Effect of Natural Killer Cells on Syngeneic Bone Marrow: In Vitro and In Vivo Studies Demonstrating Graft Failure Due to NK Cells in an Identical Twin Treated by Bone Marrow Transplantation

By Glenwood D. Goss, Michelle A. Wittwer, Werner R. Bezwoda, Jean Herman, Arthur Rabson, Lesley Seymour, Denis P. Derman, and Barry Mendelow

Bone marrow transplantation for severe idiopathic aplastic anemia was undertaken in a patient, using his monozygotic twin brother as the donor. In spite of the use of syngeneic bone marrow, failure of engraftment occurred on two occasions. In vitro studies demonstrated that natural killer (NK) cells from the recipient markedly inhibited the growth of donor bone marrow granulocyte progenitor cells. On a third attempt, successful bone marrow engraftment was achieved following high-dose cyclophosphamide, which has previously been shown to be inhibitory to NK cells. We conclude that NK cell activity may play an important role in bone marrow failure as well as being responsible for at least some cases of aplastic anemia.

DIOPATHIC aplastic anemia may reflect a disorder of the hemopoietic stem cells, a defect in the supporting microenvironment, an abnormality of regulation of hemopoietic cell growth and/or active suppression of hemopoiesis.1 Aplastic anemia and its treatment by bone marrow transplantation between monozygotic twins affords a unique opportunity to study these abnormalities without the limiting factor of genetically determined antigenicity. This report describes a monozygotic twin who developed aplastic anemia and who underwent repeated bone marrow transplantation with engraftment occurring only after the third transplant, when cyclophosphamide pretreatment was used. In vitro culture studies helped to identify both the reason for failure of engraftment and the probable mechanism for the patient’s aplastic anemia. In particular, autologous natural killer cells (NK) (large granular lymphocytes)2,3 caused marked inhibition of syngeneic granulocytic–macrophage colony-forming cell (GM–CFC) growth. Because cyclophosphamide has previously been shown to suppress natural killer cell activity4,5 the clinical course is in keeping with the results of the in vitro studies. These findings appear to be the first instance in which NK cells have been shown to be responsible for human bone marrow graft failure, and they demonstrate the potential importance of NK cells in human transplantation biology.

MATERIALS AND METHODS

Case Report

A 19-year-old man, S. M., was diagnosed as suffering from severe acute aplastic anemia in January 1983. The presenting features were recurrent hemorrhage and septicemia. Hematological investigations showed: hemoglobin concentration of 6.8 g/dL; a WBC count of 0.8 to 1.4 x 10^9/L; a neutrophil count of <5%; a lymphocyte count of 95%; a platelet count of 7 to 14 x 10^9/L; and a reticulocyte count of <0.2%. Bone marrow examination revealed marked hypocellularity; the few particles that were obtained consisted mainly of tissue basophils. Bone marrow trephine biopsy confirmed severe hypoplasia of all hemopoietic elements and also showed marked stromal edema. There was a history of possible chloramphenicol exposure some years prior to the time when the patient was seen, but no other drug, occupational exposure, or viral infection could be incriminated as an etiologic factor. Initial treatment was supportive with leukocyte-poor RBC transfusion and antibiotics. Platelet transfusions were given on two separate occasions. These platelet transfusions were pooled units from multiple donors (16 donors), and were irradiated prior to administration. Shortly after initial diagnosis, therapy with oxymetholone 150 mg/d was begun and was continued for six weeks. Because of the availability of an identical twin donor, bone marrow transplantation was undertaken as quickly as possible. Identity between donor and recipient was confirmed by a variety of investigations, including: HLA typing; mixed lymphocyte culture (MLC) nonreactivity; RBC antigens; RBC enzyme and serum protein phenotype analysis; and morphometric analysis (Table 1).

Details of the transplant procedures are summarized (Table 2). The first transplant attempt was made without immunosuppression. Adequate numbers of nucleated bone marrow cells (6 x 10^9/kg) were infused,4 but there was no evidence of engraftment by day 28. The recipient was then prepared with a rabbit antithymocyte globulin preparation (ATG) (Fresenius, Freiburg, West Germany) 10 mg/kg/d for four days; ATG is predominantly active against T helper and T cytotoxic cells. In response to ATG administration, the WBC count fell from 1.0 x 10^9/L (100% lymphocytes) to 0.2 x 10^9/L, and the patient was again transplanted using the same donor. Again an adequate number of nucleated marrow cells were infused, but engraftment failed to take place by day 35. A third transplant following pretreatment with high-dose cyclophosphamide, (50 mg/kg/d for four days) has been successful with a granulocyte count >1.0 x 10^9/L being achieved on day 20 posttransplant. In vitro bone marrow culture and immunological studies provided some insight into the reasons for initial graft failure and are detailed below. Bone marrow grafting and bone marrow culture studies were performed after consent had been obtained from the patient and the marrow donor following explanation of the procedures and attendant risks as required by the Ethics Committee of the University of the Witwatersrand.

