated) and time after BMT Apart from the practical need to evaluate the effects of lymphocyte depletion on immune reconstitution, this methodology has also permitted us to dissect the relative contributions of the various components that can potentially influence the reconstitution of antibody responses These include passively transferred antibody, residual recipient responses, donor immune memory cells adoptively transferred in the graft, and lymphocytes of donor origin that have differentiated within the host environment

Materials and methods

Mismatch definitions

For the purpose of analysis, donor and recipient ABO disparities were divided into two groups depending on the direction of potential immune recognition Major ABO incompatibility was defined as a mismatch in which the recipient had the potential to express hemagglutinins directed against donor ABO antigens (i e , recipient antidonor antibody) In minor ABO mismatch, the donor had the potential to express hemagglutinins directed against ABO antigens on recipient erythrocytes (donor antirecipient antibody) Donor and recipient ABO blood group typing and expected antibody specificities are given for the different combinations of ABO mismatched BMT (Table I)

Patient population

Between December 1985 and January 1989, 64 patients underwent allogeneic BMT with an elutriated (CCE) HLA matched graft In 17 of these patients there was an ABO incompatibility between recipient and donor, 10 cases with major, six cases with minor, and one case with both major and minor incompatibility (Table I) The hemagglutinin titers of these 17 patients were compared with those of 28 recipients of HLA matched, ABO mismatched, nonelutriated grafts between 1983 and 1989 (Table 1) These 28 patients were participants in an immune reconstitution study, and frozen (-70°C) serial serum samples were therefore available All 17 recipients of elutriated bone marrow received cyclosporin A (CsA) to prevent graft rejection and graft-versus-host disease (GVHD) Nineteen of the patients with nonelutriated grafts received CsA and nine patients were treated with cyclophosphamide (Cy) for posttransplant GVHD prophylaxis [6] The median ages in these patient groups were 36, 21, and 22 years, respectively Patients were transplanted for chronic myelogenous leukemia (CML, eight CCE, seven nonCCE), acute myelogenous leukemia (AML, three CCE, eight nonCCE), acute lymphocytic leukemia (ALL, two

CCE, nine nonCCE); non-Hodgkin's lymphoma (NHL; three CCE, one nonCCE); Hodgkin's disease (HD: one CCE), and aplastic anemia (AA; three nonCCE). Patients in CCE and nonCCE groups could not be matched on age or disease status because the CCE group represented a high risk population participating in phase 1 trials.[2,3] The pretransplant preparative regimen was determined by the patient's admission diagnosis. Patients with AML were treated with busulfan 4 mg/kg orally for 4 days followed by Cy 50 mg/kg/d for 4 days.[7] Patients with AA were treated with Cy 120 mg/kg plus total body irradiation (TBI) or 200 mg/kg with or without TBI (12 Gy). The conditioning regimen for ALL, CML, HD, and NHL consisted of Cy 50 mg/kg/d for 4 days followed by TBI (12 Gy over 4 days). An additional 12 recipients of ABO matched CCE grafts (with CsA chemoprophylaxis) and 12 patients who received ABO matched, nonCCE grafts plus CsA were also studied (Table 1).

Table I. Definition of expected antibody specificity by patient and donor blood group

Blood group		Expected antibody sources and specificity			No of patients		
		Antirecipient	Antidonor	Donor and/or			
Donor	Recipient	(donor-derived)	(recipient-derived)	Recipient-derived	CCE	nonCCE+CsA	nonCCE+Cy
Major ABO incompatible							
A	O	NA*	Anti-A	Anti-B	5	7	
B	O	NA	Anti-B	Anti-A	4	2	1
AB	A	NA	Anti-B	NA	1		
AB	B	NA	Anti-A	NA		2	2
Major + minor ABO incompatible							
A	B	Anti-B	Anti-A	NA			1
B	A	Anti-A	Anti-B	NA	1	1	
Minor ABO incompatible							
O	A	Anti-A	NA	Anti-B	3	5	4
O	B	Anti-B	NA	Anti-A	2		
O	AB	Anti-A, Anti-B	NA	NA		1	
A	AB	Anti-B	NA	NA	1		
B	AB	Anti-A	NA	NA		1	1
Totals					17	19	9
ABO matched							
A	A	NA	NA	Anti-B	5	5	
B	B	NA	NA	Anti-A	2		
O	O	NA	NA	Anti-A, Anti-B	5	7	
Totals					12	12	0

*NA, not applicable. Antibodies of these specificities are not expected for the given recipient and donor ABO blood groups

For recipients of ABO incompatible transplants, samples were obtained before BMT and 2 weeks, 4 weeks, 3 months, 6 months, and 1 year after transplant. For recipients of ABO matched grafts, recipient serum samples were examined before BMT, at 2 intervals early after BMT (2 weeks and 4 weeks), and at one interval late after BMT (1 year in 22 of 24 cases). Sera were stored at -70°C before assay; all sera from a given patient were tested simultaneously. Donor sera obtained before marrow harvest were also assayed at the same time. A total of 362 patient and donor sera were assayed.

Bone marrow processing.

All elutriated grafts were quantitatively depleted of erythrocytes (99.94% reduction) and contained no plasma.[2,8] A predetermined number of lymphocytes was intentionally added to the elutriated grafts. Twenty-two patients included in this study received $1x10^6$ morphologic lymphocytes/kg ideal body weighty and seven received $0.5x10^6$ lymphocytes/kg.[3] Immunophenotypic analysis of 11 grafts formulated at $1x10^6$ lymphocytes/kg indicated a 97% reduction in T-cells (CD3 +) and a 96% reduction in B-cells (CD19+, HLA DR+) compared with the harvested marrow. Nonelutriated grafts were prepared according to the ABO status of the donor and recipient. To prevent massive hemolysis in ABO major mismatch, more than 95% of the erythrocytes were removed from the graft by apheresis, after which the bone marrow buffy coat was suspended in 50 to 100 mL of donor plasma.[9] For minor ABO incompatibility, erythrocytes were not depleted, but approximately 90% of the plasma volume was removed from the graft and replaced with saline. Nonelutriated ABO compatible grafts contained approximately $3x10^{12}$ erythrocytes and 600 to 700 mL plasma (mean harvest volume 1100 mL ± 300).

Hemagglutinin titers.

Hemagglutination was performed in 96-well U-bottom microtiter plates (Linbro 76-103-05, Flow Laboratories, McLean, VA). Sera were diluted ½ with saline (0.9%) or saline-dithiothreitol (DTT, 0.01 mol/L) in 13 x 75 mm polypropylene tubes and incubated for 30 minutes at 37°C. Serial twofold dilutions (eight steps, highest concentration = ¼, volume = 50 µL/well) were made 12 times for each serum sample. Sera were titrated in duplicate against three reference cell types (A1, B, and O) under reducing (DTT) and nonreducing conditions. After addition of 50 µL of reagent red blood cells (RBCs; 3% in Alsever's solution, Gamma Biologicals, Houston, TX), the plates were incubated at room temperature for 1 hour. The immunoglobulin M (IgM) titer was determined by direct agglutination in the nonreduced wells immediately after incubation. Endpoints were determined microscopically as the dilution giving an even carpet of cells with a slight ring at the edge. Sera remaining positive at the highest dilution (1/512) were retitrated with an initial serum

concentration of 1/128 Sera were considered positive for IgM only if incubation with DTT resulted in a four-fold or greater decrease in titer in the reduced wells After determination of the IgM titer, the plates were incubated for 30 minutes at 37°C and washed 6 times with saline, after which rabbit antihuman IgG (γ chain) antibody (BioRad, Rockville Center, NY, no 170-1222) was added to reduced and nonreduced wells (50 µL diluted 1/1000 in phosphate-buffered saline) The cells were gently pelleted by centrifugation (60g) for 15 seconds, and endpoint titers were determined microscopically The presence of IgG was confirmed if the addition of antihuman IgG to the reduced wells resulted in a four-fold or greater increase in titer The efficacy of washing to remove unbound IgG was confirmed in each assay by the addition of IgG sensitized Rh(D) positive erythrocytes (Coombs control cells, strong, 50 µL, 3% suspension/well, Gamma Biologicals) to negative wells containing the highest serum concentration Agglutination of sensitized RBCs indicated removal of extraneous IgG and confirmed the activity of the antiglobulin

The absence of agglutination of O erythrocytes ruled out the presence of "irregular" hemagglutinating antibodies A standard reference O+ serum was run with each assay to ensure comparability of results Titers were adjusted according to the known titer of the standard These corrections were never more than one two-fold step

Statistical analysis

Statistical analysis was performed using SYSTAT [10] Hemagglutinating antibody titers were approximately log-normally distributed as determined by probability plots (SYSTAT GRAPH module) Accordingly, log transformations were made before the calculation of descriptive statistics or application of parametric analyses Arbitrary two-fold units were used, where 1 represents a titer of 1/4, 2 a titer of 1/8, etc The generalisation for this relationship is reciprocal titer = $2^{(x-1)} \times 4$, where x is the titer in arbitrary two-fold units and 4 is the reciprocal initial serum concentration For parametric analyses (means, Student's t-test, analysis of variance, linear regression) undetectable antibody levels (i e , titers less than 1/4) were treated as 1/2 (0 in two-fold units) Analysis of variance (ANOVA) and linear regression were performed using the MGLH module, descriptive statistics and Student's t-test were performed using the STATS module Pearson's χ^2 was calculated in the TABLES module Graphics were created using SYGRAPH [11]

Results

Reference ranges for the hemagglutinin assay.

The sensitivity and specificity of the assay were characterized with a series of 132 paired donor/recipient sera obtained before BMT. The distribution of donor and recipient titers by specificity and isotype are shown in Fig 1. IgM titers of both specificities were log-normally distributed. For both anti-A and anti-B antibodies, IgM was a better indicator of subject ABO phenotype than IgG. Of 75 sera expected to be positive for anti-A antibody, 74 (99%) had IgM titers ≥ 1/4 and 63 (84%) had IgG titers ≥ 1/4. Of 101 expected anti-B sera, 96 (95%) had detectable IgM titers, and 63 (62%) had detectable IgG. The geometric mean reciprocal titers (minus and plus the sample standard deviations) were 45 (10, 196), 39 (5, 277), 31 (7, 127), and 11 (2, 60) for anti-A IgM, anti-A IgG, anti-B IgM, and anti-B IgG, respectively.

No "irregular" or inappropriate antibodies were detected in this series. None of 132 sera agglutinated O erythrocytes. Similarly, in none of 89 possible instances were inappropriate specificities detected (e.g., anti-A antibody in an A type individual).

