Engraftment of Dogs With Ia-Positive Marrow Cells Isolated by Avidin-Biotin Immunoadsorption

By Ronald J. Berenson, William I. Bensinger, Dale Kalamasz, Friedrich Schuening, H. Joachim Deeg, Theodore Graham, and Rainer Storb

Previous work has shown failure of engraftment in lethally irradiated dogs when autologous marrow was depleted of Ia-positive cells with an anti-Ia antibody and complement before infusion. In the current study, we have utilized an avidin-biotin immunoadsorption procedure to obtain a population of highly enriched Ia-positive cells for autologous bone marrow transplantation in dogs given lethal irradiation. Dog marrow cells (2.4 to 7.0 x 10^6 cells) that contained 8.6% to 19.9% Ia-positive cells were treated successively with monoclonal antibody 7.2, which reacts with a framework determinant of Ia-antigen, and biotin-conjugated goat antiserum to immunoglobulin. These treated cells were passed over a column of avidin-Biogel (polyacrylamide) and the adherent cells removed by mechanical agitation. Seven lethally irradiated dogs were transplanted with 5.9 to 33.4 x 10^6 recovered adherent cells per kilogram of which 69.0% to 88.0% were Ia-positive. All dogs had hematologic recovery; six are alive and well with durable engraftment and one died on day 15 posttransplant. They are immunologically normal as determined by lymph node and bone marrow biopsies, lymphocyte function, and immunophenotyping of peripheral blood and bone marrow cells. These data provide further evidence that canine hematopoietic stem cells express Ia-like antigens and that these cells are capable of complete hematopoietic and immunologic reconstitution in an autologous model.

MATERIALS AND METHODS

Dogs

Beagles, hounds, and mongrels from the Fred Hutchinson Cancer Research Center kennels were dewormed, vaccinated against distemper, leptospirosis, hepatitis, and parvovirus, housed in single cages, and observed for disease for at least two months. The dogs weighed 6.7 to 22.5 (median 15.0) kg and their ages were 9 months to 2+ years old at the time of transplantation. Research was conducted according to the principles enunciated in the "Guide for Laboratory Animal Facilities and Care" prepared by the National Academy of Sciences-National Research Council. Dogs were observed for 2 to 6 months post-BMT.

Total Body Irradiation, Marrow Infusion, and Animal Care

Bone marrow was obtained from anesthetized dogs as previously described.9 After marrow aspiration, dogs were given 9.2 Gy of total body irradiation at 7 cGy/min from two opposing 60Co sources.10 11

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The positively selected (Ia-positive) marrow cells were infused within two to four hours of irradiation. Postgrafting parenteral fluids, electrolytes, platelet transfusions, and antibiotics were administered as indicated. All blood products used for transfusion were irradiated in vitro (15 Gy) to inactivate pluripotent stem cells and immunologically competent cells. Hematocrit, leukocyte, and platelet counts were obtained daily and an autopsy with histological examination was performed on the dog that died.

Antibodies

A purified murine monoclonal IgG2b antibody 7.2 was kindly provided by Dr John Hansen. McAb 7.2 reacts with human HLA-DR antigens and cross-reacts with similar Ia-like antigens (p 29/34) on canine cells.13 Biotinylated goat antimouse immunoglobulin (B-GMAIg), fluorescein isothiocyanate conjugate (FITC) of swine anti-goat immunoglobulin, FITC-swine immunoglobulin, and FITC-goat antimouse immunoglobulin were obtained from Tago (Burlingame, CA). McAb 9E8 (donated by Genetic Systems, Seattle, WA) recognizes the p(13)E antigen of murine leukemia virus and is not reactive with dog or human tissues, including bone marrow and peripheral blood. Antibodies reactive with dog cells have been previously described. McAb DT2 react with canine helper T lymphocytes, while E11 recognizes suppressor T lymphocytes.13,14 McAb DLY1 reacts with both lymphocytes and monocytes, while DLY6 recognizes lymphocytes.13,14 McAb Thy1 recognizes lymphocytes and natural killer cells in dogs.14

