P

REVIOUS STUDIES in the dog indicate that hematopoietic stem cells express major histocompatibility complex (MHC) class II molecules, as recognized by the anti-class II monoclonal antibody (MoAb) 7.2.12 Treatment of canine marrow in vitro with MoAb 7.2 and rabbit complement prevents autologous engraftment after otherwise lethal total body irradiation (TBI), whereas selected 7.2-positive marrow cells are capable of permanently repopulating the hematopoietic system of irradiated dogs. The genetic region of the canine MHC that codes for class II molecules is termed DLA-D, and is analogous to the H-2I region of the mouse3 and the human HLA-D region.4-5

Class II molecules are differentially expressed on hematopoietic progenitor cells of man. Committed hematopoietic progenitor cells assayed as colony-forming unit-granulocyte-monocyte (CFU-GM), CFU-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM), and burst-forming unit-erythroid (BFU-E) express HLA-DR and -DP antigens, whereas CFU-erythroid (CFU-E) are predominantly HLA-DR-negative.6 Further maturation in the granulocyte series results in the loss of cell surface expression of class II antigens, while functionally mature monocytes and macrophages retain class II expression.7 Class II antigens in dogs are expressed on most unstimulated T lymphocytes in addition to monocytes, B lymphocytes, and activated T lymphocytes.15 Canine CFU-GM and CFU-GEMM express both DLA-DR and -DP antigens.16 H. Greinix, unpublished observations. Several investigators report low HLA-DR expression on murine and human long-term marrow culture-initiating cells.13-15 This finding does not necessarily contradict the observations in 7.2-positive canine marrow transplants, since cells from long-term marrow cultures may differ from pluripotent stem cells in their ability to permanently reconstitute irradiated marrow spaces. It has not yet been possible to determine if human pluripotent stem cells express class II antigens, because a suitable assay system is not available.19 The presence of class II molecules on stem cells could have significant clinical implications, because several investigators have described a possible role of HLA-D-restricted cell interactions in hematopoiesis.10,14,15 Such restrictions could be important for interactions between transplantable cells and as yet undetected accessory cells in the hematopoietic microenvironment.

The present study further investigated the function of class II antigens in autologous reconstitution following lethal TBI, autologous marrow transplantation, and intravenous (IV) injection of anti-class II MoAbs specific for different class II epitopes in a well-established canine model.17-19 Strikingly, all dogs infused with MoAb H81.98.21 for 5 to 10 days after grafting showed transient marrow repopulation, followed by a profound "late" secondary marrow failure. In contrast, MoAb B1F6 specific for another class II epitope did not interfere with hematopoietic recovery. The development of graft failure suggests a role for class II molecules in the engraftment and/or regulation of pluripotent stem cells required for sustained marrow function.

MATERIALS AND METHODS

Experimental animals. Twenty-six beagle or mongrel dogs of both sexes, 6- to 10-months-old, were either raised at the Fred Hutchinson Cancer Research Center or obtained from Hematologic Institute, National Institutes of Health, Department of Health and Human Services. H. T. G. was supported by fellowships from the Max Kade Foundation and the Aplastic Anemia Foundation.

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Hutchinson Cancer Research Center (FHCRC; Seattle, WA) or purchased from commercial dealers licensed by the US Department of Agriculture. All were observed for disease for 2 months before use, dewormed, and vaccinated for rabies, distemper, leptospirosis, hepatitis, and parvovirus. They were housed per guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health. The protocols used for this study were approved by the Institutional Animal Care and Use Committee of the FHCRC.

TBI, marrow infusion, and postgrafting care. Marrow for transplantation was obtained immediately before TBI by needle aspirations from femora and humeri of anesthetized dogs and stored at 4°C for no more than 4 hours. Irradiation consisted of a single dose of 920 cGy TBI delivered at a rate of 7 cGy/min from two opposing 60Co sources. This dose is more than twice the LDo50 dose for dogs and consistently prevents endogenous marrow recovery. An IV infusion of 0.6 to 4.4 \times 10^9 marrow cells/kg body weight was administered immediately after TBI. Daily posttransplantation care included parenteral fluids, electrolytes, irradiated (16 Cy) platelet and red blood cell transfusions, and systemic and oral nonabsorbable antibiotics. Complete peripheral blood counts were obtained before and daily after TBI. Autopsies with histologic examinations were performed on all dogs that died. Marrow engraftment was assessed by increasing and sustained granulocyte (>1,000/mm^3) and platelet counts (>40,000/mm^3) following the postirradiation nadir, and by marrow cellularity at autopsy.

