Mixed chimeric hematopoietic stem cell transplant reverses the disease phenotype in canine leukocyte adhesion deficiency

Kate E. Creevy a, Thomas R. Bauer Jr. a, Laura M. Tuschong a, Lisa J. Embree a, Andrew M. Silverstone a, John D. Bacher b, Chris Romines b, Julie Garnier b, Marvin L. Thomas III b, Lyn Colenda b, Dennis D. Hickstein a, *

a Experimental Transplantation and Immunology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA
b Office of Research Services, Veterinary Resource Program, National Institutes of Health, Bethesda, MD 20892, USA

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Abstract

The genetic disease canine leukocyte adhesion deficiency (CLAD) is characterized by recurrent, severe bacterial infections, typically culminating in death by 6 months of age. CLAD is due to a mutation in the leukocyte integrin CD18 subunit, which prevents surface expression of the CD11/CD18 leukocyte integrin complex. We demonstrate that stable mixed donor:host hematopoietic chimerism, achieved by a non-myeloablative bone marrow transplant from a histocompatible littermate, reverses the disease phenotype in CLAD. Donor chimerism following the transplant was demonstrated both by flow cytometric detection of donor-derived CD18-positive leukocytes in the peripheral blood of the recipient, and by the demonstration of donor-derived DNA microsatellite repeats in the peripheral blood leukocytes of the recipient. These results indicate that mixed hematopoietic chimerism reverses the clinical phenotype in CLAD and represents a potential therapeutic approach for the human disease leukocyte adhesion deficiency.

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1. Introduction

Canine leukocyte adhesion deficiency (CLAD) is an autosomal recessive, genetic immunodeficiency disease first described in Irish Setters (Renshaw et al., 1975), and later in Irish Red and White Setters (Debenham et al., 2002; Foureman et al., 2002). CLAD has been shown to be due to a single point mutation resulting in an amino acid substitution in the leukocyte integrin CD18. This mutation prevents the CD18 protein from forming a heterodimer with the individual leukocyte integrin CD11 subunits, thereby,
preventing cell surface expression (Giger et al., 1987; Kijas et al., 1999). The lack of CD11/CD18 surface expression in CLAD leads to a clinical syndrome of recurrent, life-threatening bacterial infections of such severity that, by 6 months of age, most CLAD dogs have died, or a decision has been made to euthanize them to prevent further suffering (Trowald-Wigh et al., 1992; Trowald-Wigh et al., 2000). A notable feature of the disease is that CLAD-affected dogs display a persistent and marked leukocytosis even in the absence of clinically-apparent infection (Trowald-Wigh et al., 1992).

In humans, the analogous disease to CLAD is leukocyte adhesion deficiency (LAD), which results from heterogeneous mutations in the leukocyte integrin CD18 subunit (Kishimoto et al., 1987). LAD has been categorized as severe or moderate, according to quantitative differences in expression of the leukocyte integrins on the cell surface (Anderson and Springer, 1987). The severity of the clinical complications in LAD correlates with the degree of deficiency (Anderson et al., 1985). Children with severe deficiency typically display <1% of normal levels of CD11/CD18, and frequently experience lethal bacterial infections within the first few years of life (Anderson and Springer, 1987). The clinical phenotype of CLAD is most similar to the severe deficiency form of LAD.

To date, myeloablative hematopoietic stem cell transplantation remains the only definitive therapy for LAD (LeDiest et al., 1989). The myeloablative regimen prior to hematopoietic stem cell transplant in LAD is designed to result in 100% donor chimerism. However, it is not clear that full donor chimerism is required for correction of the clinical phenotype in LAD. This question is relevant to the therapy of LAD because the toxicity associated with myeloablative transplant regimens has restricted the widespread use of stem cell transplantation in LAD. Recently, it has been suggested that a non-myeloablative transplant regimen might provide a less toxic and more effective approach to genetic leukocyte disorders, such as LAD (Horwitz et al., 2001).