Production of Avidin–Biogel

Avidin obtained from Sigma Chemical (St Louis, MO) was conjugated to Biogel P-30 (Bio-Rad, Richmond, CA) using a modification of our previously described procedure.7 Carboxylation of Biogel P-30 was first performed by incubation of gel in 0.5 mol/L sodium carbonate and 0.5 mol/L sodium bicarbonate at pH 10.5 for two hours at 60°C. The gel was cooled, washed with phosphate buffered saline (PBS), and stored in PBS with 0.1% sodium azide at 4°C. Activation of the carboxylated gel was accomplished with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC-HCI). For avidin linkage 100 mL of carboxylated gel was incubated with 400 mg EDC-HCl (4 µg EDC-HCl/mL gel) in a total volume of 500 mL distilled water at pH 5 for five minutes at room temperature. Ten milligrams avidin (100 µg avidin/mL gel) was then directly added to this mixture and the incubation continued for two hours. The efficiency of conjugation exceeded 98% as determined by measurement of protein concentration of this solution after incubation with the gel. Glycine at a final concentration of 0.2 mol/L was added to block unconjugated sites with a two-hour incubation, followed by washing with PBS and storage with 0.1% sodium azide at 4°C. The column containing avidin–Biogel was sterilized with ethylene oxide gas sterilization. Microbiologic cultures performed on small samples of eluate before and after column use demonstrated absence of bacteria or fungi in all experiments.

Selection of Ia-positive Cells

Cell preparation. For the first transplanted dog, C239, cells were obtained by Ficoll-Hypaque (specific gravity, 1.077; Pharmacia, Piscataway, NJ) density gradient centrifugation at 900 g for 25 minutes. However, subsequent in vitro studies demonstrated less nonspecific binding when a buffy coat marrow cell preparation rather than Ficoll-Hypaque-separated marrow cells were utilized in the avidin–biotin immunoadsorption procedure.4 All other dogs therefore had the cells obtained from a buffy coat preparation by centrifugation at 250 g for 15 minutes.

Antibody treatment and column separation. Marrow cells at a concentration of 50 x 10⁶ cells/mL were incubated in PBS with 2% bovine serum albumin (PBS/BSA) with 20 µg/mL McAb 7.2 for 30 minutes at 4°C. The cells were washed twice before and after incubation at an identical cell concentration in PBS/BSA with 1:100 dilution of B-GAM Ig for an additional 30 minutes at 4°C. The cells were adjusted to a concentration of 25 x 10⁶ cells/mL in PBS/BSA and 1.5 to 2.0 x 10⁶ cells passed over a Chromaflex (Kontes, Vineland, NJ) 15-cm x 2.5-cm column containing 25 mL avidin–Biogel at a flow rate of 20 mL/minute until a total volume of 150 to 175 mL of eluate was collected. Approximately 50 mL of PBS was passed at the same flow rate through the gel to wash out BSA. The adherent cells were dislodged by mechanical agitation with a 10-mL pipette until 150 mL total volume had been collected. After a small sample of the recovered cells was obtained for immunofluorescence studies to determine the purity of the recovered cell population, the recovered Ia-enriched cells were resuspended in 20 to 30 mL PBS and infused into the irradiated dog. Marrow preparation, antibody treatment, and column separation were carried out with sterile technique in a laminar airflow hood. The entire marrow separation procedure required approximately three hours from start to finish.

Immunofluorescence studies. The enrichment of Ia-positive cells was determined by immunofluorescence staining and FACS analysis. Briefly, 5 x 10⁶ cells were treated with hemolytic buffer, washed twice, and then incubated with a 1:20 dilution of FITC–swine anti-goat immunoglobulin in PBS/BSA with 0.1% sodium azide for 20 minutes at 4°C. The stained cells were washed twice and analyzed with a FACS IV and computer 440 (Becton Dickinson, Mountain View, CA). The starting and adsorbed cells labeled with McAb 7.2 and B-GAM Ig were stained with FITC–swine anti-goat immunoglobulin. As a negative control, the unlabeled marrow cells were stained with FITC–swine anti-goat immunoglobulin or incubated successively with McAb 9E8 and B-GAM Ig and stained with FITC–swine anti-goat immunoglobulin. The percentage of Ia-positive cells was determined by subtracting cells labeled with the control reagents from the antibody-labeled starting and adsorbed cells. As an additional control in selected experiments, we demonstrated that neither the starting nor the adsorbed cells stained with FITC–swine immunoglobulin without antigen reactivity. For immunophenotyping of transplanted dogs, samples of peripheral blood and bone marrow mononuclear cells were stained with the McAb (DT2, E11, 7.2, DLY1, DLY6, or Thy1) and FITC–goat antimouse immunoglobulin and then subjected to FACS analysis using established methods.

