Prevention and Induction of Occlusive Coronary Vascular Disease in Autoimmune (W/B)F₁ Mice by Haploidentical Bone Marrow Transplantation: Possible Role for Anticardiolipin Autoantibodies

By Hajime Mizutani, Robert W. Engelman, Koken Kinjoh, Yoshiyuki Kurata, Susumu Ikehara, and Robert A. Good

Male (NZW X BXSB)F₁ (W/BF₁) mice develop systemic autoimmune involving autoantibodies, thrombocytopenia, lupus nephritis, and coronary vascular disease with myocardial infarction (CVD). To determine whether this murine lupus-associated CVD could be transferred to otherwise autoimmune-resistant (C57BL/6 X C3H/He)F₁ (B6C3F₁) mice via W/BF₁ T-cell–depleted marrow (TCDM) transplants, or conversely whether the CVD of W/BF₁ mice could be prevented by the reciprocal transplant, reciprocal haploidentical transplants of TCDM were performed. CVD developed only in mice with systemic autoimmunity. Mice that developed lupus had glomerulonephritis and thrombocytopenia and also had elevated titres of autoantibodies to double-strand DNA, cardiolipin, and platelets and elevated levels of circulating immune complexes. Of control W/BF₁ mice, 80% developed lupus, and of these, 81% developed CVD with a mean grade of 2.5 ± 0.8. Engraftment of W/BF₁ mice with B6C3F₁ marrow protected 90% of the recipients from the development of lupus, and none developed CVD. Engraftment of B6C3F₁ mice with W/BF₁ marrow induced lupus in 60% of the recipients, and of those, 33% developed CVD with a mean grade of 1.3 ± 0.3. The B6C3F₁ recipients of W/BF₁ marrow which developed CVD had significantly higher titres of autoantibodies to cardiolipin (aCL; P < .01). These findings show that genetic abnormalities present in the W/BF₁ hematopoietic stem cells contribute to autoantibody development, including aCL, and suggest that thrombogenic mechanisms induced by aCL may contribute to the development of CVD in this form of murine lupus erythematosus. © 1993 by The American Society of Hematology.

Occlusive coronary vascular disease (CVD) contributes to the morbidity and mortality of patients with systemic lupus erythematosus (SLE). Although the pathogenic mechanisms of SLE-associated cardiovascular injury have not been clearly delineated, endothelial injury by circulating immune complexes (CIC) and the subintimal deposition of immune reactants or the thrombogenic that developed lupus had glomerulonephritis and thrombocytopenia, and increases the incidence of CVD in the recipient females. Thyroid autoantibodies in the pathogenic mechanisms of SLE-associated cardiovascul...

MATERIALS AND METHODS

Animals. Male (NZW X BXSB)F₁ (W/BF₁, H-2K), (C57BL/6 X C3H/He)F₁ (B6C3F₁, H-2K), and Balb/c mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited, specific pathogen-free conditions in accordance with NIH publication 85-23. A total of 20 W/BF₁ mice and 20 B6C3F₁ mice served as controls; 20 B6C3F₁ mice...
Chimeric analysis. Spleen cells of mice injected with TCDM were prepared 35 weeks posttransplantation for immunostaining and flow cytometry (Epics Elite, Coulter, Hialeah, FL) with antimouse anti-H-2K<sup>k</sup> MoAb conjugated with fluorescein isothiocyanate (FITC; mouse IgG<sub>2a</sub>; Pharmigen, San Diego, CA) or with mouse IgG<sub>1</sub> conjugated with FITC (Pharmigen) as control.

Platelet counts. Blood (20 µL) from mice was diluted in buffer containing ammonium oxalate (Unopette kits; Becton Dickinson). Platelets were counted using a hematocytometer under a phase-contrast microscope.

Platelet isolation. Whole blood from mice was obtained in acid-citrate-dextrose (pH 4.5, 0.01 mol/L) as previously described. Platelets were isolated by differential centrifugation at 300g for 5 minutes to obtain platelet-rich plasma and were washed 3 times at 1,000g for 15 minutes. Contaminating red blood cells were removed by centrifugation at 100g for 5 minutes.