Transfusions.

Packed RBCs contain less than 10 mL of plasma per transfusion unit and were therefore considered not to be of influence on hemagglutinin titers. Intravenously administered gamma globulins, plasma, and platelet transfusions have higher Ig content and therefore could conceivably affect hemagglutinin titers through passive antibody transfer. To determine whether antibody titers could be affected by transfusion history, we examined the frequency of blood product administration in the first 3 months after BMT, the period during which maximal blood product support was required. A total of 45 patients receiving ABO mismatched BMT were studied. Platelet transfusions were divided in two categories, first choice or washed platelets and second choice or platelets of unknown ABO phenotype. First choice platelets are usually of AB phenotype or of an ABO phenotype lacking the antidonor antibody in major ABO incompatible BMT or the antirecipient antibody in minor ABO incompatible BMT. For washed platelets, 95% of the plasma volume is replaced by saline. For some blood products, preparations were either of pooled donor origin or the ABO phenotype was not available retrospectively; these are designated "unknown." There were no significant differences in platelet transfusion history (both categories) between the CCE group, the nonCCE + CsA group, or the nonCCE + Cy group (Pearson's χ^2 $p \geq 0.111$, all comparisons). However, in the nonCCE + Cy group a significantly higher proportion of patients received plasma transfusions (7 of 9, 78%) than in the other two groups (5 of 36, 14%, p=0 .001).

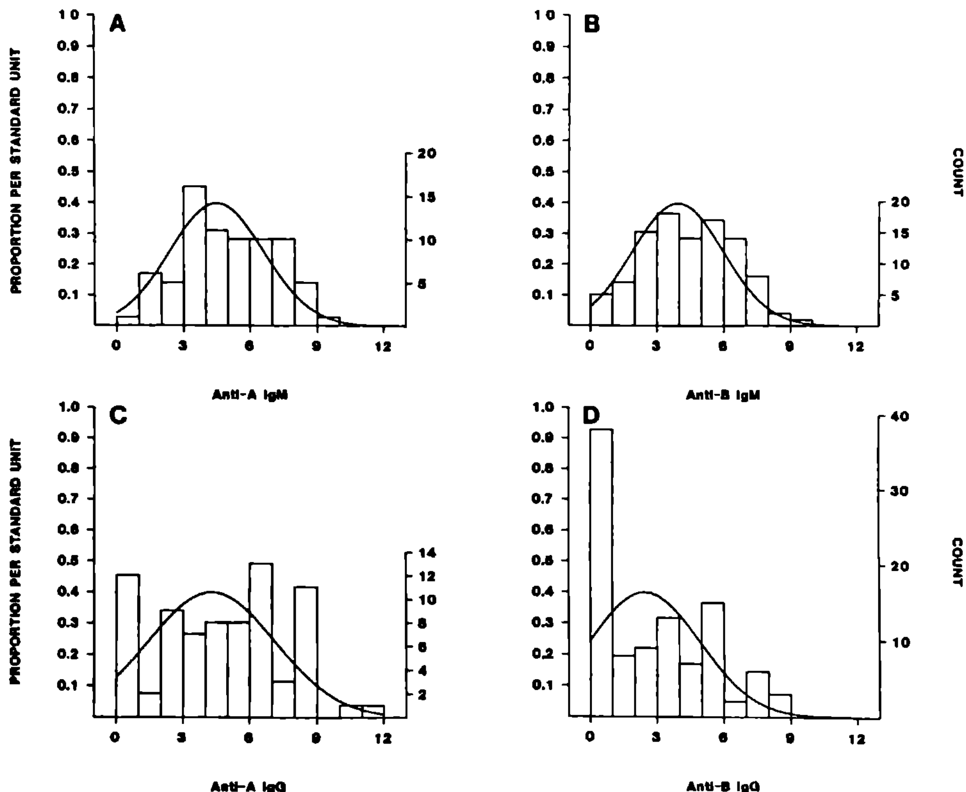

Fig 1 Distribution of hemagglutinin titers in BMT donor and recipient sera obtained before BMT (A and C) Distribution of anti-A antibody in subjects of relevant phenotype (IgM and IgG, respectively) (B and D) Distribution of anti-B antibody Antibody titers are expressed in arbitrary two-fold units (see Materials and Methods) Y axes show proportion per standard unit (count divided by the sample standard deviation) and the number of cases falling in each bar (count) Scaling to the proportion per standard units helps to compare the superimposed normal curves, which are based on the sample mean and standard deviation

To determine whether administration of unwashed platelets elicited a rise in hemagglutinin titer corresponding to the ABO phenotype of the platelet product transfused, we evaluated the correlation between platelet dose and the difference in titers (IgM and IgG) before and after platelet administration. Data from all recipients of ABO mismatched grafts were divided into three time intervals: 0 to 2 weeks, 2 to 4 weeks, and 4 weeks to 3 months after BMT (Fig 2).

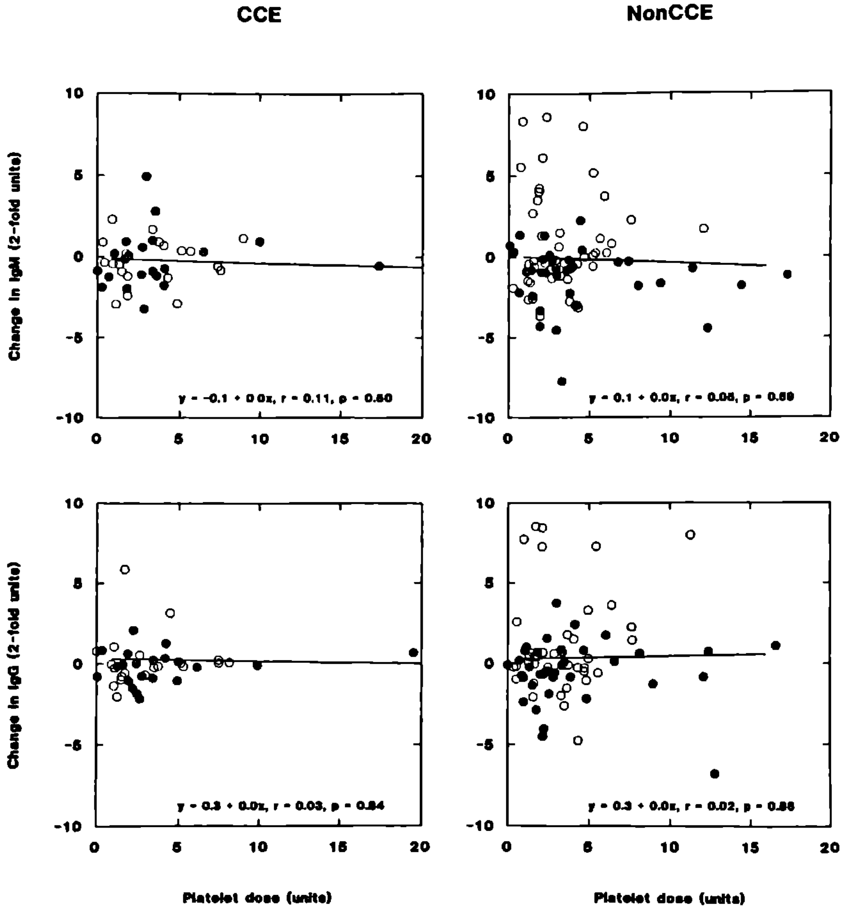

Fig 2. Lack of correlation between platelet dose and difference in relevant hemagglutinin titer after and before platelet administration. All platelets were of known blood type and were suspended in plasma. Changes in titer are expressed in two-fold steps. (O), Changes in titer measured between time 0 and 2 weeks; (⊗) 2 and 4 weeks; and (●), 4 weeks to 3 months. To prevent data points from overlapping, a small uniform random error (jitter) was introduced to the location of each point. The parameters of the least squares linear regression are indicated. Exact (nonjittered) values were used for the regression analyses. The lines of best fit are shown.

Platelet dose and change in relevant antibody titer (IgM or IgG) were uncorrelated. The greatest changes in titer were observed in recipients of nonCCE grafts in the first 2 weeks after BMT.

The same methodology was used to look for a correlation between plasma dose and change in antibody titer. Because the ABO phenotype of plasma products was unknown, the influence

of each product on anti-A and anti-B titers was evaluated (IgM and IgG) In agreement with the platelet data, no correlation was found (n=30, r=0 065, p=0 734, and r=0 026, p=0 892 for IgM and IgG, respectively) In fact, the recipient of the greatest number of plasma transfusions (122 units from 2 to 3 months after BMT) had undetectable hemagglutinins (anti-A and anti-B, IgM and IgG) at both 1 and 3 months after BMT

Five recipients of ABO mismatched grafts (two CCE, three nonCCE) received intravenous gamma globulins in the first 3 months after BMT in a dose ranging from 7 to 90g No effect of this product could be detected in any of these five patients

Major ABO incompatibility Recipient-derived antibody

In major ABO mismatch, antidonor type antibody is of recipient origin and either represents preformed antibody or is the product of persistent host origin B-cells (Table I) When the donor is A or B and the recipient is O, a second specificity may be produced, but its origin cannot be uniquely ascribed to either recipient or donor In the case of major plus minor ABO mismatch (e g , A into B), recognition occurs in both directions (donor antirecipient and recipient antidonor) Therefore, a unique donor specificity may also occur

Because major mismatched ABO specificities can be ascribed uniquely to the recipient, analysis focused on the change in titer relative to pre-BMT levels In all patient groups, antidonor antibody fell as a function of time after BMT (Fig 3) The magnitude of antibody decrease in the first 3 months after BMT did not differ between groups (p>0 2, Student's t-test, all comparisons) Neither was the proportion of patients with persistent recipient derived antibody (IgM or IgG, ≥ 3 months after BMT) significantly different between groups (p=0 176, Pearson χ^2 test) One of the CCE patients demonstrated persistently high IgM and IgG antidonor antibody titers up to 1 year after BMT Among all patients receiving nonelutriated grafts, five of 11 patients with an available serum sample at 6 months still had detectable antibody titers at that time point (IgM, IgG, or both) In no case could antidonor antibody be found at 1 year after BMT

Minor ABO incompatibility Donor-derived antibody

In minor ABO mismatch, antibody specificities can be detected that are uniquely attributable to donor B-cells (Table I) In none of the seven patients who received an elutriated, minor ABO incompatible graft could an antirecipient antibody be detected, either early (2 to 4 weeks, 14 observations) or late after BMT (3 to 12 months, 13 observations) (Table II) In eight of 14 patients in the minor ABO incompatible, nonelutriated group, a transiently detectable antirecipient antibody was found early after BMT (28 observations) Donor derived antibody was IgM, IgG, or both and was independent of GVHD chemoprophylactic regimen Anti-A was detected in 8 of 13 possible

instances (62%), and anti-B in 0 of 2 possible instances. Antirecipient antibody, when detected, was short-lived; none of the 14 patients had detectable titers 3 months or later after BMT (19 observations).

