Characterization of Host Cells Involved in Resistance to Marrow Grafts in Dogs Transplanted From Unrelated DLA-Nonidentical Donors

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A canine model of marrow transplantation was used to further define the host cells mediating resistance to marrow engraftment. Recipient dogs were given 9.2 Gy of total body irradiation followed by marrow infusion from unrelated DLA-nonidentical donors. No postgrafting immunosuppression was given. At three and ten days posttransplantation recipient marrow and peripheral blood cells were obtained and characterized by the following in vitro studies: (a) morphologic analysis; (b) phenotypic analysis with monoclonal antibodies; (c) assays for natural killer cell (NK) activity; and (d) cocultures with donor marrow to study the effect on donor CFU-GM growth. Daily differential cell counts revealed a proliferation of peripheral blood mononuclear cells approximately eight days posttransplant. By day 10 surviving host cells were uniformly large granular lymphocytes which were phenotypically of T cell lineage, had NK activity, and were capable of suppressing donor marrow CFU-GM growth. Mononuclear cells from dogs given total body irradiation only and no marrow infusion (radiation control group), did not suppress CFU-GM growth when cocultured with marrow from unrelated DLA-mismatched dogs. These results suggest that radiotolerant host cells with the morphology of large granular lymphocytes and NK activity and which proliferate in response to the infused donor marrow cells mediate resistance to DLA-nonidentical marrow grafts. It remains to be determined, however, whether in vivo functional studies reflect the mechanisms involved in vivo.

Selection of Donor-Recipient Pairs

Selection of unrelated, DLA-nonidentical pairs was based upon mutual stimulation of their peripheral blood mononuclear cells in mixed leukocyte culture assay (MLC) and nonidentity of serologically determined DLA antigens. In all cases donor and recipient differed phenotypically for both DLA-D alleles as defined by homozygous cell typing. Sixteen donor-recipient pairs were entered into the study. Ten of sixteen donor-recipient pairs were of different sex.

Total Body Irradiation and Marrow Transplantation

Recipients were prepared for grafting by 9.2 Gy of total body irradiation (TBI) delivered at 7 cGy/minute from two opposing 60Co sources. Recipients were infused with 1.3 to 6.7 x 106 (median 3.9 x 106, mean 3.98 ± 1.5) marrow cells/kg intravenously within four hours of TBI (day 0). No postgrafting immunosuppression was given and no donor buffy coat cells were infused. Postgrafting care and the marrow aspiration procedure have been described elsewhere. Complete blood counts (CBC) were obtained on day 0, before irradiation, and then daily from day 3 postgrafting until death. Cytogenetic analysis of recipient marrow and peripheral blood cells was performed three and ten days after transplantation as described with the only modifications being the use of concanavalin A (Con A, 200 mg/mL Calbiochem-Behring, LaJolla, Calif) to induce mitogenesis. Cultures were harvested on day 3 after exposure to 0.3 μg colchicine for 1½ hours.

Complete autopsies, including marrow specimens for evaluation of cellularity, were performed on ten of 16 recipients.

MATERIALS AND METHODS

Dogs

Beagles and various crossbred hounds were obtained from commercial kennels in Washington and Virginia and through a breeding program at the Fred Hutchinson Cancer Research Center. The dogs weighed 6.0 to 35.5 kg and were 6 to 16 months old (median 12 months) at the time of transplantation. They were dewormed, vaccinated against distemper, leptospirosis, hepatitis, and parvovirus, and housed in single cages.
Irradiation Controls

Two dogs received TBI without subsequent marrow infusion. The complete blood counts obtained on these dogs and on six irradiation control dogs from a previous study were combined to generate the hematologic data for irradiation controls.

Posttransplant In Vitro studies

In vitro studies were generally performed on days 3 and 10 after transplant.