Mitogenic assay and mixed lymphocyte reaction. These experiments were performed as previously described.12,13 In the mitogenic assay, 10⁷ peripheral blood mononuclear cells (PBMC) were incubated in the presence (or absence) of 20 µg/mL concanavalin A (Con-A, Calbiochem, San Diego, CA) in Waymouth’s medium with 20% heat-inactivated canine serum for three days at 37°C in a 5%-CO₂-in-air incubator. In the mixed lymphocyte reaction (MLR), 10⁵ PBMC were cultured with 10³ irradiated allogeneic (or autologous) stimulator PBMC for six days at 37°C in a 5% CO₂/air atmosphere. One microcurie of H-thymidine (Amersham, Arlington Heights, IL) was added to each sample 16 hours before harvesting. Triplicate samples were harvested onto filter paper, placed in scintillation fluid, and counted in a scintillation counter.

RESULTS

Immunofluorescence Studies

Figure 1 shows immunofluorescence staining with FITC–swine anti-goat immunoglobulin and FACS analysis of marrow cells (from dog BC128) treated with McAb 7.2 and B-GAM Ig before (Start) and after adsorption onto avidin–Biogel (Adsorbed). The starting cell population was 15.0% Ia-positive, while the recovered adsorbed cells given to the dog were 85.0% Ia-positive. Further studies were performed

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to determine the type of cells present that were Ia-positive and Ia-negative in the recovered adsorbed cell population. FACS sorting and subsequent light microscopic examination of the adsorbed cell fraction demonstrated that the Ia-positive cells were predominantly mononuclear cells, while the nonspecifically bound Ia-negative cell fraction contained greater than 90% granulocytes.

**Marrow Transplants Using Ia-positive Cells**

A total of seven dogs was transplanted with Ia-positive cells obtained using the avidin—biotin immunoadsorption procedure (Table 1). The first transplanted dog, C239, received cells obtained by Ficoll-Hypaque separation, treated successively with McAb 7.2 and B-GAM Ig, and passed over avidin—Biogel. From a starting cell population of 2.56 x 10⁷ cells of which 14.8% were Ia-positive, we recovered 2.28 x 10⁶ adsorbed cells that were 69% Ia-positive. In the six other dogs, buffy coat preparations of dog marrow were obtained containing 2.4 to 7.0 x 10⁷ cells that were 8.6% to 19.9% Ia-positive. These cells were treated successively with McAb 7.2 and B-GAM Ig and passed over avidin—Biogel. A total of 1.15 to 6.68 x 10⁶ adherent cells was recovered by mechani-

![Table 1. Engraftment Data](image)

<table>
<thead>
<tr>
<th>Dog</th>
<th>Before Treatment</th>
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<th>Post-BMT Day</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Total Cells x 10⁷</td>
<td>Total Cells x 10⁶/kg</td>
<td>Ia⁺ Cells x 10⁶/kg</td>
</tr>
<tr>
<td>C239</td>
<td>25.6 14.8</td>
<td>2.28 19.9</td>
<td>69.0 13.7</td>
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<tr>
<td>BB259</td>
<td>26.5 8.6</td>
<td>1.86 23.2</td>
<td>75.0 17.4</td>
</tr>
<tr>
<td>BC125</td>
<td>24.0 11.2</td>
<td>1.93 9.9</td>
<td>87.5 8.7</td>
</tr>
<tr>
<td>C234</td>
<td>29.4 18.0</td>
<td>2.22 19.0</td>
<td>77.0 14.6</td>
</tr>
<tr>
<td>C240</td>
<td>70.0 19.9</td>
<td>6.68 33.4</td>
<td>77.3 25.8</td>
</tr>
<tr>
<td>BC126</td>
<td>25.0 15.0</td>
<td>1.16 5.9</td>
<td>88.0 5.2</td>
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<td>BC128</td>
<td>27.1 14.5</td>
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<td>85.0 6.1</td>
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<td>Normal Unseparated (4.0 x 10⁷/kg)</td>
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*Cells obtained from buffy coat preparation except dog C239, which received Ficoll-Hypaque separated cells.
†Day after transplant when absolute neutrophil count greater than 10³/μL.
‡Day after transplant when platelet count greater than 20 x 10⁹/μL.
§Dogs were sacrificed (except dog C234) at day post-BMT listed; dog C234 died at day 15 post-BMT from sepsis with bone marrow at autopsy showing trilineage engraftment.