Circulating autoantibodies to platelets. Sera were screened for circulating platelet-binder IgG (PB IgG) by micro enzyme-linked immunosorbent assay (ELISA) as previously described with minor modifications. Briefly, 2.5 × 10<sup>6</sup> platelets from healthy BALB/c mice in 50 µL of phosphate buffered saline (PBS) were added to each well of a 96-well microtitre plate (Dynatech, Chantilly, VA), fixed with 2% paraformaldehyde (PFA) in PBS, treated with 5% bovine serum albumin (BSA) in 0.05% Tween-20 PBS (PBS-T) to prevent nonspecific protein absorption, and washed 3 times with PBS-T. A 50-µL sample of serum diluted 1:80 in PBS-T was added to each well containing PFA-fixed platelets, and the wells were incubated at 37°C for 1 hour. After washing, alkaline phosphatase (ALP)-conjugated goat antimouse γ-chain-specific IgG (Sigma, St Louis, MO) was added and the ALP reaction developed as previously described. The optical absorbance was measured at 405 nm using a microELISA autoreader, MR 580 (Dynatech), and antibody titres were determined by plotting the optical absorbance on a standard curve. A standard curve was drawn using pooled sera from male W/BF<sub>1</sub> mice. The pooled sera were serially diluted 1:80 through 1:2560, and the optical absorbance of each dilution was plotted on a log linear scale. The optical absorbance of the 1:2560 dilution was recorded as one (1) ELISA unit (U), and that of the 1:80 dilution as 10 U.

Platelet-associated IgG (PAIgG). To detect PAIgG, platelets of control and transplanted mice were prepared 35 weeks posttransplantation for immunostaining and flow cytometry (Epics Elite) with goat antimouse IgG antibody conjugated with FITC (Becton Dickinson) or with goat IgG conjugated with FITC (Becton Dickinson) as control. Details of method have been described.

Circulating autoantibodies to ss-DNA or ds-DNA. Calf thymus ss-DNA or ds-DNA (Sigma) was dissolved (10 µg/mL) in 0.5 mol/L
Table 1. Incidence and Severity of Coronary Vascular Lesions and Association With Murine Lupus

<table>
<thead>
<tr>
<th>Group</th>
<th>No. With Fatal Lupus</th>
<th>No. Killed</th>
<th>No. CVD (%)</th>
<th>CVD Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>W/BF1</td>
<td>16</td>
<td>4</td>
<td>13/20 (65)</td>
<td>RVFW</td>
</tr>
<tr>
<td>W/BF1 → B6C3F1</td>
<td>9</td>
<td>6</td>
<td>3/15 (20)</td>
<td>VS</td>
</tr>
<tr>
<td>B6C3F1</td>
<td>0</td>
<td>20</td>
<td>0/20 (0)</td>
<td>LVFW</td>
</tr>
<tr>
<td>B6C3F1 → W/BF1</td>
<td>2</td>
<td>18</td>
<td>0/20 (0)</td>
<td></td>
</tr>
</tbody>
</table>

Hearts from control W/BF, or B6C3F1, mice or from mice undergoing reciprocal haploidentical BMT were serially sectioned and evaluated for CVD (n = 20/group, except B6C3F1 recipients of W/BF1, TCDM, n = 15). Number of mice developing fatal lupus and those killed at the end of the 45-week study are indicated. Number and percent of mice with CVD and location of vascular lesions are indicated as either right ventricular free wall (RVFW), ventricular septum (VS), or left ventricular free wall (LVFW).

carbonate buffer (pH 9.6), and 100 μL of this solution was incubated in each well of a microtitre plate for 12 hours at 4°C. After blocking with 2% BSA-PBS, plates were washed 3 times with PBS-T. Sera (1:80, 50 μL) were incubated in DNA-coated wells for 1 hour at 37°C. Plates were washed, and ALP-conjugated goat antiserum IgG was added; the plates were then incubated, and the ALP reaction developed. The optical absorbance was measured at 405 nm, plotted on a standard curve, and recorded as 1 to 100 U as described above.

Circulating autoantibodies to cardiolipin. Anticardiolipin antibodies were detected by solid-phase enzyme immunoassay as described. Briefly, 50 μL of a cardiolipin solution, 1 mg/mL cardiolipin (Sigma) in 0.01 mol/L PBS (pH 7.4), was incubated in each well of a microtitre plate for 12 hours at 4°C. To block nonspecific binding of IgG, 200 μL of 10% fetal calf serum was added to each well, incubated for 1 hour at 37°C, and washed 5 times with PBS. Serum samples diluted 1:80 (50 μL) were added to wells, incubated for 1 hour at room temperature, and washed 5 times. ALP-conjugated goat antiserum IgG (100 μL) was added to each well; the ALP reaction developed, and the optical absorbance was measured, plotted on a standard curve, and recorded as 1 to 100 U as described above.