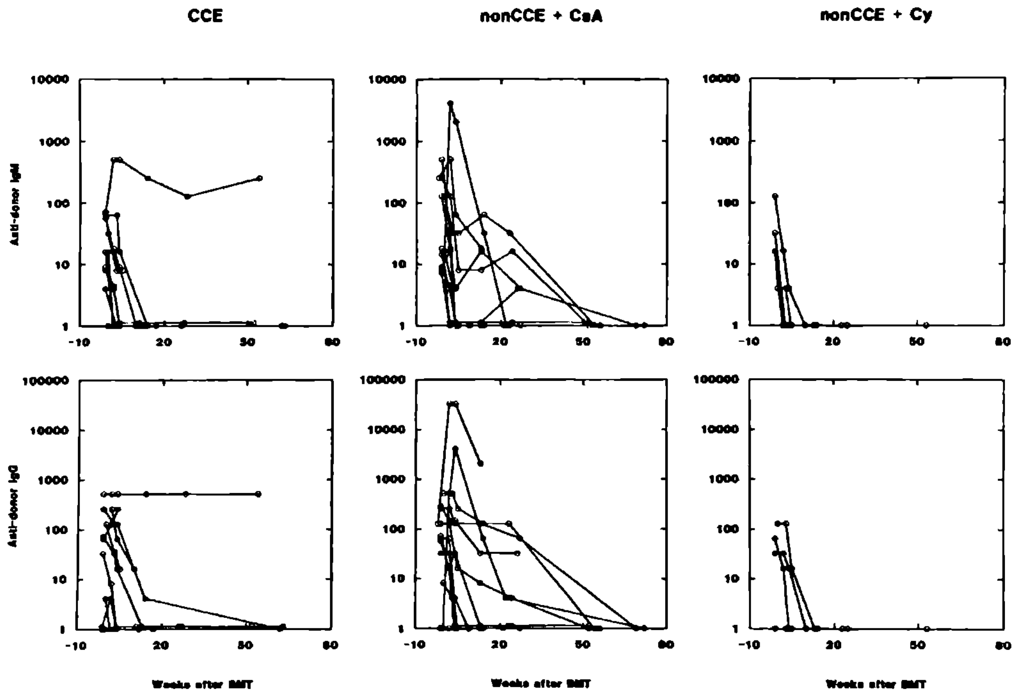

Fig 3 Course of antidonor hemagglutinins in major ABO mismatched BMT Results are plotted for individual recipients grouped by graft type and GVHD chemoprophylaxis Reciprocal titers are shown on a log scale where negative samples (i e , titer <¼) are indicated as 1 (O), Anti-A and (●), anti-B antibodies The locations of overlapping symbols were shifted slightly.

Matched specificities: Antibodies derived from the donor, recipient, or both.

In certain ABO mismatched combinations, antibody of a given specificity can be made by both donor and recipient, and thus cannot be uniquely attributed to either. In this respect they are comparable with antibodies elicited in ABO matched BMT (Table I). In the present analysis, these are referred to as donor and/or recipient-derived (DOR) antibodies. The effect of the graft type (CCE or nonCCE), GVHD chemoprophylaxis (CsA or Cy), or ABO match (match or mismatch)

on DOR antibody was determined at intervals before and after transplant by ANOVA. At no time interval did GVHD chemoprophylaxis or ABO match exert a significant influence on DOR titer (IgM or IgG, all p values >0.176). The DOR titers are shown as an function of time after BMT in Fig 4. Early after BMT (2 to 4 weeks), recipients of nonCCE grafts had significantly higher DOR IgM titers compared with the CCE group (113 observations in 26 CCE and 31 nonCCE patients, p=0.001 by ANOVA). Although not statistically significant, a similar effect was seen in DOR IgG titers. Graft type dit not exert a significant effect on DOR IgM titers at other time intervals. In both graft types, the time to maximal DOR IgM titer preceded the time to maximal IgG titer. Maximal DOR titers (IgM and IgG) occurred later in recipients of elutnated grafts.

Table II. Antirecipient antibody early after minor ABO incompatible BMT

	Uniq*	Specificity†	IgM/IgG‡ (2 wk)	IgM/IgG‡ (4 wk)	Patient with antirecipient antibody / total No.§
CCE‖		4 x Anti-A			
		3 x Anti-B	<4/<4	<4/<4	0/7
NonCCE + CsA	491	Anti-A	<4/<4	<4/<4	
	546	Anti-A	128/512	32/256	
	562	Anti-A	<4/8	<4/<4	
	630	Anti-A	8/<4	<4/<4	
	706	Anti-A	<4/<4	<4/<4	
	713	Anti-A	4/<4	<4/<4	
	857	Anti-A	<4/<4	<4/<4	
	969	Anti-A	<4/8	<4/<4	5/8
NonCCE + Cy	412	Anti-B	<4/<4	<4/<4	
	418	Anti-A	4/8	<4/8	
	464	Anti-A	<4/<4	<4/<4	
	477	Anti-A	<4/<4	<4/8	
	498	Anti-A	32/<4	<4/<4	
	779	Anti-A	<4/<4	<4/<4	3/6

* Patient unique number

† Expected specificity of donor-derived, antirecipient antibody

‡ Reciprocal titer of antirecipient antibody

§ Number of patients having titers equal to or greater than 1 4 (IgM or IgG)

‖ CCE 4 patients with expected phenotype of anti-A and 3 patients with expected phenotype of anti-B, all had titers <1 4

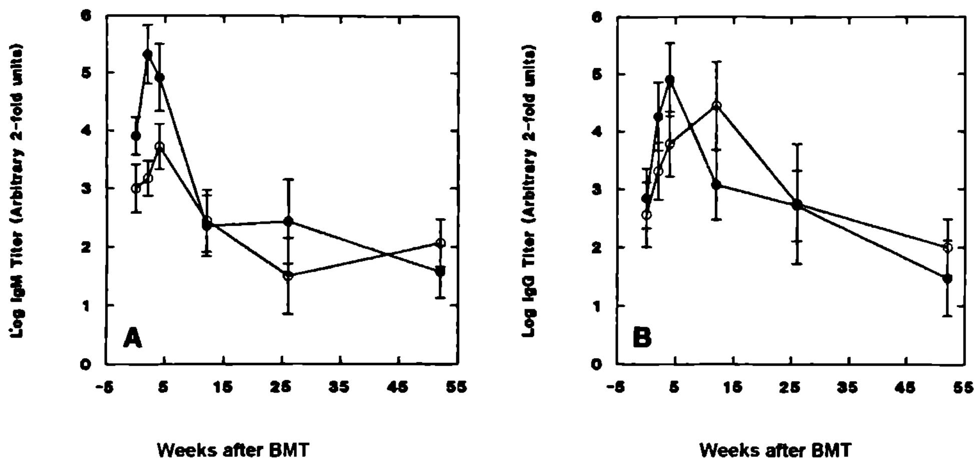

Fig 4 Course of DOR hemagglutinins in recipients of CCE and nonCCE ABO matched and mismatched grafts Symbols represent mean titers, and bars indicate standard error of the means (●), NonCCE, (O), CCE Mean titers are expressed in arbitrary two-fold units (see Materials and Methods) (A) IgM, (B) IgG

To assess the possibility that passive transfer of antibody present in the graft could account for the higher DOR titers observed in recipients of nonCCE grafts, the change in DOR antibodies from time 0 to 2 weeks after BMT was compared between recipients of ABO matched and mismatched nonCCE grafts. The grafts administered to the former group contained 600 to 700 mL donor plasma, whereas the latter contained little or no plasma. That no significant difference (IgM or IgG, p=0.178 and 0.378, respectively) was observed indicates that the infusion of several hundred milliliters of plasma did not detectably affect hemagglutinin titer. In the absence of stable B-cell mixed chimerism, DOR antibody should be exclusively of donor origin by 1 year after BMT. To determine whether normal hemagglutinin levels were attained, we compared DOR titers 1 year after BMT with the pretransplant donor titers of relevant specificity. In the case of O into O transplants where both A and B specificities counted as DOR, both anti-A and anti-B titers for a given donor/patient pair were considered in the analysis. In recipients of both graft types DOR IgM was significantly lower than the relevant donor IgM titers (6.4-fold, n=46, p=0.000, paired t-test) In contrast, late DOR IgG levels and the relevant donor IgG titers were more comparable in both groups (1.7-fold difference, n=46, p=0.078). Despite the similarity between mean group DOR IgG levels, individual late DOR IgG titers were highly correlated with those of their respective donors in recipients of nonelutriated, but not elutriated, grafts (Fig 5). In recipients of nonCCE grafts, the intercept (-0.11 ± 0.76) and slope (0.82 ± 0.19) of the linear least squares line of best

fit are consistent with a direct correspondence between the magnitude of the pretransplant donor IgG hemagglutinin titer and that of the recipient late after BMT (Fig 5).

Irregular antibodies.

