Brief Definitive Report

(NZW × BXSB)F_1 MOUSE

A New Animal Model of Idiopathic Thrombocytopenic Purpura

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There has recently been an increase in data indicating that autoimmune mechanisms are involved in the etiopathogenesis of idiopathic thrombocytopenic purpura (ITP) (1, 2). Although antibodies that react with platelets are found in most patients with ITP, the pathogenetic nature of the antibodies remains to be clarified. The discovery of an animal model for ITP has therefore been long-awaited. Here we have found that (NZW × BXSB)F_1 (W/B F_1) mice, which develop lupus nephritis with myocardial infarction (3), show thrombocytopenia with age, and that this is due to the presence of both platelet-associated antibodies (PAA) and circulating antiplatelet antibodies.

Recently, we have demonstrated that allogeneic bone marrow transplantation (ABMT) has curative effects on autoimmune diseases in (NZB × NZW)F_1, BXSB, MRL/MP-*lpr/lpr* (MRL/*lpr*), and NOD mice (4–6). These results prompted us to examine whether ABMT can be used to treat ITP. In the present study, we provide evidence that the transplantation of bone marrow from BALB/c mice to W/B F_1 mice does indeed have preventative and curative effects on ITP.

Materials and Methods

Mice. Mice of the inbred strain BALB/c *nu/nu*, BALB/c, C57B/6, C3H/HeN, BXSB, NZW were raised under specific pathogen-free conditions in our animal facility. W/B F_1 males were obtained from the Nippon Shinyaku Research Laboratories, Kyoto, Japan.

Staining Procedure and Data Analysis. Platelet-rich plasma was obtained as described previously (7). The platelets were suspended in 1% paraformaldehyde solution for 5 min. After

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TABLE I

Counts of Platelets and Megakaryocytes in (NZW × BXSB)F_1 (W/B F_1) Mice

Mouse	Age	Sex	Number examined	Platelet ($\times 10^{-4}$/μl) Mean ± SD	Megakaryocyte (/mm^2) Mean ± SD
BALB/c	2–4 mo	♂ and ♀	15	54.0 ± 10.8	132.5 ± 19.1
NZW	4 mo	♀	1	46.5	128
BXSB	8 mo	♂	14	57.9 ± 12.0	127.2 ± 11.1
W/B F_1	1–2.5 mo	♂	19	52.5 ± 10.7	118.0 ± 12.6
W/B F_1	4.5–7 mo	♂	19	15.0 ± 5.9*	150.8 ± 10.0*

* $p < 0.001$ vs. data in young (1–2.5 mo) W/B F_1 mice.

washing twice, the platelets were resuspended in EDTA-PBS to a platelet count of 2×10^6/100 μl. To detect the circulating antibodies, aliquots of platelets were incubated with 100 μl of plasma (1:4 dilution) for 30 min at room temperature. As a postive control, the platelets of BALB/c mice were treated with anti-H-2^d sera. After washing twice, they were then incubated with a 1:10 dilution of FITC-conjugated antisera (goat anti-mouse Ig; Cappel Laboratories, West Chester, PA; goat anti-mouse IgG, IgA, IgM; Behring Institute, Marburg, Federal Republic of Germany) for 30 min at room temperature. After final washing, samples were then analyzed on a FACS analyzer (Becton Dickinson & Co., Sunnyvale, CA) by gating to exclude debrils. The nonstained negative control (autofluorescence) was set at channels 0 to 8. The percentage of positive cells was calculated from the total platelet count above channel 9.

Transplantation of Bone Marrow Cells. 3–5-mo-old W/B F_1 males were exposed to 9.5 Gy from a ^{60}Co source and then reconstituted by intravenous injection of 1.4×10^7 bone marrow cells from BALB/c *nu/nu* mice, as previously described (4). The mice were killed 5.5 mo after ABMT.

Platelet and Megakaryocyte Count. Platelet counts in the peripheral blood were made in heparin blood sample on a hemocytometer. For megakaryocyte count, femur and tibia were obtained at sacrifice, and sections stained with hematoxylin and eosin. The number of megakaryocytes per square millimeter was counted under a microscope.