In this study we describe the reversal of the disease phenotype in CLAD with the establishment of stable mixed donor:host hematopoietic chimerism using a non-myeloablative conditioning regimen, followed by the transplantation of dog leukocyte antigen (DLA) histocompatible littermate bone marrow cells. These results demonstrate that mixed donor:host chimerism is sufficient to reverse the disease phenotype in CLAD, and suggest this approach should be applicable to humans with LAD.

2. Materials and Methods

2.1. Dogs

Dogs were housed in facilities on the NIH campuses in Bethesda, MD and Poolesville, MD, which are approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animal Study Protocols were approved by the Institutional Animal Care and Use Committee of the National Cancer Institute, National Institutes of Health, Bethesda, MD.

The CLAD dog in this study resulted from the breeding of two CLAD heterozygotes derived from a breeding colony established from two Irish Setter carrier males (Creevy et al., 2003). All episodes of fever in the CLAD dog were treated with antibiotics. The CLAD dog was housed with his littermates until 4 weeks of age when he was separated from them for parenteral antibiotic and fluid therapy. Photographs were taken at several time points before and after transplant to document the clinical signs of CLAD. Diagnostic radiographs were taken as needed to investigate clinical symptoms.

2.2. Dog histocompatibility testing

Dog histocompatibility testing was performed as previously described (Wagner et al., 1996; Creevy et al., 2003).

2.3. Bone marrow harvest and transplantation

Prior to the infusion of donor bone marrow cells on the day of transplant, designated day 0, the recipient CLAD dog received total body irradiation (TBI) at a sub-lethal dose of 200 cGy delivered from a 60Co source. The dog was turned 180° halfway through the irradiation. TBI was administered 2 h before the donor cell infusion.

Bone marrow cells were harvested under general anesthesia from the histocompatible littermate donor on the day of transplant. For the collection procedure,
bone marrow was aspirated into heparinized syringes from the marrow cavities of the long bones and pelvis. The bone marrow aspirate was passed through 850, 500 and 200 μm filters connected in series using a commercial bone marrow collection kit (Fenwal, Baxter Healthcare Corp., Deerfield, IL). An aliquot of the bone marrow harvest was removed prior to the infusion to allow for quantitation of the nucleated cell number in the donor marrow and for assay of CD34-positive cell number as described later. The bone marrow cells were then infused into the recipient CLAD dog intravenously over 15 min.

Standard immunosuppression with cyclosporine (CSP) (Sandimmune, Novartis, East Hanover, NJ) and mycophenolic acid mofetil (MMF) (CellCept, Roche, Nutley, NJ) was administered (Storb et al., 1999). The CSP was given at a dose of 15 mg/kg orally twice daily from day -1 to day 35, then 7.5 mg/kg orally twice a day from day 36 to day 60. MMF was administered at 10 mg/kg orally twice daily from day 0 to day 28.

2.4. Laboratory studies

White blood cell (WBC) counts were performed on peripheral blood samples at a commercial laboratory (Antech Diagnostics, Lake Success, NY). Baseline WBC counts were measured prior to transplant and post-transplant at selected time points. Routine serum chemistries were performed monthly.

2.5. Flow cytometry

At the time of transplantation, the percentage and number of cells in the bone marrow harvest expressing the CD34 antigen were determined using a modification of standard assays (McSweeney et al., 1998). Bone marrow cells were incubated in ACK Lysis buffer (Biosource International, Los Angeles, CA) to remove the red blood cells, washed with PBS, and stained with a phycoerythrin (PE)-conjugated anti-canine CD34 antibody 1H6 (Pharmingen, San Diego, CA). The cells were then analyzed by flow cytometry on a Becton Dickinson FACS Caliber (San Jose, CA).

