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BRIEF COMMUNICATION

Effect of Human Cord Blood Transfer on Survival and Disease Activity in MRL-*lpr/lpr* Mice

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The MRL-*lpr/lpr* mice have a genetic defect and by 6 months of age usually die after developing severe autoimmune disease and massively enlarged lymph nodes (Cohen, P. L., and Eisenberg, R. A., *Annu. Rev. Immunol.* 9, 243–269, 1991). Similarly as in some genetic diseases in humans, these mice, if given an appropriate marrow transplant from a mouse not having the defect, will have partial correction of the disease process and an extension of life (Cohen, P. L., and Eisenberg, R. A., *Annu. Rev. Immunol.* 9, 243–269, 1991; Lenarsky, C., Kohn, D. B., Weinberg, K. I., and Parkman, R., *Hematol. Oncol. Clin. N. Am.* 4, 589–603, 1990). Utilizing human umbilical cord blood as a donor source for marrow transplantation, we have been able to obtain significant correction of the MRL-*lpr/lpr* genetic defect and double the life span. By 11 months of age, 5 months beyond their usual life span, both the animals receiving congenic marrow (MRL-+/+) and the animals with human cord blood had mild lymphadenopathy, a decrease in double-positive T cells, and an increase in double-negative T cells. Granulomatous vasculitis could be identified in the animals receiving human cells and could not be found in the animals receiving MRL-+/+ marrow.

INTRODUCTION

The possibility of using transplantation of bone marrow to correct genetic defects goes back to the early days of marrow transplants (2). The possibility of using human cord blood for clinical and experimental marrow transplantation was suggested in 1972 (3). Recently with the use of human cord blood we have been able to have mice survive lethal irradiation (900 cGy) (4) and also survive the combination of both irradiation (800 cGy) and chemoablation (5). The survival of the animals in these studies was equal to or better than that of the controls that received syngeneic mouse marrow (6).

We have postulated in these cases that the human cells serve as a temporary graft or bridge, thus allowing the mice to survive the immediate lethal effect of the therapy and permitting the animals' own marrow to recover. We have, however, found evidence of human DNA persisting in these animals many months afterward (4). Consequently, we have attempted to determine what effect human cord blood would have on MRL-*lpr/lpr* mice that have a genetic defect and a severe autoimmune disease.

The MRL-*lpr/lpr* mice have a 50% mortality due mainly to renal disease and vasculitis (1). These mice have progressive enlargement of lymph nodes, moderate splenic enlargement, and proliferative glomerulonephritis mainly due to the disposition of anti-DNA antibodies. The MRL-*lpr/lpr* mice may develop mild arthritis with erosive synovitis and hypergammaglobulinemia, particularly IgG. These mice serve as a murine model of autoimmunity, particularly systemic lupus erythematosus, with certain SLE-specific autoantibodies such as anti-SM, anti-P, and anti-DNA antibodies. However, the T-cell defects, particularly excessive lymphoproliferation, may be dissociated from autoantibody production (1). The defect in MRL-*lpr/lpr* is due to a genetic abnormality in *fas* expression, a molecule involved in apoptosis (7).

From published material, whole-body irradiation and syngeneic marrow (MRL-*lpr/lpr*) transplants do not significantly (from 1–2 months) improve survival of these animals (8), while congenic marrow (MRL-+/+) results in a partial correction of the defects (8).

The MRL-+/+ congenic mice still carry the genetic defect, and they develop a much later onset of autoimmune disease and lymphadenopathy than MRL-*lpr/lpr* mice. Thus, if MRL-*lpr/lpr* mice are given a marrow transplant from congenic MRL-+/+ mice, they still have evidence of the disease, but with a marked decrease in the disease process and a significant prolongation of life. This is a preliminary report utilizing human cord blood on MRL-*lpr/lpr* mice.

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MATERIALS AND METHODS

Animals

Five-week-old MRL-*lpr/lpr* female mice obtained from Jackson Memorial Labs were irradiated (900 cGy) and given human cord blood cells and anti-natural killer cell antisera, congenic MRL-+/+ BM, or nothing.

