

## THE CONTRIBUTION OF NZW GENES TO LUPUS-LIKE DISEASE IN (NZB × NZW)<sub>F1</sub> MICE

BY BRIAN L. KOTZIN<sup>\*§</sup> AND ED PALMER<sup>‡§</sup>

*From the \*Department of Medicine, Veterans Administration Medical Center, Denver, Colorado 80220; the ‡Departments of Medicine and Pediatrics, the National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206; and the <sup>§</sup>Departments of Medicine and Microbiology/Immunology, University of Colorado Health Sciences Center, Denver, Colorado 80262*

New Zealand Black (NZB) × New Zealand White (NZW)<sub>F1</sub> hybrid mice exhibit a markedly different autoimmune process compared with that seen in the parental NZB or NZW strains. The <sub>F1</sub> disease is characterized by the production of antinuclear antibodies and a fatal immune-complex glomerulonephritis, features similar to those observed in human systemic lupus erythematosus (SLE)<sup>1</sup> (1). Unlike the parental strains, (NZB × NZW)<sub>F1</sub> mice develop markedly elevated serum levels of IgG antibodies to histones (2) and double-stranded DNA (dsDNA) (3–6), of which the latter has been causally linked to the renal disease (3, 7–9). Female mice develop an earlier switch from IgM to IgG autoantibodies and an earlier and more severe renal disease compared with their male counterparts (1, 4, 5). Most female <sub>F1</sub> mice die from disease within the first year of life. In contrast, parental NZB mice develop an autoimmune hemolytic anemia as the major feature of their autoimmune disease (1). Renal disease is usually mild and rarely is clinically manifest before 1 yr of age. NZB mice frequently produce IgM antinuclear antibodies and occasionally produce IgG antibodies to single-stranded DNA (ssDNA); however, high levels of IgG antibodies to dsDNA or histones are unusual (6). Although NZW mice may produce antibodies to ssDNA, this strain rarely demonstrates clinical evidence of autoimmune disease during the first 18 mo of life (1, 10–12).

Since the major features of (NZB × NZW)<sub>F1</sub> autoimmune disease are not present in the parental strains, genes from each parent most likely act in concert to produce the <sub>F1</sub> phenotype. While it has been hypothesized (13–15) that a gene or genes in the NZW background accelerate and worsen the mild lupus-like autoimmunity of NZB mice, the precise nature of the NZW genetic contribution to (NZB × NZW)<sub>F1</sub> disease remains unclear. Results from previous backcross studies (6, 11, 16) have been difficult to interpret in terms of the number of involved NZW genes, although one dominant gene appears to be

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<sup>1</sup> *Abbreviations used in this paper:* dsDNA, double stranded DNA; SLE, systemic lupus erythematosus; ssDNA, single stranded DNA.

linked to the H-2<sup>z</sup> locus. As noted above, the switch from IgM autoantibodies (an NZB-like trait) to IgG autoantibodies in F<sub>1</sub> hybrids has suggested an NZW influence on T cell function, and T cells of the L3T4 subset appear to be required for IgG anti-dsDNA antibody production in F<sub>1</sub> mice (17). We and others (18, 19) recently identified an unusual allele of the T cell receptor  $\beta$  chain gene complex in NZW mice that is distinguished by the deletion of 8.8-kb of DNA containing the C $\beta$ 1, D $\beta$ 2, and J $\beta$ 2 gene segments. In more recent studies, we have investigated T cell receptor  $\alpha$  chain genes in NZW mice. Examination of genomic DNA with probes specific for different  $\alpha$  chain variable gene families revealed multiple restriction fragment length polymorphisms compared with NZB and many nonautoimmune strains (Kotzin, B. L., V. Appel, and E. Palmer, unpublished observations).

