

## CBA/N X-LINKED B-CELL DEFECT PREVENTS NZB B-CELL HYPERACTIVITY IN F<sub>1</sub> MICE

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New Zealand Black (NZB)<sup>1</sup> mice spontaneously develop autoimmune hemolytic anemia, excessive lymphoproliferation, and immune-complex deposition (1-4). In addition to anti-erythrocyte autoantibodies, NZB mice produce autoantibodies to nucleic acids and to T lymphocytes (4-7). Although the mechanism responsible for the spontaneous autoimmune disease remains unknown, evidence has accumulated that suggests that NZB B lymphocytes are spontaneously activated very early in life, before the appearance of detectable serum autoantibodies. Spleen cells from very young NZB mice have been shown to contain an abnormally high proportion of cells spontaneously producing antibody to several synthetic haptens (8-10) and to secrete excessive amounts of pentameric IgM in culture (11). NZB B lymphocyte surface membranes bear an abnormally low proportion of IgD relative to IgM (12, 13); this pattern is characteristic of mitogenically (14) or antigenically (10) stimulated B lymphocytes from mice of nonautoimmune strains. Young NZB mice make an abnormally large antibody response to sheep erythrocytes (SRBC) (15, 16), and are abnormally resistant to tolerance induction (17, 18). The role of T lymphocytes in the development of the B lymphocyte hyperactivity of NZB mice is unclear. Although considerable evidence points to T-cell abnormalities in NZB mice (19-26), other evidence suggests that the B-cell hyperactivity begins early in life in the absence of T-cell influence (10-12, 27, 28).

CBA/N mice bear an X-chromosome-linked defect in the immune response to T-independent antigens such as pneumococcal polysaccharide (29), polyinosinic-polycytidylic acid (rI·rC) (30), and haptenated Ficoll derivatives (31). All CBA/N mice and the F<sub>1</sub> male offspring of CBA/N females are thus affected. The CBA/N defect is associated with low serum IgM levels (32), reduced numbers of splenic B cells, and a paucity of B cells with a low density of surface-membrane IgM (33, 34). In contrast, the response of CBA/N mice to T-dependent antigens is relatively normal (35), as are CBA/N serum IgG levels (32).

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<sup>1</sup> *Abbreviations used in this paper:* NZB, New Zealand Black; NZW, New Zealand White; rI·rC, polyinosinic-polycytidylic acid; SIII, pneumococcal polysaccharide type III; SRBC, sheep erythrocytes.

F<sub>1</sub> hybrids of NZB mice, particularly NZB × New Zealand White (NZB × NZW), but also NZB × BALB/c, NZB × DBA/2, and C3H × NZB share some autoimmune features with NZB mice (3, 7, 17, 36). Evidence has accumulated that male sex hormones retard the expression of autoimmune disease in NZB F<sub>1</sub> hybrids (37–40).

To gain insight into the role of T-independent immune responses in the autoimmune disease of NZB mice and their hybrids, we have undertaken a series of investigations of CBA/N × NZB mice. To ensure that chromosomal rather than hormonal effects would account for differences observed between CBA/N × NZB male and female mice, and to minimize the effect of androgens on immune phenomena of NZB F<sub>1</sub> hybrid males, all mice used in these studies were prepubertally castrated. We have found that the CBA/N X-linked B-cell defect is fully expressed in CBA/N × NZB F<sub>1</sub> male mice.

### Materials and Methods

*Mice.* All F<sub>1</sub> hybrid mice are designated by the maternal strain first. CBA/N, NZB/N, CBA/N × NZB, NZB × CBA/N, C3H/HeN × NZB, BALB/c × NZB, and CBA/N × DBA/2 mice were obtained from the animal production facilities of the National Institutes of Health. CBA/J mice, obtained from the Jackson Laboratory, Bar Harbor, Maine, served as normal controls for CBA/N mice and the various F<sub>1</sub> hybrids. CBA/J × NZB/N mice were bred in our laboratory.

*Surgical Procedures.* All mice, except as noted, underwent surgical castration under ether anesthesia at 3–4 wk of age. Testes were removed through either a scrotal or an abdominal incision; ovaries were removed through bilateral flank incisions. Incisions were closed with 6-0 silk sutures. Identical incisions were made in sham-castrated animals, but the gonads were left intact.

*Spleen Cell Preparation.* Each mouse was killed by cervical dislocation and its spleen removed and placed in 10 ml of chilled Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) containing 1% nonessential amino acids; 2 mM glutamine; 15 mM Hepes, pH 7.2; 6 µg/ml penicillin; 25 µg/ml gentamicin; and 2% fetal calf serum (Grand Island Biological Co.). A single-cell suspension was prepared by repeated passage of teased spleen through a 25-gauge needle, and the cells were washed in the same medium.

In most experiments, cells from individual spleens were simultaneously assayed for spontaneous in vitro IgM secretion and for subclass-specific Ig-producing cells. In some experiments, cells were also simultaneously analyzed by flow microfluorometry for surface- and cytoplasmic-Ig characteristics.