Human Cord Blood

Human umbilical cord blood (20–40 cc) was collected directly into 8 cc of CPD (citrate, phosphate, dextrose) anticoagulant and stored at room temperature for less than 24 hr. Ficoll–Hypaque density gradient centrifugation was used to isolate mononuclear cells from the whole cord blood.

Bone Marrow

The donor bone marrow was obtained from the femur of 5-week-old MRL-+/+ female mice. Cell viability was assessed by trypan blue dye exclusion.

Irradiation

All animals received 900 cGy of irradiation from the 8-MeV X-ray beam of a linear accelerator (Philips SL 75-20) using a 25 × 25-cm field with a rate of about 400 Gy/min.

Antisera

Approximately 8 hr prior to irradiation, the animals received 100 μl of rabbit anti-Asialo Gm antisera. Injection of 100 μl of this antibody iv depletes NK effector cells, as measured by the inability of splenic effector cells to cause lysis of the NK-susceptible target cell YAC-1, for a period of 2 weeks after injection (6).

Transfer Procedure

The recipient MRL-*lpr/lpr* mice receiving human cord blood, within 2 hr of irradiation, were adoptive transferred with 18.3×10^6 human cord blood mononuclear cells, half given intravenously and half intraperitoneally. One day later these animals received a second transfer of 3×10^6 human cord blood mononuclear cells intraperitoneally. In the recipient MRL-*lpr/lpr* mice receiving MRL-+/+ bone marrow, within 2 hr of irradiation, they received 17×10^6 nucleated bone marrow cells obtained from MRL-+/+ congenic donors, half given intravenously and half intraperitoneally.

At various times post-transfer, surviving recipients were bled and killed and thymus, lymph node, and spleen were removed to obtain single-cell suspensions. Nucleated cells were counted with a Coulter counter.

Flow Cytometry

Cells (1×10^6) were stained with phycoerythrin or FITC-labeled mAbs (anti-mouse monoclonal antibodies anti-CD4, anti-CD8, or anti-CD3 (Caltag, San Francisco, CA)).

Dual staining (anti-CD4, anti-CD3; anti-CD8, anti-CD3; or anti-CD4, anti-CD8) was performed and appropriate isotype controls were employed. The number of $CD4^-$ $CD8^-$ (DN) T cells was determined by flow cytometric analysis of dual-stained cells. The number of T cells which were DN T cells was obtained by determining the ratio of $CD4^-/CD8^-$ cells to the number of $CD3^+$ cells (total T cells). Cells exhibiting morphologic characteristics of live lymphocytes as assessed by forward and 90° scatter parameters were gated and analyzed on a FACScan (Becton–Dickinson, Mountain View, CA). The numbers reported are a percentage of that observed in nontreated MRL-*lpr/lpr* mice at 5 months of age (% CTR, Fig. 1).

Anti-ss DNA

Sera were analyzed for the presence of anti-ss DNA antibodies. Anti-DNA IgG antibodies were detected with an enzyme-linked immunosorbent assay using single-stranded DNA (10 mg/ml)-coated plates. Positive and negative sera were employed. The optical density was determined by measuring absorbance at 450 nm.

RESULTS

Of eight animals receiving human cells, three (37%) survived lethal irradiation; of eight MRL-*lpr/lpr* receiving MRL-+/+ congenic mouse bone marrow, five survived lethal irradiation (63%). Of two animals receiving 900 cGy of irradiation and no further treatment, none survived (0%). Five controls received no irradiation or treatment.

Animals receiving only irradiation were dead by 3 weeks. By 6 months of age the MRL-*lpr/lpr* control mice (untreated and with no radiation) developed massive lymphadenopathy (average 349 mg, per lymph node), microscopic evidence of vasculitis, and electron-dense deposits in the kidneys. By comparison, at 5 months of age (4 months post-transfer), the irradiated animals that received human cord blood or bone marrow from MRL-+/+ mice showed no lymphadenopathy and no evidence of vasculitis. Similar findings were noted at 8 months postirradiation. Evidence of glomerulonephritis with subendothelial electron-dense deposits was present in both the animals receiving human cord blood and the animal receiving congenic marrow at 8 months post-transfer.