Thus, at least three unlinked genetic loci have been distinguished in NZW mice that conceivably contribute to T cell dependent autoantibody responses in (NZB  $\times$  NZW)F<sub>1</sub> mice. These include the T cell receptor  $\alpha$  and  $\beta$  chain loci described above as well as the unusual MHC haplotype in NZW (H-2<sup>z</sup>, reference 20). We undertook an (NZB  $\times$  NZW)F<sub>1</sub>  $\times$  NZB backcross to determine the relative contribution of these NZW genes to lupus-like renal disease and autoantibody production in the F<sub>1</sub>. We now report a strong correlation between the presence of the H-2<sup>z</sup> haplotype and lupus-like disease in backcross mice. This MHC or gene(s) linked to this locus appears to be the only dominant NZW genetic contribution to F<sub>1</sub> disease, and there appears to be no association between the presence of NZW T cell receptor alleles and autoimmune disease.

### Materials and Methods

*Mice.* Parental NZB and NZW mice, BALB/c, and C57BL/6 mice were obtained from the NCI Frederick Cancer Research Center, Fort Detrick, Frederick, MD. (NZB  $\times$  NZW)F<sub>1</sub> hybrids and (NZB  $\times$  NZW)F<sub>1</sub>  $\times$  NZB backcross mice were bred in the animal facilities of the V. A. Medical Center, Denver, CO. Only female animals were used for the present experiments. All groups of mice were housed in the same animal room, fed the same diet, and analyzed concurrently. At the beginning of the study, 178 backcross mice (mean age, 4 mo) were randomized to be followed until 12 mo of age. Four of these mice died of unknown causes before 11 mo of age with no evidence of proteinuria, and were excluded from the analysis. Before 12 mo of age, severely ill animals were killed for collection of tissues. At 12 mo of age, all backcross mice were killed.

*Collection of Serum and Tissue Samples.* Experimental mice were usually bled at monthly intervals beginning at 6 mo of age. Blood was obtained from the retroorbital sinus, clotted at room temperature, and the serum was stored at  $-20^{\circ}\text{C}$  until further use. Liver and kidneys were obtained from killed mice and then frozen at  $-70^{\circ}\text{C}$  until further use.

*Proteinuria.* Proteinuria was measured with tetrabromphenol paper (21) on freshly collected urine samples. This colorimetric assay, which is relatively specific for albumin, was graded 1–4<sup>+</sup> and approximated protein concentrations were as follows: 1<sup>+</sup>,  $\sim 30$  mg%; 2<sup>+</sup>,  $\sim 100$  mg%; 3<sup>+</sup>,  $\sim 300$  mg%; and 4<sup>+</sup>,  $\geq 1,000$  mg%. Mice with negative or trace determinations at 12 mo of age were classified as having no evidence of renal disease. Severe proteinuria was defined as  $>2^+$  ( $>100$  mg%). In previous studies (21, 22), mice with this level of proteinuria usually died from renal failure within a period of 4–8 wk.

*Southern Analysis of Genomic DNA.* Liver and kidney DNA from individual mice were isolated as described (23). DNA was then digested with the restriction enzyme Pvu II (2 U/ $\mu\text{g}$  DNA for 1 h at  $37^{\circ}\text{C}$ ); this procedure was repeated once. Digested DNAs were subjected to electrophoresis through 0.7% agarose gels, transferred to nitrocellulose filters, and hybridized with a <sup>32</sup>P-labeled probe as described (24), except that the acid

