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Nonmyeloablative Bone Marrow Transplantation of BXSB Lupus Mice Using Fully Matched Allogeneic Donor Cells from Green Fluorescent Protein Transgenic Mice

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Male BXSB mice, a mouse model of systemic lupus erythematosus, were given bone marrow transplants (BMT) at 20 wk of age using MHC-matched donor cells and nonmyeloablative conditioning (550 cGy irradiation). Transplanted mice and irradiation controls were followed for a period of 20 wk. Mice transgenic for green fluorescent protein were used as donors to allow tracking of donor cells and a determination of chimerism. Radiation controls had reduced renal pathology at 10 wk posttransplant, but not at 20 wk compared with untreated mice, while nonmyeloablative BMT mice had significantly reduced pathology at both time intervals. The monochorionic characteristic of older BXSB mice was also reduced by BMT, but the treatment did not prevent production of Ab to dsDNA. A stable chimerism of 24–40% donor CD45-positive cells was achieved in spleen and bone marrow, and there was no evidence of clinical graft vs host disease. Donor cells were detected in most recipient organs, notably the thymus and renal glomeruli. The results suggest that complete depletion of mature lymphocytes or of progenitor stem cells is not required to control lupus nephritis in BXSB mice. *The Journal of Immunology*, 2004, 172: 5415–5419.

BXSB is a well-characterized mouse model that spontaneously develops autoimmune disease similar to human systemic lupus erythematosus (1, 2). The disease is associated with positive serology to self Ags (dsDNA, ssDNA, anti-erythrocyte, and platelet Abs) and immune complex-mediated nephropathy. Males are affected more severely than females due to the Yaa gene (Y chromosome-associated accelerator gene) that potentiates the Ab responses to Ags with weak affinity (3, 4). Immune complex-mediated glomerulonephritis is the hallmark of disease in BXSB mice. Histopathological changes are evident by 10 wk of age, leading to end stage renal disease and 70% mortality by 40 wk of age (5). Male BXSB also develop a characteristic monocytosis that escalates over time (6). Although the relationship of this monocytosis to disease progression is not well understood, a decrease was associated with reduced renal injury (7).

Historically, BXSB was one of the first mouse models of systemic lupus erythematosus to be treated by allogeneic bone marrow transplantation (BMT) (8). It was found that lethally irradiated old male BXSB (H-2b) transplanted with bone marrow cells from young athymic (nu/nu) BALB/c (H-2b) achieved long-term survival compared with untreated mice, a marked improvement of renal pathology, and a significant decrease in anti-dsDNA Ab titers at 12 wk post-BMT. Attempts at treatment of other BXSB mice with syngeneic BMT were unsuccessful. A second approach for treatment of disease in BXSB included generation of a stable mixed chimerism (9). A mixture of healthy allogeneic (H-2d) and age-matched syngeneic bone marrow cells (3:1) was infused into lethally irradiated aged BXSB male mice. This approach produced 50% donor chimerism and reversal of the renal disease along with diminished anti-dsDNA titers when tested 34 wk posttransplant. Using this approach, it was also possible to prevent lupus activity when the treatment was applied to young BXSB males (10). Mixed chimerism also improved immunocompetency compared with that of lethally irradiated mice that were only given allogeneic stem cells. These results suggest improvement in disease can occur even in the presence of disease-prone host stem cells. However, the complete ablation of host bone marrow that was used could limit the application of this approach for some human patients. In the current studies, chimerism was generated by nonmyeloablative bone marrow transplantation using low dose total body irradiation and fully MHC-matched healthy donor cells from green fluorescent protein transgenic (GFP-Tg) mice (11). The results suggest that complete depletion of mature lymphocytes or of progenitor stem cells is not required to control lupus nephritis.

**Materials and Methods**

**Mice**

Male BXSB (H-2b) bred in our facilities from stock obtained from The Jackson Laboratory (Bar Harbor, ME) (stock 000740) were the recipient mice. The donor mice were C57BL/6 GFP-Tg (H-2b) bred from Jackson stock (stock 003291).