Recipient marrow and peripheral blood cells. Donor bone marrow was aspirated from the humerus head into a 10-mL syringe containing 5 mL of Waymouth medium (Waymouth-MB752/1, Fred Hutchinson Cancer Research Center media room) and 400 U of preservative-free heparin (Heparin, Sodium, 1,000 USP units/mL, O'Neal, Jones and Feldman, St Louis). Mononuclear marrow cells (MC) were isolated by layering the diluted marrow onto a Ficoll-Hypaque density gradient (1.074 specific gravity), and centrifuging at 1,000 g for 25 minutes. Interface cells were washed once with hemolytic buffer, three times with Waymouth medium, and then resuspended in Waymouth medium for cell counting. All washes were at 200 g for ten minutes. Donor peripheral blood mononuclear cells (PBMC) were isolated from venous blood as previously described.

Recipient marrow cells were obtained using a vacuum pump aspiration procedure that permitted the procurement of 50 to 80 mL of marrow mixed with an equal volume of heparinized TC199 medium (GIBCO, Grand Island, NY). This diluted marrow was centrifuged at 300 g for 15 minutes to remove the red cells. The resulting cell pellet was washed three times and resuspended in Waymouth medium for cell counts. Viability was determined using trypan blue exclusion. Recipient peripheral blood (PB) cells were obtained by mixing two parts of heparinized venous blood (from 300 to 600 mL) with one part of Plasmagel (Cellular Products, Buffalo). This mixture was incubated for 45 minutes at 37 °C, 7% CO2 in air. The result of supernatants were centrifuged at 500 g for 15 minutes; residual red cells were lysed with a hemolytic buffer wash. The cell pellet was washed three times and resuspended in Waymouth medium for cell counting. Obtaining sufficient cell numbers from the recipients for the in vitro studies was a consistent problem necessitating the procurement of large volumes of marrow and peripheral blood.

Morphologic analysis of cells. Cytospin preparations (Cytospin, Shandon-Elliot, Sewickley, Penn) were made of cells obtained on days 3 and 10. These preparations were stained with a standard Wright-Giemsa fixative, postfixed in 0504 in S. collidine buffer and Wright-Giemsa stain and evaluated for percentage of LGL.

For ultrastructural analysis, cells were fixed in a half-strength Karnofsky's fixative, postfixed in 0504 in S. collidine buffer and centrifuged at 39 °C prior to use. The marrow culture medium was equilibrated at 36 °C prior to use. 2 x 10^6 donor marrow cells were added to 2.0 mL of marrow culture medium, then either no other cells, donor PBMC, recipient marrow, or peripheral blood cells were added at ratios of 1:1, 1:4, and 1:6, respectively. One mL of these cell mixtures was then added to 35-mm plastic Petri dishes (Falcon #1008, Oxnard, Calif) in duplicate. After the agar solidified at room temperature, the cultures were incubated for ten days in 100% humidified atmosphere at 37 °C under 7% CO2 in air. Aggregates of more than 50 cells were counted as colonies using a dissection microscope (Olympus) at a magnification of 25X. As a control, marrow cocultures were also done in 5 donor-recipient pairs prior to TBI and marrow transplantation. Results of CFU-GM growth in the various cocultures were compared statistically by nonparametric tests for the comparison of two unpaired samples.

NK assays. NK activity of recipient marrow and peripheral blood cells was determined and compared to that of donor marrow and peripheral blood mononuclear cells. A 51Cr (New England Nuclear, NaCrO4, 300 μCi/mL)-labelled canine thyroid adenocarcinoma cell line (CTAC) was used as the target in an 18-hour NK assay using effector-to-target ratios of 20:1 and 50:1, with the results reported as percent specific target cell lysis as previously described.

RESULTS

Peripheral Blood Cell Counts Post-TBI and Marrow Transplantation

Of the sixteen dogs transplanted, two had sustained engraftment and no further studies were done with these two dogs. The other fourteen dogs (88%) failed to engraft and died with aplasia. Figure 1 shows the peripheral white blood cell counts (WBC), including mean absolute granulocyte and mononuclear cell counts, available from eleven recipients. There was a rapid fall in WBC with the mean absolute granulocyte number falling from 8,200 cells/μL pretransplant to 3,000 cells/μL at day 5, to 210 cells/μL at day 7.
Peripheral white blood cell counts from the eight irradiation control dogs are shown for comparison in Fig 2. Similar to the transplanted dogs, there was a rapid fall in the WBC with the mean absolute granulocyte count falling to zero by day 7 post-TBI, with no subsequent rise in counts observed. Of note, the increase in mean absolute mononuclear cell numbers observed from day 3 to day 8 in the transplanted dogs was not seen in the radiation controls. In both transplanted and radiation control dogs, there was a constant decline in mean platelet numbers to less than 1,000/μL at day 10 post irradiation.