MoAbs. MoAb H81.98.21 was generated against mouse IgA and is reactive with human HLA-DR and canine DLA-DR framework antigen. B1F6 was produced against canine cells and is reactive with HLA- and DLA-DR and -DP framework antigen. The isotypes of both MoAbs are IgG,. MoAb 6.4, used as an irrelevant MoAb, is an IgG, murine MoAb directed at the Thy 1.1 antigen in mice and does not cross-react with canine cells. The MoAbs were purified antibodies was confirmed by gel filtration chromatography. The monomeric structure of the MoAbs, is an IgG, murine MoAb directed at the Thy 1.1 antigen in mice and does not cross-react with canine cells. The MoAbs were purified by affinity chromatography on a Staphylococcus aureus protein A sepharose column. The monomeric structure of the purified antibodies was confirmed by gel filtration chromatography.

MoAbs H81.98.21 and B1F6 showed reactivity of greater than 80% with peripheral blood lymphocytes (PBL) and of 14% to 20% with marrow cells, identified by low forward angle and right angle light scattering properties, in indirect immunofluorescence assays and flow microfluorometry. In competitive inhibition assays, preincubation of PBL in H81.98.21 decreased the intensity of staining with fluorescein isothiocyanate (FITC)-labeled B1F6 by 30% (Table 1). However, binding of H81.98.21 was unaffected by preincubation of cells with MoAb B1F6. PBL preincubated with the irrelevant antibody 6.4 showed a fluorescence intensity of 100% when either FITC-labeled H81.98.21 or B1F6 was added. These data provide evidence that MoAbs H81.98.21 and B1F6 recognize different epitopes on class II molecules or different class II molecules.

In Western blot analysis, both antibodies identified the heterodimeric structure of class II molecules on canine PBL or marrow cells. However, MoAb H81.98.21 did not identify any monomer, whereas MoAb B1F6 strongly reacted with the β-monomer of class II molecules.

Cells. PBL and marrow cells were obtained by separation of heparinized blood or marrow over a Ficol-Hypaque (F/H) gradient. Thoracic duct lymphocytes were obtained via thoracic duct cannulation and cryopreserved until use.

Determination of anti-mouse MoAb levels by enzyme-linked immunosorbent assay. Assays were performed as described, with minor modifications. Briefly, polyvinyl plastic microtiter plates (Becton Dickinson, Oxnard, CA) were coated with MoAb H81.98.21. Appropriately diluted sera, collected before and weekly up to 50 days after transplant, were added to respective wells and incubated for 60 minutes followed by another 1-hour incubation with peroxidase-conjugated goat anti-dog IgG (Sigma Chemical, St Louis, MO). Then, ABTS (2,3-azinodi-[3-ethybenzthiazoline-6-sulfonicacid)] enzyme substrate (Sigma) was added, and the resultant color change was read within 15 minutes on an enzyme-linked immunosorbent assay (ELISA) plate spectrophotometer at 405 nmol/L. A sample was considered positive for dog anti-mouse antibodies if the test antibody level was greater than the pretreatment level.

Ex vivo treatment of marrow with MoAbs and/or complement. In some studies, marrow was exposed to antibody ex vivo before autologous transplantation. Briefly, F/H-separated marrow cells were incubated with MoAb (1 mg/10^9 cells) at 4°C for 30 minutes, followed by incubation with rabbit complement (C') (Pelfreeze, Rogers, AR) (1 \times 10^9 cells/50 mL) or canine serum for 60 minutes at room temperature. The incubation with C' was repeated. Then, cells were washed, resuspended in medium, and infused IV into the TBI-treated dog. After antibody incubation, supernatants were tested for residual antibody activity by flow cytometry, using PBL as targets.

Ex vivo treatment of marrow with 1-leucyl-1-leucine ortho-methyl ester. In some experiments, marrow cells were treated with 1-leucyl-1-leucine o-methyl ester (Leu-Leu-OMe). Equal volumes of marrow buffy coat cells (20 \times 10^9/mL) and Leu-Leu-OMe solution at a concentration of 1,000 μmol/L were incubated for 15 minutes at room temperature. Then, marrow cells were washed twice, resuspended in Waymouth medium (GIBCO, Grand Island, NY), counted, and cryopreserved at −80°C. The elimination of alloantigen responsiveness and mitogen-induced lymphocyte blastogenesis by Leu-Leu-OMe were shown, as was the elimination of natural killer activity.

