Thirty-two patients with acute leukemia, chronic granulocytic leukemia, or multiple myeloma received a T lymphocyte-depleted HLA-identical marrow. After being treated with pan-T monoclonal antibodies (MoAbs) and one round of baby rabbit complement, the mean percentage of T cell depletion was 94% ± 4%. The number of residual viable T cell infused to the patient was 0.99 ± 0.65 \times 10^6 per kg body weight. The patients were conditioned with fractionated total body irradiation (TBI) (12 Gy) preceding high doses of cyclophosphamide (120 mg/kg). Methotrexate was used as an additional immunosuppressant in the first ten patients. For the following 22 patients no posttransplant immunoprophylaxis was administered. Eight patients died within three months due to complications related to cytolysis is well adapted to clinical use. As a general rule, transplants following cyclophosphamide and total body irradiation (TBI) are not rejected from the HLA-matched siblings, whereas the same is not true after an in vitro depletion of T cells prior to a bone marrow transplantation. According to several papers that have been published recently, an unusual percentage of graft failures occurs in recipients of a T cell-depleted marrow graft.

The results of an open pilot study on 32 patients transplanted with a HLA-identical bone marrow in vitro treated with pan-T MoAbs and one round of baby rabbit complement are reported here. Ten patients, included in a previously reported pilot study, were treated with methotrexate plus T cell depletion. Acute GVHD was prevented by T cell depletion alone in 22 patients. Our aims were to evaluate the efficacy of incomplete T cell depletion as a method of preventing acute and chronic GVHD and evaluating the reconstituting ability of the T cell-depleted marrow. Our T cell depletion protocol was sufficient in preventing GVHD in most cases, and an engraftment occurred in all the study patients.

**MATERIALS AND METHODS**

**Monoclonal antibodies and complement.** According to their availability, we used different pan-T MoAbs with the intention of evaluating, in a nonrandomized study, two pan-T antibody combinations of different origins (both include a CD2 antibody). The first cocktail (OKT-pool) consisted of two murine pan-T antibodies OKT11 (CD2) and OKT3 (CD3) from Ortho (Raritan, NJ). Both MoAbs used were of IgG2 class and they were reactive with almost all mature T lymphocytes. The second cocktail (CD-pool) was composed of two IgG2 class (CD5/A50 and CD7/I21) and one IgM class (CD2/D66) MoAbs (A. Bernard, Institut Gustave Roussy, Villejuif, France). Neomural rabbit complement (23 to 28 days) was prepared in our Institution (Immunological Department, Blood Transfusion Center, Besançon, France). The characteristics of the rabbit complement are detailed in Table 1.

**Detection of residual T cells.** Identification of the T cells coated by pan-T MoAbs was performed with indirect immunofluorescence; cells were counted either by microscope or flow cytometry. Cell viability was evaluated with trypan blue or ethidium bromide.
GVHD PREVENTION WITH T CELL-DEPLETED MARROW

Table 1. Sources and Characteristics of Pan-T MoAbs and Complement Used in This Study

<table>
<thead>
<tr>
<th>MoAbs</th>
<th>First combination (Ortho)</th>
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<tbody>
<tr>
<td>CD3/OKT3 (lgG2a)</td>
<td>0.2 µg to 1 µg/1 x 10⁷ NC</td>
</tr>
<tr>
<td>CD2/OKT11 (lgG2)</td>
<td>0.7 µg to 1 µg/1 x 10⁷ NC</td>
</tr>
</tbody>
</table>

| CD2/OKT6 (lgGM2)       | 10 µg/L x 10⁷ NC          |
| CD7/42 (lgG2b)         |                           |

Rabbit Complement
White baby rabbits (23 to 28 days)
Anti-A anti-B heterohemagglutinins negative
Hematopoietic stem cell recovery (>70%)
Hemolytic activity (CH50 = 29.6 units ± 6.9)
Calcium level = 3.06 ± 0.156 mmol/L
T cell lysis (standardized conditions with pan-T MoAbs)
Limulus test negative
Bacteriologic and virologic controls negative

Measurements indicate mean ± 1 SD.

Approximately 200 to 500 cells were examined and counted under a Leitz fluorescent microscope.

Bone marrow processing and ex vivo treatment. Bone marrow was aspirated from the iliac crests and from the sternum (2 mL per aspirate to avoid peripheral blood contamination). Our protocol has been previously reported and is summarized in Fig 1. This procedure takes approximately six hours and 30 minutes to complete.