Table 1 summarizes the autopsy findings available for ten of the fourteen marrow recipients evaluated in this study. The degree of marrow cellularity in these dogs was expressed as a fraction of normal and ranged from 0% to 10%. In those recipients in whom hemopoietic cell lineage could be evaluated, only focal areas of myeloid, erythroid, or plasma cells were observed.

Cytogenetic Analysis of Recipient Marrow and Peripheral Blood Cells Posttransplant

Cytogenetic analysis on marrow and peripheral blood cells was available in two dogs which failed to achieve sustained engraftment. In both cases only host type cells (19/19 and 20/20) were observed.
3/3 metaphases, respectively, from Con A-stimulated PBC) were present at day 10 post transplantation. Cytogenetic analysis was hindered by the poor proliferative ability of recipient cells in response to mitogen stimulation. It is possible that the metaphases seen were spontaneous and that the surviving host cells did not possess the capacity to undergo mitogenesis with Con A stimulation.

**Morphology of Recipient Peripheral Blood Cells at Days 3 and 10 Posttransplant**

Table 2 summarizes the differential cell counts of recipients at three and ten days posttransplantation. LGLs were the predominant lymphoid cells observed on days 3 and 10, 88 ± 9% and 82 ± 13% respectively. Of the total cells on day 3, only 30 ± 27% were LGL, while on day 10, 80 ± 16% were LGL.

Figures 3 and 4 are light and ultrastructural photomicrographs of representative population of LGLs seen in recipient peripheral blood cells at day 10 posttransplant. In Fig 3 the morphological characteristics of LGLs (eg, an eccentric nucleus, relatively large cytoplasmic/nuclear ratio and cytoplasmic granules) are evident. Figure 4 reveals ultrastructural features of canine LGL, including electron dense granules as previously described.28

**Cytofluorometric Analysis of Recipient Cells**

Figure 5 shows representative dot plot profiles of recipient marrow and peripheral blood cells three and ten days posttransplant and of those from the radiation control group at day 10 post-TBI. The population of marrow and peripheral blood cells that showed significant shifts in fluorescence in response to labeling with monoclonal antibodies also showed a forward light scatter characteristic of canine lymphoid cells.13 On day 3 posttransplant both forward light scatter and the degree of autofluorescence were consistent with the presence of large numbers of granulocytes. This type of profile was also seen with marrow and peripheral blood cells from the radiation control dogs at day 3 post-TBI (dot plots not shown). By day 10, in both recipients and radiation control dogs the cell populations were essentially devoid of granulocytes and highly enriched for lymphoid cells. These findings are consistent with the differential white blood cell counts illustrated in Figs 1 and 2.

On days 3 and 10 posttransplant, recipient marrow and peripheral blood cells were 7.2*, DT2*, 1A1*, Thy 1*, and 1S1g*. Recipient peripheral blood cells were D1y6* and E11* on day 3, but by day 10 posttransplant were D1y6*, and approximately 28% of the peripheral blood cells were E11*. In contrast, the day 3 and 10 marrow cells were D1y6*, with a conversion from E11* on day 3 to 34% of the marrow cells E11* by day 10 posttransplant. The percentage of marrow and peripheral blood cells that were found to be positive in their reactivity with E11 was calculated by integrating that portion of the fluorescence peaks obtained from histograms that exceeded the fluorescence obtained using the irrelevant control McAb (9.6). The dot plot patterns for Thy 1* cells (not shown) from recipient marrow and peripheral blood were comparable to 1A1*. The phenotypes of the radiation

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**Table 2. Differential Cell Counts of Recipient Peripheral Blood Cells Obtained at Days 3 and 10 After Marrow Transplant**

<table>
<thead>
<tr>
<th>Days post-BMT</th>
<th>Differential (%)</th>
<th>LGL as % of Total</th>
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<tbody>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>Granulocytes</td>
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<tr>
<td>3</td>
<td>34 ± 32</td>
<td>65 ± 32</td>
</tr>
<tr>
<td>10</td>
<td>97 ± 4</td>
<td>0 ± 0</td>
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*BMT, bone marrow transplant; Day 3 and day 10. Five dogs evaluated.