RESULTS

MoAb infusion in canine autologous marrow recipients. Twenty dogs were given 920 cGy TBI followed by infusion of unmodified autologous marrow and IV injection of anti-class II MoAb. Results shown in Table 2 and Fig 1 are contrasted to those in 16 control dogs given 9.2 Gy TBI, autologous marrow infusion, and no MoAb injections.

All dogs experienced the typical granulocyte nadirs within 5 to 7 days of TBI (Fig 1A), not different from the 16 control dogs. Subsequently, granulocyte counts began to increase both in experimental and control dogs. Granulocyte counts in control dogs reached pretransplant levels after day 35 to 40, where they remained. In contrast, the two dogs in group 1, given MoAb H81.98.21 at 0.6 mg/kg/d from days...
MoAb-INDUCED MARROW GRAFT FAILURE IN DOGS

Table 2. Results in Dogs Given 9.2 Gy TBI, Autologous Marrow Infusion, and Anti-Class II MoAb IV After Transplant

<table>
<thead>
<tr>
<th>Group</th>
<th>Dog No.</th>
<th>MoAb Dose (mg/kg/d)</th>
<th>Treatment Days (after TBI)</th>
<th>MoAb Dose (mg/kg/d)</th>
<th>Treatment Days (after TBI)</th>
<th>Marrow Cells Engraftment Sustained</th>
<th>Marrow Cellularity (% normal)</th>
<th>Survival (d)</th>
<th>%normal</th>
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<td>1</td>
<td>C717</td>
<td>H81.98.21</td>
<td>0.6</td>
<td>0-9</td>
<td>3.1</td>
<td>26</td>
<td>0</td>
<td>No</td>
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<tr>
<td></td>
<td>C745</td>
<td>H81.98.21</td>
<td>0.6</td>
<td>0-9</td>
<td>4.1</td>
<td>31</td>
<td>0</td>
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<td>2</td>
<td>C690</td>
<td>B1F6</td>
<td>0.6</td>
<td>0-9</td>
<td>2.3</td>
<td>&gt; 78</td>
<td>100</td>
<td>Yes</td>
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<td></td>
<td>C797</td>
<td>B1F6</td>
<td>0.6</td>
<td>0-9</td>
<td>3.2</td>
<td>&gt; 60</td>
<td>100</td>
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<td></td>
<td>D190</td>
<td>B1F6</td>
<td>0.6</td>
<td>0-4</td>
<td>3.8</td>
<td>&gt; 40</td>
<td>100</td>
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<td>3</td>
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<td>0.3, 0.3</td>
<td>0-9</td>
<td>4.4</td>
<td>&gt; 70</td>
<td>100</td>
<td>Yes</td>
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<td>C687</td>
<td>H81.98.21, B1F6</td>
<td>0.3, 0.3</td>
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<td>3.9</td>
<td>&gt; 90</td>
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<td>H81.98.21</td>
<td>0.3</td>
<td>0-9</td>
<td>2.9</td>
<td>&gt; 105</td>
<td>100</td>
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<td>4</td>
<td>C684</td>
<td>H81.98.21</td>
<td>0.6</td>
<td>6-15</td>
<td>3.8</td>
<td>&gt; 70</td>
<td>100</td>
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<td>C688</td>
<td>H81.98.21</td>
<td>0.6</td>
<td>6-15</td>
<td>4.0</td>
<td>&gt; 100</td>
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<td>0.6</td>
<td>0-4</td>
<td>2.4</td>
<td>26</td>
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<td>D096</td>
<td>H81.98.21</td>
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<td>0-4</td>
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<td>19</td>
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<td>D094</td>
<td>H81.98.21</td>
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<td>H81.98.21</td>
<td>0.6</td>
<td>0-2</td>
<td>4.0</td>
<td>30</td>
<td>20</td>
<td>No</td>
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<td>D079</td>
<td>H81.98.21</td>
<td>0.6</td>
<td>0-2</td>
<td>3.8</td>
<td>&gt; 175</td>
<td>100</td>
<td>Yes</td>
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<td>7</td>
<td>D037</td>
<td>H81.98.21</td>
<td>0.6</td>
<td>0</td>
<td>4.0</td>
<td>&gt; 50</td>
<td>100</td>
<td>Yes</td>
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<td>D037</td>
<td>H81.98.21</td>
<td>0.6</td>
<td>0</td>
<td>1.6</td>
<td>&gt; 75</td>
<td>100</td>
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0 to 9 after grafting, experienced a second granulocyte nadir, with counts decreasing to less than 100/mm³ by day 19. Granulocytopenia persisted in the two dogs, and their platelet counts remained low. They died of infections on days 26 and 31, respectively. Marrow histology at autopsy failed to show hematopoietic cells. The three dogs in group 2, given MoAb B1F6 at 0.6 mg/kg/d from days 0 to 9 or 0 to 4, showed sustained hematopoietic engraftment similar to that seen in controls (Fig 1A).

