Rejection of Marrow From DLA-Identical Canine Littermates Given Transfusions Before Grafting: Antigens Involved are Expressed on Leukocytes and Skin Epithelial Cells but not on Platelets and Red Blood Cells

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Previous studies in dogs given 1200 R and a hemopoietic graft from DLA-identical littermates have shown that marrow graft rejection generally does not occur when the recipient is untransfused (58 of 59 achieved sustained engraftment) but is seen after a single transfusion of blood from the marrow donor on day -10 (13 of 18 rejected) and even after multiple transfusions of random blood (3 of 11 rejected). This study extends those initial observations by determining the incidence of rejection of DLA-identical littermate marrow grafts following administration of different cells from the marrow donor on days -24, -17, and -10 prior to transplantation.

Using this protocol, the following results and conclusions were established. (1) All 19 dogs given transfusions of whole blood rejected their grafts. This 100% incidence of rejection after three transfusions indicates that more than one minor histocompatibility system outside of DLA is involved in transfusion-induced sensitization and subsequent marrow graft rejection. (2) Six dogs given subcutaneous injections of cultured skin epithelial cells rejected their subsequent marrow grafts. Hence, antigens mediating rejection are not restricted to hemopoietic cells but are also expressed on at least one other tissue. (3) Seven of 15 dogs given transfusions of platelet concentrates ("free" of white and red blood cells) rejected the marrow graft, while 8 showed sustained engraftment. (4) Five of 14 dogs given transfusions of red blood cells (free of white blood cells and platelets) rejected the marrow graft, while 9 had sustained engraftment. This suggests that only some (and perhaps none) of the non-DLA antigens responsible for rejection reside on platelets and red blood cells. To definitively answer this question, the current blood cell separation techniques must be improved further to provide pure red blood cells and platelets for transfusion studies. Assays of lymphocytotoxic antibodies, mixed leukocyte culture reactivity, and survival of donor platelets in the recipient did not predict the fate of the subsequent marrow graft. These findings in DLA-identical canine marrow graft recipients are of potential practical importance for planning platelet and red blood cell support of human marrow graft candidates.

Graft rejection is a major cause of failure in patients with severe aplastic anemia treated by marrow transplantation from HLA-identical siblings. Previous studies in DLA-identical canine littermates given hemopoietic grafts after
1200 R have led us to suggest that graft rejection is most likely the result of transfusion-induced sensitization of the marrow recipient against minor histocompatibility antigens of the donor.9,10 Rejection rarely occurred in untransfused dogs but was seen after a single transfusion of whole blood from the marrow donor on day –10 and after multiple transfusions from unrelated dogs. The present study extends these previous observations in DLA-identical littermates and provides evidence that (1) more than one minor histocompatibility antigen system outside of DLA is involved in sensitization and marrow graft rejection, (2) the antigens involved are not only located on hemopoietic cells but also on skin epithelial cells, and (3) only some (perhaps none) of the non-DLA antigens responsible for rejection reside on platelets and red blood cells (RBC).

MATERIALS AND METHODS

Canine litters were obtained from kennels in the states of Washington, Virginia, and Oregon. The dogs, weighing 7.5–27.4 kg and 6–12 mo of age, were observed for disease for 2 mo before grafting. They were typed for dog erythrocyte antigens, dewormed, and immunized against distemper, leptospirosis, and hepatitis. The pairs used in this study were beagles, German shorthairs, black and tan hounds, Basenji/Labrador crossbreeds, Dalmatians, labradors, and mongrels.

Pairs were chosen on the basis of matching for the serologically detectable canine histocompatibility antigens DLA-A and B using a microlymphocytotoxicity test.11,12 Pairs were mutually nonreactive in mixed leukocyte culture (MLC), carried out as previously described.13 Recipients were prepared for grafting by exposure to 1200 R (midline air exposure) of total-body irradiation (TBI) given at a rate of 9.3 R/min from 2 opposing 60Co sources. The midplane tissue exposure was calculated to be approximately 850–1000 rad.14 All dogs studied were given marrow, 2.8 ± 2.1 (SD) x 10⁶ cells/kg body weight infused intravenously within 4 hr of irradiation. In addition, all recipients except those in groups 1a and 3a were given peripheral blood leukocytes from the marrow donor, 18.2 ± 7.0 (SD) x 10⁶ cells/kg infused intravenously on days 1 and 2 after irradiation.15 No postgrafting immunosuppression was administered. The postgrafting care has been described.15 The day of TBI and marrow grafting is designated day 0. Days before grafting are indicated by a minus sign.