CIC. Determination of CIC levels was made using an antiserum C3 F(ab')2 (Cappel, Durham, NC) solid-phase immunoassay, as previously described. Antimouse C3 F(ab')2 (10 μg/mL, 200 μL/well) in 0.1 mol/L carbonate buffer was added to microtitre wells and incubated at 4°C overnight. Wells were washed for 1 hour with 1% BSA-PBS, and then 3 times with PBS-T. To determine CIC levels, a 1:80 dilution of serum was added to each serum sample and incubated at 37°C for 60 minutes. Wells were washed 3 times with PBS-Tween. Alkaline-phosphatase-conjugated rabbit antimouse IgG (200 μL, 1:1000) was added to each well; the ALP reaction developed, and the optical absorbance was determined, plotted on a standard curve, and recorded as 1 to 100 U as described above.

Histological analysis. Formalin-fixed tissues were sectioned at 2 to 4 μm and stained with either hematoxylin and eosin or periodic acid Schiff (PAS). Each heart was cut transversely into 3 blocks, and each block was serially sectioned. The degree of CVD and myocardial infarctions (MI) were classified from 0 to 3+, according to the method used by Berden et al. Grade 1 indicates minimal PAS-positive deposits along and within 1 coronary vascular wall; grade 2 indicates PAS-positive deposits with narrowing of the lumen in 2 or 3 coronary vessels; and grade 3 indicates 4 or more affected coronary vessels. MI were often associated with CVD grades 2 and 3.

Fig 3. Representative histopathologic sections of CVD, grade 3 from (A) a W/BF1 mouse and (B) a B6C3F1 recipient of W/BF1, TCDM. Note PAS-positive deposits within the media of the coronary arterial walls, presence of thrombi, presence of only a few mononuclear inflammatory cells, and absence of extensive inflammatory infiltrate (A, X630; B, X1000, PAS).
Immunohistochemistry. Heart and kidney sections were evaluated for the deposition of Ig within microvasculature using either biotinylated goat antimouse IgG or IgM, an avidin-biotin-peroxidase complex, and diaminobenzidine as substrate in a Vectastain Kit (Vector Laboratories, Burlingame, CA).

Statistical analysis. Development of systemic autoimmunity by mice of each experimental group was compared by Kaplan-Meier analysis, using a log-rank test with multiple contrasts between experimental groups examined. Statistical analysis of autoantibody or CIC levels was performed using the Student’s t-test.

RESULTS

Chimeric analysis. Reciprocal transplantations of TCDM were made between 10-week-old W/BF₁ and B6C3F₁ mice, and the recipients and control mice were followed up for the development of autoantibodies, thrombocytopenia, and glomerulonephritis and for the incidence and severity of CVD. As expected, chimeric analysis of representative 45-week-old control mice showed that approximately 94% of B6C3F₁ splenocytes were H-2Kk positive, whereas among W/BF₁ mice only approximately 12% seemed reactive in this way. Thirty-five weeks posttransplantation, similarly aged B6C3F₁ recipients of W/BF₁ marrow had only approximately 8% H-2Kk-positive splenocytes, and W/BF₁ recipients of B6C3F₁ marrow had elevated numbers, approximately 99% H-2Kk-positive splenocytes. Representative flow cytometry profiles of H-2Kk-positive spleen-cell fluorescence among control and experimental mice are shown in Fig 1.

Survival analysis. Within 30 weeks posttransplantation, 80% of control autoimmune-prone W/BF₁ mice had developed fatal systemic autoimmunity (Fig 2). Control autoimmune-resistant B6C3F₁ mice remained healthy throughout the 45 weeks of study. Haploidentical BMT prevented the development of fatal systemic autoimmunity in W/BF₁ recipients, with 90% of the W/BF₁ recipients of B6C3F₁ marrow surviving beyond age 40 weeks (Fig 2). This protection afforded W/BF₁ mice by engraftment with B6C3F₁ marrow was nearly complete (P < .001). Conversely, the reciprocal haploidentical transplant of W/BF₁ marrow induced fatal systemic autoimmunity in 65% of B6C3F₁ recipients within 30 weeks posttransplantation. This development of systemic autoimmunity by B6C3F₁ recipients of W/BF₁ marrow approached the tendency of control W/BF₁ mice to spontaneously develop systemic autoimmunity, but was somewhat less (P < .05).