At 10 months post-transfer (approximately twice the normal life span of these animals), there was some lymphadenopathy in the animals that received human

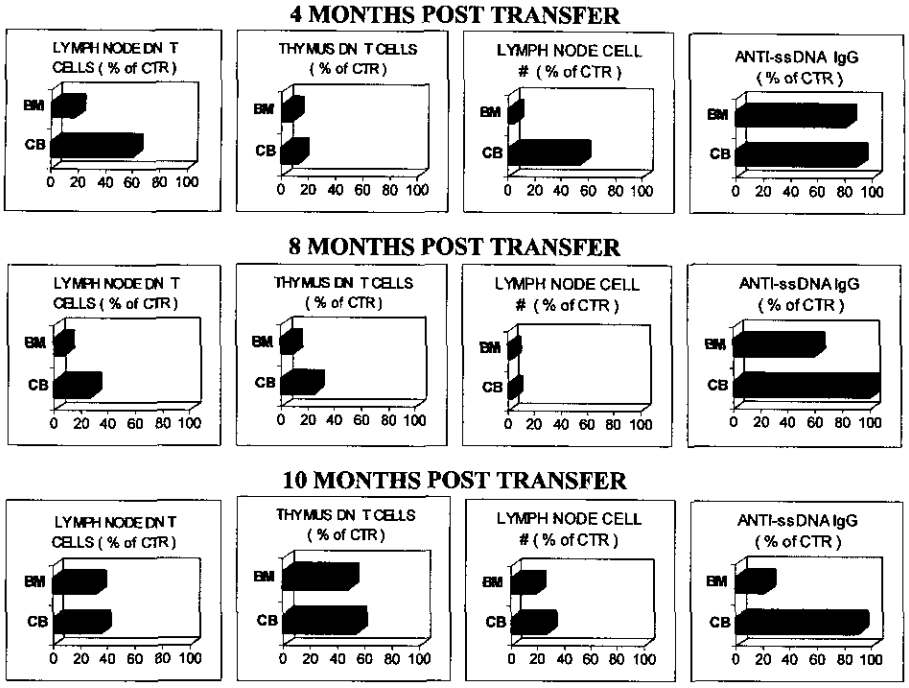

FIG. 1. Analysis of MRL-*lpr/lpr* recipient mice at various times postrepopulation. Double-negative T cells and cell numbers in the lymph nodes of MRL-*lpr/lpr* recipient mice and serum levels of anti-ss DNA IgG antibodies were compared to control MRL-*lpr/lpr* values. The data are presented as a percentage of that observed in control nontreated MRL-*lpr/lpr* cells and sera obtained at 5 months of age. MRL-*lpr/lpr* mice were irradiated and reconstituted with MRL-+/+ bone marrow (BM) or human cord blood (CB). The mice were killed at 4, 8, and 10 months post-transfer. Axillary lymph node cell values were determined and compared to that observed in MRL-*lpr/lpr* mice (5 months of age). The presence of double-negative ($CD4^-$,$CD8^-$) T cells (DN T cells) determined by flow cytometric techniques was compared to that observed in MRL-*lpr/lpr* animals 5 months of age. The recipient mice were bled prior to death and the sera were analyzed for the presence of anti-ss DNA antibodies of the IgG isotype. These values were reported as a percentage of anti-ss DNA observed in the sera of 5-month-old MRL-*lpr/lpr* mice. CTR, MRL-*lpr/lpr* 5-month-old control; DN, CD4,CD8 double-negative T cell.

cord blood, while the animals receiving bone marrow from MRL-+/+ mice had only slight enlargement. A granulomatous vasculitis could be identified at 10 months in the animal receiving human cord blood but not in animals receiving congenic marrow.