Six groups of recipients were studied. In group 1, 59 recipients were not given preceding blood transfusions. A number of these 59 transplants have been reported previously.16,17 In group 2, 11 recipients were given a single intravenous transfusion of 50 ml of heparinized whole blood from the intended marrow donor on day –10. Dogs in groups 3a–c were given three transfusions of 50 ml of whole blood from the intended marrow donor on days –24, –17, and –10. Marrow donors in group 3c were exposed to the marrow recipient’s histocompatibility antigens by simultaneous transfusions of 50 ml of whole blood from the intended recipient on days –24, –17, and –10.

Dogs in group 4 were given multiple intradermal and subcutaneous injections of skin epithelial cells from the marrow donor. Numbers of epithelial cells injected ranged from 0.6 to 2.3 (median 2.0) x 10⁶ cells/kg of which 0.05–1.80 (median 1.10) x 10⁶ cells/kg were viable as determined by trypan blue exclusion. Skin epithelial cells were obtained by treatment of a 12 sq cm piece of full-thickness skin with 0.5% trypsin for 3–5 hr at 37°C, as described elsewhere.18 The epithelial layer was removed with a forceps, disrupted, and cultured overnight in a plastic dish in Dulbecco’s modified minimum essential tissue culture medium with insulin (Grand Island Biological Company, Santa Clara, Calif.).

Dogs in group 5 were given intravenous infusions of “leukocyte and red-cell-poor” platelets from the intended marrow donor on days –24, –17, and –10. A platelet concentrate was obtained from 50 ml of ACD anticoagulated whole blood by centrifuging at 270 g for 10 min at room temperature. After transfer of the platelet-rich plasma (PRP) to a transfer bag, it was acidified to pH 6.5 with 0.15 M citric acid. The PRP was reinfused at 1700 g for 15 min to form a platelet button, which was gently resuspended in 10 ml of residual platelet-poor-plasma, and platelets were then labeled with ⁵¹Cr for survival studies.19 The total number of platelets injected at the three occasions ranged from 0.7 to 3.0 (median 1.8) x 10⁹ cells/kg body weight of the recipient. White blood cell (WBC) contamination of the platelet concentrate ranged from 0 to 27 (median 6.7) x 10⁸ WBCs/kg of recipient body weight. More than 98% of these WBCs were mononuclear cells.
MINOR ANTIGENS AND MARROW GRAFT REJECTION

Dogs in group 6 were given infusions of "leukocyte- and platelet-poor" RBC from the intended marrow donor on days -24, -17, and -10. The RBC were prepared as previously described with some modifications. Fifty milliliters of heparinized blood were centrifuged at 350g for 25 min in a plastic tube. Then, the bottom of the tube was perforated with a sterile needle, and the RBC drained off into a second 50-ml plastic tube. RBC were resuspended in cell-free plasma and layered on a cotton-wool column consisting of 2g of pure cotton (USP) packed to the 10-ml mark of a 20-ml plastic syringe barrel. The RBC were filtered through by gravity. The column was then washed with 20 ml of Ringer's solution, and the filtrate washed twice with phosphate-buffered saline. The RBC so treated were then injected intravenously into the intended marrow recipients. The packed RBC volume injected at the three occasions ranged from 1.6 to 4.3 ml/kg body weight (median 3.1). WBC contamination of the RBC concentrate ranged from 0 to 10 x 10^6 cells/kg (median 0.17) and platelet contamination from 0 to 36 x 10^3/kg (median 0.9). These WBCs were also predominantly lymphocytes.

Marrow engraftment was assessed by promptly rising WBC and platelet counts following the postirradiation nadir, histologic features of the marrow at autopsy, demonstration of donor sex karyotypes in cells from marrow obtained 7 or more days after transplantation, the demonstration of presence of peripheral blood RBCs of donor isoenzyme or antigen type, and the development of graft-versus-host disease (GVHD). Clinical and histologic findings of GVHD in the dog have been described previously and are similar to those in other species. Marrow graft rejection was defined as either failure of recovery of peripheral blood cell counts following the postirradiation decline or, following initial evidence of engraftment, decline of WBC counts to near zero and platelet counts to less than 10,000/cu mm. Marrows in dogs with rejection showed extreme hypocellularity. Absence of GVHD lesions at autopsy was additional evidence for marrow graft failure.