Incidence and histopathology of CVD. Hearts from all mice were serially sectioned and examined. Eighty-one percent of control W/BF₁ mice that developed systemic autoimmunity (or 65% of all control W/BF₁ mice including those that were healthy and were killed at the termination of the study) had CVD (Table 1). In contrast, none of the control
B6C3F1 mice developed CVD. Engraftment of W/BF1 mice with B6C3F1 marrow prevented the development of CVD in all of the recipients, including the 2 mice which developed other aspects of autoimmunity (Table 1). The CVD of W/BF1 mice was transferred to 33% of B6C3F1 recipients of W/BF1 marrow. These mice also developed systemic autoimmunity and died within 30 weeks after transplantation, and these mice represented 20% of all engrafted B6C3F1 mice. B6C3F1 recipients of W/BF1 marrow that did not develop thrombocytopenia or lupus nephritis had normal hearts. Post mortem changes of the hearts of 5 B6C3F1 recipients of W/BF1 marrow prevented accurate histologic evaluation and these were excluded from CVD scoring (Table 1).

Lesions of murine lupus-associated CVD occurred typically within ventricular walls and not within the septa and consisted of PAS-positive subintimal depositions in small intramyocardial arterioles, scant mononuclear inflammation, infiltrate, and partially to fully occlusive thrombi with accompanied regional myocardial necrosis (Fig 3). A subintimal brown precipitate, indicative of IgG and IgM deposition, was evident in immunohistologic preparations of heart sections from mice with CVD (data not shown). CVD of control W/BF1 mice had a mean grade of 2.5 ± 0.8, whereas the CVD of B6C3F1 mice engrafted with W/BF1 marrow were less severe, with a mean grade of 1.3 ± 0.3 (Table 1). Mitral valvulitis was also evident in a single heart of a B6C3F1 recipient of W/BF1 marrow.

Development of anticardiolipin and other autoantibodies. The sera of all experimental mice were evaluated for the presence of circulating autoantibodies to ds-DNA, the presence of cardiolipin or platelets, and the presence of CIC (Fig 4). Titres of autoantibodies that reacted with ds-DNA, platelets, or cardiolipin and the levels of CIC among W/BF1 recipients of B6C3F1 marrow were all lower than those of control W/BF1 mice, with mean autoantibody titres to ds-DNA or to cardiolipin and mean CIC levels of W/BF1 recipients all significantly reduced by engraftment with B6C3F1 marrow (P < .05). Conversely, transplanting B6C3F1 mice with marrow from autoimmune-prone W/BF1 mice led to increases in the titres of autoantibodies to cardiolipin, platelets, or ds-DNA. Further, levels of CIC among B6C3F1 recipients of W/BF1 marrow were also increased; the mean CIC level and mean ds-DNA autoantibody titre of B6C3F1 recipients was significantly greater than those of control B6C3F1 mice (P < .05). The mean level of anticardiolipin antibodies was higher among B6C3F1 recipients that developed CVD (75 ± 4 U) as compared with B6C3F1 recipients free of CVD (27 ± 15 U; P < .01) (Fig 5). No clear association between the development of CVD in B6C3F1 recipients and titres of autoantibodies to ds-DNA or to platelets or the levels of CIC among B6C3F1 recipients of W/BF1 marrow was evident.

Platelet counts and antplatelet autoantibodies. Autoimmune thrombocytopenia in W/BF1 mice is not only accompanied by increased titres of circulating antplatelet autoantibodies and reduced platelet lifespan, but is also accompanied by increased PAIgG. B6C3F1 recipients of W/BF1 marrow became progressively thrombocytopenic so that by 20 weeks posttransplantation platelet counts of B6C3F1 mice did not differ significantly from those of W/BF1 controls (Fig 6). In contrast, W/BF1 recipients of B6C3F1 marrow had normal platelet counts throughout the 45-week course of the study. Presence of PAIgG was reduced in platelet samples of W/BF1 recipients of B6C3F1 marrow compared with those of control W/BF1 mice (Fig 7A). Conversely, PAIgG was elevated in platelet samples of B6C3F1 recipients of W/BF1 marrow as compared with those of control B6C3F1 mice (Fig 7B).