**Nonmyeloablative BMT**

Twenty-week-old male BXSB mice were conditioned by 550 cGy of nonlethal irradiation 4 h before an i.v. (tail vein) infusion of unfractionated bone marrow cells (1 × 107/mouse) obtained from femurs of 10-wk-old male GFP-Tg mice. Ten or 20 weeks later, blood and tissues were collected.
Histopathology

Tissues were fixed in 10% buffered formalin, and 3-μm sections were processed for staining by H&E or periodic acid-Schiff. Kidney pathology was blindly scored as 0–4 (0 = no pathology, 4 = severe pathology) using a system modified from that described previously (9). Replicate unstained sections were examined with a UV microscope for cells expressing GFP using an autofluorescence occlusion method (A. Steele, manuscript in preparation). Briefly, slides of paraffin-embedded tissue sections were treated with monochromic blocking wash composed of sodium borate, azure II, and toluidine blue in an alcohol-free solution and mounted with nonfluorescent medium. Fluorescent green signal was of sufficient intensity to discern morphology and localization when background autofluorescence noise was occluded in this manner. Results were confirmed by collateral examination of replicate sections stained with anti-GFP mAb (Santa Cruz Biotechnology, Santa Cruz, CA). Some sections were further stained using mAb to CD45 (Santa Cruz Biotechnology). Primary Abs were localized using a mouse-on-mouse kit (Vector Laboratories, Burlingame, CA). Tissues from GFP-Tg mice served as control material.

Flow cytometry

Single cell suspensions of blood, spleen, lymph nodes, thymus, and bone marrow from BXSB, GFP-Tg, or transplanted mice were stained with fluorochrome-conjugated mAbs (BD Biosciences, San Diego, CA) for surface markers: pan leukocytes (CD45), B cells (B220), T cells (CD4, CD8, TCR β-chain, TCRδ), monocytes/macrophages (CD11b), and granulocytes (Gr1). Cell suspensions were fixed with paraformaldehyde and analyzed with a four-color BD FACSCalibur flow cytometer. GFP-positive cells were estimated using the FITC gate, and percentage of engraftment was calculated from the ratio of percentage of FITC positivity of transplanted mice compared with that found for the same tissues from GFP-Tg mice.

Serum autoantibodies

Abs to dsDNA were measured using ELISA kits (Alpha Diagnostic, San Antonio, TX), as described by the company (serum diluted 1/100). The assays used a solid-phase coated with dsDNA and a peroxidase-labeled anti-mouse Ig detection system. Each kit contained positive and negative controls, and the use of the same positive controls in separate assays allowed semiquantitative estimates of dsDNA Ab proportional to the OD (OD450).

Statistics

Groups were compared using Student’s t, and p values <0.05 were considered significant.

Results

Histopathology

Renal pathology scores are summarized in Table I. There was improvement (lower score) in both irradiation controls and transplanted mice at 10 wk posttransplant (30 wk of age) compared with untreated BXSB mice. By 20 wk posttransplant (40 wk of age), continued improvement was observed only in transplanted mice, while irradiation controls had reverted to the characteristic diseased state. Although some untreated mice had a higher renal score at 30 wk than at 40 wk, this difference is somewhat artificial because more than half of such mice die of renal disease by 40 wk of age and there is no evidence of spontaneous recovery (5). There was a significant difference between irradiation controls and BMT mice at 40 wk of age (p < 0.01). None of the transplanted mice showed evidence of clinical graft-vs-host disease (GVHD) such as skin rash or diarrhea, and at necropsy, none had evidence of accumulation of inflammatory cells in the liver. In addition, spleen cells from BXSB and GFP-Tg did not respond to each other in mixed lymphocyte cultures, while both responded to third party cells (C3H or BALB/c; data not shown).