To define effective antibody doses, three dogs in group 3 were given MoAb H81.98.21 at a dose of 0.3 mg/kg/d from days 0 to 9, administered either alone or in combination with MoAb B1F6, also at a dose of 0.3 mg/kg/d. All three showed prompt increases in granulocyte counts to a mean of 4,500/mm³ by day 14. This was followed by a decline in counts to a nadir of 800/mm³ by day 26, followed by subsequent complete recovery of counts and survival of dogs (Fig 1B). Dogs needed platelet transfusions through

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Fig 1. Recovery of peripheral blood granulocyte counts after 9.2 Gy TBI and autologous marrow infusion with and without MoAb infusion. Shaded area represents the range of granulocyte counts from 16 control dogs not receiving MoAb infusion. Lines represent the mean granulocyte counts in antibody-treated dogs. (A) MoAb H81.98.21 was given for 5 or 10 days (N = 5), and B1F6 for 5 or 10 days (N = 3), each at a dose of 0.6 mg/kg/d. (B) MoAb H81.98.21 was given at a dose of 0.3 mg/kg for 10 days (N = 1) or 0.6 mg/kg/d for 5 to 10 days (N = 5). A combination of MoAb H81.98.21 and B1F6 consisted of 0.3 mg/kg/d of each MoAb for 10 days (N = 2). (C) MoAb H81.98.21 was given at a dose of 0.6 mg/kg/d for either 5 and 10 days (N = 5), 1 day (N = 2), or 3 days (N = 2), respectively, starting on day 0 or from day 6 to 15 (N = 2).
day 25 before eventual complete recovery of platelet counts. Thus, infusion of MoAb H81.98.21 at 0.3 mg/kg/d was not sufficient to produce the fatal late graft failure observed in dogs of group 1 given MoAb H81.98.21 at 0.6 mg/kg/d.

In four subsequent experiments, we determined the time frame during which injection of MoAb H81.98.21 was effective in causing late graft failure. Two dogs in group 4 given MoAb at 0.6 mg/kg/d from days 6 to 15 showed sustained hematopoietic engraftment (Fig 1C). Three dogs in group 5 given MoAb H81.98.21 at 0.6 mg/kg/d from days 0 to 4 showed initial granulocyte recovery after the post-TBI nadir (Fig 1C). In all three, counts subsequently decreased to less than 100/mm³ by day 12, and dogs died 19 to 26 days after grafting with infections and the finding of complete marrow aplasia at autopsy. Two dogs in group 6 were given MoAb at 0.6 mg/kg/d from days 0 to 2. Both dogs had initial increases in granulocyte counts by day 8, followed by a decrease to less than 100/mm³ by day 16 (Fig 1C). One showed a gradual recovery of granulocyte counts and survived, but the other died on day 30 with infection; marrow cellularity at autopsy was 20% (Table 2). Both dogs in group 7 given a single 0.6-mg/kg dose of MoAb on day 0 experienced granulocyte nadirs within 5 days after TBI, and showed complete and sustained recovery of granulocytes by day 17 and of platelets by day 34 (Fig 1C). Comparing results of the five dogs given MoAb H81.98.21 for at least 5 days after transplant with those of the three dogs treated with MoAb B1F6 shows the difference in sustained engraftment to be statistically significant (P = .02 by two-sided Fisher's exact test).