*Spleen Cell Cultures.* The washed cells from each spleen were centrifuged three times through fetal calf serum at 400 g for 5 min, resuspended at  $1.0 \times 10^7$  cells/ml in the medium described above, and cultured in a 65-mm plastic Petri dish (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) for 4 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. A baseline sample of each cell supernate obtained before culture was assayed for IgM along with a sample of the 4-h-culture supernate. Viability of the cells immediately before culture was 80–92% as measured by trypan blue exclusion.

*Immunoradiometric Assay of IgM.* The assay method previously described (11) was used, with two modifications. In the first modification, a 96-well microtiter plate (Cooke Engineering Co., Alexandria, Va.) coated with affinity purified-rabbit-anti-mouse Fab was substituted for the previously described anti-IgM coated beads. In the second modification, both the incubation of the IgM-containing sample with the microtiter plate, and the subsequent incubation of <sup>125</sup>I-labeled anti-IgM with the microtiter plate, were shortened from the previously described 4 h, to 1 h at room temperature. The plates were washed after each incubation, and, finally, each well counted in a gamma spectrometer (Beckman Instruments, Inc., Fullerton, Calif.). All manipulations were performed in 0.01 M phosphate-buffered saline, pH 7.6; containing 0.01 M EDTA, 0.015 M sodium azide, 0.1% Triton X-100 and 0.2% bovine serum albumin.

*Assay for Immunoglobulin-Secreting Cells.* Total subclass-specific Ig-secreting cells per spleen were enumerated by the reverse hemolytic-plaque method of Molinaro and Dray (41). Briefly,

SRBC, to which sheep anti-mouse Ig had been coupled, served as targets, while subclass-specific rabbit anti-mouse-Ig reagents (the gift of Dr. Richard Asofsky, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.) served as developers. All assays were performed using the Cunningham and Szenberg modification (42) of the hemolytic-plaque assay.

*Antibody Response to rI·rC.* Castrated mice were injected intraperitoneally with rI·rC (P-L Biochemicals, Inc., Milwaukee, Wis.), 100  $\mu$ g in saline without adjuvant; they were bled 4 d later by retroorbital sinus puncture, and the serum was assayed for antibody to rI·rC as previously described (43). Briefly, 25 or 50  $\mu$ l serum was incubated overnight with 28 ng [ $^{14}$ C]rI·rC (1.8 nCi/ $\mu$ g, Miles Laboratories, Inc., Kankakee, Ill.); ammonium sulfate was then added to make a 35% saturation, and radioactivity counted in both supernate and precipitate in a liquid scintillation counter (Nuclear Chicago Corp., subsid. of G. D. Searle & Co., Des Plaines, Ill.).

This 4-d antibody response to rI·rC, given without protein carrier or adjuvant, has been previously shown to be almost completely of the IgM class (43) and to be absent in CBA/N mice and the F<sub>1</sub> male offspring of CBA/N females (30).

*Immune Response to Pneumococcal Polysaccharide Type III (SIII).* Castrated mice were injected intraperitoneally with 0.5  $\mu$ g of SIII (the gift of Dr. P. J. Baker, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.) in saline without adjuvant. 5 d later, their spleens were removed and cells forming direct hemolytic plaques in a slide assay employing SIII-coated SRBC were counted. Net plaques were calculated by subtracting background plaques obtained using plain SRBC.

*Flow Microfluorometry.* To study B-cell surface-membrane-Ig characteristics, spleen cells were depleted of erythrocytes by ammonium chloride lysis, then suspended at  $5.0 \times 10^7$  cells/ml in Hanks' balanced salt solution containing 3 mM sodium azide and 0.1% bovine serum albumin. 0.1-ml aliquots of the cell suspensions were incubated for 30 min with fluoresceinated F(ab)<sub>2</sub>' fragments of affinity purified-goat-anti-mouse Fab (F1 anti-Fab) or anti-mouse  $\mu$ -chain (F1 anti- $\mu$ ). The cells were then washed twice and subsequently analyzed on a fluorescence activated cell sorter (FACS II, Becton, Dickinson & Co., Mountain View, Calif.). Light-scatter criteria were used to gate on viable cells.

In some experiments, a separate aliquot of erythrocyte-depleted spleen cells was analyzed for cytoplasmic IgM after fixation in a 5% acetic acid, 95% ethanol solution at  $-20^{\circ}\text{C}$ , washed, and stained with F1 anti- $\mu$ . Gating in this case was on all cells.

*Direct Coombs' Assay of Anti-Erythrocyte Autoantibodies.* Blood from each mouse was collected in preservative-free heparin and centrifuged at 10,000  $g$  for 5 min. The erythrocytes were washed three times with phosphate-buffered saline, pH 7.2, and resuspended at a final concentration of 1%. Polyvalent sheep-anti-mouse-Ig serum was heated at  $56^{\circ}\text{C}$  for 30 min and absorbed with an equal volume of packed mouse erythrocytes overnight at  $4^{\circ}\text{C}$ . The direct Coombs' assay was performed by adding to microtiter wells, 25  $\mu$ l of 1% erythrocyte suspension and 25  $\mu$ l of serial twofold dilutions of sheep anti-mouse Ig. The wells were then examined for agglutination after an incubation of 1 h at  $37^{\circ}\text{C}$ . A positive result was assigned to erythrocyte samples that showed agglutination at a serum dilution of 1:40. Known positive and negative controls were performed with each assay.

