Growth In Vitro of Donor Marrow Cultured With Recipient Lymphocytes Predicts the Fate of Marrow Grafts in Transfused DLA-Identical Dogs

By Beverly J. Torok-Storb, Rainer Storb, H. Joachim Deeg, Theodore C. Graham, Cathy Wise, Paul L. Weiden, and John W. Adamson

It has been shown previously that peripheral blood lymphocytes from normal dogs cocultured with DLA-identical littermate marrow increased the number of erythroid colonies over that obtained with marrow alone. In contrast, preceding blood transfusion from the littermate reduced the ability of lymphocytes to enhance erythroid colony growth. The current study correlates similar findings in vitro with the observations made in vivo following marrow transplantation after lethal total body irradiation. Twenty pairs of DLA-identical littermates were studied. One member of each pair served as the blood and marrow donor and the other as the recipient. Each recipient was "sensitized" by transfusion of blood products from their prospective donor. Of 20 transfused recipients, 14 rejected the marrow graft; 13 of these 14 rejections were predicted by observation in vitro of either lack of stimulation or inhibition of donor marrow by recipient lymphocytes. Successful and sustained marrow engraftment occurred in 6 dogs; 5 of these were associated with significant stimulation of donor marrow in coculture. These results were significant at $p = 0.002$ and suggest that transfusion-induced sensitization and marrow graft rejection can be predicted by reduced erythroid colony growth of donor marrow cocultured with recipient lymphocytes.

Recently we reported that normal dog peripheral blood lymphocytes (PBL) significantly increased the number of erythroid colonies (EC) grown from DLA-identical littermate marrow. Following transfusion-induced sensitization of the lymphocyte donor this stimulating ability was abolished, resulting in most cases in either a failure to increase or a decrease in EC numbers. This lymphocyte-mediated suppression of EC growth following transfusion is presumably related to an immune reaction against minor tissue antigens.

Previous studies have shown that dogs given blood transfusions from the marrow donor or from random donors frequently rejected a subsequent marrow graft from a DLA-identical littermate following 1200 R total body irradiation (TBI). In contrast, all dogs not given preceding transfusions showed sustained marrow engraftment. Presumably, rejection was the result of immunization of the recipient to minor histocompatibility antigens in the marrow donor. The purpose of the present study was to investigate whether or not the inhibition of growth in vitro of
donor marrow by "sensitized" recipient lymphocytes might predict subsequent marrow graft rejection in vivo.

**MATERIALS AND METHODS**

**Dogs.** Canine litters were obtained from kennels in the states of Washington, Oregon, and Virginia. Dogs used were beagles, German shorthaired pointers, dalmations, and basenji-Labrador retriever-greyhound-golden retriever crossbreds. Twenty pairs of littermates were selected on the basis of identity for DLA (as determined by serotyping and mutual nonreactivity in mixed leukocyte culture) and dog erythrocyte antigen 1 described previously.

**Transfusions.** Six dogs were injected intravenously with 50 ml heparinized whole blood from their prospective marrow donor on each of three occasions, 24, 17, and 10 days before transplantation (group 1). Five recipients were given 50 ml donor blood on day 10 before transplantation (group 2). Nine recipients were given infusions of 5.5 ± 1.5 (1 SD) X 10^8 platelets separated from 60 ml donor blood on each of three occasions, 24, 17, and 10 days before transplantation. Platelets were separated as previously described. The total number of white blood cells "contaminating" the platelet preparations per dog ranged from 0 to 4.5 (median 0.2) X 10^3; approximately half of these were lymphocytes.

**TBI and hemopoietic transplantation.** Recipients were conditioned for transplantation by exposure to 1200 R TBI. Hemopoietic grafts and posttransplantation supportive care were carried out as previously described. Prompt and sustained rises in peripheral white blood cell and platelet counts after the postirradiation decline and marrow histology were used as criteria for marrow engraftment. Additional evidence for engraftment were the development of graft-versus-host disease and the demonstration of donor sex chromosomes in cells from marrow and peripheral blood. Graft rejection was established in those dogs with a severely hypocellular marrow either without recovery of peripheral blood counts after the postirradiation nadir or, after an initial recovery, a subsequent decline of white blood cell counts to <300/mm^3 and platelet count to <10,000/mm^3.