Indirect immunofluorescence studies were performed to monitor serum levels of MoAb after infusion. Lymphocytes from normal dogs were incubated with serum from untreated and MoAb-treated dogs collected 24 hours after each MoAb injection. All samples with serum from MoAb-treated dogs showed increases in the fluorescence intensity of 70% to 80% compared with samples with serum from untreated dogs. MoAbs were no longer detectable in the serum and on PBL of MoAb-treated dogs 48 hours after the last MoAb injection. All dogs given MoAb H81.98.21 and B1F6 IV had marrow aspirations before and on days 2 and 6 after marrow grafting to test for presence of MoAb-coated cells by flow cytometry. Marrow cells from MoAb-treated dogs incubated with goat anti-mouse IgG-FITC alone showed 50% to 70% fluorescence staining during days of antibody treatment, compared with 0.5% to 1% in untreated dogs. However, in dogs given MoAb from days 0 to 4, MoAb was no longer detectable on either PBL or marrow cells on day 6. Circulating serum antibodies against any of the murine MoAbs used could not be detected by weekly sampling up to 7 weeks after marrow grafting.

**Autologous transplants with ex vivo manipulated marrow.** To examine several possible explanations for the late marrow graft failure seen in our model, F/H-separated marrow was treated ex vivo with MoAb and/or C' before infusion into dogs given 920 cGy TBI. Results are shown in Table 3.

Two dogs in group 8, given density gradient separated and otherwise unmanipulated marrow, showed sustained engraftment. Three dogs in group 9, given marrow incubated ex vivo with two cycles of rabbit C' without antibody treatment, also showed prompt and sustained marrow recovery. Thus, F/H separation alone or in combination with exposure to rabbit C' did not affect the ability of the marrow to restore hematopoiesis.

In two dogs (C796 and C765, group 10), the marrow was incubated ex vivo for 1 and 4 hours, respectively, with MoAb H81.98.21 and autologous serum containing canine C' to address the possibility of lysis of marrow cells either through canine C'-mediated cytotoxicity or antibody-mediated cellular cytotoxicity (ADCC). Both dogs experienced the typical granulocyte nadirs within 5 to 7 days of TBI, followed by complete and sustained hematopoietic recovery indistinguishable from that seen in controls. This result also argues against removal of antibody-coated hematopoietic cells through cells of the reticuloendothelial system (RES) following transplantation. Additionally, in vitro ADCC assays showed that PBL harvested from antibody-treated dogs and used as effector cells failed to show significant ⁵¹Cr release from untreated or antibody-coated autologous or allogeneic marrow target cells (data not shown). Also, if ADCC were operative in vivo, the observed early recovery of granulocytes, presumably derived from committed progenitors expressing class II antigens, should not take place.

**Ex vivo treatment of the marrow with either MoAb H81.98.21 or B1F6 in the presence of rabbit C'.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dog No.</th>
<th>Ex Vivo Treatment</th>
<th>Marrow Cells Infused/kg Body Weight (× 10⁹)</th>
<th>Survival (d)</th>
<th>Marrow Cellularity (% Normal)</th>
<th>Sustained Marrow Engraftment</th>
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<tr>
<td>8</td>
<td>C101</td>
<td>Ficoll separated BM only</td>
<td>1.7</td>
<td>&gt; 60</td>
<td>100</td>
<td>Yes</td>
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<tr>
<td></td>
<td>B900</td>
<td>Ficoll separated BM only</td>
<td>0.6</td>
<td>&gt; 55</td>
<td>100</td>
<td>Yes</td>
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<tr>
<td>9</td>
<td>C392</td>
<td>C'</td>
<td>0.27</td>
<td>&gt; 50</td>
<td>100</td>
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<td></td>
<td>C432</td>
<td>C'</td>
<td>0.55</td>
<td>&gt; 120</td>
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<td></td>
<td>C46</td>
<td>C'</td>
<td>1.7</td>
<td>&gt; 70</td>
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<tr>
<td>10</td>
<td>C796</td>
<td>H81.98.21 + autologous serum</td>
<td>1.14</td>
<td>&gt; 50</td>
<td>100</td>
<td>Yes</td>
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<td>C765</td>
<td>H81.98.21</td>
<td>2.18</td>
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<td></td>
<td>C782</td>
<td>H81.98.21</td>
<td>1.82</td>
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<td>C741</td>
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<td>&gt; 90</td>
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<td>11</td>
<td>C737</td>
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<td>D120</td>
<td>B1F6 + C'</td>
<td>1.0</td>
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of group 11 led to death on day 17 with complete marrow aplasia; neither dog showed increases in granulocyte counts after the postirradiation nadirs. After cytolytic treatment, less than 1% residual class II antigen-positive cells were identifiable by flow microfluorometry, and 5% to 8% CFU-GM were recovered in agar cultures after 14 days. This result shows that class II antigens detected by either of the two MoAbs are expressed on cells in the graft that are required both for initial and for sustained hematopoietic recovery.