purination step was omitted and the gel was irradiated for 7 min with shortwave UV light before denaturation (18). Filters were washed several times in 2× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate) at room temperature and once in 0.1× SSC at 55°C for 35 min. Hybridizing fragments were detected by autoradiography. A cDNA probe (pH-2IIA) derived from the 3' untranslated exon of a class I MHC gene (kindly provided by Dr. L. Hood) was used to identify MHC haplotypes (25). This probe hybridizes to multiple class I genes within the MHC of all strains tested. The T cell receptor  $\alpha$  chain allele was identified using a cDNA clone ( $V_\alpha$  7.2) containing  $V_\alpha$ ,  $J_\alpha$ , and  $C_\alpha$  gene sequences (Yague, J., M. Blackman, W. Born, P. Marrack, J. Kappler, and E. Palmer, manuscript submitted for publication). The  $V_\alpha$  7.2 sequence falls into a previously identified  $V_\alpha$  multi-gene family (26). The T cell receptor  $\beta$  chain allele was identified with a  $C_\beta$  probe, pDO $\beta$ 2, derived from a  $C_\beta$ 2 cDNA clone (18, 27). This probe hybridizes to restriction fragments containing  $C_\beta$ 1 and  $C_\beta$ 2 sequences.

**Measurement of Antihistone and Anti-DNA Antibodies.** Antibodies to histones, ssDNA, and dsDNA were quantitated using an ELISA as described previously (2, 22, 28, 29). To measure antihistone antibody activity, total histones were obtained by acid extraction of calf thymus chromatin, diluted in PBS to a concentration of 2.5  $\mu$ g/ml and then 0.2 ml of antigen solution were added per well of an Immulon II microtiter plate (Dynatech Laboratories, Inc., Alexandria, VA). After overnight incubation at 4°C, wells were coated with 0.2 ml gelatin (1 mg/ml in PBS) for at least 16 h at 4°C. Control wells were coated with gelatin only. After washing, 0.2 ml of sera that were diluted 1/500 to 1/2,000 in PBS-Tween with 5 mg/ml bovine  $\gamma$ -globulin and 1 mg/ml gelatin were added and incubated for at least 1.5 h at room temperature. After washing, peroxidase-coupled antibodies to mouse IgM or to mouse IgG (both from Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) were added at saturation levels. After 2 h incubation at room temperature, the wells were washed with PBS-Tween and substrate solution was added. The optical density (OD) was then read at various times thereafter with an automated spectrophotometer (Dynatech Laboratories, Inc.) at 405 nM. All samples were run in triplicate and standard positive and negative control serum samples were run on each ELISA plate. OD values were converted to units of antibody activity (described below).

Antibodies to ssDNA were measured similarly, except that heat-denatured DNA was initially coated to wells at 2.5  $\mu$ g/ml (28, 29). dsDNA was extracted from mouse liver as described (23) and stored at 4°C for no greater than 1 wk before use in the ELISA assay. To successfully attach dsDNA, microtiter wells were first coated with poly-D-lysine (Sigma Chemical Co., St. Louis, MO) at 5.0  $\mu$ g/ml in H<sub>2</sub>O overnight at 4°C. After washing, dsDNA was added at 5.0  $\mu$ g/ml in PBS for ~20 h. Wells were then washed in PBS and then incubated with 5 U/ml sodium heparin diluted in PBS-Tween for 1 h at 20°C. After washing, sera or standards were added as described above. We have demonstrated previously that mAbs specific for histone do not crossreact with DNA in these assays and vice versa (28). In the current study, nearly half of the (NZB  $\times$  NZW) $F_1$   $\times$  NZB backcross mice were negative for antibodies to dsDNA or histones, yet nearly all had markedly elevated antibodies to ssDNA. Thus, this assay for anti-dsDNA antibodies demonstrates very low sensitivity for antibodies to ssDNA.

To allow for interassay comparisons, OD values were converted to a unit scale using antihistone and anti-DNA mAbs described in a previous report (28). Known concentrations of purified mAbs were run with every assay to create a standard curve. OD values from sera were then converted to a unit scale; one unit was defined as that level of antibody activity observed with 1  $\mu$ g/ml of the appropriate mAb.