**Bone marrow and lymphocyte preparation.** Bone marrow cells (BMC) were obtained for culture prior to transfusion and again on the day of irradiation. Cells were aspirated from the humoral head of the donor into a 10-ml syringe containing 4 ml ^99m^Tc tissue culture medium (Microbiological Associates, Bethesda, Md. and 200 units of preservative-free heparin. Buffy-coat cells were separated, washed three times, and suspended in supplemented alpha medium (Flow Laboratories, Rockville, Md.). Lymphocytes from the recipient were obtained by layering 10 ml heparinized blood over Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo, Norway). Mononuclear cells were washed three times and then suspended in supplemented alpha medium. All cells were counted on a hemocytometer using trypan blue exclusion to measure viability.

**EC assay.** In all experiments 2 X 10^4 donor BMC were cultured alone and in the presence of 5 X 10^4 recipient lymphocytes in 0.1-ml plasma clots using the technique described by Stephenson et al. as modified in this laboratory for dog cells. All cultures contained erythropoietin (step III sheep plasma, Connaught Laboratories, Willowdale, Ontario) at a final concentration of 1.0 unit/ml. The clots were harvested after 72 hr, fixed with 5% glutaraldehyde on glass slides, and stained with benzidine and aggregates of eight or more hemoglobinized cells were counted as EC. There were at least six replicates of each culture.

**Data analysis.** The results were expressed as ratios derived by dividing the mean number of EC grown in the presence of lymphocytes by the mean number obtained in control cultures without lymphocytes. Ratios greater than unity indicated that lymphocytes increased the number of EC, while ratios less than unity indicated a reduced number of EC. The distribution of ratios >1 versus those ≤1 between dogs with successful engraftment or dogs with graft rejection was analyzed by Fisher's exact probability test. The mean ratios of EC obtained with lymphocytes were compared to control ratios by the standard t test.

**RESULTS**

The mean number of EC obtained from 2 X 10^4 BMC in control cultures without lymphocytes was 65 ± 14.17. The effects of lymphocytes from the marrow recipient on the growth of EC from the DLA-identical marrow donor are expressed as ratios in Table 1. All lymphocyte populations tested before transfusion-induced
Table 1. Effect of Recipient Lymphocytes Tested Before and After Transfusion on the Growth of Donor EC. Correlation With the Results of Subsequent Marrow Grafts

<table>
<thead>
<tr>
<th></th>
<th>Marrow Graft Result</th>
<th>Ratio*</th>
<th>Marrow Recipient No.</th>
<th>Experiment</th>
<th>Group 1: three preceding transfusions of whole blood</th>
<th>Group 2: one preceding transfusion of whole blood</th>
<th>Group 3: three preceding transfusions of platelets</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before Transfusion</td>
<td></td>
<td>B299 3.44t 1.00 Rejection</td>
<td>B321 1.39t 0.62t Rejection</td>
<td>B450 ND 0.73t Rejection</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>After Transfusion</td>
<td></td>
<td>B336 9.00t 0.55t Rejection</td>
<td>B388 1.07 0.76t Rejection</td>
<td>B414 1.60t 0.79t Rejection</td>
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<td></td>
<td>B364 1.91t 0.79 Rejection</td>
<td>B394 1.46t 0.81t Rejection</td>
<td>B423 ND 0.83t Rejection</td>
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<td></td>
<td>B305 3.82t 0.88 Rejection</td>
<td>B361 3.57t 1.00 Rejection</td>
<td>B415 1.20 0.88 Engraftment</td>
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<td>B365 1.02 0.89 Rejection</td>
<td>B322 1.51t 1.57t Engraftment</td>
<td>B429 1.42t 1.24t Engraftment</td>
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<td></td>
<td></td>
<td>B398 2.03t 1.00 Rejection</td>
<td>B382 1.27t 1.99t Engraftment</td>
<td>B413 ND 1.46t Engraftment</td>
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<td></td>
<td>B449 1.57t 3.29t Rejection</td>
<td>B488 1.10 3.67t Engraftment</td>
</tr>
</tbody>
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* Ratio = Mean no. of EC grown with littermate lymphocytes divided by the mean no. of EC grown without lymphocytes.