Results

Male W/B F_1 mice at the age of 1–2.5 mo showed platelet counts similar to those of BALB/c, NZW, and BXSB mice (Table I). W/B F_1 mice at the age of >4.5 mo, however, showed a marked reduction in platelet count. In contrast, bone marrow megakaryocyte counts increased in these mice with age, though they retained their normal shape.

To elucidate the cause of thrombocytopenia in peripheral blood of W/B F_1 mice, we first examined PAA on the platelets. As shown in Fig. 1, PAA were found in the platelets of 4.5-mo-old W/B F_1 mice with thrombocytopenia (10.5×10^4/μl). BALB/c platelets treated with anti-H-2^d serum were used as a positive control.

The next step was to examine whether circulating antiplatelet antibodies are present in the plasma of aged W/B F_1 mice. As shown in Table II, circulating antiplatelet antibodies were found in the plasma of W/B F_1 mice that were >5 mo old.

The characterization of the antiplatelet antibodies revealed that both PAA and circulating antiplatelet antibodies belong to the IgG and IgM classes, but not IgA (Table III).

Since we know that ABMT has curative effects on autoimmune diseases (4–6), W/B F_1 mice at the age of 3–5 mo were lethally (9.5 Gy) irradiated and then recon-

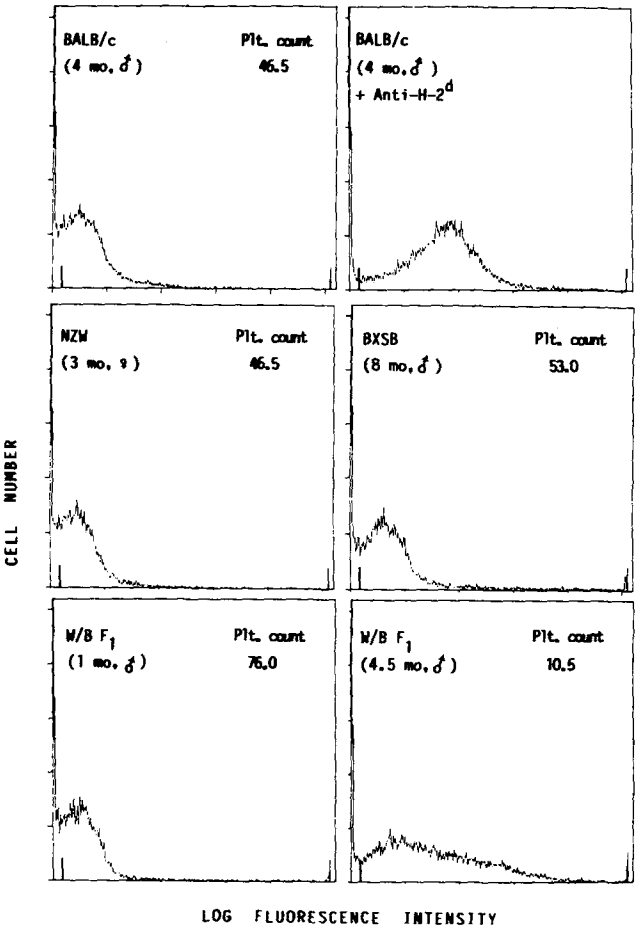

FIGURE 1. Platelet-associated antibodies in W/B F_1 mice. Aliquots of the platelets (2×10^6) were labeled with FITC-anti-mouse Ig and analyzed on a FACS analyzer. As a positive control, BALB/c platelets treated with anti-H-2^d antisera were used. Platelet counts (Plt. count: $\times 10^{-4}$/ml) for each mouse are shown.

stituted with bone marrow cells from BALB/c *nu/nu* mice. The mice were killed 5.5 mo after ABMT. W/B F_1 mice reconstituted with BALB/c *nu/nu* bone marrow cells showed normal platelet counts even at the age of 10.5 mo, and the levels of antiplatelet antibodies were reduced to those of BALB/c mice (Table IV).