## Results

**Expression of Renal Disease in (NZB  $\times$  NZW) $F_1$   $\times$  NZB Backcross Mice.** To determine the contribution of NZW genes to (NZB  $\times$  NZW) $F_1$  autoimmune disease, (NZB  $\times$  NZW) $F_1$   $\times$  NZB backcross mice were bred and then analyzed for expression of lupus-like renal disease. Because of the known influence of sex on disease (1, 4, 5), only female backcross,  $F_1$  (NZB  $\times$  NZW), and parental (NZB

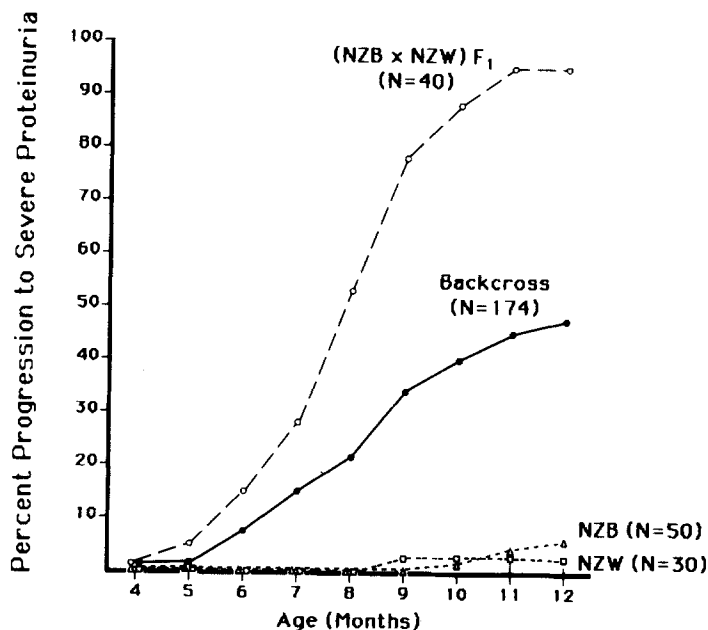


FIGURE 1. Expression of severe proteinuria in (NZB  $\times$  NZW) $F_1$   $\times$  NZB backcross, (NZB  $\times$  NZW) $F_1$ , NZB, and NZW mice. All mice were female, housed in the same area, and analyzed concurrently. Severe proteinuria was defined as  $>2^+$  ( $>100$  mg%) by spot urine colorimetric analysis (21, 22).

and NZW mice) were used for analysis. Fig. 1 shows the percentage of each group that developed severe proteinuria over 12 mo. Beginning at 5–6 mo of age, the percentage of  $F_1$  and backcross mice demonstrating severe proteinuria progressively increased. At 11 mo of age, 95% of  $F_1$  and 45% of backcross mice had developed severe proteinuria. Nearly all of these mice died within 4–6 wk of the initial detection of severe proteinuria, presumably from renal failure (21). At 12 mo of age, 84 of 174 backcross mice (48%) demonstrated evidence of severe renal disease. In contrast to  $F_1$  and backcross mice, parental NZB and NZW mice showed a very low incidence of severe proteinuria throughout the observation period. At 11 mo of age, only one NZW (3%) and two NZB (4%) were positive for severe proteinuria, and no parental animal died before 12 mo of age. Thus the incidence of renal disease in the backcross mice was approximately half of that observed in (NZB  $\times$  NZW) $F_1$  mice, suggesting that a single NZW locus contributes to the  $F_1$  renal disease.

*Production of IgG Antinuclear Antibodies in Backcross Mice.* Beginning at 6 mo of age, backcross and parental mice were serially bled and the sera were analyzed for IgG and IgM antibodies to ssDNA, dsDNA, and histones. NZW, NZB, and nearly all backcross mice had markedly elevated peak IgG anti-ssDNA antibody levels (data not shown) and thus this autoantibody could not be used to discriminate parental from  $F_1$  disease. IgM antibodies to all three antigens were also frequently present in NZB mice. In contrast, no nonautoimmune mice and only rare parental NZB or NZW mice demonstrated elevated levels of IgG anti-dsDNA or antihistone antibodies (Fig. 2). The data are presented as peak values