In Vitro Studies

Preparation and identification of large granular lymphocytes (LGLs) and T cell subsets. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density centrifugation.7 Nonadherent cells were obtained by recovering cells nonadherent to
OKT, (Ortho) monoclonal antibody: 0.2 mL of rabbit complement serum and 0.2 mmol/L of glutamine and antibiotics were incubated obtained after treatment of T cells with either OKT8 or OKT4 cells. Incubation of PBMC fractions consisting predominantly of clonal antibody. Viability was assessed by trypan blue exclusion and cells as demonstrated by complement lysis using the OKT3 monoclonal antibody, respectively, together with complement. In standard chromium release assay with the K562 cell line as target exceeded 90%. The cytotoxic ability of the LGLs was confirmed in a 107 LGLs as described above, with the ATG used for conditioning left 0.8:1.

Table 1. Data Confirming Monozygosity

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA typing</td>
<td>A29; - B12 (W45); - CW2; CW7</td>
</tr>
<tr>
<td>RBC antigens</td>
<td>A1</td>
</tr>
<tr>
<td>Rhesus</td>
<td>ccDee</td>
</tr>
<tr>
<td>MNs</td>
<td>MMss</td>
</tr>
<tr>
<td>Duffy</td>
<td>Fy (a + b -)</td>
</tr>
<tr>
<td>Kidd</td>
<td>JK (a + b -)</td>
</tr>
<tr>
<td>Kell</td>
<td>K (-)</td>
</tr>
<tr>
<td>RBC enzyme phenotype</td>
<td>Identical</td>
</tr>
<tr>
<td>Serum protein</td>
<td>Identical*</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>2</td>
</tr>
<tr>
<td>Transferrin</td>
<td>C</td>
</tr>
<tr>
<td>Properdin</td>
<td>S</td>
</tr>
<tr>
<td>Factor</td>
<td>S</td>
</tr>
<tr>
<td>Mixed lymphocyte culture (stimulation index)</td>
<td>Recipient v donor (irradiated)</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td>Donor v recipient (irradiated)</td>
</tr>
<tr>
<td></td>
<td>0.8:1</td>
</tr>
</tbody>
</table>

*Fourteen RBC enzymes were typed.
†No cytogenetic evidence to exclude homozygosity.

RESULTS

At the time of the first transplant, the patient’s lymphocyte count was 1 x 10⁹/L. The OKT₄⁺--OKT₈⁺ cell ratio was then added to the cell suspension, which was incubated for a further 60 minutes at 37 °C and then washed. The viable cell count was then determined by the trypan blue method and adjusted to the final working concentration. Bone marrow co-culture. Bone marrow cultures were performed using the method of Pike and Robinson. Molter 10⁶ PBMCs obtained from normal volunteers. Donor target bone marrow was mixed with peripheral blood mononuclear effector cells or specifically enriched subfractions to a final ratio of effector-target cell ratio of 2:1 and was incubated in suspension culture in 1 mL McCoy’s 5A medium containing 20% fetal calf serum (GIBCO, Grand Island, NY). Thereafter, cells were washed, and triplicate aliquots of cell suspensions (2 x 10⁶ cells per plate) were plated over feeder layers in McCoy’s SA medium and 0.3% agar plus 20% fetal calf serum and were incubated at 37 °C in humidified 5% CO₂. Colonies (aggregates of >40 cells) were counted after seven days of culture. Culture studies were performed using donor marrow collected prior to transplantation and with marrow from a normal volunteer (G. G.). The studies (Table 3) included control cultures and cultures to which either PBMCs, OKT₄⁺ enriched cells, OKT₈⁺ enriched cells, or cells from the 40% Percoll gradient depleted of the OKT₄ and OKT₈ populations and consisting of >80% LGLs were added. When PBMCs or specifically enriched cell populations were co-cultured with bone marrow, the cell concentrations were adjusted to obtain a ratio of peripheral blood-bone marrow cells of 2:1.

Table 2. Bone Marrow Transplantation Data

<table>
<thead>
<tr>
<th>Transplant 1</th>
<th>Transplant 2</th>
<th>Transplant 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretransplant immunosuppression</td>
<td>None</td>
<td>ATG (rabbit)</td>
</tr>
<tr>
<td>Lymphocyte count x 10⁶/L</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Percentage of LGLs</td>
<td>5.0</td>
<td>40</td>
</tr>
<tr>
<td>Marrow cell dose (x 10⁶/kg)</td>
<td>6.0</td>
<td>6.6</td>
</tr>
<tr>
<td>Buffy coat augmentation</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Posttransplant</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Engraftment</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>50 mg/kg/d x 4 days</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>Daily x 3 days</td>
<td></td>
</tr>
<tr>
<td>Cytoxic drugs on alternate doses x 3 doses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 20 neutrophils &gt; 1 x 10⁹/L</td>
<td></td>
<td></td>
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</tbody>
</table>
cells prior to transplant 3. After cyclophosphamide therapy cell viability and the persistent isolation of K562 reactive
This finding was further confirmed by the in vitro studies, own control. Experiment 1 was performed at the first trans-
geneic twin donor. Experiment three was performed using
bone marrow from a normal volunteer (author G. G.).