None of 230 post-BMT patient sera agglutinated O erythrocytes. Similarly, in no case were inappropriate specificities detected (e.g., anti-A antibody in an A or AB recipient with an A or AB donor)

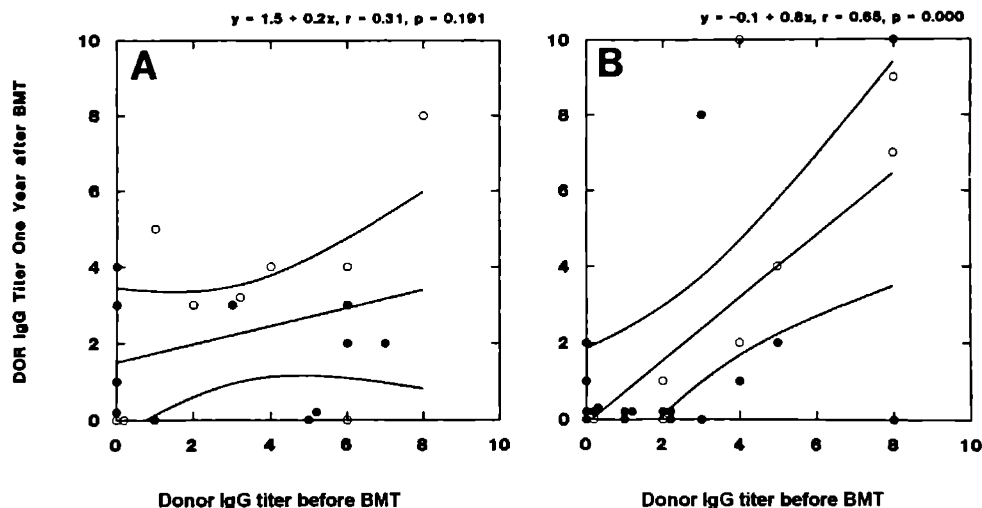

Fig 5 DOR IgG hemagglutinins in recipients of CCE (A) and nonCCE (B) grafts Correlation of DOR hemagglutinin measured at 1 year after BMT (y axis) with pretransplant donor IgG titers of the relevant specificity (x axis) (O), Anti-A IgG, (●), anti-B IgG. The locations of overlapping symbols were shifted slightly. The parameters of the least squares linear regression are indicated The lines of best fit and 95% confidence intervals are shown

DISCUSSION

Hemagglutinins provide an excellent model for studying the reconstitution of antibody responsiveness after BMT. They are directed against well-characterized oligosaccharide units attached to carrier molecules,[12] are absent in infants, and are elicited by exposure to antigens homologous to the ABH specificities found on human erythrocytes.[13] These ubiquitous determinants are also present on a variety of gram-negative bacterial strains[13] which are regularly

encountered by immunogenic routes,[13] resulting in the generation of IgM, IgG and IgA isotypes.[14] Consistent with previous results,[15] we found the prevalence of anti-B hemagglutinins somewhat lower than that of anti-A, in subjects of the relevant respective phenotypes (Fig 1).

Applied to the problem of immune reconstitution after BMT, the use of ABO mismatched donor/recipient pairs allows one to distinguish between donor- and recipient-derived antibodies. Further, comparison of the generation of donor-derived responses in recipients of unmanipulated and elutriated grafts permits one to differentiate between donor-derived antibody responses conferred on the recipient by adoptive transfer of immune memory cells, and those generated de novo in the recipient environment.

Hemagglutinin titers are not significantly influenced by passive transfer of antibody present in blood products. This was conclusively demonstrated by the lack of correlation between platelet (Fig 2), plasma, and gamma globulin doses with changes in antibody titers during the first 3 months after BMT. Similarly, although significantly more patients in the nonCCE + Cy group received plasma transfusions than those in the nonCCE + CsA group, their hemagglutinin titers were not elevated.

Major ABO incompatible BMT provided the opportunity to study the tempo of disappearance of recipient-derived antidonor antibody. This parameter is of concern because lymphocyte depletion could result in prolonged survival of host immunity, a phenomenon that has been suggested to account for the increased incidence of graft rejection associated with this procedure.[16] The rate of disappearance of antidonor hemagglutinins is also of interest in itself, since significant immunohematologic problems have been associated with its persistence.[17] Although the number of patients available for study was small, we found no evidence for prolonged survival of recipient-derived antibody in recipients of elutriated grafts. Only one recipient of elutriated marrow retained antidonor hemagglutinin by 1 year after BMT. Restriction fragment length polymorphism analysis of his peripheral blood showed mixed chimerism at that time. In recipients of nonCCE grafts, the use of CsA for aGVHD chemoprophylaxis may have enhanced survival of host origin antibody, but the small number of observations in the Cy group precluded a definitive comparison.

After minor ABO incompatible BMT we found an early transient appearance of donor-derived, antirecipient antibody in eight of 14 recipients of conventional bone marrow grafts. This contrasted sharply with recipients of elutriated grafts in whom we never detected this specificity. The transient appearance of antirecipient hemagglutinins in recipients of nonCCE grafts was not due to passive transfer because the plasma was removed from all minor ABO incompatible grafts. Thus, it reflects the activity of mature donor lymphocytes infused with the nonelutriated graft. These data are

consistent with our previous studies, which demonstrated that successful adoptive transfer of humoral- and cell-mediated responses to recall antigens required both donor-immune memory and early encounter with antigen in the recipient environment [4] Both of these conditions are fulfilled in the nonCCE group Transient donor antirecipient hemagglutinins have been described previously in minor ABO incompatible conventional BMT [18] It has also been seen in minor ABO incompatible solid-organ transplants where it has been attributed to passenger lymphocytes present in the graft [19,20] In contrast to our findings using elutriation as a means of lymphocyte depletion, investigators who used T-cell-specific monoclonal antibodies failed to abrogate the transient expression of donor-derived antirecipient hemagglutinins [21,22] Thus, elimination of this response in recipients of elutriated marrow most likely reflects the removal of B- as well as T-memory lymphocytes Although it has been suggested that treatment with CsA favors the production of antirecipient antibody,[18] our results and the results of others [23] do not support this conclusion antirecipient antibody was found in three of six patients who received Cy rather than CsA immunosuppression In agreement with the results of others,[18,24] we did not detect antirecipient antibody in any of our patient groups at 3 months or later after BMT The disappearance of this specificity in recipients of nonCCE grafts, and its complete absence in recipients of CCE grafts, can be explained by the development of high-zone tolerance[18,24] for the recipient ABO antigen due to its wide distribution in the tissues [12] Indeed, tolerance has been experimentally induced in O type infants by repeated parenteral challenge with purified A substance from birth to 8 months [25] The alternative explanation, that antirecipient antibody is produced but subsequently absorbed by recipient tissues, is less likely because this antibody was detected at maximal levels early after BMT when recipient ABO antigens are expressed on residual host erythrocytes as well as other tissues

The existence of matched ABO specificities in ABO mismatched BMT permits the immune reconstitution of this relatively limited population to be compared with that of recipients of ABO compatible grafts Although these matched (DOR) specificities cannot in theory be uniquely ascribed to donor or recipient, the kinetics of disappearance of recipient derived antibody (Fig 3) suggest the donor origin of DOR antibody late after transplant. Early after transplant, DOR titers increased above pretransplant levels in both groups, with the rise in IgM titer preceding that of IgG The early increase in IgM titer was far more pronounced in the nonCCE group, most likely reflecting the adoptive transfer of mature B- and T-memory lymphocytes in the nonelutriated grafts This increase could not be attributed to passive transfer of antibody in the graft because DOR antibody was indistinguishable in the ABO-matched and -mismatched nonCCE groups, despite a substantially greater plasma volume in the nonCCE matched grafts Late after BMT, both IgM

and IgG levels decreased, but IgG levels remained comparable with donor pretransplant titers in both groups The striking correlation between late DOR IgG in the nonCCE group, and the relevant pretransplant titers of their respective donors, suggests that even late after BMT, DOR IgG is still the product of adoptively transferred memory cells That DOR IgG titers in the CCE group were of comparable magnitude, yet uncorrelated with individual donor titers, suggests the de novo generation of the immunologic components required for antigen-specific IgG response in this group

The primary goal of this study was to determine the influence of graft lymphocyte depletion by CCE on the reconstitution of antibody responses Taken together, these data bear on four important aspects (1) the fate of recipient origin B-lymphocytes, (2) the significance of memory lymphocytes present in the graft, (3) the contribution of T- and B-lymphocytes of donor origin differentiating within the new host environment, and (4) the induction of immunologic tolerance to host antigens The removal of mature T- and B-lymphocytes from the graft did not result in a detectable prolongation of recipient-derived antibody production However, it did entirely abrogate adoptive transfer of antibody uniquely attributable to the donor, and retarded the expression of DOR antibody Despite this delay, recipients of lymphocyte depleted and conventional allografts had comparable DOR IgG titers by 25 weeks after BMT We have presented evidence suggesting that recipients of elutriated grafts generated this response de novo, while those who received unmanipulated grafts used memory lymphocytes transferred in the graft Finally, the adoptive transfer and subsequent loss of donor antirecipient antibody in recipients of unmanipulated grafts strongly suggests the induction of tolerance That these specificities were never observed in recipients of elutriated grafts highlights the fact that such "autoimmune" responses are far less likely when the majority of immune cells have matured within the recipient environment

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Chapter 7

Summary

Samenvatting

In Chapter 1, the introduction to this thesis, graft versus host disease (GVHD) is described as the major posttransplant complication of allogeneic bone marrow transplantation (BMT) GVHD is caused by donor T-lymphocytes in the marrow graft and its incidence can be clearly reduced by in vitro depletion of these lymphocytes Therefore all patients, transplanted at the University Hospital Nijmegen since 1981, have received allogeneic bone marrow partially depleted of T-lymphocytes by counterflow centrifugation

T-cell depletion is associated with an increased risk of leukemic relapse, especially in patients with chronic myeloid leukemia (CML) Patients with relapse of CML after BMT can be successfully treated with infusion of lymphocytes from the marrow donor, resulting in cytogenetic and molecular remission of CML in the majority of the patients

The two possibilities of allogeneic BMT across the ABO barrier are explained In major ABO incompatible BMT the recipient has the potential to express anti-A and/or anti-B antibodies against donor ABO antigens This can cause immunohematologic complications like delayed hemolytic anemia, delayed onset of erythropoiesis and pure red cell aplasia In minor ABO incompatible BMT anti-A and/or anti-B antibodies of the donor may cause hemolysis of recipient erythrocytes early after BMT

Mixed chimerism is the coexistence of recipient and donor lymphohematopoietic cells after BMT The incidence of mixed chimerism is clearly increased after BMT with T-cell depleted marrow compared to an unmanipulated graft The various techniques to distinguish donor and host lymphohematopoietic cells are briefly described In this thesis a sensitive fluorescent microsphere technique has been applied to measure recipient and donor erythropoiesis after BMT and donor lymphocyte infusions This requires determination of red cell antigenic differences between host and donor prior to BMT and subsequently an adapted erythrocyte transfusion policy

Chapter 2 describes an analysis of host and donor erythrocyte repopulation patterns after BMT, measured with the fluorescent microsphere assay All 91 patients, transplanted between 1981 and 1988, received donor marrow, depleted of 98% of lymphocytes by counterflow centrifugation Complete red cell phenotyping of patient and donor was performed before BMT to determine differing antigens, if present Both recipient and donor markers, recipient marker only, donor marker only or no markers were available in 64, 16, 9 and 11 percent of the BMT patients respectively The patients subsequently received erythrocyte transfusions of blood group O lacking the marker antigens The sensitivity level of the fluorescent microsphere assay is 0 01%

Thirty-seven patients were evaluable to monitor donor erythrocyte repopulation at one or more time points between 0 5 to 6 months after BMT Thanks to the sensitivity of the applied technique,

donor erythrocytes could be detected in 19 out of 22 evaluable patients (86%) two weeks after BMT in contrast to 15% using conventional agglutination techniques. At 6 months after BMT donor erythrocytes were detectable in 100% of the evaluable patients. From 0.5 to 6 months after BMT the percentages of donor red cells steadily increased from a median of 0.04% at 0.5 month to 88.8% at 6 months. Recipients of major ABO mismatched transplantations showed a trend for delayed donor erythrocyte repopulation, which was significant at 3 months after BMT ($p=0.03$). A gradual disappearance of autologous erythrocytes in the first 6 months after BMT was observed in patients whose course was not complicated by relapse. At 6 months after BMT the percentage of autologous erythrocytes varied from 0 to 0.1% in the majority of these patients.