We next sought to exclude the possibility that dog anti-mouse immunoglobulin antibody is involved in the functional inactivation of cells important for sustained engraftment. Therefore, one dog (C782, group 10) was hyperimmunized by injecting MoAb H81.98.21 daily IV for 10 days at a dose of 0.6 mg/kg body weight 6 weeks before transplantation. Anti-mouse antibody, detected by ELISA at a titer of 1:128, was present in the serum from day 28 after the first MoAb injection until after transplantation. After ex vivo incubation with MoAb H81.98.21 for 1 hour, the marrow was infused. The dog showed prompt and sustained hematopoietic engraftment. An additional dog (C741, group 10) also engrafted uneventfully following infusion of marrow incubated ex vivo with MoAb H81.98.21 and dog anti-mouse serum from dog C782. In both dogs, marrow cells were examined for class II antigen expression after ex vivo treatment, and they showed 14% to 20% of fluorescence intensity in flow microfluorometry.

Additional infusion of hematopoietic cells on day 6 after marrow grafting. Late graft failure can be induced with five daily injections of MoAb H81.98.21 from days 0 to 4 after transplantation. Presumably as a result of MoAb injections, the pluripotent stem cells infused along with the graft have failed to replicate in numbers sufficient for sustained hematopoietic recovery, even though MoAb H81.98.21 was cleared from blood and marrow already on day 6 after transplantation. Persistent stem cell failure could be explained in either of two ways. First, pluripotent stem cells are still present after day 6, but the marrow microenvironment (ME) and accessory cells (AC), necessary to provide signals for stem cell replication, could be "permanently" defective. Alternately, stem cells present on day 6 are no longer pluripotent and have lost their ability to replicate. This could be due to a block of replication initiating signals from the ME during MoAb infusions, or to MoAb-induced stem cell differentiation at the expense of replication. We performed experiments to distinguish between the two possibilities. Accordingly, three dogs were given 9.2 Gy TBI, an autologous marrow graft on day 0, and an IV infusion of MoAb H81.98.21 at 0.6 mg/kg/d on days 0 to 4 (Table 4). One dog (C984, group 12) was given an IV infusion of 5 × 10^8 cryopreserved autologous thoracic duct lymphocytes/kg on day 6, when antibody clearance from marrow and peripheral blood was documented by cytofluorometric analysis. Thoracic duct lymph is known not to contain hematopoietic stem cells, and, therefore, could serve as a source of accessory cells. The dog showed initial granulocyte engraftment beginning on day 8, followed by a decline of granulocytes to less than 100/mm^3 by day 17. Granulocytes remained at less than 100/mm^3, and the dog died on day 25 from infection. Marrow histology at autopsy failed to show evidence of hematopoietic cells, a finding identical to that in dogs given H81.98.21 antibody infusions without additional thoracic duct lymphocytes (Table 2, group 5). Two dogs (group 13) were given an infusion of 1.5 and 3.6 × 10^8 cryopreserved marrow cells/kg, respectively, on day 6. The marrows were treated ex vivo with Leu-Leu-OMe, a lysosomotropic agent, with the aim of removing cytotoxic cells and monocytes/macrophages from the marrow inoculum. Both dogs showed initial granulocyte engraftment beginning on day 8, followed by a decrease of granulocytes to less than 100/mm^3 by day 17. However, counts recovered slowly, reaching 500/mm^3 by day 31, and 1,000/mm^3 by day 36. Both granulocyte and platelet counts subsequently recovered completely and marrow histology obtained on day 50 showed trilineage engraftment in both dogs. These results are consistent with the notion that early administration of MoAb H81.98.21 results in a loss of the replication potential of the grafted pluripotent stem cells.