Because these were separate experiments, the degree of
inhibition of GM–CFC in each experiment is related to its
own control. Experiment 1 was performed at the first trans-
plant attempt and demonstrated that the patient’s PBMC
significantly inhibited syngeneic donor GM–CFC. This in
vitro suppression of colony growth was reflected by failure of
engraftment 28 days later. Experiment 2 was carried out
prior to the second transplant attempt and shows that donor
GM–CFC inhibition was mediated by the patient’s LGLs
and not by the patient’s OKT8-depleted T cells or OKT4-depleted T cells. Studies using bone marrow from a normal
volunteer (author G. G., experiment 3) unrelated to the
patient undergoing marrow transplant and co-cultured with
autologous LGLs and LGLs from the bone marrow graft
recipient at the same concentration as those used in experi-
ments 1 and 2 demonstrated that bone marrow GM–CFC
were not inhibited by autologous LGLs, whereas the LGLs
isolated from the patient caused marked suppression of
GM–CFC.

DISCUSSION
Bone marrow transplantation for aplastic anemia between
identical twins would appear at first sight to be a simple
procedure. However, the results reported in the literature
show an initial graft failure rate of 50% (including our own
patient) which is considerably higher than that encountered
in HLA-compatible but nonidentical marrow transplants
following marrow ablation for acute leukemia and even
for nonidentical transplants in other patients with aplastic
anemia. This high graft failure rate has largely been
unexplained, although there has been speculation about
immunological factors which may be responsible for both the
development of aplastic anemia11,12 and the failure of
engraftment.13,14 Indeed, it has been considered that the high
bone marrow rejection rate in aplastic anemia is due to less
intensive cytotoxic/immunosuppressant pretreatment15 of
these patients as compared with subjects receiving bone
transplantation as part of the treatment for acute leukemia.
In this regard, it should be pointed out that of the 23 bone
marrow transplants for aplastic anemia using identical twin
donors that have been reported in the literature,15 and in
patients in whom no immunosuppression was used in the
initial attempt, 11 grafts failed to take. Subsequently, eight
of these patients were successfully retransplanted while
receiving various cytostatic/immunosuppressant regimes.
Current speculation as to the cause of failure has largely
centered on either T cell-mediated or B cell-mediated spe-
cific immune mechanisms.16-19

In the patient reported here, failure of bone marrow
engraftment appears to have been due to endogenous NK cell
activity, demonstrating the potential importance of this
subset of effector cells in human transplantation biology.
Evidence for the role of NK cells came from the in vitro
studies which demonstrated suppression of bone marrow
colony growth in co-cultures with an enriched LGL cell
population as well as the clinical response to bone marrow
infusion. The second attempt at bone marrow grafting
following immunosuppression with ATG, which was under-
taken before the results of the in vitro studies became
available, also failed, adding weight to the evidence that a
cell population other than T lymphocytes was responsible
for the failure of engraftment. Moreover, it was subsequently
demonstrated that, although the ATG used for transplant
conditioning caused a fall in total lymphocyte count, this
reduction was due to depletion of T lymphocytes; large
granular lymphocytes (further identified as NK cells)
remained present and functionally intact, as demonstrated
by persistent isolation of cells with K562 killing ability.
These cells were also the subset responsible for maximal
inhibition of in vitro granulocyte–macrophage colony
growth. Although inhibition of in vitro granulopoiesis20 and
also of erythroid stem cell proliferation21 by NK cells has
been described previously, this phenomenon can only be
demonstrated after either prolonged liquid phase coincuba-
tion of bone marrow precursor cells and NK cells prior to plating in agar or after liquid phase coincubation with interferon-activated NK cells. In the investigation of Hansson and co-workers, 20 two hours of coincubation of bone marrow precursor cells and unstimulated NK cells resulted in <5% inhibition of GM–CFC. These results contrast with the findings of the present investigation in which marked inhibition (as much as 85% inhibition) was shown after only two hours of coincubation of syngeneic donor marrow and recipient NK cells as well as allogeneic marrow plus recipient NK cells, whereas NK cells from a normal volunteer coincubated with autologous bone marrow for two hours produced no such inhibition. Furthermore, the in vitro inhibition of GM–CFC was paralleled by in vivo failure of engraftment. Thus, although an in vitro effect on hemopoietic precursor cell growth by NK cells has been described previously, the present study is the first instance in human bone marrow transplantation in which evidence for NK cell-mediated aplastic anemia has been documented by both in vitro and in vivo studies.

REFERENCES


Effect of natural killer cells on syngeneic bone marrow: in vitro and in vivo studies demonstrating graft failure due to NK cells in an identical twin treated by bone marrow transplantation

GD Goss, MA Wittwer, WR Bezwoda, J Herman, A Rabson, L Seymour, DP Derman and B Mendelow