Taking into account a 150-day survival time of recipient erythrocytes generated before BMT, mixed erythrocyte chimerism was defined by the coexistence of host and donor erythrocytes from 6 months after BMT onward. In the 38 evaluable patients the incidence of mixed chimerism varied from 50 to 71% at various time points from 6 months onward. This high incidence can be explained by the T-cell depletion of the graft and the sensitivity of the fluorescent microsphere technique. No clinical evidence of leukemic relapse was found in 10 stable mixed chimeras with low percentages (<1) of autologous erythrocytes at different moments of analysis or in 6 mixed chimeras, who converted to complete donor chimeras. Mixed chimeras with high (>10), sometimes even gradually increasing, percentages of host erythrocytes did not relapse necessarily.

In Chapter 3 the influence of the conditioning regimen on erythrocyte chimerism, GVHD and relapse after BMT has been studied. From May 1981 to January 1991 three consecutive conditioning regimens were applied in 144 patients transplanted with marrow from a HLA identical sibling. The marrow graft was always depleted of lymphocytes by counterflow centrifugation. All three conditioning regimens consisted of cyclophosphamide and fractionated total body irradiation (TBI). In regimen A, applied in 49 patients, the total dose of TBI was 9 Gy. In regimen B, used in 65 patients, the dose rate of TBI was increased and anthracyclines were added. In regimen C the total dose of TBI was increased to 12 Gy but no anthracyclines were administered ($n=30$).

Age distribution was significantly different between the three patient groups with age at BMT increasing during the years. The absolute number of T-cells in the marrow grafts differed significantly between the patients treated with regimen A, B and C. However, compared to unseparated grafts the absolute number was very low in all patients in this study.

In a multivariate analysis a significant influence of the conditioning regimen on the incidence of mixed erythrocyte chimerism was found ($p=0.0004$). Neither age nor the number of T-cells in the graft had a significant influence on this incidence. Intensification of the pretransplant regimen

resulted in a decrease of the incidence of mixed erythrocyte chimerism with incidences at 6 months after BMT of 73, 33 and 20% for regimen A, B and C respectively.

The incidence and severity of acute GVHD could be assessed in 139 out of 144 patients. The incidence of acute GVHD grade I or more was 15, 29 and 77% for regimen A, B and C respectively. Both age and the conditioning regimen influenced significantly the incidence of acute GVHD ($p=0.017$ and 0.0001 respectively). No significant influence of age, the conditioning regimen or the number of T-cells in the graft on the incidence of chronic GVHD could be detected by multivariate analysis.

Combining the results of the three conditioning regimens, a relation between the presence of acute and chronic GVHD and the incidence of mixed erythrocyte chimerism could be established. The incidence of both acute and chronic GVHD was significantly higher in the group of complete donor than in the group of mixed chimeras ($p<0.001$ and $p<0.01$ respectively).

The significance of mixed erythrocyte chimerism in CML and acute leukemia was studied. In CML patients a relation was found between the percentages of autologous erythrocytes from 6 months after BMT onward and the occurrence of relapse. Patients, who did not relapse, were either complete donor chimeras or stable mixed chimeras with low (<0.5) percentages of recipient erythrocytes. High (>10) or increasing percentages of autologous erythrocytes were indicative of cytogenetic relapse. In acute leukemia this relation was not evident.

To compare the probability of relapse between the conditioning regimens only good risk patients (acute myeloid or lymphoblastic leukemia in first complete remission and CML in first chronic phase) were studied. The probability of relapse was 43% in 32 and 18% in 43 good risk patients treated with regimens A and B respectively ($p=0.07$). Follow up was too short to draw conclusions about relapse in regimen C.

We conclude that intensification of the conditioning regimen resulted in a lower incidence of mixed erythrocyte chimerism, a higher incidence of acute GVHD and a trend to a lower relapse rate.

Chapter 4 describes the treatment of seven patients with CML who relapsed after allogeneic BMT with a T-cell depleted graft. One patient received a second BMT with unseparated marrow from the same sibling donor 3 years after the first BMT. The other six patients were treated with donor leukocyte infusions from the original marrow donor. Five of them had a hematologic relapse of CML, one patient only a cytogenetic relapse. The interval between BMT and time of the infusions varied from 13 to 65 months in these patients. Nucleated cells from the donor were obtained by leukapheresis using a blood cell separator. The nucleated cells consisted mainly of

lymphocytes (70 to 85%) and monocytes (10 to 15%). The total dose of nucleated cells infused during the first course varied from 0.34 to 1.8 x 10^8/kg in the six patients. Two patients, who did not respond to the first course received a second course with a double dose of donor leukocytes. Chimerism was studied using erythrocyte and cytogenetic markers. Monitoring of residual leukemic cells was performed by cytogenetic analysis of the Philadelphia (Ph) chromosome and by polymerase chain reaction (PCR) of the breakpoint cluster region/Abelson (BCR-ABL) fusion gene.

The patient treated with a second BMT achieved cytogenetic remission 6 months after BMT. At that time point she was a complete donor chimera by cytogenetic analysis. PCR of the BCR/ABL breakpoint molecules was negative 12 months after BMT. Five patients with hematologic relapse achieved hematologic and cytogenetic remission 1 to 3 months after the donor lymphocyte infusions. Four out of five patients achieved molecular remission 3 to 12 months after the infusions. All five responding patients became complete donor chimeras by cytogenetic analysis and/or in situ hybridization. Four out of 5 patients had a recipient erythrocyte marker. A decrease in the percentage of autologous erythrocytes was observed in these patients with a disappearance in two of them.

All responding patients developed GVHD which was extensive in two patients. The patient with cytogenetic relapse did not respond to two courses of donor lymphocyte infusions. No signs of GVHD were observed in this patient.

We conclude that infusion of donor leukocytes is an effective treatment in patients with CML relapsed after BMT with a T-cell depleted graft. Response always coincided with GVHD in our patients.

Chapter 5 is an analysis of erythrocyte repopulation in patients transplanted with major ABO incompatible, lymphocyte-depleted marrow. Major ABO incompatibility between patient and donor existed in 44 out of 285 patients transplanted in Nijmegen from 1981 to 1994. Donor erythrocyte repopulation was evaluable in 30 out of 44 patients. Fifty-eight patients transplanted with an ABO compatible or minor incompatible graft served as a control group. All marrow grafts were depleted of lymphocytes and erythrocytes by counterflow centrifugation. Less than 10^8 residual erythrocytes were present in the marrow graft. Plasma exchange or plasma immunoadsorption to reduce anti-A or anti-B antibody titers before BMT were never applied. Erythrocyte repopulation was measured using the fluorescent microsphere method previously mentioned.

Donor erythrocyte repopulation was significantly delayed in the major ABO incompatible group compared to the control group up to 3 months after BMT ($p \leq 0.03$). Significantly more erythrocyte transfusions were required in the ABO incompatible group ($p<0.001$). In the study group 62% and

33% of the evaluable patients had no donor red cells detectable at 0 5 and 1 month after BMT respectively In the control group these percentages were 19% and 0% respectively (p<0 01) Eight patients in the major ABO incompatible group had no detectable donor erythrocytes 2 months after BMT All eight patients had blood group O in contrast to 12 out of 22 patients in the major ABO incompatible group with timely onset of erythropoiesis Six patients (20%) developed pure red cell aplasia which resolved in five of them without therapeutic intervention 3 5 to 7 months after BMT

Pretransplant anti-A and anti-B titers and the course of these antibodies after BMT were compared between the eight patients with delayed onset of erythropoiesis and the remaining 22 patients in the major ABO incompatible group No significant difference in IgM titers before BMT could be detected between the two groups, while the IgG titers before BMT were significantly higher in the eight patients (p<0 01) In the 22 patients a steady decrease of anti-A and/or anti-B antibodies IgM as well as IgG was observed after BMT This was in contrast with the eight patients with delayed onset of erythropoiesis, whose anti-A titers were persistently high the first 3 months after BMT

We conclude that the incidence of immunohematologic complications after major ABO incompatible BMT was not increased in our patients compared to data reported on recipients of unmanipulated grafts despite the higher incidence of mixed chimerism Serious morbidity related to major ABO incompatibility did not occur Counterflow centrifugation is an effective technique to remove erythrocytes in case of major ABO incompatible BMT

Chapter 6 describes the results of a study performed at the Johns Hopkins Oncology Center In this study the effect of lymphocyte depletion of the marrow graft by counterflow centrifugation on the expression of hemagglutinins after transplantation was studied ABO incompatibility existed in 17 out 64 patients transplanted with an elutriated graft between December 1985 and January 1989 Anti-A and/or anti-B antibodies of these recipients before and at various time points after BMT were compared with the antibodies of 28 recipients of ABO incompatible non-elutriated grafts transplanted between 1983 and 1989 In addition anti-A and/or anti-B antibodies were studied in 24 patients before and after ABO matched transplantation, 12 cases with elutriated and 12 cases with non-elutriated marrow grafts Donor anti-A and/or anti-B antibodies were measured in all cases Plasma volume in the graft and in blood transfusion products was uncorrelated with changes in anti-A and/or anti-B titers and could be excluded as a significant source of antibody

In both patient groups (elutriated n=11 non-elutriated n=16), recipient anti-donor, anti-A and/or anti-B antibody fell as a function of time after major ABO incompatible BMT Although the number

of evaluable patients was small, no evidence for prolonged survival of host-derived hemagglutinins in recipients of elutriated grafts was found.