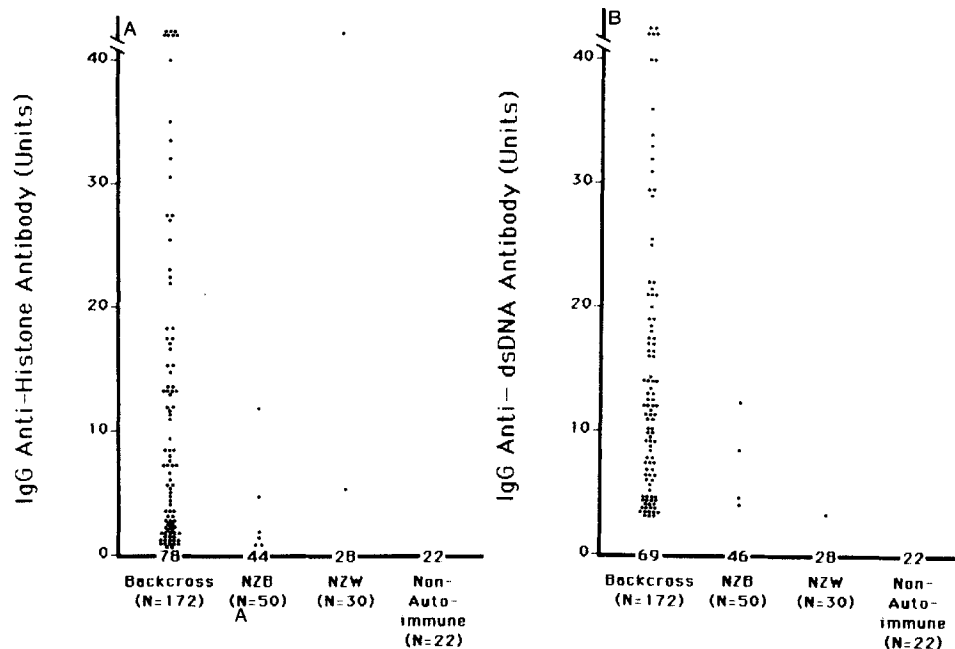


FIGURE 2. Serum levels of IgG antihistone and anti-dsDNA antibody levels in backcross, NZB, NZW, and nonautoimmune (BALB/c and C57BL/6) mice. (A) For antihistone antibody values, the number at the bottom of the column indicates the number of mice with levels  $\leq 0.5$  U. IgG antihistone antibody levels (mean  $\pm$  SE) were  $6.60 \pm 0.86$ ,  $0.36 \pm 0.24$ ,  $1.6 \pm 1.4$ , and  $0.10 \pm 0.04$  for backcross, NZB, NZW, and nonautoimmune mice, respectively. (B) For anti-dsDNA antibody values, the number at the bottom of the column indicates the number of mice with levels  $\leq 3.0$  U. Anti-dsDNA antibody levels (mean  $\pm$  SE) were  $9.9 \pm 1.2$ ,  $0.70 \pm 0.32$ ,  $1.84 \pm 1.53$ , and  $0.28 \pm 0.11$  for backcross, NZB, NZW, and nonautoimmune mice, respectively. All values represent the peak levels for individual mice between 6 and 11 mo of age.

for individual mice determined between 6 and 11 mo of age. Whereas only 4% of NZB and 6% of NZW mice had IgG antihistone antibody levels  $>2$  U, 42% of backcross mice had at least this level. In a similar analysis, 4% of NZB and 3% of NZW mice had IgG anti-dsDNA antibody levels  $>5$  U; in contrast, 47% of the backcross mice had peak values  $>5$  U. These threshold values to categorize mice with elevated levels were determined arbitrarily. However, similar percentages were obtained using a cutoff of 4 SD above the mean level for nonautoimmune mice. Although not measured concurrently, nearly all (NZB  $\times$  NZW) $F_1$  mice routinely developed elevated levels of these autoantibodies in previous analyses (2, 21, 22). Thus, excessive production of these IgG lupus-like antinuclear antibodies in approximately half of the backcross mice also suggests that a single NZW genetic locus contributes to  $F_1$  autoantibody production.