†Differential on Wright-Giemsa-stained cytopsin cell preparations obtained from recipient PBC. Values represent mean ± SEM (%) obtained from eight recipients.

‡LGL, large granular lymphocyte.
Fig 5. Representative dot plot profiles of recipient marrow (MC) and peripheral blood (PBC) cells on days 3 and 10 post TBI and transplant (BMT), and from the radiation control group on day 10 post TBI. The horizontal axis provides a relative measure of cell size as determined by forward light scatter (smaller cells on the left, larger cells on the right). The vertical axis provides a relative measure of fluorescence intensity. All samples contained approximately 10^6 cells, prepared for cytofluorometric analysis as described under "Materials and Methods."

control dogs' marrow and peripheral blood cells at day 10 post-TBI were 7.2*, DT2*, IA1*, D1y6*, E11* and S1g*.

These results suggested that recipient marrow and peripheral blood cells were of lymphoid lineage with the appearance, by day 10, of a subpopulation with the surface antigen phenotype (E11*) previously found to be characteristic for canine suppressor/cytotoxic cells, and also expressed on canine NK cells.

Effects of Recipient Marrow and Peripheral Blood Cells on CFU-GM Growth From Donor Marrow In Vitro

The effect on donor marrow CFU-GM growth by coculturing with donor peripheral blood mononuclear cells, recipient peripheral blood, or recipient marrow cells is illustrated in Fig 6. In all experiments the addition of recipient marrow or peripheral blood cells to donor marrow cells resulted in a decrease in the number of CFU-GM colonies as compared to cultures of donor marrow, either alone or cocultured with donor peripheral blood mononuclear cells. On day 3 post-transplant, the percent inhibition (62% to 67%) of donor marrow CFU-GM growth when cocultured with recipient peripheral blood cells was greater than that obtained from cocultures of recipient marrow cells (49% to 62% inhibition). On day 10, inhibition of donor CFU-GM growth was more profound than on day 3, with cocultures of recipient marrow cells being as potent (70% to 80% inhibition) as peripheral blood cells (62% to 84% inhibition). This increased inhibition coincided with a significant enrichment for large granular lymphocytes by day 10. Cocultures of donor marrow cells with recipient peripheral blood cells obtained before TBI and transplantation did not yield any significant suppression of donor CFU-GM growth.

Similar CFU-GM cultures were carried out using marrow and peripheral blood cells from two dogs in the radiation control group. Cells were obtained on days 3 and 10 after TBI and were cocultured with normal marrow cells from an unrelated, DLA-nonidentical dog. No suppression of CFU-GM growth was observed (data not shown).

NK Activity of Recipient Marrow and Peripheral Blood Cells

Results of NK assays are summarized in Fig 7. Recipient marrow and peripheral blood cells obtained on day 3 post-transplant did not show any detectable NK activity. On day
10 posttransplant NK activity was observed in recipient marrow and peripheral blood cells at the 50:1 effector-to-target ratio (16 ± 5% and 20 ± 8% 51Cr release, respectively).

Marrow and peripheral blood cells obtained from radiation control dogs ten days after TBI also showed NK activity at the 50:1 effector-to-target ratio (59 ± 23% and 21 ± 0% 51Cr release, respectively).

DISCUSSION

Previous studies have shown failure of engraftment in 90% of dogs receiving 9.2 Gy of TBI followed by the infusion of DLA-nonidentical donor marrow. The current results (88% failure of engraftment) are in keeping with those data. These and additional studies suggests that in nonsensitized transplant recipients, a relatively radiation-resistant host cell population not requiring pretransplant exposure to donor antigen is involved in mediating resistance to engraftment of DLA-nonidentical marrow. The present study was designed to further characterize these cells.