In eight out of 14 recipients of a minor ABO mismatched non-elutriated graft, an antirecipient antibody could be detected early (2 and/or 4 weeks) after BMT. As plasma was removed from the graft the appearance of this antibody can not be explained by passive transfer. It reflects the activity of mature donor lymphocytes in the graft. This is supported by the fact that the antirecipient antibody was never detected in seven recipients of a minor ABO mismatched elutriated graft. Antirecipient antibody was never detected 3 months or later after elutriated nor after non-elutriated minor ABO mismatched BMT. This suggests the induction of tolerance for the recipient ABO antigen due to its wide distribution in the tissues.

In certain ABO mismatched combinations, antibody of a given specificity can be made by both donor and recipient (DOR) antibody. This is a situation comparable with ABO matched BMT. No significant influence of ABO match (match or mismatch) on DOR titers before and at various time points after BMT could be detected. After pooling the data of ABO matched and mismatched BMT, DOR titers were compared between recipients of elutriated and non-elutriated grafts. Early after BMT (at 2 and 4 weeks) DOR antibody titers increased more clearly in the non-elutriated group which reached significance in IgM but not in IgG titers. This difference is probably caused by the adoptive transfer of mature lymphocytes in the non-elutriated grafts. One year after BMT DOR antibody is most likely completely of donor origin. At this time point no significant difference of DOR antibody titers, IgM as well as IgG, could be detected between recipients of elutriated and non-elutriated grafts suggesting a de novo response in the elutriated group.

General conclusion

The microsphere technique is a very sensitive, reproducible method to monitor erythrocyte chimerism after allogeneic BMT. We used this technique in combination with other methods to study the influence of T-cell depletion and the intensity of the conditioning regimen on mixed chimerism and to relate mixed chimerism with the relapse risk.

Moreover we studied kinetics of donor and recipient erythropoiesis in ABO incompatible allogeneic BMT and after donor lymphocyte transfusion for treatment of relapse after allogeneic BMT.

Samenvatting

In hoofdstuk 1 van dit proefschrift wordt de omgekeerde afstotingziekte (GVHD) beschreven als de belangrijkste complicatie van allogene beenmergtransplantatie (BMT) Deze omgekeerde afstotingsziekte wordt veroorzaakt door de T-lymfocyten in het beenmerg van de donor De incidentie ervan kan duidelijk worden verminderd door in vitro verwijdering van deze lymfocyten uit het donorbeenmerg vóor toediening aan de ontvanger Om deze reden hebben alle patienten, die sedert 1981 in het Academisch Ziekenhuis Nijmegen zijn getransplanteerd, donorbeenmerg ontvangen waaruit het merendeel van de T-lymfocyten met behulp van tegenstroomcentrifugatie is verwijderd

Verwijdering van T-lymfocyten leidt tot een verhoogde kans op recidief van de leukemie, vooral bij patienten met chronisch myeloide leukemie (CML) Patienten met een recidief CML na BMT kunnen vaak met goed resultaat worden behandeld door toediening van lymfocyten van de beenmergdonor Deze behandeling leidt in het merendeel van de patienten tot een cytogenetische en moleculaire remissie van de CML

De 2 mogelijke vormen van allogene BMT, waarbij een ABO bloedgroepverschil bestaat tussen ontvanger en donor, worden uitgelegd In het geval van een "major" ABO incompatibiliteit vormt de ontvanger anti-A en/of anti-B antistoffen tegen de bloedgroep van de donor Dit kan leiden tot immuunhematologische complicaties zoals een uitgestelde hemolytische reactie, leidend tot bloedarmoede, vertraagd op gang komen van de donor erytropoiese en het volledig ontbreken van erytropoiese In het geval van een "minor" ABO incompatibiliteit is het mogelijk dat anti-A en/of anti-B antistoffen van de donor in de vroege fase na transplantatie hemolyse van erytrocyten van de ontvanger veroorzaken

Gemengd (mixed) chimerisme na BMT is het verschijnsel van gelijktijdig aanwezig zijn van lymfohematopoietische cellen van zowel ontvanger als donor Na BMT met beenmerg waaruit de T-lymfocyten grotendeels zijn verwijderd, komt mixed chimerisme duidelijk vaker voor dan na transplantatie met onbehandeld beenmerg De verschillende technieken met behulp waarvan donor en ontvanger lymfohematopoietische cellen onderscheiden kunnen worden, worden in het kort beschreven

In dit proefschrift wordt een gevoelige techniek beschreven die gebruik maakt van fluorescerende microsferen (kleine bolletjes) om ontvanger- en donor-erytropoiese na BMT en infusie met donorlymfocyten te meten Dit vereist het vastleggen van de verschillen in bloedgroep-antigenen tussen ontvanger en donor vóór BMT en vervolgens een aangepast erytrocyten-transfusiebeleid

Hoofstuk 2 beschrijft een analyse van het verloop van de erytropoiese van de ontvanger en de donor na BMT, gebruikmakend van de fluorescerende microsferentechniek Alle 91 patienten, die tussen 1981 en 1988 werden getransplanteerd, ontvingen donorbeenmerg waaruit met behulp van tegenstroomcentrifugatie 98% van de lymfocyten waren verwijderd Uitgebreide fenotypering van patiènt- en donorerytrocyten werd voor transplantatie uitgevoerd om, indien aanwezig, verschillen tussen patient en donor aan te tonen In 64% van de patienten was zowel voor de ontvanger als voor de donor een kenmerkend antigeen aantoonbaar, in 16% alleen een ontvanger-kenmerk en in 9% alleen een donorkenmerk In 11% konden er geen onderscheidende kenmerken worden vastgesteld De patienten werden vervolgens getransfundeerd met erytrocyten met de bloedgroep O, waarop de kenmerkende antigenen ontbraken Het gevoeligheidsniveau van de fluorescerende microsferentechniek bleek 0,01%
Het opgang komen van de donorerytropoiese kon in 37 patienten vastgesteld worden op een of meer tijdstippen tussen 0,5 en 6 maanden na transplantatie Dankzij de gevoeligheid van de gebruikte techniek was het al 2 weken na transplantatie mogelijk in 19 van de 22 evalueerbare patienten (86%) donorerytrocyten aan te tonen Dit in tegenstelling tot de conventionele agglutinatie-techniek, waarbij dit slechts in 15% van de patienten mogelijk was Zes maanden na transplantatie waren donorerytrocyten aantoonbaar in alle evalueerbare patienten Het percentage donorerytrocyten nam geleidelijk toe van 0,04% na 2 weken tot 88,8% na 6 maanden De donorerytrocyten repopulatie kwam vertraagd op gang in ontvangers van een "major" ABO incompatibel transplantaat, indien vergeleken met de overige patienten Alleen 3 maanden na transplantatie bleek dit verschil significant (p=0,03) Bij patienten, bij wie het beloop niet gecompliceerd werd door een recidief van de oorspronkelijke ziekte, nam het percentage autologe erytrocyten in de eerste 6 maanden na transplantatie geleidelijk af Zes maanden na transplantatie varieerde het percentage autologe erytrocyten in het merendeel van deze patienten van 0 tot 0,1%

Rekening houdend met een overlevingstijd van ontvanger-erytrocyten aangemaakt voor BMT van 150 dagen, definieerden wij mixed erytrocytenchimerisme als de coexistentie van ontvanger- en donorerytrocyten vanaf 6 maanden na transplantatie De incidentie van mixed chimerisme, vastgesteld op meerdere tijdstippen vanaf 6 maanden na BMT, varieerde van 50 tot 71% in 38 evalueerbare patienten De verklaring voor deze hoge incidentie is enerzijds de verwijdering van T-lymfocyten uit het transplantaat, anderzijds de hoge gevoeligheid van de fluorescerende microsferentechniek De 10 stabiele mixed chimeren met lage percentages (<1%) autologe erytrocyten op verschillende tijdstippen na transplantatie toonden geen aanwijzingen voor recidief Dit was evenmin het geval in 6 mixed chimeren die in het verdere verloop complete donor-chimeren werden Ook de mixed chimeren met hoge (>10%) of zelfs stijgende percentages

autologe erytrocyten ontwikkelden niet altijd een recidief

In hoofdstuk 3 is de invloed van het conditioneringschema op het optreden van erytrocyten chimerisme, GVHD en recidief na transplantatie bestudeerd Vanaf mei 1981 tot januari 1991 werden achtereenvolgens 3 conditioneringsschema's toegepast bij 144 patienten die getransplanteerd werden met beenmerg van een HLA-identieke broer of zus Het donormerg werd in alle gevallen met behulp van tegenstroomcentrifugatie ontdaan van het merendeel van de lymfocyten Alle 3 de conditioneringsschema's bevatten cyclofosfamide en gefractioneerde totale lichaamsbestraling De totale dosis lichaamsbestraling in schema A, toegepast in 49 patienten, bedroeg 9 Gy In schema B, toegediend aan 65 patienten, werd het dosistempo van de totale lichaamsbestraling verhoogd Bovendien werden in dit schema antracyclines toegevoegd Schema C bevatte geen antracyclines doch in dit schema was de totale dosis lichaamsbestraling 12 Gy Dit schema werd toegepast bij 30 patienten Er was een significant verschil in de leeftijdsverdeling tussen de 3 groepen, aangezien de leeftijdsgrens voor transplantatie door de jaren heen toenam Het absolute aantal T-lymfocyten in het transplantaat verschilde eveneens significant tussen de 3 patienten groepen Vergeleken met onbewerkt beenmerg was het absolute aantal T-lymfocyten echter zeer laag bij alle bestudeerde patienten

Een significante invloed van het conditioneringsschema op de incidentie van gemengd erytrocytenchimerisme werd vastgesteld met behulp van multivariant analyse ($p<0,0004$) Intensivering van het conditioneringsschema leidde tot een daling in de incidentie van gemengd erytrocyten chimerisme De leeftijd van de patient en het aantal T-lymfocyten in het transplantaat hadden geen invloed op deze incidentie Zes maanden na BMT was de incidentie respectievelijk 73, 33 en 20% voor schema A, B en C

De incidentie en mate van acute GVHD kon bestudeerd worden in 139 van de 144 patienten De incidentie van GVHD graad I of meer bedroeg 15, 29 en 77% respectievelijk voor schema A, B en C Zowel leeftijd als het conditioneringsschema hadden een significante invloed op het optreden van acute GVHD ($p=0,017$ en 0,0001) De incidentie van chronische GVHD werd noch door de leeftijd, noch door het conditioneringsschema of het aantal T-lymfocyten in het donormerg beinvloed