Fig. 1 compares representative renal sections from untreated BXSB and GFP-Tg mice, irradiation controls, and transplanted mice at indicated time intervals. The renal profile of donor GFP-Tg mice (A) was normal. BXSB mice showed a worsening renal profile over time (B–D). At 20 wk (B), there were loss of capillary loop architecture, membranous thickening, hypercellularity, and increased mesangial matrix. By 30 wk (C), there were segmental sclerosis and focal inflammatory infiltrates progressing to global glomerular sclerosis by 40 wk (D). Irradiation controls (550 cGy only) had less renal pathology compared with untreated BXSB at 30 wk (E) with decreased membrane thickening and discernible capillary lumens. By 40 wk (F), however, the pathology was similar to that of B. At 30 wk, transplanted mice (G) had discernible capillary loops and normal appearing membranous profiles and

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age (weeks)</th>
<th>Number Examined</th>
<th>Average ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>30</td>
<td>6</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>6</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>550 cGy</td>
<td>30</td>
<td>3</td>
<td>0.5 ± 0.4†</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>550 cGy + BMT</td>
<td>30</td>
<td>7</td>
<td>0.6 ± 0.4†</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>6</td>
<td>0.8 ± 0.5†</td>
</tr>
</tbody>
</table>

* For treated groups, 30 and 40 wk are 10 and 20 wk after treatment.
† Injected i.v. with 1 × 10⁹ unfractionated bone marrow cells from GFP-Tg mice 4 h after the nonmyeloablative irradiation.
‡ Value of p < 0.02 compared with untreated control of same age group.

FIGURE 1. Periodic acid-Schiff-stained sections of kidney (all at ×10). A, Untreated GFP-Tg mouse (20 wk old); B, untreated BXSB mouse (20 wk old; this is age at time of treatment); C, untreated BXSB (30 wk old); D, untreated BXSB (40 wk old); E, irradiation control BXSB (30 wk old, 550 cGy); F, irradiation control (40 wk old); G, BMT-transplanted BXSB (30 wk old: 10 wk posttransplant); H, BMT BXSB (40 wk old; 20 wk posttransplant). A, Shows normal renal tissue; B–H, exhibit various degrees of pathology, as described in the text.
mesangial matrix. Even at 40 wk (H) there remained marked improvement with discernible capillary loops and no evidence of sclerosis.

Direct fluorescent microscopic examination of paraffin-embedded tissues after immunohistochemical staining or autofluorescence occlusion treatment (Fig. 2) showed presence of GFP-tagged donor cells in the renal tissue (Fig. 2A) and spleen (Fig. 2, A (inset) and D) of transplanted BXSB mice. In the kidney, donor cells were concentrated in the glomeruli (arrows, Fig. 2A). These cells morphologically resembled stromal (mesangial) cells (Fig. 2C) and were CD45 negative (Fig. 2B). When stained sequentially for CD45, followed by autofluorescent occlusion, these cells were individually positive for GFP. Infrequently, CD45-positive cells were observed within the tubular interstitium, but were negative for GFP reactivity. Numerous cells in spleen were CD45 positive (inset, Fig. 2B) and presumed to be of both donor and recipient origin. The absence of GFP-positive cells in untreated BXSB mice is obvious (Fig. 2, insets, C and D). GFP-positive cells were also detected in liver, thymus, and bone marrow of transplanted recipient mice (data not shown). In tissues of GFP-Tg donor mice, virtually all cells, except RBC, were green, as expected.

Chimerism

The percentage of chimerism of transplanted mice was stable over the 20-wk observation period, and was most prominent in thymus tissue, as summarized in Table II. In comparison, two mice given BMT after lethal irradiation had chimerism of >95% CD45-positive donor cells in bone marrow and 82% in spleen at 20 wk posttreatment.

Monocytosis

The monocytosis characteristic of BXSB mice was significantly decreased in spleen and lymph nodes 20 wk after transplantation (Table III). It is of interest that an average of >76% of these cells was of recipient origin. The percentiles of total T (CD4+ plus CD8+) and B (B220+) cells in the spleens (25.2% T; 40.2% B) and lymph nodes (40.7% T; 34.9% B) of transplanted mice were similar to untreated age-matched BXSB controls (17.5% T; 39.2% B in spleen, and 40.0% T; 32.5% B in lymph nodes).