Following the transplant, peripheral blood leukocytes from the recipient were analyzed for CD18 expression by flow cytometry. Briefly, RBC were lysed and the leukocytes were stained with a FITC-labelled mouse anti-human CD18 monoclonal antibody (MHM23; Dako Corp., Carpinteria, CA) which cross-reacts with the canine CD18 molecule (Jacobsen et al., 1993). A FITC-labelled IgG1 isotype control antibody served as the negative control for staining (DAK-GO1; Dako Corp., Carpinteria, CA). T-lymphocytes were identified by staining with a mouse anti-canine anti CD3 antibody (CA17.2A12; Dako Corp., Carpinteria, CA) which cross-reacts with canine CD14 (Jacobsen et al., 1993). Neutrophils were identified by staining with an anti-neutrophil antibody (CAD048A; VMRD, Inc., Pullman, WA) labelled with Cy5 using a commercial kit based on succinimidyl ester amine modification chemistry (Amersham Biosciences, Piscataway, NJ). Dead cells were gated out using 7-amino actinomycin D (Sigma–Aldrich, St. Louis, MO).

2.6. DNA chimerism analysis

The percentage of donor:host chimerism following the transplant was also determined using DNA microsatellite repeat markers. These microsatellite or short tandem repeat (STR) markers were identified prior to transplant. A large number of STR markers, which were described previously as exhibiting a high degree of polymorphism in dogs, were screened to identify those which were polymorphic for this specific donor:host pair (Breen et al., 2001) (Canine Mappairs® Microsatellite Markers, Resgen, Huntsville, AL). An informative STR marker (FH2199) was selected for the donor and host dogs. DNA chimerism analysis was performed both on the total leukocyte population, and on leukocyte subsets sorted using a FACSVantage SE (Becton Dickinson Immunocytometry Systems, San Jose, CA). Genomic DNA was isolated from leukocytes or leukocyte subpopulations and amplified by PCR using a 6-Fam-end-labelled forward primer (Synthegen LLC, Houston, TX) and an unlabelled reverse primer. Products were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The chimerism was then calculated as the summed average of the heights.
and/or areas of the donor DNA PCR peaks divided by the average of the heights and/or areas of the summed donor plus host DNA PCR peaks. The CD3, CD14, and Neutrophil percentages were calculated from DNA PCR peaks from flow-sorted cells, respectively. The peak heights and areas were adjusted for PCR amplification bias by comparison to a 1:1 donor/recipient DNA mix.

3. Results

3.1. Bone marrow transplant

At 4 months of age, the CLAD dog received a bone marrow transplant from his histocompatible littermate donor. The bone marrow donor was identified as a tissue-type match using microsatellite repeats which are tightly linked to the class I and class II major histocompatibility loci (Wagner et al., 1996). The infusion of marrow cells was preceded by a non-myeloablative dose of 200 cGy TBI. The marrow infusion consisted of unfraccionated bone marrow containing 1.95 × 10^8 nucleated cells/kg of which 1.3% of cells were CD34-positive by flow cytometry. This resulted in a dose of 2.53 × 10^6 CD34+ cells/kg. Immunosuppression consisted of CSP and MMF as described (see Section 2).

3.2. Clinical phenotype of CLAD-affected dog

The CLAD dog exhibited symptoms typical of the disease with the early onset of severe infections including neonatal omphalitis. This initial episode responded to treatment with broad-spectrum antibiotics. Subsequently, episodes of fever, anorexia and lethargy continued and were accompanied by joint pain, lameness, metaphyseal swelling and radiographic lesions consistent with hypertrophic osteodystrophy (HOD) (Fig. 1, panels A and B). HOD is a clinical diagnosis manifesting as migratory metaphyseal pain, swelling, tenderness, fever and lameness (Abeles et al., 1999). Radiographic lesions provide definitive diagnosis of the syndrome, and include radiolucent metaphyseal intramedullary regions, with a radiopaque band bordering the physis (double physis sign), and flared metaphyses, with deposition of periosteal new bone in one or more concentric layers (periosteal cuffing sign) (Fig. 1). All episodes of fever, anorexia, lethargy, and lameness were treated symptomatically with non-steroidal anti-inflammatory drugs (NSAIDs), opioid analgesics, and intravenous fluids and antibiotics. On the day of bone marrow transplantation, the dog displayed marked lameness, fever, lethargy, and generalized pain.