† Mean number of EC grown with littermate lymphocytes significantly different (p < 0.05) from the mean number grown without lymphocytes.

sensitization stimulated EC growth. In 13 of 17 experiments the degree of stimulation by normal lymphocytes was significant (Table 1). In contrast, lymphocytes obtained after the dogs were transfused failed to stimulate in 14 of 20 cases and in 11 instances decreased EC numbers. The variation in the degree of stimulation/inhibition was probably due to the variation in the lymphocyte–target cell ratio between experiments. In our previous study normal lymphocytes gave stimulation ratios >1 in 50 of 52 experiments. Consistency of the stimulation was subsequently shown in 5 dogs tested on three separate occasions. In all instances stimulation ratios were >1.0. For these reasons ratios >1 are considered normal and those ≤1 are grouped together as abnormal.

Of the 20 recipients, 14 rejected their marrow graft. Rejection occurred in all 6

Table 2. Distribution of EC Ratios > 1 Versus Those ≤1 in Relation to the Outcome of Marrow Transplantation

<table>
<thead>
<tr>
<th>Result of Marrow Graft (No. of Dogs)</th>
<th>&gt;1</th>
<th>≤1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rejection (14)</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Engraftment (6)</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>
recipients given three blood transfusions, in 4 of 5 given one blood transfusion, and in 4 of 9 given three platelet transfusions.

The distribution of ratios $>1$ versus those $\leq 1$ in relation to engraftment or graft rejection is shown in Table 2. Of the 14 dogs with graft rejection, 13 had ratios $\leq 1$. Of the 6 dogs with sustained engraftment, 5 had ratios $>1$. The correlation of results in vitro and in vivo was significant at $p = 0.002$.

**DISCUSSION**

We previously showed that normal dog lymphocytes cultured with littermate marrow increased EC numbers. In contrast, lymphocytes from dogs presumably sensitized by blood transfusions to “minor” histocompatibility antigens of a DLA-identical littermate lost their ability to stimulate EC growth from that littermate. In most cases the sensitized lymphocytes actually decreased colony growth, suggesting that sensitization in vivo could be detected in vitro by coculturing donor marrow with sensitized lymphocytes. Since marrow graft rejection in dogs has been shown to occur if the recipient has been previously exposed to minor histocompatibility antigens, we sought to determine if the inhibition in vitro of donor marrow growth would predict marrow graft rejection in vivo. The results clearly indicate that transfusion-induced sensitization and subsequent rejection of a DLA-identical marrow graft are predicted by reduced EC growth of donor marrow when it is cultured with recipient lymphocytes.

These results are of potential importance for the multiply transfused human patient with severe aplastic anemia who is a candidate for marrow transplantation from an HLA-identical family member. Marrow graft rejection occurs in 20%–30% of patients and is presumably related to transfusion-induced sensitization. The availability of tests documenting sensitization and predicting rejection is important, since sensitized patients can then be treated with different immunosuppressive conditioning regimens aimed at preventing rejection. It has been suggested that the presence before transplantation of cytotoxic antibodies against random panel lymphocytes predicts graft rejection in patients; however, this could not be confirmed in a large series of patients or in dogs.

Observations in patients with aplastic anemia treated by HLA-identical sibling marrow grafts suggest that tests of cell-mediated immunity might be more useful. The results of two tests in vitro are predictive of marrow graft rejection, one a positive relative response index in mixed leukocyte culture and the other a positive lymphocyte-mediated $^{51}$Cr release assay. This significant association between test results in vitro and rejection of the graft was sustained in an analysis simultaneously considering 23 additional possible predictive factors using a binary-logic regression model. It is also clear, however, that the two tests in vitro currently in use fail to predict the fate of the subsequent graft in all instances. In particular, “false-negative” tests in vitro are obtained in approximately 15% of patients. Hence additional and perhaps more accurate tests in vitro are needed. One of these may be the interaction between recipient lymphocytes and donor CFU-C. The current study indicates that the interaction between recipient lymphocytes and EC from donor marrow is predictive of marrow graft rejection or engraftment with a high
degree of accuracy. Whether or not this test will be applicable to human patients with aplastic anemia remains to be determined.

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REFERENCES

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