Autoimmune Disease and Lymphadenopathy

The recipient MRL-*lpr/lpr* mice were evaluated for their lymphadenopathy and autoimmune disease at 5, 9, and 11 months of age (4, 8, and 10 months postirradiation and transplant). Since MRL-*lpr/lpr* rarely survive beyond 6 months, age-matched comparison could not be made with the 8- and 10-month post-transplantation animals. Therefore, the disease progression in the transplanted animals was compared to that in MRL-*lpr/lpr* mice at 5 months of age (Fig. 1).

The most immature cell in the thymus is considered to be the double-negative $CD4^-CD8^-$ T cell which gives rise to the double-positive T cells (the major type of thymocyte). Neonatal thymectomies in MRL-*lpr/lpr* mice eliminated the accumulation of double-negative T cells and lymphadenopathy and greatly increased survival (9–11).

The lymphadenopathy noted in MRL-*lpr/lpr* is due mainly to the accumulation of $CD4^-CD8^-CD3^+$ double-negative (DN) T cells (1). Analysis of lymph node and thymus of recipient animals for cell number and presence of double-negative T cells as a percentage of that observed in control MRL-*lpr/lpr* mice (at 5 months of age) is shown in Fig. 1. Double-negative T cells and lymph node cell number in the animals receiving cord blood were found to be somewhat higher than those receiving congenic marrow, but both were considerably less than the untreated controls of 5 months. Although there is a slight elevation of cell number in lymph nodes in cord blood recipients compared to +/+ bone marrow recipients, the cord blood animals' values are greatly reduced in comparison to 5-month-old MRL-*lpr/lpr* control animals.

Anti-ss DNA levels remained comparable between the two types of recipients and the control MRL-*lpr/lpr* at 4 months post-transfer. At 10 months post-transfer, the +/+ bone marrow recipients' serum anti-DNA levels were reduced in comparison to the human cord blood recipient and control animals.

In the normal adult mouse, the thymus consists predominantly of double-positive T cells with a small percentage (<5%) of double-negative T cells. The MRL-*lpr/lpr* mice as they approach the end of their life span

(5–6 months) show an increase in double-negative cells in the thymus (Fig. 2). At the same point in time (4 months post-transplantation) the recipient MRL-*lpr/lpr* of both human cord blood and bone marrow (MRL-++) had very few double-negative T cells, but a predominance of double-positive T cells (CD^+CD8^+) similar to that seen in the normal adult thymus. At 9 months of age (8 months post-transfer) the MRL-*lpr/lpr* recipients of human cord blood differ substantially from the recipients of MRL-+/+ bone marrow such that their thymic subpopulations are greatly reduced in the percentage of double-positive T cells. By 11 months of age (10 months post-transplant) both recipient mice of cord blood and MRL-+/+ bone marrow had a decrease in double-positive cells in the thymus and an increase in double-negative cells. This finding is also consistent with moderate lymphadenopathy noted in both bone marrow and cord blood recipients at 11 months of age. Thus, human cord blood was as effective as repopulation with congenic bone marrow in retarding the accumulation of double-negative thymocytes, typically found in 5-month-old MRL-*lpr/lpr* mice.

DISCUSSION

The overall findings indicate that human umbilical cord blood given to mice with a severe genetic defect was able to control the disease process and extend the duration of life almost as well as the animals receiving congenic mice bone marrow transplant.

For many years there have been scattered reports of human genetic defects modified or cured by means of allogeneic transplants of bone marrow and fetal liver (2). Total lymphoid irradiation has long been felt to have potential application in the treatment of certain autoimmune disease (12). While low-dose irradiation is felt to have a beneficial effect, whole-body irradiation alone on MRL-*lpr/lpr* followed by receipt of a syngeneic marrow (*lpr/lpr*), however, has not significantly prolonged the life span or significantly altered the disease of MRL-*lpr/lpr* mice (8).