*Statistical Methods.* Geometric means and standard errors were calculated from the results of the IgM secretion and reverse plaque experiments. Comparisons were made by a two-tailed Student's  $t$  test. Chi-square and Student's  $t$  test were applied to the results of the direct Coombs' assay.

## Results

*Antibody Response to T-Independent Antigens.* Castrated mice of eight strains and crosses were injected with rI·rC and bled 4 d later. In Fig. 1, the results of the assay for antibodies to rI·rC in the serum of these mice are shown. The male mice bearing only the CBA/N X chromosome (CBA/N, CBA/N  $\times$  NZB, and CBA/N  $\times$  DBA/2) all failed to make a significant antibody response, although all of the mice bearing at least one normal X chromosome made a significant response. In particular, both the

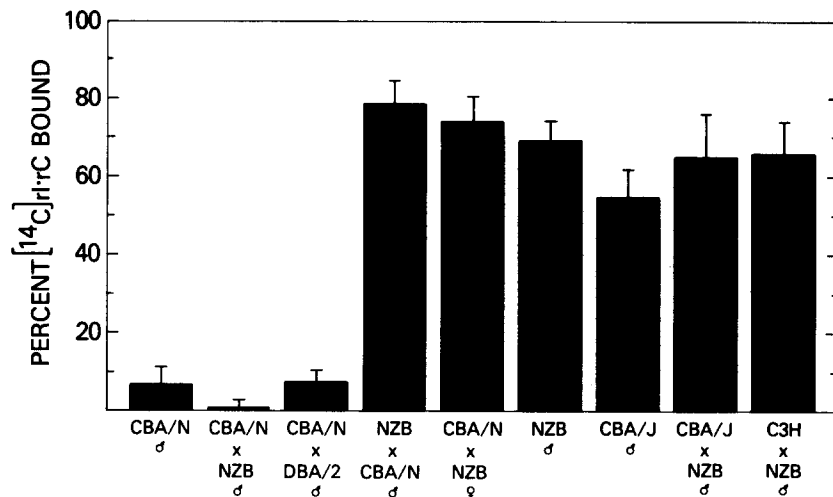


FIG. 1. Antibody response to polyinosinic-polycytidylic acid in mice of different strains and crosses. Mice were immunized with 100  $\mu$ g of rI·rC and bled 4 d later. The percentage of [ $^{14}$ C]rI·rC bound by 50  $\mu$ l of serum (100% is 28 ng) was assayed. Each bar with error bracket represents arithmetic mean  $\pm$  SEM of four or five mice, 7–12 wk of age.

NZB  $\times$  CBA/N males and CBA/N  $\times$  NZB females, which differed from the CBA/N  $\times$  NZB males only in their sex chromosomes, had normal responses.

Similarly, castrated NZB, CBA/N, CBA/N  $\times$  NZB, NZB  $\times$  CBA/N, and CBA/N  $\times$  DBA/2 male mice, aged 12–17 wk, each were injected with 0.5  $\mu$ g of SIII; 5 d later, each spleen was assayed for the direct PFC response to SIII-coated SRBC. None of the mice bearing only a CBA/N X chromosome made a detectable net response to SIII; the NZB and NZB  $\times$  CBA/N males made large responses (data not shown).

**IgM Secretion In Vitro.** Spleen cells from castrated mice of different strains and crosses were incubated in vitro for 4 h and their spontaneous secretion of IgM assayed. The results, summarized in Fig. 2, indicate that spleen cells of mice bearing only a CBA/N X chromosome secreted negligible amounts of IgM ( $P < 0.001$ , compared with CBA/J). NZB spleen cells secreted 10 times more IgM than those of CBA/J mice ( $P < 0.001$ ). Spleen cells of NZB hybrids with at least one non-CBA/N X chromosome secreted quantities of IgM intermediate between those of NZB and CBA/J mice, and significantly different from each ( $P < 0.001$ ).

The CBA/N  $\times$  NZB male spleen cells secreted no more IgM than did those of either CBA/N or CBA/N  $\times$  DBA/2 males. In contrast, the IgM secretion of the CBA/N  $\times$  NZB female and NZB  $\times$  CBA/N male spleen cells was comparable to that of other NZB F<sub>1</sub> hybrids.