By 2 weeks following the infusion of donor bone marrow cells, the CLAD dog became persistently afebrile, and displayed increased activity and appetite. He experienced one episode of fever, anorexia, lethargy,
and signs of HOD 12 weeks following the bone marrow transplant. However, this episode responded within 48 h to treatment with antibiotics, fluids, and NSAIDs. Since that single episode, the dog has been free from all clinical signs of CLAD. At more than 1 year post-transplant, he is fully-grown, active, and in good health. The HOD lesions have completely resolved clinically and radiographically (Fig. 1, panels C and D).

3.3. Peripheral white blood cell count

The infusion of donor bone marrow cells also resulted in the return of the elevated WBC count to the normal range (Fig. 2). CLAD-affected dogs typically display a profound leukocytosis characterized by a mature neutrophilia, even in the absence of clinically apparent infection (Trowald-Wigh et al., 2000). The CLAD pup displayed a leukocytosis during the pre-transplant period that reached a maximum of 60,000/µl (Fig. 2). Two weeks after the bone marrow transplant, the total peripheral WBC count decreased to 9200/µl.

The total WBC count and differential have remained within the normal range since transplant, except for one time point 7 weeks after transplant when the dog developed a mild, transient leukocytosis that was not associated with any clinical signs of disease.

3.4. Flow cytometric analysis of hematopoietic chimerism

Flow cytometric analysis of peripheral blood leukocytes was used to detect the CD18-positive leukocyte populations. Pre-transplant flow cytometric analysis revealed no CD18-positive cells in the peripheral blood of the CLAD dog (Fig. 3, upper left panel). Within 2 weeks following the infusion of donor cells, CD18-positive leukocytes were present in the peripheral blood, although the percentage of leukocytes that were CD18-positive represented only 1.57% (Fig. 3, upper middle panel). The number of CD18-positive leukocytes increased progressively, reaching a level of 10.5% of the total leukocytes by 1 month...
post-transplant (Fig. 3, upper right panel), and 23.3% of the total leukocytes by 6 months post-transplant (Fig. 3, lower left panel). The number of CD18-positive leukocytes then persisted in the 15–20% range through the subsequent 6 months (Fig. 3, lower middle and lower right panels) (Table 1). Of note, the percentage of CD18-positive T-lymphocytes remained higher than the percentage of CD18-positive neutrophils in the peripheral blood at all time points (Table 1), with levels of 51.8 and 10.9%, respectively, at the 12-month time point.

3.5. DNA analysis of hematopoietic chimerism

Donor:host chimerism was assessed by PCR of genomic DNA with a STR marker that differed between the donor and recipient. Both the total peripheral blood leukocyte pools, and leukocyte subpopulations sorted by flow cytometry, were analyzed. The STR marker assay performed on DNA extracted from total leukocytes and from leukocyte subpopulations correlated with the donor chimerism measured by flow cytometry (Table 1). DNA chimerism assays also supported the difference in the donor and host contribution to the myeloid and lymphoid compartments in the peripheral blood. The DNA chimerism assays demonstrated that the peripheral blood neutrophils were less than 10% donor-derived at 12 months, whereas the peripheral blood T-lymphocytes were approximately 45% donor-derived at this same time point.
4. Discussion

This study demonstrates that stable mixed donor:host hematopoietic chimerism sufficient to reverse the disease phenotype in a CLAD-affected dog was achieved using a non-myeloablative bone marrow transplant from a histocompatible littermate. In addition, the markedly elevated white blood cell count that serves as a hallmark of the disease also reverted to normal following the transplant.