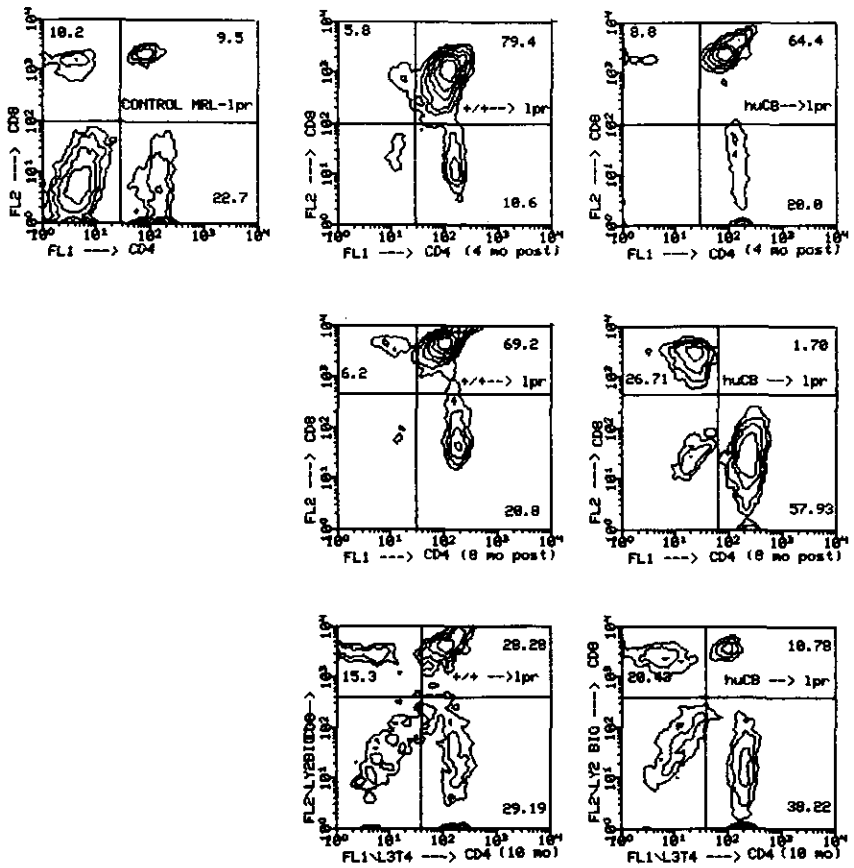

FIG. 2. Flow cytometric analysis of thymocytes from MRL-*lpr/lpr* control and recipient mice. Flow cytometric analysis of thymocytes from either MRL-*lpr/lpr* control at 5 months of age or irradiated reconstituted MRL-*lpr/lpr* recipients killed at various times post-transfer. The percentage of single-positive cells (either $CD4^+$ or $CD8^+$) as well as the percentage of double-positive cells ($CD4^+$ and $CD8^+$) is indicated in the appropriate quadrant. (Top) Histograms obtained from the thymus of 5-month-old mice (either nontreated control or recipients 5 months of age but 4 months post-transfer); (middle) histograms of stained cells obtained from the thymus of recipients 8 months post-transfer; (bottom) histograms from recipients 10 months post-transfer. The *X* axis is increasing CD4 expression and the *Y* axis is increasing CD8 expression.

By the technique described herein, using lethal irradiation of another species of mice (SJL/J) and reconstitution with human cord blood treated with anti-natural-killer cell antisera, we usually obtain a survival of 70% (6). The lower survival rate of MRL-*lpr* mice, when comparing SJL/J mice with irradiated MRL-*lpr/lpr* mice reconstituted with human cord blood, may be due to the fact that MRL mice may be more sensitive to irradiation than the SJL/J. Currently we have not attempted to optimize reconstitution in MRL mice. Certainly, increased survival in irradiated recipients is of considerable significance if applied to the clinical situation. With an increase in cell numbers, however, and the use of cord blood buffy coat cells we have been able to increase survival of SJL/J mice to 85% (13).