Host lymphoid cells surviving supralethal doses of TBI were predominantly LGLs capable of suppressing in vitro donor bone marrow cell proliferation as measured by inhibition of CFU-GM growth. It is tempting to speculate that these host cells were responsible for the destruction of the marrow graft in vivo, similar to the mechanisms suggested for allogeneic or hybrid resistance in mice. These host cells expressed NK activity when tested in vitro; they had the morphologic appearance previously described for canine NK cells and, except for some minor differences, the phenotype of these LGLs on day 10 posttransplant, Dly 6, 1AI  and E11 (28% to 34%), was in agreement with that previously described for canine NK cells. Staining with additional monoclonal antibodies recognizing T cell determinants (DT2, Thy 1) suggested a T cell lineage.

Of interest was the shift from an E11 to an E11* subpopulation in both recipient marrow and peripheral blood cells (34% and 28% positive cells, respectively) between days 3 and 10 posttransplant. Whether this transition indicates a maturation of host LGLs to functional NK cells is conjectural but such a development would be in agreement with the
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increased NK activity observed on day 10 postransplant. Additional, circumstantial evidence for the NK nature of these cells comes from the lack of Con A-induced mitogenesis observed during cytogenetic analysis of these cells. It has been shown that HNK-1^+ human peripheral blood lymphocytes (NK cells) could not be induced to proliferate when stimulated with various mitogens.39 Alternatively, the presence of a subpopulation of E11^+ lymphoid cells may indicate the differentiation of NK cells into cytotoxic T lymphocytes. Such a mechanism for the activation of NK-derived cytotoxic T lymphocytes regulated by macrophage and prostaglandin has been described in the mouse.34 Perhaps intact macrophages are involved in the development of NK-derived cytotoxic lymphocytes in the present model in agreement with observations by other investigators of the beneficial effect of macrophage inhibitors on engraftment in recipients of unrelated, DLA-nonidentical marrow.35 The increase of recipient mononuclear cells observed in the present study approximately eight days postransplant may indicate such an activation with in vivo proliferation and priming of hosts cells against donor marrow. It is conceivable that a non-primed (NK type) cell population is operative initially and interferes with engraftment.30 This may allow for another radiation-resistant host T cell population to be primed and amplified, resulting finally in graft destruction. The transition from E11^+ to E11^+ host lymphoid cells may signal such an event in the present dog model.

Evidence against the preceding postulated mechanisms is provided by the results of two previous studies which indicated that host cells not of T lymphocyte lineage mediated marrow graft resistance in unrelated DLA-nonidentical transplants.18 In one study, the treatment of recipients with antithymocyte serum alone or in combination with procarbazine before TBI and marrow infusion did not abrogate graft resistance in six of seven recipients.1 In the second study recipients received cyclosporine after TBI and marrow infusion with eight of 13 recipients failing to show sustained engraftment.38 These results argue against the involvement of T lymphocytes in marrow graft resistance, but they do not conclusively rule out NK cells or the postulated mechanisms of macrophage-regulated NK-derived cytotoxic T lymphocytes as the effector cells mediating graft resistance. The cells involved in resistance are apparently metabolically active, since treatment of recipients after TBI and marrow infusion with methotrexate abrogates resistance.6,7

Recently, resistance to marrow grafts in the mouse was shown to be the result of antibody-dependent cell-mediated cytotoxicity (ADCC), in which NK cells were operating in responder mice in conjunction with a target-specific antibody, resulting in marrow graft destruction.39 If a similar mechanism is operational in the canine model, in vitro tests of host LGLs carried out early postransplant might fail to detect significant NK/ADCC-like activity if the appropriate antibody is not present in the culture system. So far no such antibodies have been described in dogs.

The present findings provide additional background data for the design of conditioning regimens capable of overcoming resistance. Preliminary data in the canine model suggest that treatment of recipient dogs with the anti-Ia monoclonal antibody 7.2 in addition to 9.2 Gy of TBI allows for sustained engraftment in a number of dogs.40 Conceivably, additional more specific monoclonal antibodies (eg 1A1 and E11) or a mixture of antibodies could be even more effective. Whether this information can be applied in clinical marrow transplantation remains to be determined.

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