**DISCUSSION**

We have previously shown that MHC class II antigens are expressed on canine hematopoietic cells that are important for sustained recovery of transplanted autologous marrow. This finding was made in otherwise lethally irradiated dogs in which marrow depleted of class II antigen-bearing cells failed to engraft, whereas positively selected class II antigen-bearing cells gave rise to hematopoietic reconstitution. The current study extended these initial observations and sought to determine a functional role of class II MHC antigens in the events that lead to hematopoietic failure. The infused marrow was infused on day 6. The dog showed prompt and sustained hematopoietic engraftment. The dog showed initial granulocyte engraftment beginning on day 8, followed by a decrease of granulocytes to less than 100/mm^3 by day 17. Granulocytes remained at less than 100/mm^3, and the dog died on day 25 from infection. Marrow histology at autopsy failed to show evidence of hematopoietic cells, a finding identical to that in dogs given H81.98.21 antibody infusions without additional thoracic duct lymphocytes (Table 2, group 5). Two dogs (group 13) were given an infusion of 1.5 and 3.6 × 10^8 cryopreserved marrow cells/kg, respectively, on day 6. The marrows were treated ex vivo with Leu-Leu-OMe, a lysosomotropic agent, with the aim of removing cytotoxic cells and monocytes/macrophages from the marrow inoculum. Both dogs showed initial granulocyte engraftment beginning on day 8, followed by a decrease of granulocytes to less than 100/mm^3 by day 17. However, counts recovered slowly, reaching 500/mm^3 by day 31, and 1,000/mm^3 by day 36. Both granulocyte and platelet counts subsequently recovered completely and marrow histology obtained on day 50 showed trilineage engraftment in both dogs. These results are consistent with the notion that early administration of MoAb H81.98.21 results in a loss of the replication potential of the grafted pluripotent stem cells.

**Table 4. Additional Infusion of Thoracic Duct Lymphocytes and Marrow Cells Treated With Leu-Leu-OMe on Day 6 After 9.2 Gy TBI, Autologous Marrow Infusion, and Anti-Class II MoAb IV**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dog No.</th>
<th>MoAb</th>
<th>MoAb Dose (mg/kg/d)</th>
<th>Treatment Days (after TBI)</th>
<th>Marrow Cells Infused (x10^8/kg)</th>
<th>Additional Cell Infusion Day 6 (% normal)</th>
<th>Survival (d)</th>
<th>Marrow Cellularity (x10^8/kg)</th>
<th>Sustained Marrow Engraftment</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>C984</td>
<td>H81.98.21</td>
<td>0.6</td>
<td>0-4</td>
<td>3.9</td>
<td>TLD 5.0</td>
<td>25</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
<td>D211</td>
<td>H81.98.21</td>
<td>0.6</td>
<td>0-4</td>
<td>4.0</td>
<td>Leu-Leu-OMe 3.6</td>
<td>&gt;70</td>
<td>170</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>D167</td>
<td>H81.98.21</td>
<td>0.6</td>
<td>0-4</td>
<td>0.6</td>
<td>Leu-Leu-OMe 1.5</td>
<td>&gt;40</td>
<td>120</td>
<td>Yes</td>
</tr>
</tbody>
</table>

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etnic recovery after autologous marrow transplantation. We studied two isotype-matched MoAbs directed at HLA-DR and HLA-DP framework antigens. Daily injection of MoAb H81.98.21 for 5 days after transplant led to failure of sustained hematopoietic engraftment, whereas injection of MoAb B1F6 in identical dose and schedule failed to affect hematopoietic recovery. The early postgrafting recovery of granulocytes (days 7 to 15) in antibody-treated dogs was comparable to that of control dogs not receiving antibody. This presumably reflects granulocyte production generated by already committed hematopoietic progenitor cells, which is not impaired by antibody treatment even though progenitor cells are known to bind this MoAb. The late marrow failure observed in the H81.98.21-treated dogs during the third or fourth week after transplantation appears to be due to the failure of transplanted pluripotent stem cells to replicate and then differentiate after the committed progenitor cell compartment has been exhausted. This apparent inability to replenish the committed progenitor cell compartment resulted in marrow aplasia, pancytopenia, and death.

The observed effects of MoAb H81.98.21 on hematopoiesis were dose-dependent; a reduction of the antibody dose to 0.3 instead of 0.6 mg/kg body weight/d led only to transient secondary declines in granulocyte counts, but did not prevent ultimate sustained hematopoietic engraftment. The critical interaction between antibody and hematopoietic cells takes place during the first 5 days after marrow grafting. A delay of the first infusion of antibody until day 6, or a reduction of the infusion time to less than 5 days, did either not at all or only inconsistently affect ultimate hematopoietic recovery.