Anti-dsDNA Ab titers

Although anti-dsDNA Ab decreased in transplanted mice between 10 and 20 wk posttransplant, there were no significant differences among treatment groups or untreated BXSB mice (Table IV). Also

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Weeks Posttransplant and % Chimerism&lt;sup&gt;a&lt;/sup&gt;</th>
<th>10 wk</th>
<th>20 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>23.6 ± 3.0</td>
<td>35.5 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>42.7 ± 2.4</td>
<td>73.5 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>26.2 ± 4.1</td>
<td>39.7 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>Lymph node</td>
<td>22.5 ± 5.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.7 ± 6.3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Calculated percentage of chimerism (percentage of GFP-positive donor cells in CD45 gate) of seven mice at 10 wk or six mice at 20 wk posttransplant; average ± SE.

<sup>b</sup>At 10 wk, lymph node cells from only three mice tested.
included are results from three female BXSB mice, which, as a rule, do not develop anti-dsDNA Ab.

Discussion

The preclinical studies of Eisenberg et al., Good, and Ikehara et al. (12–15) have indicated that spontaneous autoimmune disease in lupus-prone mice is derived from the information encoded in hemopoietic stem cells, suggesting that interventions should target these progenitor cells. Our results further indicate that modulation of immune networks is possible by introducing healthy stem cells using a nonmyeloablative protocol. The results suggest that donor progenitor cells, even when in a minority in numbers, can establish a functional dominance in a chimeric BXSB host. One current clinical trend favors autologous hemopoietic stem cell transplantation for treatment of patients with severe autoimmune diseases based on the assumption that mature autoreactive lymphocytes must be depleted by conditioning. It has been suggested that the lymphocytes regenerated by autologous stem cells will no longer be autoreactive or that a prolonged disease-free period can occur before such cells reappear (16, 17). It was also argued (18) that stem cell rescue is not even needed and that high dose cyclophosphamide can eliminate offending autoimmune lymphocytes while sparing stem cells. However, this approach can have a side effect of prolonged immunosuppression that some patients may not tolerate. The differing views regarding spontaneous autoimmune disease of mice and autoimmune disease that occurs in humans raise the possibility that elements of both may be correct. Depending on the degree of genetic involvement and environmental triggers needed, basic mechanisms may differ and it may be necessary to formulate different treatments tailored for different patients (19).

Nonmyeloablative BMT with matched cells provides an option that avoids some of the disadvantages of each of the other approaches described above.

In our model, we purposely used fully matched donor mice to mimic a clinical setting. There are minor, but uncharacterized antigenic differences between GFP-Tg and BXSB, but we saw no evidence of clinical GVHD or MLR. In other studies, mixed chimerism without GVHD has been achieved in mice and humans, including treatments for autoimmune disease (20–23). It has been suggested (24) that a graft vs autoimmune effect similar to graft-vs-leukemia effect might be useful in some instances if it is necessary to eliminate remaining autoreactive lymphocytes of the recipient. Our data suggest it is not necessary to eliminate all recipient cells (neither mature lymphocytes nor stem cells) for treatment of autoimmune renal disease in BXSB mice. Our results on anti-dsDNA Ab levels suggest some continuing activity of self-reactive B cell clones after nonmyeloablative BMT. Anti-dsDNA has been suggested to be involved in the disease progression (25), but it is not the only pathogenic Ab, and our study did not examine other candidates or attempt to characterize the anti-dsDNA Ab detected.

Nonmyeloablative BMT has been the treatment of choice for illnesses of the hemopoietic system, including immune deficiencies, metabolic disorders, and chronic malignancies (reviewed in Refs. 26 and 27). With the use of fully matched donor cells, the nonmyeloablative protocols are considered to pose less mortality and morbidity compared with total myeloablative protocols. There have been only few reports on the applications of nonmyeloablative hemopoietic stem cell transplantation for the treatment of autoimmune diseases in humans (23, 27) or in mice (28–30).