Patients' characteristics. A total of 32 patients (18 males, 14 females: 19 adults, 13 children) were included in our open pilot study. The median age was 20 years (ranging from 2 to 40 years). Ten previously reported patients are included in this group.

The diagnoses were: nine acute myelogenous leukemia (AML), 16 acute lymphocytic leukemia (ALL), five chronic granulocytic leukemia (CGL), and two multiple myeloma. Table 2 indicates the patients' status at the time of the BMT. All the patients were HLA-A, B, DR identical with their sibling donors. Mixed lymphocyte cultures were bidirectionally negative for all of them. There were seven major blood group incompatibilities. High risk of acute GVHD was expected in ten patients over 30 years of age, in two patients with CGL in acute or accelerated phase, and in four patients with acute leukemia in relapse or subsequent remission. Ten patients exhibited all the criteria of malignant disease with high risk, ie, major chromosome abnormalities in six patients, high WBC count (>50 x 10⁹/L) in nine patients, first relapse on therapy in the course of an acute lymphoblastic leukemia (fast disease) in eight patients, and subsequent remission or relapse in four patients. The patients were conditioned by fractionated TBI (2 Gy for six fractions for a total dose of 12 Gy) preceding high dose of cyclophosphamide (60 mg/kg body weight x 2). Irradiation was delivered through a linear accelerator with an exposure rate of 5 to 6 cGy per minute. Protocol and consent forms were approved by the institutional review board of the University of Besancon.

Five ALL patients and one AML patient received reinforced chemotherapy in the pre-transplant regimen, according to their high risk of relapse (cytostine arabinoside 100 mg/m² for five days and/or etoposide 100 mg/m² per day for three days). In two patients high dose melphalan (180 mg/m²) was used as a substitute for cyclophosphamide. One multiple myeloma patient was given high-dose melphalan (140 mg/m²) combined with cyclophosphamide preceded by a fractionated TBI.

All but three patients were nursed in closed plastic tent isolators (Iroom, La Calhène, France) with a total nonselective gut decontamination using non absorbable antibiotics. All the patients were given low-dose heparin (1 mg/kg/d) for prophylaxis of venoocclusive disease of the liver.

During the initial feasibility study, posttransplant immunosuppression with methotrexate was maintained until day 100 in four patients and stopped at day 11 post-BMT in six patients (group 1). In 22 patients, no posttransplant immunosuppression was administered (group 2). No maintenance chemotherapy was given following BMT. The median duration of isolation from BMT was 30 days (ranging from 17 to 49 days). Eighteen patients received bone marrow treated with the first MoAb combination (OKT-pool) and 14 with the second (CD-pool). The day of engraftment was defined as the third consecutive day on which the WBC count was >1 x 10⁹/L. Acute GVHD is defined as beginning <90 days after transplant. Severity was defined according to the Seattle criteria.

Genetic markers were available to prove engraftment in 25 patients (sex chromosomes in 12 patients and red blood cell phenotype disparities in 13 patients). No marker was available in seven patients. Chromosome studies were performed by direct processing of the unstimulated bone marrow specimens. An average of 16 mitoses (3 to 110) were analyzed in detail at three, six, and 12 months post-BMT.

Cellular immune function. Immune status was evaluated through surface cell markers by using a panel of seven specific T and B cell MoAbs, lymphocyte proliferative responses to mitogens (PHA, con A) and allogeneic cells (mixed lymphocyte culture). The periods tested were pretransplant and one, three, six, and 12 months posttransplant.

RESULTS

Marrow transplants for the study patients were performed between January 1983 and August 1985.

Early mortality. Of the 32 patients who underwent BMT, eight (five of whom were children) died within three months (mean 35.6 days) due to transplantation-related complications (six had ALL, two had AML). Early toxicity was significantly higher in the patients who had received additional chemotherapy in the preparative regimen (5/8). The causes of death were one fungal septicemia, three interstitial pneumonitis, one acute pancreatitis, one toxoplasmosis, one pseudomonas infection that localized in the neck, and one cardiac and renal failure. The patients who died within 21 days and 40 days post-BMT were not evaluable for engraftment of acute GVHD prevention. Thus 28 patients were evaluated for engraftment and 27 for acute GVHD prevention.