Een relatie tussen het optreden van acute en chronisch GVHD enerzijds en de incidentie van gemengd erytrocytenchimerisme anderzijds kon aangetoond worden, na bijeenvoegen van alle data van de drie schema's Zowel acute als chronisch GVHD kwamen significant vaker voor in de groep met compleet donorchimerisme dan in de patiënten met mixed chimerisme ($p<0,001$ en $p<0,01$)

De klinische relevantie van het optreden van mixed erytrocytenchimerisme werd bestudeerd bij patienten met CML en patienten met een acute leukemie Bij patienten met CML bleek een relatie te bestaan tussen het percentage autologe erytrocyten vanaf 6 maanden na BMT en het optreden van een recidief van de ziekte Patienten die geen recidief ontwikkelden, waren of compleet donorchimeren of stabiele mixed chimeren met lage (<0,5%) percentages ontvanger-erytrocyten Hoge (>10%) of stijgende percentages ontvanger-erytrocyten duidden op het ontstaan van een cytogenetisch recidief Voor patienten met een acute leukemie was deze relatie niet aantoonbaar

Ter beoordeling van de recidiefkans na de verschillende conditioneringsschema's werden alleen patienten met een gunstige prognose (acute myeloide of lymfatische leukemie in eerste complete remissie en CML in eerste chronische fase) bestudeerd In 32 patienten behandeld met schema A was de recidiefkans 43%, vergeleken met een kans van 18% in 43 patienten na schema B (p=0,07)

Gezien de korte follow-up konden wij geen uitspraak te doen over de resultaten van schema C

Wij concluderen dat intensivering van het conditioneringsschema leidt tot een lagere incidentie van gemengd erytrocytenchimerisme, een hogere incidentie van GVHD en een tendens tot afname van de recidiefkans

In Hoofdstuk 4 wordt de behandeling beschreven van zeven patiënten met een recidief CML na transplantatie met beenmerg waaruit de T-lymfocyten grotendeels waren verwijderd Een patient onderging een tweede BMT, dit keer met onbewerkt beenmerg van dezelfde donor, 3 jaar na de eerste transplantatie De overige zes patienten kregen infusies met lymfocyten van de oorspronkelijke beenmergdonor Vijf van hen hadden een hematologisch recidief van de CML, de zesde patient had slechts een cytogenetisch recidief De periode tussen BMT en de toediening van lymfocyten varieerde in deze patienten van 13 tot 65 maanden De donorcellen werden door middel van aferese verzameld en bestonden voornamelijk uit lymfocyten (70-85%) en monocyten (10-15%) In de eerste cyclus van infusies varieerde de totale dosis kernhoudende cellen van 0,34 tot 1,8 x 10^8/kg bij de zes beschreven patienten Twee patienten, die geen reactie vertoonden op de eerste cyclus, werden opnieuw behandeld met een tweede cyclus infusies waarbij de dosis toegediende cellen twee maal zo hoog was Analyse van chimerisme van de erytroide reeks vond plaats met behulp van de fluorescerende microsfeertechniek Chimerisme werd ook bestudeerd gebruik makend van cytogenetisch onderzoek

Het effect van de therapie op de CML werd vervolgd door cytogenetisch onderzoek naar het Philadelphia chromosoom en door moleculair onderzoek van de BCR/ABL translocatie De patient,

die een tweede BMT onderging was 6 maanden later in cytogenetische remissie. Cytogenetisch onderzoek toonde verder een compleet donor chimerisme aan. Twaalf maanden na BMT was bij moleculair onderzoek geen BCR/ABL translocatie aantoonbaar. De vijf patiënten met een hematologisch recidief kwamen 1 tot 3 maanden na de lymfocyten infusies in hematologische en cytogenetische remissie. Een moleculaire remissie werd in vier van de vijf patiënten bereikt 3 tot 12 maanden na de lymfocyteninfusies. Alle vijf patiënten werden na de behandeling compleet donor chimeren bij cytogenetisch onderzoek en/of in situ hybridisatie. Bij vier patiënten met een kenmerkend antigeen voor ontvanger erytrocyten, daalde het percentage autologe erytrocyten na de lymfocyteninfusies. Dit leidde tot het volledig verdwijnen van autologe erytrocyten in twee van hen. Alle vijf patiënten die in remissie kwamen ontwikkelden GVHD waarvan twee in ernstige vorm. De patiënt met het cytogenetisch recidief toonde geen reactie op de behandeling.

Wij komen tot de conclusie dat infusie van donorlymfocyten een effectieve therapie is voor patiënten die een recidief CML ontwikkelen na BMT met lymfocyten-arm beenmerg. Het anti-leukemie effect ging bij onze patiënten altijd samen met GVHD.

Hoofdstuk 5 beschrijft een analyse van het herstel van de erytropoiese in patiënten na transplantatie met "major" ABO incompatibel beenmerg. "Major" ABO incompatibiliteit deed zich voor bij 44 van de 258 patiënten, die tussen 1981 en 1994 in Nijmegen getransplanteerd werden. In 30 van deze 44 patiënten kon de donorerytrocyten-repopulatie geëvalueerd worden. De controlegroep bestond uit een vergelijkbare groep van 58 patiënten die een ABO compatibele of "minor" ABO incompatibele transplantatie ondergingen. Door middel van de tegenstrooncentrifugatie werden zowel lymfocyten als erytrocyten uit het transplantaat verwijderd met als gevolg dat in het transplantaat na elutriatie minder dan 10^8 erytrocyten resteerden. Geen van de patiënten onderging voor BMT een behandeling met het doel de anti-A of anti-B titer te verlagen. De erytrocytenrepopulatie werd gemeten door gebruik te maken van de fluorescerende microsferen techniek.

Het herstel van de donorerytropoiese bleek de eerste 3 maanden na BMT significant vertraagd in de "major" ABO incompatibele patiëntengroep, vergeleken met de controlegroep ($p<=0,03$). De patiënten in de "major" ABO incompatibele groep kregen ook significant meer erytrocytentransfusies ($p<0,001$). In de studiegroep hadden 62% en 33% van de evalueerbare patiënten geen aantoonbare donorerytrocyten 0,5 en 1 maand na BMT, vergeleken met 19% en 0% in de controlegroep ($p<0,01$). Acht patiënten in de "major" ABO incompatibele groep hadden 2 maanden na BMT nog geen meetbare donorerytropoiese. Al deze acht patiënten, bij wie de donorerytropoiese vertraagd op gang kwam, hadden bloedgroep O in tegenstelling tot 12 van de 22 patiënten

in de "major" ABO incompatibele groep bij wie de donor erytropoiese wel tijdig op gang kwam. Bij zes patiënten was er sprake van "pure red cell" aplasie, een beeld dat zich in vijf patienten 3,5 tot 7 maanden na BMT spontaan herstelde.

De anti-A en anti-B titers voor BMT en het verloop van deze antistofspecificaties na BMT, werden vergeleken tussen de acht patienten met vertraagd op gang komende donorerytropoiese en de overige 22 patienten in de studiegroep. Vóór transplantatie bestond geen significant verschil in de IgM titers tussen de twee groepen, terwijl de IgG titers vóor BMT wel significant hoger waren in de acht patienten (p<0,01). Na BMT werd in de groep van 22 patienten een gestage daling van zowel IgM als IgG anti-A en/of anti-B titers geobserveerd. Dit was duidelijk in tegenstelling met de andere acht patiënten van wie de anti-A titers de eerste 3 maanden na BMT hoog bleven.

De incidentie van immuunhematologische complicaties na transplantatie met "major" ABO incompatibel beenmerg in onze patienten, ondanks het frequenter voorkomen van "mixed" chimerisme, bleek niet hoger dan de gerapporteerde incidentie bij patienten getransplanteerd met onbewerkt beenmerg. Wij zagen geen ernstige morbiditeit gerelateerd aan de "major" ABO incompatibiliteit. De tegenstroomcentrifugatie techniek bleek een effectieve methode om erytrocyten uit het transplantaat te verwijderen in het geval van "major" ABO incompatibiliteit.

Hoofdstuk 6 beschrijft de resultaten van een studie die uitgevoerd werd in het "Johns Hopkins Oncology Center". In dit onderzoek werd het effect van lymfocytenverwijdering uit het beenmerg door tegenstroomcentrifugatie op de expressie van anti-A en/of anti-B antistoffen bestudeerd. In 17 van de 64 patienten, die van december 1985 tot januari 1989 getransplanteerd werden met beenmerg waaruit het merendeel van de lymfocyten was verwijderd, bleek sprake van ABO incompatibiliteit tussen donor en ontvanger. De anti-A en/of anti-B titers van deze patienten voor en na BMT werden vergeleken met die van 28 patienten die tussen 1983 en 1989 een ABO incompatibel, onbewerkt transplantaat ontvingen. Tenslotte werden de anti-A en/of anti-B titers voor en na BMT bepaald van 24 ontvangers van een ABO compatibel beenmerg (12 onbewerkt en 12 met lymfocyten verwijdering). In alle gevallen werden ook de anti-A en/of anti-B titers van de donors bepaald. Er kon geen correlatie worden vastgesteld tussen het plasmavolume in het transplantaat en in de diverse bloedprodukten enerzijds en veranderingen in anti-A en/of anti-B titers anderzijds.

Na "major" ABO incompatibele BMT werd in beide groepen (elutriatie n=11, geen elutriatie n=16) een daling van de ontvanger anti-A en/of anti-B antistoffen gezien. Er waren geen aanwijzingen voor persisterende ontvangerantistoffen in de elutriatie patientengroep. Hierbij moet aangetekend worden dat dit effect slechts in kleine patientengroepen werd bestudeerd.

In de vroege fase na transplantatie (2 tot 4 weken) met een "minor" ABO incompatibel, onbewerkt beenmerg kon in acht van de 14 patiënten een antistof gericht tegen de ontvanger aangetoond worden. Passieve overdracht van deze antistof met het transplantaat kon uitgesloten worden, daar het plasma hieruit verwijderd was. De antistof is een produkt van volwassen donorlymfocyten in het transplantaat. Na "minor" ABO incompatibele transplantatie met beenmerg waaruit het merendeel van de lymfocyten verwijderd was (n=7), werd deze antistof gericht tegen de ontvanger nooit aangetoond. Het feit dat vanaf 3 maanden na BMT in geen van de patiënten deze antistof nog aantoonbaar was, zou verklaard kunnen worden door het ontstaan van tolerantie voor ontvanger A en/of B antigeen.