DISCUSSION
In the present report, murine lupus-associated CVD was induced, and autoantibody and CIC titres were elevated in otherwise autoimmune-resistant mice by the transplantation of W/BF1 marrow. Using the reciprocal transplantation of B6C3F1 marrow to W/BF1 recipients, the murine lupus-associated CVD of W/BF1 mice was prevented, and levels of autoantibodies and CIC remained low. These results imply that the cardiovascular aspects of murine lupus are at least partially attributable to genetic abnormalities that reside within the radiosensitive hematopoietic stem cells. These results also suggest that its development may involve features other than those responsible for the pathogenesis of ATP, and that W/BF1 CVD is not dependent on intrinsic coronary vascular abnormalities. The reduced incidence and degree of CVD in B6C3F1 recipients of W/BF1 marrow compared with those for W/BF1 controls might infer that full manifestation of lupus-associated CVD can involve ab-
normalities and mechanisms other than those intrinsic to the stem cell influence. Anticardiolipin antibodies appear to contribute to occlusive thrombosis in humans by various mechanisms.6-10 Of the B6C3F1 recipients of W/BF1 marrow, those that developed CVD had the highest titres of aCL. The variable elevation of aCL among B6C3F1 recipients of W/BF1 marrow may explain, in part, the irregular development of CVD in B6C3F1 recipients of W/BF1 marrow. Furthermore, variability may exist between mouse strains in the sensitivity to influences of aCL.

Although the morbidity and mortality attributable to cardiovascular aspects of human SLE have been acknowledged, a mechanism that describes the formation of vascular lesions in systemically autoimmune patients is lacking. Vascular endothelial injury by CIC and the subintimal deposition of immune reactants may contribute to the CVD of SLE patients.11 In our described experiments, Ig deposition was shown in the coronary vascular lesions of both the W/BF1 control and B6C3F1 recipient mice that developed CVD. Others have suggested that SLE patients develop CVD because of the effects of corticosteroid treatment and resultant hyperlipidaemia and/or hypertension.11,12 In addition, the thrombogenic consequences of elevated titres of autoantibodies, such as aCL, may contribute significantly to CVD in SLE patients.13-15 Anticardiolipin antibodies are found in 30% to 60% of SLE patients,13-15 and half of SLE patients with high-titre aCL develop vascular thrombosis.14 Anticardiolipin antibodies may activate platelets and cause abnormal platelet aggregation, thereby contributing to occlusive thrombosis and CVD. In contrast, we have previously shown that monoclonal aCL and pathogenic antiplatelet MoAbs can be distinct.20 In these prior experiments, we produced several clones of antiplatelet MoAbs using W/BF1 splenocytes and evaluated them for their reactivity with platelets and cardiolipin and for their ability to induce thrombocytopenia in nude mice. Of the 7 antiplatelet MoAbs produced, only 1 reacted with cardiolipin, and this antiplatelet MoAb did not induce thrombocytopenia in nude mice.20 These findings suggest that the pathogenicity of autoantibodies that react with platelets or with cardiolipin may be separable.

Our results indicate that murine lupus-associated CVD is an aspect of systemic autoimmune and, as such, is transferable by TCDM transplantation. A total of 33% of those B6C3F1 recipients of W/BF1 marrow that developed systemic autoimmune also developed CVD, and those that developed CVD had the highest aCL titres, suggesting that cardiolipin autoantibody-mediated thrombogenic mechanisms contribute to lupus-associated CVD in mice. Alternatively, aCL antibodies may have arisen as a result of CVD development. Current experiments in our laboratory using the passive transfer of aCL MoAbs to mice may clarify the potential pathogenic nature of aCL antibodies in autoimmune CVD. Furthermore, these findings show that similarities in the pathogenesis of human and murine lupus-associated CVD exist and also show that the W/BF1 male mouse may prove to be an ideal model for clarifying details of autoimmune-associated cardiovascular disease.

ACKNOWLEDGMENT
We thank Robert McCoobery and Ann Lynch for excellent technical assistance with chimeric and PAIgG analyses using flow cytometry, and Dr C.H. Brown (Department of Biostatistics and Epidemiology, University of South Florida) for assistance with statistical analyses.

REFERENCES


Prevention and induction of occlusive coronary vascular disease in autoimmune (W/B)F1 mice by haploidentical bone marrow transplantation: possible role for anticardiolipin autoantibodies

H Mizutani, RW Engelman, K Kinjoh, Y Kurata, S Ikehara and RA Good