The mechanisms involved in the findings presented are not known. There may be a threshold effect, and if recipient cells are diluted with sufficient numbers of donor cells, there is reduction in disease severity. Central modulation of immune repertoire in chimeric mice could be involved, as GFP⁺ donor-derived cells were found in the thymus (Table II). Previous studies on mixed chimeras, investigating models of host alloreactive (31)- or mammary tumor virus-specific T cells (32), have shown changes in host T cell repertoire in concord with emergence of donor-derived class II⁺ dendritic cells in the thymus of the host. Of note, normal splenic B cells from healthy allogeneic or MHC-matched strains were shown to inhibit autoimmunity and autoantibody production in (New Zealand Black × New Zealand White)F₁ mice when infected periodically (33). It is not clear whether this was secondary to cotransfer of regulatory cells or dilution of host peripheral lymphocytes. Immune modulation at peripheral sites such as involvement of professional APCs or tissue parenchymal cells is also important. Although the role of monocytosis that occurs naturally in BXSB mice is not clear, the reduction in the monocyte numbers we found after BMT may indicate reduced availability of potential APCs or diminished inflammation. Stem cells from BXSB mice are more responsive to M-CSF than stem cells from normal mice (34), and transplanted stem cells may compete for this and for other intrinsic factors.

Our results are compatible with a view that disease outcome depends on a balance between the kinetics of the tissue damage induced by the autoimmune reactions and the kinetics of self-repair mechanisms in the target tissues (healing). Thus, not only the progeny of donor hemopoietic stem cells, but also the progeny of donor mesenchymal stem cells are likely to be crucial. We found donor cells in the renal glomeruli, which raises the possibility of an involvement in tissue repair. It is known that mesenchymal stem cells are included in crude bone marrow preparations similar to the ones we used, and it has been documented that such cells can differentiate in vitro or in vivo into several types of stromal cells.

Table III. Monocytosis in untreated or transplanted BXSB mice 20 wk posttransplant (40 wk old)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Untreated</th>
<th>Transplanted</th>
</tr>
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<tbody>
<tr>
<td>Spleen</td>
<td>24.1 ± 3.2</td>
<td>6.7 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>17.1 ± 3.0</td>
<td>7.4 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of cells in CD45 gate positive for CD11b and negative for Gr1; average of four to seven mice ± SE.

<sup>ab</sup> Value of p < 0.03.

Table IV. dsDNA Ab values in serum<sup>b</sup>

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (weeks)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unt BXSB male</td>
<td>30</td>
<td>41.7 ± 19.1</td>
</tr>
<tr>
<td>Unt BXSB male</td>
<td>40</td>
<td>21.3 ± 9.7</td>
</tr>
<tr>
<td>Unt BXSB female</td>
<td>40</td>
<td>4.7 ± 3.2</td>
</tr>
<tr>
<td>10 wk post-550</td>
<td>30</td>
<td>37.9 ± 16.7</td>
</tr>
<tr>
<td>20 wk post-550</td>
<td>40</td>
<td>49.0 ± 20.9</td>
</tr>
<tr>
<td>10 wk post-BMT</td>
<td>30</td>
<td>62.0 ± 27.1</td>
</tr>
<tr>
<td>20 wk post-BMT</td>
<td>40</td>
<td>38.6 ± 11.1</td>
</tr>
</tbody>
</table>

<sup>b</sup> Assayed by ELISA. Values are percentage of positive control determined from OD values, average of three to seven mice ± SE. Unt = untreated; 550 = irradiated with 550 cGy; BMT = transplanted with 10<sup>7</sup> bone marrow cells from 12-wk-old GFP-Tg mice 4 h after irradiation.

<sup>5</sup> O. Y. Jones, R. A. Good, and R. A. Cahill. Treatment of a childhood overlap syndrome and small vessel vasculitis with fully matched allogeneic bone marrow transplantation using nonmyeloablative preparatory regimen. Submitted for publication.
including renal tissue stroma (35, 36). Repair mechanisms could include production by infiltrating donor cells of cytokines that suppress local inflammation or fibrosis. In a study of correcting LPS-induced emphysema by BMT, it was suggested (37) that bone marrow progenitor cells may replace dead cells or differentiate and become incorporated into the structure of damaged tissue. As an initial step, the levels of immunosuppression provided by the conditioning regimen are likely to be important to reduce the size of the effector cell population. The observed improvement of renal pathology in mice treated with radiation alone is compatible with such a conclusion, but this effect appeared to be transient and was not apparent after 20 wk, while the beneficial effects of BMT were still obvious.

Acknowledgments

We gratefully acknowledge the expert assistance of Jo Pascual.

References