In sommige situaties van ABO incompatibiliteit kan antistof met een bepaalde specificiteit zowel door ontvanger- als door donorlymfocyten (DOR antistof) geproduceerd worden. Deze situatie is vergelijkbaar met een ABO compatibele BMT. De DOR titers voor en na BMT bleken niet significant te verschillen tussen ontvangers van een ABO compatibel en een ABO incompatibel transplantaat. Na bijeenvoegen van alle data (ABO compatible en incompatibel) werden de DOR titers van ontvangers van onbewerkt en lymfocyten-arm beenmerg met elkaar vergeleken. In de vroege fase na BMT (2 tot 4 weken) werd met name in de ontvangers van onbewerkt beenmerg een stijging van de DOR titers gezien. Dit zou verklaard kunnen worden door de aanwezigheid van volwassen lymfocyten in het transplantaat. Een jaar na transplantatie lijkt het redelijk te veronderstellen dat de DOR antistof door donorlymfocyten geproduceerd wordt. Op dit tijdstip was er geen significant verschil meer in DOR titers (IgM en IgG) tussen beide groepen. Dit feit suggereert dat de produktie van antistof ook in de elutriatie groep op gang was gekomen.

Algemene conclusie

De fluorescerende microsferentechniek is een gevoelige, reproduceerbare methode om erytrocytenchimerisme na BMT te onderzoeken. Wij pasten deze techniek naast andere technieken toe om de invloed van lymfocytenverwijdering uit het transplantaat en van de conditionering op het optreden van mixed chimerisme te bestuderen en om mixed chimerisme te relateren aan de recidiefkans.

Verder bestudeerden wij de kinetiek van donor- en ontvanger-erytropoiese in het geval van ABO incompatibele BMT en na infusie van donorlymfocyten ter behandeling van een recidief CML na BMT.

Dankwoord

Graag wil ik allen bedanken die hebben meegewerkt aan de tot stand koming van dit proefschrift

In het bijzonder wil ik mijn dank uitspreken aan Prof dr Theo de Witte die de basis heeft gelegd voor dit proefschrift en mij door de jaren heen heeft begeleid en gemotiveerd

Voorts wil ik de medewerkers van de Universitaire Transfusiedienst bedanken, met name dr Chiel de Man, die de fluorescerende microsferentechniek heeft opgezet, en de analisten van het "bolletjeslab" die alle bepalingen hebben uitgevoerd Graag bedank ik apart mevrouw Ria Moors die mij zelfs nog met de anti-A en anti-B bepalingen heeft geholpen toen ik in Baltimore werkte Ook ben ik dr Bob van Dijk bijzonder dankbaar voor zijn adviezen en zijn persoonlijke steun

Ir Jan Boezeman ben ik dankbaar voor het uitvoeren van de statistische analyses Zijn eindeloze geduld en kalmte, wanneer ik hem weer om hulp kwam vragen, waren inspirerend

Dr Albert Donnenberg en zijn medewerkers hebben mij op een geweldige manier begeleid in het jaar dat ik in het Johns Hopkins Oncology Center mocht werken

Tenslotte wil ik iedereen bedanken die door te informeren hoever ik al was gevorderd met dit proefschrift, mij stimuleerde om het daadwerkelijk te voltooien

Rob, je hebt mij meer geholpen dan ik hier kan zeggen

Curriculum vitae

De auteur van dit proefschrift werd op 28 augustus 1957 te Venlo geboren
In 1975 behaalde zij het diploma Gymnasium-β aan het Augustinianum Lyceum te Eindhoven
Zij studeerde Geneeskunde aan de Rijksuniversiteit te Leiden, alwaar haar op 16 april 1982 de artsenbul werd uitgereikt Van 1982 tot 1986 werd zij opgeleid tot internist in het Sint Joseph Ziekenhuis, toentertijd te Eindhoven (opleider destijds dr P F L Deckers, internist) en trad in september 1986 in dienst van de afdeling Bloedziekten van het Academisch Ziekenhuis Nijmegen St Radboud Op 1 mei 1987 werd zij als internist geregistreerd
Zij verwierf een klinisch fellowship van 2 jaar van het Koningin Wilhelmina Fonds, waarvan het tweede jaar werd doorgebracht in het Johns Hopkins Oncology Center in Baltimore, MD, USA
Sedert 1990 is zij weer parttime werkzaam bij de afdeling Bloedziekten van het Academisch Ziekenhuis Nijmegen St Raboud en daarnaast bij de Rode Kruis Bloedbank Nijmegen en Omstreken

STELLINGEN

behorende bij het proefschrift

"Studies on host and donor erythrocyte populations in recipients of T-cell depleted bone marrow or lymphocyte transfusions from allogeneic donors".

Brigit M.A.M. Bär

Nijmegen, 26 november 1996

1. Analyse van erytrocytenpopulaties na beenmergtransplantatie is slechts mogelijk wanneer er een ruim uitgetypeerd bestand van bloeddonors beschikbaar is.

2. Door het regelmatig met behulp van de fluorescerende microsferentechniek bepalen van percentages ontvanger-erytrocyten na beenmergtransplantatie voor chronisch myeloide leukemie, kan een uitspraak worden gedaan over het ontstaan van een cytogenetisch recidief.

3. Een recidief chronisch myeloide leukemie na beenmergtransplantatie kan met succes worden behandeld door infusie met donorlymfocyten. Dit is de eerste succesvolle toepassing van immuuntherapie bij kanker.

4. De incidentie van immuunhematologische complicaties na "major" ABO incompatibele transplantatie wordt niet nadelig beïnvloed door elutriatie van het beenmerg.

5. De vraag of de afwezigheid van donor anti-A en/of anti-B antistoffen gericht tegen de bloedgroep van de ontvanger, vanaf drie maanden na "minor" ABO incompatibele beenmergtransplantatie, een gevolg is van tolerantie of absorptie, wordt ook in dit proefschrift niet opgelost.

6. Behandelingsprotocollen zijn een groot goed mits er gemotiveerd van kan worden afgeweken.

7. Mede door gebrek aan ervaren chirurgen is bij de stadiëring van maligne lymfomen de moderne beeldvormende diagnostiek te prefereren boven een laparotomie.

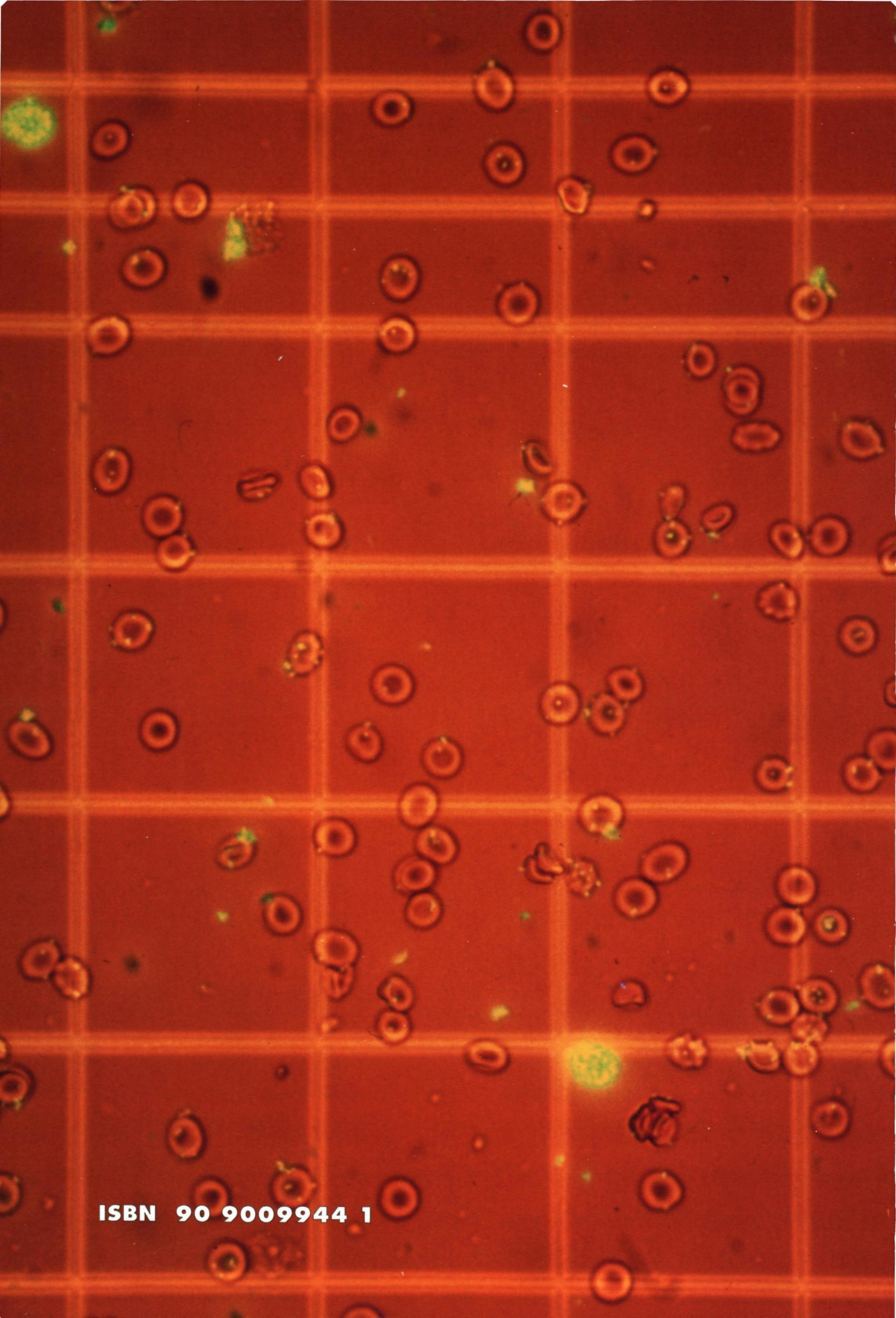
8. In tegenstelling tot een theatervoorstelling wordt onderzoek vaak gecontinueerd door gebrek aan succes.

9. Besluitvorming rond optimale versus maximale veiligheid van bloedtransfusie lijkt meer door angst voor media en publiek bepaald dan door wetenschappelijke argumenten.
(N.a.v. de Invitational Conference "Optimale versus maximale veiligheid van de bloedtransfusie-keten in Nederland", 11-9-1996)

10. Een bloeddonor doet wonderen, een beenmergdonor is een wonder.

11. Een arts zou zich regelmatig moeten realiseren wat het betekent om zelf patiënt te zijn.

12. Eén hond is leuk, twee honden nog veel leuker.



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