Similar to (NZB  $\times$  NZW) $F_1$  mice (2, 21, 22), diseased backcross mice usually demonstrated peak levels of IgG anti-dsDNA or antihistone antibodies at  $\sim 8$ – $9$  mo of age, and the elevated levels were comparable between the  $F_1$  and backcross groups (data not shown). A high correlation ( $>0.9$ ) was observed between

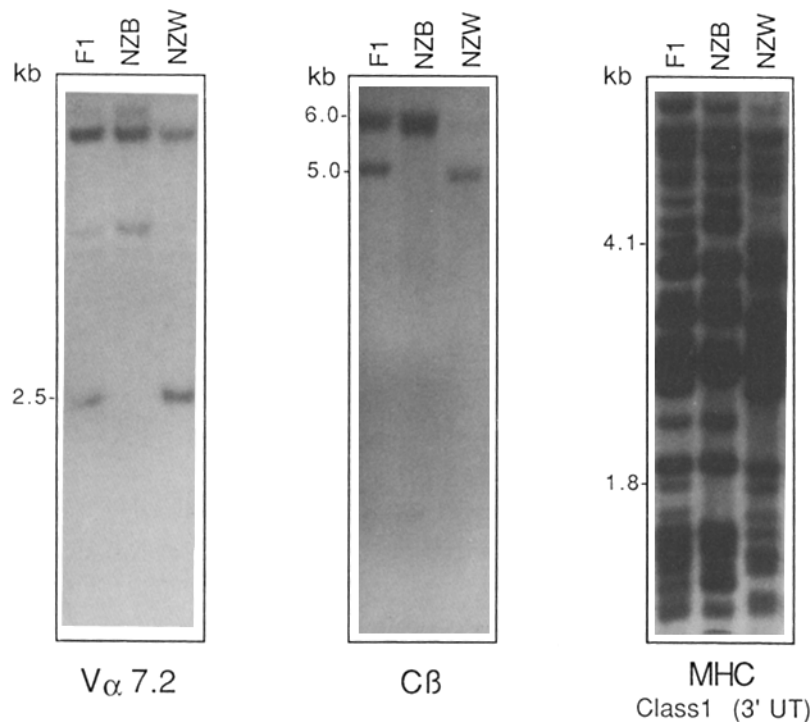


FIGURE 3. Southern analysis of liver DNA from NZB, NZW, and (NZB  $\times$  NZW) $F_1$  mice. All samples were digested with Pvu II. ( $V_\alpha$  7.2) Blots were hybridized with a cDNA probe,  $V_\alpha$  7.2, which recognizes a multigene family of murine T cell receptor  $V_\alpha$  genes. ( $C_\beta$ ) Blots were hybridized with a  $C_\beta$  probe, pDO $\beta$ 2, derived from a  $C_\beta$ 2 cDNA clone, which identifies restriction fragments containing  $C_\beta$ 1 and  $C_\beta$ 2 (18, 27). There are actually two  $C_\beta$ -containing restriction fragments (6.0 and 6.2 kb) in the Pvu II digests of NZB DNA (18). ( $MHC$ ) Blots were hybridized with a cDNA probe (pH-2IIA) derived from the 3' untranslated exon of a class I MHC gene (25). This probe hybridizes to multiple class I genes within the MHC.

elevated levels of these autoantibodies and the expression of renal disease in backcross mice.