Both antibodies H81.98.21 and B1F6 identify glycoproteins of identical molecular weights in simultaneous radioimmunoprecipitation studies. Both appear to recognize antigens expressed on pluripotent and committed progenitor cells or important accessory cells, since ex vivo incubation of the marrow before transplantation with either antibody plus rabbit C" destroyed the ability of that marrow both to initiate early granulocyte recovery and sustained engraftment. However, only antibody H81.98.21 produced late graft failure when administered in vivo after transplantation. Competitive inhibition studies and Western blot analyses showed that MoAb H81.98.21 and B1F6 recognize different epitopes of class II molecules or different class II molecules.

An important function of MHC class II molecules is to bind antigens in their native conformation as a basis for their recognition by T cells. B-cell surface Ia molecules may function as signal transducer molecules, as well as recognition molecules, which are important for B-cell activation. Anti–HLA-DR antibodies were reported to inhibit the proliferation of lipopolysaccharide-stimulated, polyclonal anti-IgM-stimulated, and unstimulated human B cells. Antibodies H81.98.21 and B1F6 did not inhibit lymphocyte activation and proliferation in mitogen-stimulated cultures. However, both antibodies showed profound inhibition of the mixed lymphocyte reaction. Since the antibodies differ significantly in their in vivo effects on hematopoiesis, it is unlikely that the observed late marrow failure is related to an effect of MoAb H81.98.21 on AC.

The precise mechanism by which MoAb H81.98.21 prevents sustained hematopoietic engraftment is as yet unknown. We favor the hypotheses that MoAb H81.98.21 either interferes with the exchange of signals between pluripotent stem cells and AC or stromal cells needed to initiate stem cell replication, or that it initiates stem cell differentiation at the expense of replication by direct action on stem cells or cells providing stimulatory signals for differentiation. We arrived at these hypotheses by excluding other possible mechanisms. Nonspecific removal of antibody-coated marrow cells by the reticuloendothelial system was considered; however, since both marrow pretreated with antibody alone and marrow infused into dogs given 1 day of antibody treatment engrafted uneventfully, this explanation seems less likely. A more definitive rejection of this mechanism would need to include blockage of reticuloendothelial function by, for example, silica particles or carageenans. Our results also do not support canine C'-mediated lysis of stem cells. Similarly, inactivation of MoAb-coated marrow cells by dog anti-mouse immunoglobulin antibody does not seem to play a role in late graft failure. An ADCC-type mechanism seems unlikely: first, since MoAb H81.98.21 fails to produce ADC in vitro; second, because marrow incubated for 4 hours at 37°C with the antibody engrafted uneventfully; and finally, because early granulocyte engraftment from committed progenitor cells was seen in all antibody-treated dogs, although previous and current in vitro and in vivo data (dogs in group 11) have shown committed progenitors to express class II antigens. However, formal exclusion of an ADCC-type mechanism has to await experiments in which F(ab') fragments are substituted for whole MoAb H81.98.21.

If MoAb H81.98.21 acted by preventing replication of pluripotent stem cells, the replication block should be over by day 6 after transplantation, when MoAb can no longer be detected in marrow or blood. Subsequent initiation of stem cell replication should lead to sustained hematopoietic engraftment, although with a delay of 6 days over controls. However, engraftment was not seen, and marrow histologies at autopsy showed complete marrow aplasia. We reasoned that the stem cells might need replication signals through “accessory” mononuclear cells and, accordingly, infused autologous thoracic duct lymphocytes on day 6, but failed to see sustained engraftment. In contrast, infusion on day 6 of autologous marrow cells that were depleted of mononuclear AC by incubation with Leu-Leu-OMe, resulted in delayed but sustained engraftment. This result indicates that the marrow ME is functional on day 6 and capable of supporting the growth of newly injected stem cells. Implicit in this observation is the conclusion that the originally transplanted pluripotent stem cells have lost their replicative potential in response to MoAb infusion. We suggest that MHC class II molecules bearing the epitope recognized by MoAb H81.98.21, appear to be of functional importance in regulation of transplanted hematopoietic pluripotent stem cells, while the role of other
epitopes, eg, the one detected by MoAb B1F6, is as yet unknown.

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Late failure of autologous marrow grafts in lethally irradiated dogs given anti-class II monoclonal antibody

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