Before and 10 days after the last transfusion (or injection of skin epithelial cells) sera of recipient dogs in these studies were tested against cells of the donors for the presence of lymphocytotoxic, leukoagglutinating and hemagglutinating antibodies using techniques previously described. Also, MLCs were repeated in 17 pairs of group 3 dogs 10 days after the last transfusion. Finally, survival of radiochromium-labeled donor platelets was determined in all recipients of group 5 before grafting, as described previously.

RESULTS

In Vivo Results

Table 1 summarizes the data. Of 21 dogs without preceding transfusion given marrow only, one rejected and 20 had sustained engraftment (group 1a). All 38 untransfused dogs given a combination of marrow and donor peripheral blood leukocytes engrafted and none rejected (group 1b). Thus, the overall rejection rate in untransfused dogs given grafts from DLA-identical littermates was less than 2%.

Of the 11 dogs in group 2 given a single whole-blood transfusion from the marrow donor on day -10, 8 rejected while 3 showed sustained engraftment.

All 19 dogs in group 3 given 3 preceding whole-blood transfusions from their intended marrow donors on days -24, -17, and -10 rejected and none showed sustained engraftment. Rejection occurred regardless of whether dogs were given marrow alone or marrow and additional leukocytes from nonsensitized or specifically sensitized (against the hosts) marrow donors.

All 6 dogs in group 4 given preceding donor skin epithelial cell injections rejected and none engrafted. However, only 7 of 15 recipients in group 5 given leukocyte-poor platelet transfusions rejected, while 8 engrafted. Similarly, only 5 of 14 dogs in group 6 given leukocyte- and platelet-poor RBC transfusions rejected, while 9 engrafted.
Table 1. Results of Marrow Grafts Between DLA-Identical Littermates After 1200 R TBI

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Donor Leukocytes in Addition to Marrow</th>
<th>Number of Dogs Studied</th>
<th>With Marrow Rejection</th>
<th>With Sustained Engraftment</th>
<th>Incidence of Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a,b</td>
<td>No preceding tx*</td>
<td>No</td>
<td>21</td>
<td>1</td>
<td>20</td>
<td>5%</td>
</tr>
<tr>
<td>2</td>
<td>One whole blood tx from marrow donor, day -10</td>
<td>Yes</td>
<td>38</td>
<td>0</td>
<td>38</td>
<td>0%</td>
</tr>
<tr>
<td>3a,b,c</td>
<td>Three whole blood tx from marrow donor, days 17, -10</td>
<td>No</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes†</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>4</td>
<td>Three injections of donor skin epithelial cells, days 17, -10</td>
<td>Yes</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>5</td>
<td>Three leukocyte-poor platelet tx from marrow donor, days 24, 17, -10</td>
<td>Yes</td>
<td>15</td>
<td>7</td>
<td>8</td>
<td>47%</td>
</tr>
<tr>
<td>6</td>
<td>Three leukocyte-poor red blood cell tx from marrow donor, days 24, 17, -10</td>
<td>Yes</td>
<td>14</td>
<td>5</td>
<td>9</td>
<td>36%</td>
</tr>
</tbody>
</table>

*tx, transfusion.
†Marrow donors were exposed to histocompatibility antigens of the recipients by whole-blood transfusions before marrow grafting (see text).
‡Statistical analyses by chi-square test. Group 1 versus groups 2, 3, 4, 5, 6: p < 0.01 each; group 3 versus groups 5 and 6: p < 0.01 each; group 5 versus group 6: p > 0.4