Discussion

In the present study we have demonstrated that W/B F_1 mice develop thrombocytopenia with age, and have found the presence of both PAA and circulating antiplatelet antibodies in such mice.

Several mechanisms that would explain the cause of thrombocytopenia in patients with ITP have been proposed. One is the presence of antiplatelet antibodies, which results in platelet destruction by complement-mediated lysis (8) or sequestration by the reticuloendothelial system (1). It has been reported that the antibodies in humans belong mainly to the IgG class (9). In W/B F_1 mice, we have found the presence of IgG and IgM (but not IgA) antibodies both in the plasma and on the platelets (Table III). Since W/B F_1 mice show high levels of circulating immune complexes

TABLE II

Circulating Antiplatelet Antibodies in Old (NZW × BXSB)F_1 (W/B F_1) Mice

1st antibody (plasma from)	Age	2nd antibody (anti-Ig)*	Percent positive (Mean ± SD)	
	mo			
–		–	0.1	
–		+	14.0 ± 3.1	
Anti-H-2^d		+	54.9 ± 1.8	
BALB/c	1.5 to 2.0	+	26.0 ± 0.3	
(W/B)F_1	1.0	+	17.8	(20.1 ± 3.5)
	1.5	+	16.5	
	1.5	+	21.7	
	1.5	+	24.9	
	2.0	+	16.8	
	2.0	+	22.9	
(W/B)F_1	5.0	+	45.3	(49.9 ± 4.8)‡
	5.5	+	49.2	
	7.0	+	57.6	
	7.0	+	50.7	
	8.5	+	46.8	

Platelets were obtained from 2-mo-old BALB/c mice.
* FITC-labeled goat anti-mouse Ig.
‡ $p < 0.001$ vs. data in young (1–2.0 mo) W/B F_1 mice.

(CICs) from the age of 2.5 mo (3), it is conceivable that CICs are involved in the development of thrombocytopenia, CICs being found to affect platelets by activating complement or platelet-aggregating factor (1, 2). However, murine platelets have

TABLE III

Characterization of Antiplatelet Antibodies in Old (NZW × BXSB) F_1 (W/B F_1) Mice

Platelet-associated antibodies				Circulating antibodies		
Source of platelet	1st antibody	2nd antibody	Percent positive	1st antibody (plasma from)	2nd antibody	Percent positive
BALB/c	–	–	2.1	–	–	0.03
				–	Anti-Ig	9.9 ± 3.7
BALB/c	Anti-H-2^d	Anti-Ig*	48.9 ± 2.5	Anti-H-2^d	Anti-Ig	50.5 ± 0.2
BALB/c	–	Anti-Ig	10.7 ± 0.7	BALB/c	Anti-Ig	27.5 ± 8.3
BXSB	–	Anti-Ig	23.6 ± 1.2	C57BL/6	Anti-Ig	22.7 ± 2.0
				BXSB	Anti-Ig	24.8 ± 3.5
W/B F_1 (1.0 mo)	–	Anti-Ig	23.1 ± 4.2	W/B F_1 (1.0 mo)	Anti-Ig	12.9 ± 1.3
W/B F_1‡	–	Anti-Ig	44.8 ± 0.8	W/B F_1§	Anti-Ig	52.8 ± 4.3
(4.5 mo)	–	IgG‖	41.7	(7.0 mo)	IgG	33.7 ± 3.0
	–	IgA	18.8		IgA	20.9 ± 2.3
	–	IgM	30.5		IgM	35.9 ± 0.4

Platelets used in circulating antibody assay were obtained from 4-wk-old BALB/c mice.
* FITC-labeled goat anti-mouse Ig.
‡ Platelet counts were 5.5 × 10^4/μl.
§ Platelet counts were 13.0 × 10^4/μl.
‖ FITC-labeled goat anti-mouse IgG.