**Subclass-Specific Ig Production.** Spleen cells from castrated mice of different strains and crosses were assayed for production of IgM, IgG1, and IgG2, and IgA by a reverse hemolytic-plaque technique. The results of the IgM-producing-cell assay are shown in Fig. 3. CBA/N, CBA/N  $\times$  NZB and CBA/N  $\times$  DBA/2 male spleens had significantly fewer IgM-producing cells per 10<sup>6</sup> spleen cells than did spleens of CBA/J males or NZB F<sub>1</sub> hybrids with at least one normal X chromosome ( $P < 0.001$ ). NZB male spleens had significantly more IgM-producing cells than did all other groups ( $P < 0.001$ ). Because CBA/N, CBA/N  $\times$  NZB, and CBA/N  $\times$  DBA/2 males had

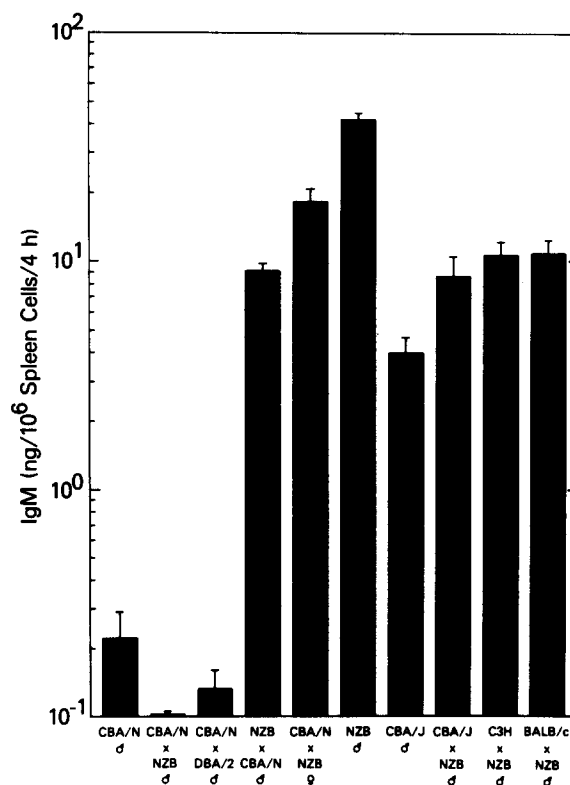


FIG. 2. Secretion of IgM during 4 h, in vitro, by spleen cells from mice of different strains and crosses. Each bar with error bracket represents the geometric mean  $\pm$  SEM of values from 8 to 15 mice, 7–11 wk of age.

reduced numbers of spleen cells, their total IgM-producing cells per spleen were proportionally even further reduced (data not shown).

The results of the IgG1-, IgG2- and IgA-producing cell assays are summarized in Fig. 4. The number of IgG1-producing cells per  $10^6$  cells (Fig. 4A) of CBA/N and CBA/N  $\times$  NZB male spleens were similar to each other and significantly ( $P < 0.001$ ) less than the corresponding number of IgG1-producing cells of the NZB and control NZB F<sub>1</sub> hybrid male spleens. However, the number of CBA/N and CBA/N  $\times$  NZB male IgG1-producing cells were not significantly different ( $P > 0.10$ ) from those of the CBA/J males. The results of the IgG2-producing-cell assay (Fig. 4B) were similar to those of the IgG1 assay. CBA/N and CBA/N  $\times$  NZB males were low and NZB males were high. However, there was overlap of the control NZB F<sub>1</sub> hybrid values with the CBA/J, CBA/N, and CBA/N  $\times$  NZB values. The IgG2-producing cells of all groups tended to be more numerous than those producing IgG1; this phenomenon has previously been observed to be due to nonspecific environmental effects.<sup>2</sup> The number of IgA-producing cells per  $10^6$  spleen cells were similar in all of the mice studied (Fig. 4C).

*Flow Microfluorometry.* Surface-membrane-Ig staining patterns of spleen cells

<sup>2</sup> Rosenberg, Y. J. Unpublished observations.

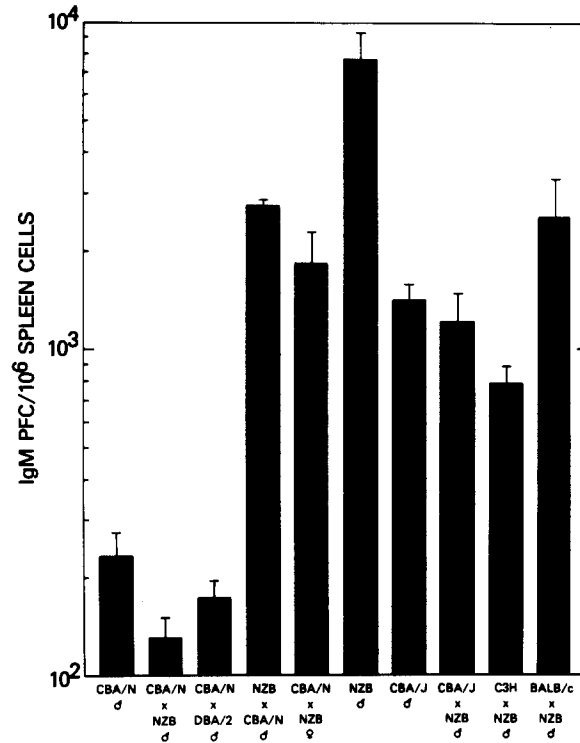


FIG. 3. Total IgM plaque-forming cells per 10<sup>6</sup> spleen cells from mice of different strains and crosses. Each bar with error bracket represents geometric mean ± SEM of values from 4 to 12 mice, 7-11 wk of age.