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**Fig 1.** Bone marrow cells from dog BC128 treated successively with McAb 7.2 and B-GAM Ig and passed over avidin—Biogel. Antibody-treated cells (Start) and recovered adherent cells (Adsorbed) stained with FITC—swine antigoat immunoglobulin and subjected to FACS analysis.

**Fig 2.** Rate of engraftment as measured by post-BMT day when absolute neutrophil count greater than 1,000/μL and platelet count greater than 20,000/μL compared with number of infused Ia-positive cells per kilogram. Values in brackets indicate dog receiving Ia-positive cells obtained from Ficoll-Hypaque-separatad marrow.
10 x 10^6 Ia-positive cells were infused into dogs. However, the dogs who received lower numbers of Ia-positive cells also received less total cells, which may in itself be responsible for slow engraftment. The single dog receiving Ficoll-Hypaque-separated marrow cells appeared to have more delayed engraftment than the other dogs receiving comparable numbers of buffy coat separated cells.

**Posttransplant Analyses of Dogs BC126 and BC128**

Histopathological studies and immunologic studies were performed on dogs BC126 and BC128 approximately 3 to 4 months after BMT. Lymph node and bone marrow biopsies were normal in these dogs (data not shown). Normal T lymphocyte function was demonstrated by both in vitro and in vivo studies. Proliferative responses to the T lymphocyte mitogen, Con-A, were excellent (Table 2). Normal stimulation of peripheral lymphocytes by irradiated allogeneic cells in MLR was documented (Table 3). In vivo studies showed that the two dogs rejected skin grafts from allogeneic donors in 12 days (normal range: 11 to 14 days). Immunophenotyping of peripheral blood and bone marrow revealed normal percentages of cells reactive with McAbs 7.2, DT2, E11, DLY1, DLY6, and Thy1 (data not shown).

**DISCUSSION**

We have previously shown with negative selection that canine stem cells express Ia antigens. In that study, dogs given lethal total body irradiation (9.2 Gy) and an autologous marrow depleted of Ia-positive cells with McAb 7.2 and complement failed to engraft.4 In contrast, Prendergast et al observed slow marrow recovery over a 2-month period in irradiated dogs receiving autologous marrow depleted of Ia-positive cells.17 However, the dogs were treated with lower doses of radiation (5 Gy), suggesting that the slow hematologic improvement may be from endogenous recovery of host marrow rather than the Ia-depleted infused marrow.

Because of the continued controversy regarding the expression of Ia-antigens on stem cells, we wished to conduct a study in which positively selected Ia-positive cells were transplanted into lethally irradiated dogs. Workers have previously been unable to perform this experiment because of the lack of a method to separate the large number of cells required for BMT. Although FACS sorting can provide relatively pure cell populations, its limited throughput makes it impractical for large-scale application.16 The "panning" technique, in which cells are separated on plastic petri dishes coated with antibodies, produces variable yield and purity and is cumbersome to use for separating large numbers of cells.19

In the current study, we used avidin–biotin immunoadsorption chromatography to purify Ia-positive cells using the same antibody (7.2) as previously employed for negative selection. This highly enriched population of Ia-positive cells was infused into seven lethally irradiated dogs. Sustained complete marrow engraftment was observed in six out of seven transplanted dogs, while a seventh dog died from sepsis at day 15 post-BMT with histologic evidence of engraftment. Detailed studies of two dogs also documented recovery of normal immunologic function after transplanting Ia-positive cells. These data suggest that marrow stem cells responsible for hematologic and immunologic reconstitution express Ia antigens.