In Vitro Test Results

Fifty-seven marrow graft recipients that were given preceding transfusions of blood products or skin epithelial cell injections from the marrow donor were tested for lymphocytotoxic antibodies against their marrow donors on the day of transplantation. Forty-eight failed to show cytotoxic antibodies. Thirty-four of these rejected, and 14 had sustained engraftment. Four had positive lymphocytotoxic antibodies. Three of the 4 rejected, while 1 showed sustained engraftment. In 5 dogs, reactions were borderline, i.e., lymphocytotoxic antibodies were found only at 1:1 and 1:2 dilutions; all 5 dogs rejected. Nine dogs in group 5 were tested for leukoagglutinating antibodies against their marrow donor, and all 9 were negative. Four of the 9 rejected, and 5 accepted the graft. None of the 41 dogs tested showed hemagglutinating antibodies against marrow donor cells on the day of transplantation, and 30 of these rejected. Eleven dogs were not tested for hemagglutinating antibodies against the marrow donor but against a random panel of at least 5 dogs. Only 1 of the 11 was positive with the 5 panel dogs. This dog rejected the marrow graft, as did all 10 that were negative with the panel cells. Thus, there was no significant correlation between presence or absence of lymphocytotoxic, leukoagglutinating, or hemagglutinating antibodies against the marrow donor and graft rejection.

In 17 dogs of groups 2 and 3a and 3b, MLC tests were repeated between donor and recipient on the day of transplantation to see whether a change of the mutual nonresponsiveness seen before transfusion had occurred after transfusion. In all 17
instances, the MLC between donor and recipient remained mutually nonreactive. Fifteen of the 17 dogs rejected, while 2 had sustained engraftment.

In the 15 dogs of group 5, radiochromium-labeled donor platelet survivals were determined in the recipient with each of the 3 sequential transfusions. Several patterns of recipient responses to donor platelets were observed. (1) Four recipients had normal platelet survivals for each of 3 infusions (5.6 days ± 0.2 [1 SEM]); 2 of these rejected and 2 had sustained engraftment. (2) Four recipients had normal survivals for the first two transfusions and modest reduction with the third (5.6 days ± 0.3 for transfusions 1 and 2 and 3.3 days ± 0.1 for the third); 3 of these rejected and 1 had sustained engraftment. (3) Five recipients had reduced survival of donor platelets for all infusions (2.4 days ± 0.3); 3 of these rejected and 2 had sustained engraftment. (4) Two dogs had no recovery of donor platelets from the first or subsequent infusions; one of these rejected and one had sustained engraftment. The pattern of response to donor platelets did not correlate with number of platelets or contaminating WBCs in the platelet concentrates transfused nor was there any apparent correlation between donor platelet survival and the fate of the subsequent marrow transplant.

DISCUSSION

This study confirms and extends our previous observations on marrow transplants in DLA-identical canine littermates with or without preceding blood transfusions. With one exception, untransfused dogs showed sustained engraftment.

In contrast to the findings in untransfused recipients, dogs given one or more transfusions of whole blood from the marrow donor before transplantation, as a rule, rejected. This must be explained by sensitization of the recipient to non-DLA antigens contained in the transfused blood. TBI, approximately 2½ times the lethal dose, was not sufficient to suppress sensitization to the grafted marrow. The addition of donor leukocytes to the marrow inoculum, thought to provide increased numbers of hemopoietic stem cells and/or lymphocytes, failed to decrease the rejection rate in the current canine studies. Furthermore, infusion of marrow and leukocytes from donors exposed to recipient antigens by preceding blood transfusions failed to overcome rejection. We had hoped to override host immunity by infusion of donor lymphocytes specifically sensitized against host lymphocytes. Apparently, administration of three transfusions from the marrow donor immediately preceding TBI and transplantation provided for a very strong immunogenic stimulus that could not be overridden by any of the manipulations tried in this study.

The 100% incidence of rejection after 3 transfusions of whole blood from the marrow donor (19 of 19 instances) suggests that at least 2 polymorphic histocompatibility systems outside of DLA are involved in sensitization. (The probability of encountering 19/19 rejections if only 1 polymorphic locus were involved is <0.008; the likelihood for more than 2 loci is <0.29, by the binomial significance test.) Therefore, attempts at unraveling the genetics of these antigen systems by in vitro tests of immunity will be difficult to interpret. Only 75% of dogs rejected after a single whole-blood transfusion from the donor, while 25% had sustained engraft-
ment. This could be compatible with the presence of one important "minor" histocompatibility system requiring less intense immunization. The conclusion that polymorphic "minor" antigen systems are involved is consistent with our previous findings that transfusions from unrelated donors can immunize a recipient against a subsequent marrow graft from a DLA-identical littermate. In that situation, rejection of the marrow might be expected only when one or more of the blood transfusion donors and the marrow donor share "minor" antigens not present in the recipient. The observations in the dog are in keeping with the findings in patients with aplastic anemia treated by marrow grafts from HLA-identical siblings. Rejection rates ranging from 30% to 60% have been reported in multiply transfused patients, while untransfused patients have generally shown sustained engraftment.