*Correlation of Genotype with the Expression of Renal Disease in Backcross Mice.* For any genetic locus, (NZB  $\times$  NZW) $F_1$   $\times$  NZB backcross mice are either heterozygous for the NZW allele (as in [NZB  $\times$  NZW] $F_1$  mice) or homozygous for the NZB allele. We attempted to correlate the genotype of individual backcross mice with the expression of lupus-like autoimmune disease. The genotypes for the MHC, T cell receptor  $\alpha$  chain, and T cell receptor  $\beta$  chain loci in individual mice were determined by Southern analysis of genomic liver DNA. Fig. 3 shows the restriction fragment length polymorphisms used to distinguish NZB, NZW, and  $F_1$  DNA for these three loci. A T cell receptor  $\alpha$  chain cDNA containing the  $V_\alpha$  7.2 gene segment (Yague, J., M. Blackman, W. Born, P. Marrack, J. Kappler, and E. Palmer, manuscript submitted for publication) hybridized to a 2.5-kb fragment in Pvu II digests of NZW DNA that was not present in similarly digested NZB DNA. To identify the NZW T cell receptor  $\beta$  chain allele, a  $C_\beta$  probe (pDO $\beta$ 2) was used that hybridizes to restriction fragments containing  $C_\beta$ 1 and  $C_\beta$ 2 (18, 27). There are actually two  $C_\beta$ -containing restriction

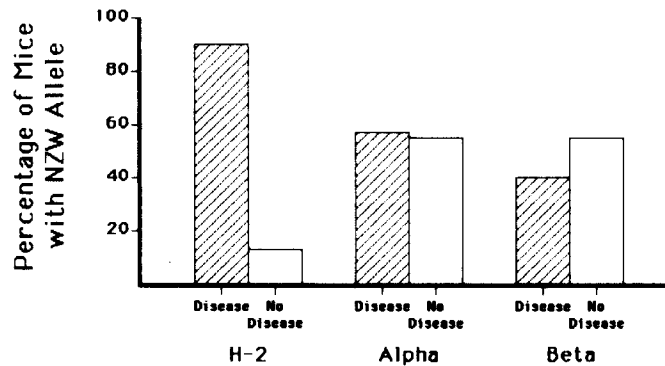


FIGURE 4. Analysis of genotype in backcross mice with severe renal disease before 10 mo of age ( $n = 47$ ) and in backcross mice with no evidence of renal disease when killed at 12 mo of age ( $n = 45$ ). The data are presented as percentage of mice heterozygous for the NZW H-2<sup>d</sup> haplotype, NZW T cell receptor  $\alpha$  chain allele or NZW T cell receptor  $\beta$  chain allele.

fragments (6.0 and 6.2 kb) in the Pvu II digests of NZB DNA that are not resolved. The NZW 5.0-kb fragment containing only C $\beta$ 2 is easily distinguished from the larger NZB fragments in the heterozygous F<sub>1</sub> or backcross mice. The probe, pH-2IIA, used to identify the H-2 region hybridizes to multiple class I loci within the MHC of all mice tested (Hood, L., personal communication, reference 25). Two Pvu II restriction fragments (4.1 and 1.8 kb) are present in NZW (H-2<sup>s</sup>) but not in NZB (H-2<sup>d</sup>) DNA and could be used to identify the NZW allele in backcross mice heterozygous at the MHC.

For genetic analysis, we randomly selected 47 backcross mice that expressed severe proteinuria at or before 10 mo of age and subsequently died from disease. The incidence of severe renal disease in parental NZB mice at 10 mo was 2% and no NZB deaths were recorded during the observation period (see Fig. 1). We also obtained DNA from a similar number of randomly selected backcross mice that showed no evidence of proteinuria (negative or trace by colorimetric analysis) when killed at 12 mo of age. Overall, in these 92 backcross mice, 53%, 57%, and 48% were heterozygous at the NZW MHC, T cell receptor  $\alpha$  chain locus, and T cell receptor  $\beta$  chain locus, respectively. This is close to the expected incidence of 50% for randomly assorting alleles.