Although dogs were transplanted with predominantly Ia-positive cells, it was also possible that contaminating Ia-negative cells infused into these dogs could be responsible for marrow engraftment. FACS sorting experiments of the recovered adherent cells showed that the nonspecifically bound Ia-negative cells consisted almost entirely of granulocytes. It thus seems unlikely that these infused Ia-negative cells represent pluripotent hematopoietic stem cells. However, we cannot exclude the possibility that a subpopulation of the infused cells (Ia-positive or Ia-negative) played an accessory role in marrow engraftment.

In humans, Ia-like antigens are found on committed hematopoietic progenitors cultured in semisolid medium, including early erythroid progenitor cells (BFU-E), late erythroid progenitor cells (CFU-E), granulocyte-macrophage progenitor cells (CFU-GM), and multipotent progenitor cells giving rise to megakaryocytes as well as erythroidcyte, granulocyte, and macrophage progenitor cells (CFU-GEMM).20,24 More recently, it has become apparent that at least three subloci (HLA-DP, HLA-DQ, HLA-DR) exist within the HLA-D region.25-27 Recent studies have detected some of these antigens (HLA-DP and HLA-DR) on both committed progenitors and potentially more primitive cells capable of sustaining marrow growth in long-term culture.28,29 Similar results have also been reported in the mouse using the CFU-S assay with I-E antigens (equivalent to human HLA-DR) detected on CFU-S and I-A antigens (equivalent to human HLA-DQ) lacking on these cells.23

The application of our results to human (or murine) hematopoietic stem cells must be done with caution.

**Table 2. Mitogenic Response to Con-A of PBMC from Transplanted Dogs BC126 and BC128**

<table>
<thead>
<tr>
<th>Dog</th>
<th>Media</th>
<th>Con-A</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC126</td>
<td>506 ± 197</td>
<td>12,613 ± 512</td>
<td>24.9</td>
</tr>
<tr>
<td>BC128</td>
<td>256 ± 140</td>
<td>24,294 ± 1,766</td>
<td>94.9</td>
</tr>
<tr>
<td>Control</td>
<td>163 ± 60</td>
<td>6,539 ± 939</td>
<td>40.1</td>
</tr>
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</table>

*PBMC from transplanted dogs BC126 and BC128 as well as a normal (control) dog were incubated in the presence or absence of Con-A (see Materials and Methods). Mean CPM of triplicate cultures are shown. A stimulation index was calculated as follows: cpm Con-A-stimulated PBMC/cpm media-incubated PBMC.

**Table 3. Mixed Lymphocyte Reaction of PBMC from Transplanted Dogs BC126 and BC128**

<table>
<thead>
<tr>
<th>Dog</th>
<th>Autologous</th>
<th>Allogeneic</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC126</td>
<td>149 ± 78</td>
<td>3,948 ± 687</td>
<td>26.5</td>
</tr>
<tr>
<td>Control</td>
<td>238 ± 74</td>
<td>4,037 ± 995</td>
<td>17.0</td>
</tr>
<tr>
<td>BC128</td>
<td>774 ± 54</td>
<td>5,011 ± 912</td>
<td>6.5</td>
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<tr>
<td>Control</td>
<td>602 ± 134</td>
<td>10,320 ± 1,082</td>
<td>17.1</td>
</tr>
</tbody>
</table>

*PBMC from transplanted dogs BC126 and BC128 as well as from two normal (control) dogs cultured with autologous or irradiated allogeneic PBMC (see Materials and Methods). Mean cpm of triplicate cultures are shown. A stimulation index was calculated as follows: cpm allogeneic stimulated PBMC/cpm autologous stimulated PBMC.
Although McAb 7.2 has been shown to react with a similar bimolecular glycoprotein complex on both human and dog cells, the DLA-D region has not been well-characterized in dogs. Furthermore, the distribution of class II antigens in cells, the DLA-D region has not been well-characterized in phocytes, monocytes, and activated T lymphocytes, while bimolecular glycoprotein complex on both human and dog Although McAb 7.2 has been shown to react with a similar bimolecular glycoprotein complex on both human and dog cells, the DLA-D region has not been well-characterized in dogs. Furthermore, the distribution of class II antigens in cells, the DLA-D region has not been well-characterized in phocytes, monocytes, and activated T lymphocytes, while

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