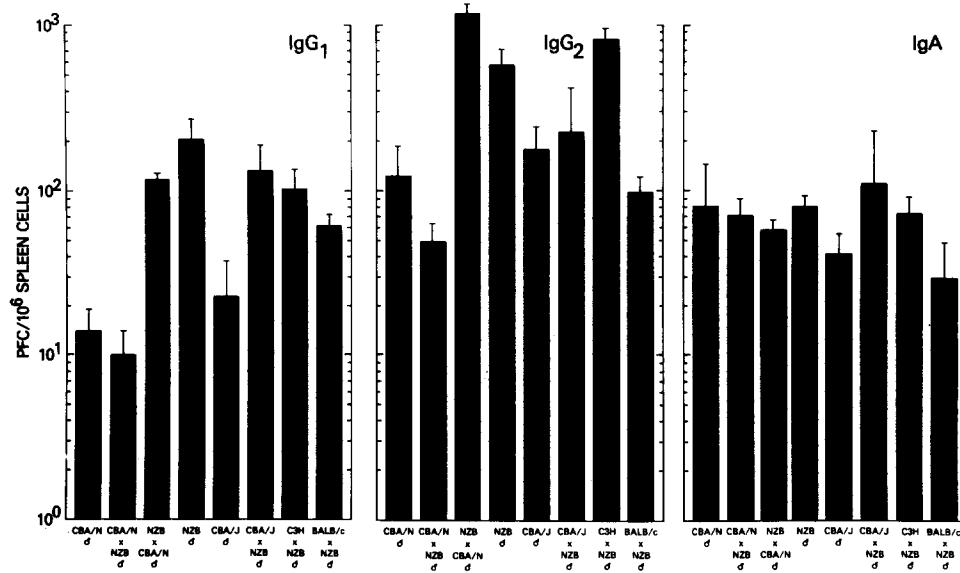


FIG. 4. Total subclass-specific plaque-forming cells per 10<sup>6</sup> spleen cells from mice of different strains and crosses; (A) IgG1, (B) IgG2, and (C) IgA. Each bar with error bracket represents geometric mean ± SEM of values from 5 to 12 mice, 7-11 wk of age.

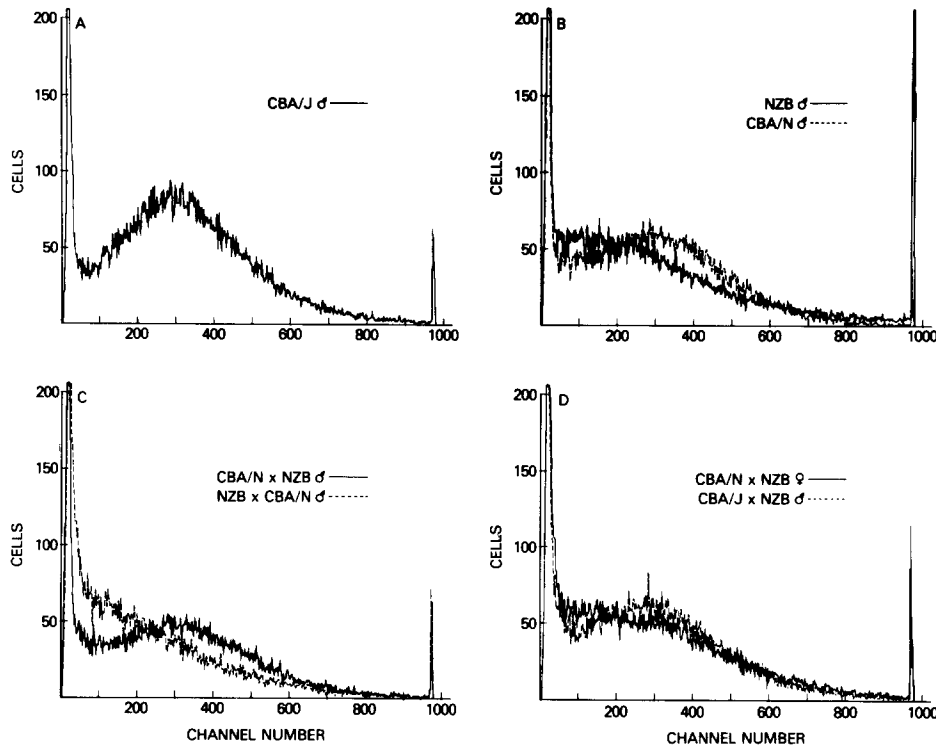


FIG. 5. Fluorescence profiles of spleen cells labeled with F1 anti-Fab from (A) CBA/J male, (B) CBA/N and NZB male, (C) CBA/N  $\times$  NZB and NZB  $\times$  CBA/N males, and (D) CBA/N  $\times$  NZB female and CBA/J  $\times$  NZB male mice, 8–11 wk old. The area under the CBA/J curve is not normalized with respect to the others.