There are a number of genetic disorders for which hematopoietic stem cell transplantation represents the definitive treatment. However, the toxicity of conditioning in a standard, myeloablative regimen represents a major deterrent to the widespread use of this approach in genetic diseases, such as LAD. The optimal therapy for LAD may consist of the creation of a state of mixed donor:host chimerism in which there is co-existence of donor and host lymphohematopoietic cells. In a study by Thomas and colleagues (1995) using myeloablative bone marrow transplantation to treat LAD, several patients became hematopoietic donor:host chimeras following the transplant. In that study, using a myeloablative conditioning regimen, the presence of greater than 20% granulocytes and monocytes post-transplant was sufficient to prevent infections. The chimeric state not only resulted in a reversal of the disease phenotype of LAD, but also appeared to be accompanied by considerably less graft-versus-host disease than full donor engraftment exhibited by other patients in the study (Thomas et al., 1995). Although the precise number of CD18-positive neutrophils required to reverse the disease phenotype in LAD is not known, it does appear that complete hematopoietic replacement by donor cells is not required for effective therapy. Thus, in LAD the best regimen to generate stable, long-term chimerism has not been identified, nor has the level of chimerism necessary to reverse the disease phenotype been established.

To determine the feasibility of mixed chimerism as a therapeutic approach to LAD, we conducted a histocompatible littermate bone marrow transplant in a CLAD pup using non-myeloablative conditioning and a brief post-transplant immunosuppressive regimen similar to that described previously in normal 6–12-month-old dogs (Storb et al., 1999). In this regimen, a short course of CSP and MMF allowed the TBI dose to be lowered from the lethal range to the sub-lethal, non-myeloablative dose of 200 cGy. Normal adult dogs transplanted with this regimen became stable, long-term mixed hematopoietic chimeras. In our study, this low-dose irradiation regimen resulted in a state of mixed chimerism in a transplanted CLAD dog, which resulted in complete reversal of the disease phenotype, including the episodes of HOD. There was an accompanying normalization of the major laboratory indicator of disease, the elevated white blood cell count. While the initial decrease in the white blood cell count post-transplant may be attributable to irradiation, the persistent normalization of the white blood cell count over the ensuing months correlated with the presence of CD18-positive donor neutrophils in the peripheral blood.

The percentage of donor hematopoietic chimerism post-transplant was evaluated both by flow cytometry

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<td>12 months</td>
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*a Proportion of donor cells in the host’s peripheral blood as measured by flow cytometry or DNA content

*b Chimerism calculated as the percentage of cells expressing CD18 among total peripheral blood leukocytes (WBC), CD3, CD14, or anti-neutrophil immunostained cells where noted.

Table 1
Mixed donor:host hematopoietic chimerism after matched littermate bone marrow transplant

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and by DNA analysis of STR markers. The percentage donor chimerism measured by flow cytometry correlated with the measurement of percentage donor chimerism using DNA analysis of the STR markers. Both analysis techniques showed that peripheral blood leukocyte subpopulations exhibited different levels of CD18 positivity. Approximately 50% of the peripheral blood T-lymphocytes were donor-derived, whereas the neutrophil compartment in the peripheral blood was only approximately 10% donor-derived at 1 year post-transplant by flow cytometric analysis. Rather than reflecting split chimerism in engraftment of the lymphocyte and myeloid compartments, this difference may reflect the selective egress of the CD18-positive neutrophils into the tissues. In addition, the fact that cells of the myeloid compartment have relatively short lifespans, typically a few days, whereas the lifespans of cells of the lymphoid compartment, particularly T-cells, are typically measured in years, might also contribute to the split chimerism. This question remains an area of active investigation. However, the presence of donor-derived myeloid cells in the peripheral blood at 1 year post-transplant also provides strong proof of the engraftment of donor stem cells.

The successful induction of stable mixed hematopoietic chimerism in the CLAD dog in this report, and the subsequent reversal of the disease phenotype, demonstrates the promise of this technique for human LAD patients, as well as the utility of this canine model.

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three patients with the leukocyte adhesion deficiency. Blood 74, 512–516.