T cell depletion. Processing of the bone marrow led to
the separation of marrow mononuclear cells on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), which was well adapted to a complement-mediated cytolysis. The harvested donor marrow volume was 470 mL ± 12% and contained a mean of 4.3 ± 1.9 x 10^8 nucleated cells per kilogram recipient weight. These data demonstrate that it is not necessary to aspirate a large volume of bone marrow to harvest sufficient nucleated cells to ensure engraftment. The small volume of collected marrow avoided a peroperative hemorrhage. A density gradient separation of the marrow resulted in a 79% ± 16% recovery of committed myeloid progenitors. Treatment with pan-T MoAbs and complement resulted in 1 to 1.5 log of T cell depletion from 30 study marrows out of 32.

The mean T cell depletion was 94% ± 4%. T cells harvested with the transplant were 74 ± 36 x 10^6. One round of complement lysis produced an incomplete T cell depletion. A total of 47 ± 3 x 10^6 residual viable T cells remained in the inoculum, and the number of viable T cells infused to the patient was 0.99 ± 0.65 x 10^6 per kilogram of the recipient’s weight. Although the use of OKT-MoAbs led to a smaller clumping than the CD2, 6, and 7 pool, we did not observe a significant difference between the two MoAb combinations in terms of T cell depletion efficacy. The T cell removal with the OKT-pool and the CD-pool was 93% ± 6% and 95% ± 3% respectively. Committed stem cell assays done before and after complement incubation indicated a 66% ± 21% CFU-GM recovery. These data are summarized in Table 3. In most patients the main side effects of the treated marrow infusion were chills and fever.
developed in this patient, and he died four months posttransplant. He was treated with two cycles of bolus intravenous (IV) high-dose methylprednisolone and rabbit antithymocyte globulins. A majority of deaths attributed to late infections occurred in the first and second complete remission (CR). One patient relapsed 16 months after BMT; following this he achieved a second remission resulting from the German ALL-BFM-82 chemotherapy protocol.

Engraftment. Engraftment was achieved in all the study patients who received a T cell-depleted transplant. At the time of this analysis (October 15, 1985), no patients had an early (no engraftment within 30 days post-BMT) or a late (graft rejection beyond 90 days post-BMT) graft failure.

The time of recovery of 1 × 10^9/L white blood cells was 14 to 28 days (mean 18.9 ± 4.7). For 65% of the patients the recovery time was under 20 days. Thirteen to 41 days were needed to recover 0.5 × 10^9/L granulocytes (mean 20.5 ± 6.4). The time required was under 20 days for 54% of the patients. The time to recover 50 × 10^9/L platelets was 16 to 65 days (mean 29.2 ± 12.5). The time required was under 30 days for 56% of the patients.

Proof of total chimerism was established in 20 patients by utilizing either genetic markers, eg, sex chromosomes in 11 patients or RBC antigen disparities in 13. Mixed chimerism was documented in two ALL patients grafted in first and second remission after low-dose prednisolone. In 19 study patients the follow-up was longer than six months.

Acute and chronic GVHD. Twenty-four of the 27 evaluable patients (88%) did not have an acute GVHD higher than grade 0 to 1. No acute GVHD occurred in the first group of ten patients. In group 2, two patients had grade 2 (skin only) confirmed by skin biopsy. Both responded promptly to 2 mg/kg/d of prednisone. A third patient developed a grade 4 GVHD. This patient received more viable T cells than the other patients because of unforeseen technical problems during the in vitro treatment. He was treated with two cycles of bolus intravenous (IV) high-dose methylprednisolone and rabbit antithymocyte globulins. Three months later evidence of extensive chronic GVHD developed in this patient, and he died four months posttransplant.

No other patient developed extensive chronic GVHD. Nevertheless, limited chronic GVHD occurred in four patients (Sicca syndrome in two patients, focal skin lesions in two others). These manifestations promptly disappeared after low-dose prednisolone. In 19 study patients the follow-up was longer than six months.

Chronic GVHD and infectious complications. It is well known that infectious complications that occur after the first three months are seen primarily in patients with a chronic GVHD. A majority of deaths attributed to late infections are reported in patients with active chronic GVHD. Seventeen study patients without extensive disease were analyzed to evaluate if the clinically significant late infection rate was reduced. Most of them had either none or only one or 2 late infections. Twelve infectious episodes were documented in the 17 study patients (mean ± SD ranged between 0 and 3). Skin and genitourinary tract infections and septicemia were the most common types. One impressive example was that of our patient with an active chronic GVHD. He suffered six severe infectious episodes before death occurred.