Initially we expected the changes that occurred in the mice receiving human cord blood to be temporary and, after a limited period of time, that the MRL-*lpr/lpr* mice would return to their usual disease state with the recovery of their own marrow, similar to what occurs in mice receiving a syngeneic marrow (6). Human umbilical cord cells would serve only as a temporary bridge during the period of reconstitution, as we have noted in SJL/J mice (4, 6). The long-lasting modification of disease in the MRL-*lpr/lpr* human cord blood recipients was unexpected. From previous studies we have been able to identify the presence of minute quantities of human DNA in SJL/J mice over 6 months after lethal irradiation and human cord blood infusion (4). It is possible that in these MRL-*lpr/lpr* human cord blood recipients, some of the most primitive of the immature human pluripotential stem cells (Berashis cells) (14) may continue to exist and function in the postirradiated mouse, producing enough corrective factors to significantly modify the animal's illness over a prolonged period.

The lymph nodes and spleen of the MRL-*lpr/lpr* mice with age develop progressive infiltration of $CD4^-CD8$ Thy-1^+ cells (DN). The double-negative population predominates in the normal neonatal thymus and is present as only a small percentage (3–5%) of the adult thymus. During thymic development, the double-negative population differentiates into double-positive T cells, which then constitute the majority of the adult murine thymocytes. These double-positive cells can then terminally differentiate into single-positive ($CD8^+$ or $CD4^+$) mature T cells. Conversely, the adult MRL-*lpr/lpr* (5 months of age) thymus contains predominantly double-negative T cells. In contrast to the findings in MRL-*lpr/lpr* mice, the thymus of recipients of human cord blood, at 4 months post-transfer, had very few double-negative T cells present, and the thymus consisted mainly of double-positive T cells. This thymocyte population is similar to that observed in MRL-*lpr/lpr* reconstituted with MRL-+/+ bone marrow and to that reported for the normal murine thymus. Interestingly, at 8 months post-transfer (9 months of age), the recipients of human cord blood began to show an abnormal thymocyte population with a marked decrease in double-positive T cells not seen in MRL-*lpr/lpr* recipients reconstituted with MRL-+/+ bone marrow. However, at 10 months post-transfer (11 months of age), both human cord blood and MRL-+/+ bone marrow recipients demonstrated the presence of an increased population of double-negative T cells in the thymus.

These findings indicate that the human cord blood was able to retard the development of double-negative T cells. The loss of double-positive T cells in the thymus of a cord blood recipient at 9 months of age appears to be due more to the terminal differentiation of these cells into $CD4^+$ or $CD8^+$ (single-positive) T cells, rather than the failure of the double-negative precursor to progress to the double-positive T cell. It is unclear if the transient population of double-positive T cells in the thymus at 4–5 months post-transfer in the human cord blood recipients represents abnormal double-positive T cells which have failed to undergo apoptosis (15) or comprises the normal population of double-positive T cells observed in the adult thymus.

Overall, these data appear to show a disappearance of double-positive T cells from the thymus followed by an increase in the percentage of double-negative T cells more rapidly in the human cord blood recipient than in the recipient of MRL-*lpr/lpr* bone marrow. However, while the human cord blood recipient appeared less competent than the MRL-+/+ bone marrow recipient in maintaining the normal thymic population, the transfer of human cord blood was comparable to MRL-+/+ bone marrow in retarding the development of the lymphoproliferative disorder in MRL-*lpr/lpr* mice.

Finally, the effect of irradiation alone cannot be totally excluded. As previously noted, low-dose irradiation is felt to modify and prolong the survival of these animals and whole-body lethal irradiation with syngeneic marrow transplant only increased survival by 1–2 months (8). Alternatively, a very small number of differentiating cord blood pluripotential cells of human origin may serve as a chronic source of antigenic stimulation which may be immunosuppressive. Previous studies have shown that human cord blood can be immunosuppressive (16).

Our own studies have indicated that marrow transplants may be successful with the use of human cord blood unmatched for HLA (3). San Dong University in China has recently reported successful long-lasting chimera established with the use of human cord blood not matched for HLA (17, 18). Although there is a considerable gulf between temporarily suppressing an autoimmune disease in an imbred strain of laboratory mice and the treatment of a specific genetic defect in human, the preliminary study reported here strongly suggests the possibility of using unmatched human

cord blood in place of bone marrow for transplantation in producing a significant clinical effect on human genetic disease involving the immune or hematopoietic system.

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