TABLE IV

Effects of Bone Marrow Transplantation on Platelet Counts and Circulating AntiPlatelet Antibodies in Old (NZW × BXSB)F_1 (W/B F_1) Mice

1st antibody (plasma from)	Age	Platelet count (Mean ± SD)		2nd antibody (Anti-Ig)*	Percent positive (Mean ± SD)	
	mo	*× 10⁻⁴/μl*				
–				–	0.1	
–				+	19.2 ± 7.6	
Anti-H-2^d				+	54.3 ± 3.9	
C3H/HeN	1.5	35.6		+	33.9	
C57BL/6	3.5	49.6		+	30.6	
BALB/c	1.5	44.8		+	35.3	
W/B F_1	1.5	42.7 ± 12.8		+	27.5 ± 8.8	
W/B F_1	5.0 to 8.5	15.5 ± 1.6		+	54.3 ± 9.5	
[BALB/c → W/B F_1]‡	8.5	33.2	(36.2 ± 10.0)§	+	35.6	(39.6 ± 4.3)‖
	9.5	26.0		+	43.6	
	9.5	28.8		+	44.1	
	10.5	50.0		+	39.9	
	10.5	43.2		+	34.8	

Platelets were obtained from 2-mo-old BALB/c mice.

* FITC-labeled goat anti-mouse Ig.

‡ The W/B F_1 mice at the age of 3–5 mo were exposed to 9.5 Gy from a ^{60}Co source and then reconstituted with 1.4×10^7 bone marrow cells of BALB/c *nu/nu* mice. The mice were killed 5.5 mo after bone marrow transplantation.

§ $p < 0.01$ vs. data in old (5–8.5 mo) W/B F_1 mice without ABMT.

‖ $p < 0.02$ vs. data in old (5–8.5 mo) W/B F_1 mice without ABMT.

no Fc receptors (2), and the sera from BXSB (Table III) and MRL/*lpr* (data not shown) mice, which show high CIC levels, did not bind to the platelets of BALB/c mice. It is therefore unlikely that antibodies bound to platelets and circulating antiplatelet antibodies exist as a form of CIC.

Hang et al. (10) investigated the etiopathogenesis of autoimmune diseases in these W/B F_1 mice by reciprocal transfer experiments of spleen cells between males that exhibit early-onset autoimmune disease and females with late-onset autoimmune diseases; the transfer of male lymphoid cells to female mice caused the development of accelerated lupus nephritis, hypertension, and myocardial infarction, whereas the transfer of female lymphoid cells to male mice delayed the onset. We have recently demonstrated that the transplantation of bone marrow cells from normal mice to autoimmune-prone mice has curative effects on autoimmune diseases, such as lupus nephritis, lupoid hepatitis, rheumatoid arthritis, and type I diabetes mellitus (4–6). In the present study, W/B F_1 mice after ABMT showed normal platelet counts and no evidence of the presence of circulating antiplatelet antibodies even at the age of 10.5 mo (Table IV). These results provide additional evidence that autoimmune mechanisms are involved in the development of thrombocytopenia in W/B F_1 mice, although the exact mechanism by which it develops remains to be clarified.

We thus think that W/B $_1$ mice serve as a useful animal model of ITP not only for elucidating the mechanism of the development of antiplatelet antibodies, but also for characterizing autoantibodies to platelets.

Summary

A decrease in thrombocyte count was observed in (NZW × BXSB)F_1 (W/B F_1) mice at the age of >5 mo, whereas megakaryocyte counts were found to increase in such mice. FACS analyses revealed the presence of both platelet-associated antibodies (PAA) and circulating antiplatelet antibodies. There is a correlation between the presence of these antibodies and the degree of thrombocytopenia. The transplantation of normal bone marrow cells from BALB/c *nu/nu* mice to W/B F_1 mice was found to have preventative and curative effects on thrombocytopenia; the mice showed normal platelet counts and no evidence of circulating antiplatelet antibodies. These results indicate that thrombocytopenia in W/B F_1 mice is due to the presence of antibodies to platelets. We therefore think that W/B F_1 mice serve as a useful animal model of idiopathic thrombocytopenic purpura (ITP) not only for elucidating the mechanism of the development of antiplatelet antibodies, but also for characterizing autoantibodies to platelets.

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