Relapse. At the time of analysis a medullary leukemia relapse occurred in 13 patients (out of 24 evaluable patients), prior to six months in eight patients and after six months in five. Nine of the 13 were previously defined as “high risk leukemia.” In this series the longest period between transplant and relapse was 364 days. Eleven patients have remained in unmaintained continuous CR to date. The mean follow-up is that of 13.3 months (ranging from three to 30 months) with only three patients below six months. The current status of the 32 study patients is reported in Table 4.

Immunologic reconstitution. Recovery of the immune function will be reported at length elsewhere (E. Racadot). In all engrafted patients there was considerable impairment of immunologic status after allogeneic BMT using incomplete T cell-depleted marrow (Fig 2). This severe immune deficiency persisted beyond one year posttransplant. These immune alterations are equivalent to those reported by Zander et al after allogeneic BMT using untreated marrow.

DISCUSSION

The removal of T cells by pan-T MoAbs and complement-mediated cytolysis is a simple and effective method available in clinical situations. The cytolytic effect of these pan-T MoAbs depends closely on the source of complement and on the anticomplementary effect of bone marrow cells. Treatment with antibodies and complement in vitro can achieve an efficient and rapid killing of donor T cells. The extent of the removal of T cells can be accurately measured. We did not observe any difference between the two MoAb combinations used in terms of engraftment kinetics and GVHD prevention. The major finding of this study was that a prompt engraftment was achieved in all the patients who had received ex-vivo treated marrow. No early or late graft failure occurred in 17 patients beyond six months after BMT. Our results differ from the clinical data that have been published recently. Martin et al reported 20 patients grafted with marrow treated with a mixture of eight MoAbs and rabbit

<table>
<thead>
<tr>
<th>Table 3. T Cell Counts Before and After Ex-vivo Treatment in HLA-Matched Situation</th>
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<tbody>
<tr>
<td>N* T cells in the transplant × 10^9 = 73.7% ± 36.1%</td>
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<tr>
<td>N* residual viable T cells × 10^9 = 4.71% ± 3.27%</td>
</tr>
<tr>
<td>% of T cell depletion = 93.7% ± 4.08%</td>
</tr>
<tr>
<td>1st MoAb association (OKT-pool) = 93.17% ± 5.81%</td>
</tr>
<tr>
<td>2nd MoAb association (CD-Pool) = 94.85% ± 2.61%</td>
</tr>
<tr>
<td>T cell infused to the patient × 10^9/kg = 0.99% ± 0.65%</td>
</tr>
<tr>
<td>CFU-GM recovery post-T cell removal = 69.2% ± 17.2%</td>
</tr>
</tbody>
</table>

Measurements indicate mean ± 1 SD.

|N* = 32

| Engraftment. Engraftment was achieved in all the study patients who received a T cell-depleted transplant. At the time of this analysis (October 15, 1985), no patients had an early (no engraftment within 30 days post-BMT) or a late (graft rejection beyond 90 days post-BMT) graft failure.

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<table>
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<th>Table 4. Current Status of the BMT Program With a T Cell-Depleted Inoculum</th>
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</thead>
<tbody>
<tr>
<td>Number of patients = 32</td>
</tr>
<tr>
<td>BMT related death (&lt;3 months) = 8 (25%)</td>
</tr>
<tr>
<td>Number of take = 28/28 EP</td>
</tr>
<tr>
<td>Late graft failure = 0</td>
</tr>
<tr>
<td>Acute GVHD &gt; grade 2 = 3/27 EP (11.1%)</td>
</tr>
<tr>
<td>Chronic GVHD = 1/19 EP (5.2%)</td>
</tr>
<tr>
<td>Relapse = 13/24 EP (54.1%)</td>
</tr>
<tr>
<td>Number of living patients = 11/24 (45.8%)</td>
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</tbody>
</table>

EP = Evaluable patient.
complement that led to a 2- to 3-log depletion of T cells. Eight cases of graft failure occurred, seven of them being among the 11 patients prepared for transplantation with 12.0 Gy of TBI, and only one out of the nine patients was prepared with 15.75 Gy.7

As part of a European cooperative study on T cell depletion with Campath-1 MoAb (lgM, which fixes human complement and binds both T and B lymphocytes), the incidence of graft failure was high while varying from one team to another (10% to 20%).28 In a prospective randomized controlled trial, clinical data from the UCLA Center showed that five of 20 patients in the CT2/CD2-treated marrow group failed to engraft (none were in the control group).31 The association of graft failure with a near complete removal of T cells from the donor marrow suggests that T cells in the marrow graft may eliminate host effector cells that survive the conditioning regimen.