treated with F1 anti-Fab are shown in Fig. 5. The fluorescence-intensity distribution of cells from a CBA/J mouse, shown in Fig. 5A, illustrates that of a normal strain. The CBA/N pattern (Fig. 5B) shows relatively fewer dull (less-intensely fluorescent) cells. In contrast, NZB spleen cells had an increased proportion of these dull cells (Fig. 5B). The CBA/N  $\times$  NZB male pattern (Fig. 5C) did not differ from that of the CBA/N male, whereas the pattern of the NZB  $\times$  CBA/N male spleen cells closely resembled that of the NZB spleen cells (Fig. 5C). CBA/N  $\times$  NZB female and CBA/J  $\times$  NZB male spleen cells (Fig. 5D), had fluorescence-intensity distributions with F1-anti-Fab staining similar to that of NZB, and NZB  $\times$  CBA/N, males.

Surface-membrane-Ig staining patterns of spleen cells treated with fluoresceinated anti- $\mu$  are shown in Fig. 6. Again, the CBA/J pattern, shown in 6A, illustrates that of a normal strain. The CBA/N pattern shows a relative deficiency of dull cells and an excess of bright cells, whereas the NZB pattern is similar to that of the CBA/J (Fig. 6B). The CBA/N  $\times$  NZB male pattern is similar to that of the CBA/N (Fig. 6C) although the NZB  $\times$  CBA/N males (Fig. 6C) CBA/J  $\times$  NZB male, and CBA/N  $\times$  NZB female (Fig. 6D) patterns, like the NZB pattern, are normal.

Results of cytoplasmic staining of fixed spleen cells with fluoresceinated anti- $\mu$  are shown in Table I. Both CBA/N and CBA/N  $\times$  NZB males had significantly fewer positive cells than did CBA/J males ( $P < 0.001$ ), whereas NZB  $\times$  CBA/N males had significantly more IgM-containing spleen cells than did CBA/J males ( $P < 0.001$ ).

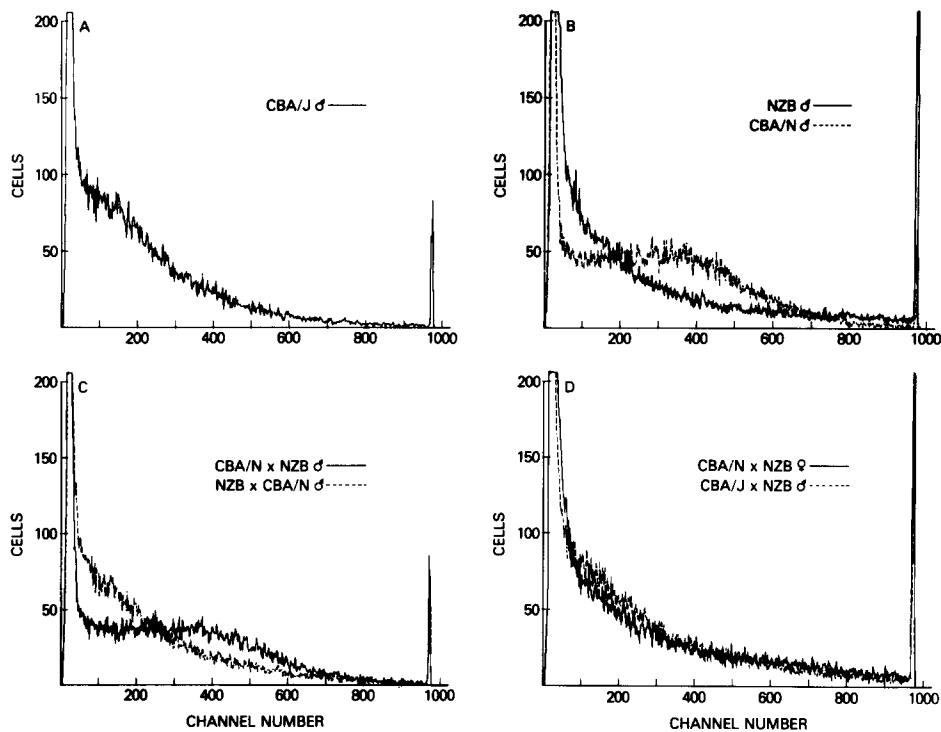


FIG. 6. Fluorescence profiles of spleen cells labeled with F1 anti- $\mu$  from (A) CBA/J male, (B) CBA/N and NZB male, (C) CBA/N  $\times$  NZB and NZB  $\times$  CBA/N male, and (D) CBA/N  $\times$  NZB female and CBA/J  $\times$  NZB male mice, 8-11 wk old. The area under the CBA/J curve is not normalized with respect to the others.