The observation that all dogs given subcutaneous and intradermal injections of skin epithelial cells from the marrow donor rejected a subsequent DLA-identical marrow graft suggests that the expression of antigens mediating rejection is not restricted to hemopoietic cells but extends to at least one other tissue. Skin epithelial cells cultured overnight in plastic dishes were chosen for this study because they would not be expected to contain nonepidermal cells in numbers sufficient to obscure the interpretation of results. Although contamination by leukocytes cannot be completely ruled out by morphological examination, canine skin epithelial cells so treated failed to stimulate lymphocytes from unrelated dogs in vitro in mixed culture (K. Atkinson, unpublished observations). On the other hand, skin cells completely free of hemopoietic cells are capable of sensitizing lymphocytes in vivo to produce GVHD against non-DLA antigens.

The finding that more than half of the recipients given transfusions of leukocyte-poor platelets or RBCs from the marrow donor showed sustained engraftment was surprising. Earlier studies in DLA-nonidentical unrelated recipients had failed to show a clear-cut reduction in the rejection rate with leukocyte-poor RBCs. Mononuclear cell contamination in the earlier studies was on the order of $5 \times 10^6$/dog. The current data could mean that platelets and RBCs exhibit only some but not all of the non-DLA antigens involved in sensitization and rejection, or that they express none of the antigens, and sensitization and rejection were due to mononuclear cell contamination that was on the order of $1.5 \times 10^5$ cells/dog. The latter explanation is supported by preliminary (unpublished) observations in the same canine model showing that rejection can be seen after injections of $1.5 \times 10^5$ mononuclear peripheral blood cells from the marrow donor. To definitively answer this question, the current blood cell separation techniques must be improved. It would be important to extend these studies to transfusions of donor granulocytes free of lymphocytes. These findings in DLA-identical canine marrow graft recipients are of potential practical importance for the platelet and RBC support of human marrow graft candidates. The incidence of rejection after marrow transplantation for aplastic anemia could perhaps be reduced if leukocyte-poor platelets and RBC were used exclusively during the phase of conventional management before transplantation.

We have reported earlier that tests of humoral immunity as well as studies of donor platelet survival did not predict the outcome of marrow grafts in previously transfused dogs. The present results agree with that conclusion. With rare
exceptions, recipients failed to show lymphocytotoxic, leukoagglutinating, or hemagglutinating antibodies against the DLA-identical marrow donor’s cells, and the results of these tests did not correlate with the fate of the marrow grafts. A similar lack of correlation was seen with marrow graft outcome in relation to pregraft survival of marrow donor platelets. The finding of shortened survival of DLA-identical platelets after only three transfusions was somewhat surprising, although similar findings have now been reported in man.26,27 We have previously presented evidence in patients with aplastic anemia given marrow grafts from HLA-identical siblings that a positive relative response index (RRI) in MLC was highly predictive for marrow graft rejection.28 None of the dogs in this study showed a positive RRI, regardless of whether or not the graft was subsequently rejected. This failure to demonstrate a positive RRI is unexplained but may be related to the technique used or to species differences.

Among the techniques used, only the results of lymphocyte marrow cocultures, reported elsewhere,29 proved to be predictive of the fate of a subsequent marrow graft. Twenty pairs of transfused DLA-identical littermates in the present study were examined. Fourteen of the 20 rejected the marrow graft. In 13 of the 14 rejection was predicted by the in vitro observation that recipient lymphocytes either failed to stimulate or inhibited erythroid colony formation by donor marrow. Successful and sustained marrow engraftment occurred in 6 dogs. Five of these were associated with significant stimulation of donor marrow in coculture. This correlation was highly significant and suggests that transfusion-induced sensitization and marrow graft rejection can be predicted by reduced erythroid colony growth of donor marrow cocultured with recipient lymphocytes.

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Rejection of marrow from DLA-identical canine littermates given transfusions before grafting: antigens involved are expressed on leukocytes and skin epithelial cells but not on platelets and red blood cells

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