Our clinical results on engraftment strongly suggest that our own conditioning regimen protocol may play a major part in the patient's immunologic status. Engraftment depends closely on the magnitude of the residual host-v-graft reactivity caused by residual host T cells, which might react against donor antigens exhibited on donor hematopoietic stem cells.24 Some recent data suggest that increased immunosuppression can overcome the risk of graft failure.7

An experimental model is under study in our laboratory in an attempt to demonstrate the influence on the immunologic status of the host at the time of BMT in relation to radiation exposure of the recipient. We would like to emphasize that the patients who received fractionated TBI a week before grafting tolerated the preparative regimen far better.

Evidence shows that acute GVHD can be effectively prevented by a depletion of T cells from the bone marrow. Our own results are in agreement with the recently published results of the Royal Free Hospital,5 the Fred Hutchinson Center,1 the University of Wisconsin,25 and the Hammer- smith Hospital.18 The difference lies at the T cell removal level. A ≥99% T cell depletion is reported in most publications, whereas in our experience a mean of 94% T cell depletion has been observed. It could be suggested that a near-to-complete removal does not appear necessary to prevent severe forms of acute GVHD. Nevertheless, we have to interpret our results with caution given the relatively small group of evaluable patients in group 2.

The outlook is even more promising insofar as chronic GVHD is concerned. Extensive chronic GVHD occurred in only one of the 22 study patients followed beyond three months. It is obvious that the late infection rate decreased significantly. To date, we can assume that, independently of its source, a pan-T MoAb cocktail is highly effective in preventing severe forms of acute and chronic GVHD, even in the absence of posttransplant immunosuppression. Other MoAbs in combination with the pan-T MoAb can be used for a T cell depletion, such as antibodies that recognize the CD4 and CD8 T cell molecules.

The suppression of severe forms (grade 3 to 4) of acute GVHD and of extensive forms of chronic GVHD should improve the patient's survival rate in the first year posttransplant, although this fact has not been demonstrated in the present study. Nevertheless, we have to point out that the quality of life is excellent given the low frequency of late infections; the Karnovsky score has always been higher than 90%. We must not, however, overlook the fact that a graft-v-leukemia effect has been closely associated with acute and especially chronic GVHD. Such close correlations were clearly shown when the allograft was done in the course of second remission or later.36 One may therefore expect to find that an improvement in the survival rate is associated with an increase in the relapse rate. However, the clinical series reported by Prentice et al17 on a homogeneous group of acute leukemia patients who had undergone a BMT in CR1 did not show that the relapse rate had increased. Nevertheless, we may wonder if a total GVHD prevention in high-risk leukemia is reasonable when the BMT is done in CR2 and subsequent remission. It is hoped that some light will be shed on this question once the randomized clinical trials in progress are completed.

In conclusion, the results of this open pilot study lead us to assume that it has been possible to prevent GVHD successfully through a T cell depletion alone and to obtain engraftment in all the study patients who did not have a late graft failure. Although an evaluation is premature at this stage, the relapse rate appeared to be relatively high in our series. Perhaps there is a close connection with the high percentage of study patients defined as "high risk leukemic."

NOTE ADDED IN PROOF
The authors report two graft rejections and one graft failure in a new series of 14 T-depleted matched BMT since the time of the analysis reported above.

ACKNOWLEDGMENT
We thank Prof G. Santos for reviewing the manuscript and for helpful suggestions. The authors gratefully acknowledge the technical assistance provided by A. Jeanroy and T. Bertrand. They are also indebted to the Besançon BMT nursing teams for the skilled and devoted nursing care given to our patients. C. Delagrange provided excellent secretarial assistance. Lastly we thank Zinat Saleh for having corrected the English translation of this text.
GVHD PREVENTION WITH T CELL-DEPLETED MARROW

REFERENCES


Successful graft-versus-host disease prevention without graft failure in 32 HLA-identical allogeneic bone marrow transplantations with marrow depleted of T cells by monoclonal antibodies and complement

P Herve, JY Cahn, M Flesch, E Plouvier, E Racadot, A Noir, Y Couteret, G Goldstein, A Bernard and R Lenys