TABLE I  
Fraction of Spleen Cells Positive for Cytoplasmic IgM by Flow Microfluorometry

Strain	Number of mice	Percentage positive for IgM, mean $\pm$ SEM	P value	
			Comparison with CBA/N	Comparison with CBA/J
		%		
CBA/N	3	0.11 $\pm$ 0.02	—	<0.001
CBA/N $\times$ NZB	5	0.18 $\pm$ 0.03	0.10	<0.001
NZB $\times$ CBA/N	8	1.70 $\pm$ 0.07	<0.001	<0.001
CBA/J	5	0.81 $\pm$ 0.06	<0.001	—

Spleen cells were fixed, then stained with F1 anti- $\mu$ , and analyzed by flow microfluorometry. Positive cells contained cytoplasmic IgM. All mice were males.

*Effect of Castration.* 15 3-wk-old C3H/HeN  $\times$  NZB males were divided into three equal groups: castrated, sham-castrated, and intact. The mice were caged together and at age 8 wk, their spleen cells were assayed for IgM secretion in vitro. The geometric means ( $\pm$  SEM) were, respectively, 162 (154-170); 156 (144-169); and 152 (135-170) ng/ $10^7$  cells/4 h, for castrated; sham-castrated; and intact mice. These values are not statistically different.



TABLE II  
*Direct Coombs' Test in 9-mo-old CBA/N × NZB Mice*

Strain	Number of mice	Number positive (titer ≥ 1:40)	Mean titer ± SEM
CBA/N × NZB, females	22	10	3.54 ± 0.90
CBA/N × NZB, males	20	2*‡	0.95 ± 0.68§
CBA/N × NZB, castrated males	17	0‡	0.00 ± 0.00§

\* These two mice, unlike any of the females tested, showed hemolysis, rather than agglutination, of erythrocytes, that may represent a false-positive result.

‡  $P < 0.05$ , compared with females, by chi-square analysis.

§  $P < 0.05$ , compared with females, by Student's  $t$  test.

Similarly, no significant differences occurred in the antibody responses of castrated, sham-castrated, and intact BALB/c × NZB males 4 d after immunization with 100  $\mu\text{g}$  rI·rC in saline.

*Anti-Erythrocyte Antibodies in Older CBA/N × NZB Mice.* 9-m-old CBA/N × NZB mice were studied for the presence of anti-erythrocyte autoantibodies by the direct Coomb's method. The results, presented in Table II, show that both castrated and intact CBA/N × NZB males had significantly less anti-erythrocyte autoantibody on their erythrocytes than did comparably aged, intact CBA/N × NZB females.

### Discussion

In this study we have analyzed, by a variety of techniques, the B-cell properties and functions of CBA/N × NZB  $F_1$  male mice and mice of several control strains and crosses. We have examined, in one series of experiments, most of the immune properties that characterize the X-linked genetic deficiency of CBA/N mice. In all experiments, there was agreement among the various assays and techniques employed: NZB mice have excessive numbers of splenic IgM-producing cells, excessive total-splenic-IgM production, vigorous immune responses to two T-independent antigens, and an increase in splenic B cells bearing a low proportion of total surface Ig. In contrast, CBA/N mice are deficient in total splenic-IgM production, in IgM response to two T-independent antigens, in numbers of splenic IgM-producing cells (by both reverse plaque analysis and cytoplasmic fluorescent staining), and in spleen cells bearing small quantities of surface-membrane IgM. Furthermore, the CBA/N X-linked defect of B-lymphocyte function is fully expressed in CBA/N × NZB  $F_1$  male mice. These hybrids closely resemble homozygous CBA/N mice and control hemizygous CBA/N × DBA/2 male mice in: (a) failure to make antibody responses to two T-independent antigens, (b) reduced numbers of total splenic IgM-secreting cells, (c) barely detectable in vitro secretion of IgM, and (d) cell-surface-Ig characteristics. In contrast, NZB × CBA/N males and CBA/N × NZB females, which lack the X-linked defect, resemble the other NZB  $F_1$  hybrids studied. Thus, despite the excessive B-cell activity of NZB mice, the CBA/N X-chromosome has the same influence on the splenic B cells of a CBA/N × NZB  $F_1$  hybrid male as on those of a CBA/N × DBA/2 or any other previously reported, CBA/N-mothered  $F_1$  hybrid male mouse.

It is remarkable that the CBA/N × NZB  $F_1$  male B-cell functions were not different from those of the CBA/N males. One might have predicted that the CBA/

N × NZB males would have greater spleen cell IgM secretion and greater numbers of splenic IgM-producing cells than CBA/N males on the basis of an effect of the NZB genes upon those B cells not affected by the CBA/N X-chromosome-mediated defect. The observed results suggest that, with regard to abnormalities of IgM production, the NZB genetic defect acts almost exclusively upon that subset of B cells upon which the X-linked CBA/N defect acts. Further work is required to assign relative positions to the CBA/N and NZB genetic influences in the pathway of B-cell differentiation and proliferation.

In contrast to the markedly reduced numbers of IgM-producing cells, the numbers of IgG1-producing spleen cells of the CBA/N-mothered males studied were comparable to those of the normal CBA/J mice. However, their IgG1-producing cells were lower than those of the NZB F<sub>1</sub> hybrids possessing at least one normal X chromosome. These results raise the possibility of a minor effect of the CBA/N X chromosome on the high IgG1 production of NZB mice and their F<sub>1</sub> hybrids. IgA-producing cells were numerically similar in all mice studied, suggesting that neither the B-cell defect of the CBA/N strain nor the B-cell hyperresponsiveness of the NZB strain influences spleen cell IgA production.

Considerable evidence has accumulated to support a prominent role for sex hormones in the expression of autoimmunity in F<sub>1</sub> hybrids of NZB mice (37–40). Therefore, the mice used in the present study were castrated to eliminate potential effects of sex hormones on the functions under investigation. We thought this particularly important in a study of CBA/N hybrids with immune responses known to be influenced by a sex chromosome. Nonetheless, prepubertal castration had no effect upon *in vitro* IgM production by spleen cells of C3H × NZB males, nor on the 4-d IgM antibody response to the T-independent antigen rI·rC in BALB/c × NZB males. These data suggest that castration had neither a positive nor a negative influence on those B-cell functions profoundly suppressed by the presence of the CBA/N X chromosome.

Uncertainty exists regarding the relative importance of B cells and T cells in the expression of autoimmunity in New Zealand mice. The finding that B cells appear to be excessively activated very early in life in NZB mice and their F<sub>1</sub> hybrids has suggested a primary role for the B cell (10, 12, 22, 28). However, T-cell defects also occur in NZB mice. In particular, defects in NZB suppressor-T-cell function not specific for antigen occur by 4 wk of age (24), a time when NZB B-cell excesses have not reached their peak (11). Studies of tolerance to heterologous gamma globulin suggest that both B cells and T cells may contribute to the observed defects (17). Both B-cell (27) and T-cell (26) abnormalities have been observed in the spontaneous anti-erythrocyte production of NZB mice. However, little is known of the specific interaction of T and B cells required for the production of these autoantibodies; less is known about T- and B-cell abnormalities leading to other autoantibody responses. The excessive IgM produced by NZB mice is known to be polyclonal, without a preponderance of specific autoantibody (11). Castration, which profoundly accelerates autoimmunity in NZB hybrids (37–39), had no short-term effect on total IgM secretion or the IgM response to rI·rC. Nevertheless, it is reasonable to assume that the excessive IgM production observed in short-term NZB-spleen-cell cultures bears an important relationship to high serum IgM levels (11) and to IgM-autoantibody production (44) in NZB mice. Our preliminary studies of the spontaneous development of anti-erythrocyte autoantibodies indicate that CBA/N × NZB males have

retarded development of this autoantibody relative to their female littermates. This observation suggests that the CBA/N X-linked gene is capable of favorably modifying the spontaneous expression of autoimmunity. Whether or not the same effects will be observed in NZB mice will be learned from studies of congenic mice bearing the CBA/N X chromosome on an NZB background.

Different spontaneously-produced autoantibodies of New Zealand mice have been shown to be under separate, although complex, genetic control (4, 7, 45). Whether such genetic control involves one subset of lymphocytes, such as B cells, remains unknown. However, we have found in the present study that the genetically conditioned, high IgM, B-cell response of NZB mice may be modified by the CBA/N X-linked gene in such a way as to completely abrogate the effect of the NZB genes. Further studies are in progress to determine the effects of the CBA/N X-linked defect upon the development of autoimmunity in NZB mice. We hope that such studies will serve to further clarify the role of T-independent immune responses in the development of spontaneous autoimmunity.

### Summary

NZB mice and their F<sub>1</sub> hybrids produce excessive polyclonal IgM and autoantibodies of both IgM and IgG classes. CBA/N mice and CBA/N-mothered F<sub>1</sub> males fail to make antibody to many T-independent antigens and have low levels of serum IgM; further, these mice lack a population of splenic B cells characterized by a low-to-intermediate density of surface IgM. We have studied male CBA/N, NZB, CBA/N × NZB, NZB × CBA/N, and CBA/J mice; female CBA/N × NZB mice; and males of several control crosses of NZB and CBA/N mice. We have found that the CBA/N X-linked defect of T-independent immune response is completely expressed in CBA/N × NZB mice. In marked contrast to NZB mice and to NZB F<sub>1</sub> hybrids bearing at least one normal X chromosome, the CBA/N × NZB males failed to respond to two T-independent antigens, had small numbers of splenic IgM-producing cells, barely detectable splenic IgM production, and splenic B-cell surface-Ig patterns resembling those of CBA/N mice. These data suggest that the NZB B-cell abnormality resulting in excessive IgM production occurs almost exclusively in that population of B cells affected by the CBA/N X-chromosome-linked defect. Preliminary studies suggest that the CBA/N X chromosome retards the spontaneous development of anti-erythrocyte autoantibodies in CBA/N × NZB males. Castration, known to accelerate autoimmune disease in certain NZB F<sub>1</sub> males, appears to have no influence on the immune functions examined in this study.

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