The data in Fig. 4 compare backcross mice with and without renal disease. In backcross mice with severe renal disease, 89% carried the NZW MHC haplotype, i.e., these mice were H-2<sup>d/z</sup> heterozygotes ( $p < 0.0001$  by  $\chi^2$  analysis compared with overall 53% frequency of H-2<sup>d/z</sup>). In mice with no evidence of disease, only 15.5% were H-2<sup>d/z</sup> heterozygotes ( $p < 0.0001$  compared with overall frequency). Thus a gene or complex of genes linked to the NZW MHC appears to be strongly associated with disease expression. Nevertheless, the association was not perfect, in that 11% of the diseased mice did not carry the H-2<sup>s</sup> haplotype. In contrast, there was no association between the presence of the NZW  $\alpha$  chain or  $\beta$  chain alleles and the expression of disease. Whereas 57% of diseased backcross mice carried the NZW allele of the T cell receptor  $\alpha$  chain locus, 56% of backcross mice without disease carried this allele. There was also no association of autoim-

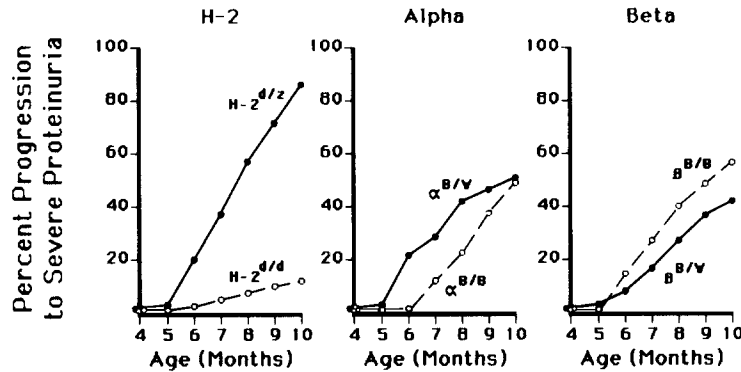


FIGURE 5. Expression of severe renal disease with age in 92 backcross mice grouped by genotype. The data are presented as the percentage of mice with severe proteinuria. There were 49, 52, and 44 mice heterozygous for the NZW H-2<sup>d</sup> haplotype, NZW T cell receptor  $\alpha$  chain locus ( $\alpha^{B/w}$ ), and NZW T cell receptor  $\beta$  chain locus ( $\beta^{B/w}$ ), respectively.

mune disease with the NZW  $\beta$  chain locus. Indeed, only 40% of mice with disease carried this allele compared with 56% of the backcross mice without disease.

Particular genotypes were also examined for correlations with the expression of severe proteinuria. As shown in Fig. 5, backcross mice with the NZW H-2<sup>d</sup> haplotype demonstrated a progressive increase in the incidence of disease during the observation period. At 10 mo of age, 86% of the H-2<sup>d/z</sup> mice had expressed severe proteinuria compared with 12% of the H-2<sup>d/d</sup> mice ( $p < 0.0001$ ). It should be noted that the disease in these five H-2<sup>d/d</sup> mice was indistinguishable from that in the H-2<sup>d/z</sup> mice. In contrast to H-2<sup>d</sup>, no significant difference in disease expression was apparent in mice heterozygous for the NZW T cell receptor  $\alpha$  chain locus compared with those homozygous for the NZB allele (52 vs. 50% at 10 mo). Mice with the NZW  $\beta$  chain allele actually showed a slightly lower incidence of renal disease compared with those homozygous for the NZB allele (43 vs. 58%).

*Correlation of Genotype with the Production of IgG Antinuclear Antibodies in Backcross Mice.* We also were able to correlate the genotype of the backcross mice with the production of IgG antihistone and anti-dsDNA antibodies. Fig. 6 shows that the only difference in mean serum levels of antihistone antibodies exists when H-2<sup>d/z</sup> mice are compared with H-2<sup>d/d</sup> mice (mean  $\pm$  SE,  $11.9 \pm 1.52$  for H-2<sup>d/z</sup> mice vs.  $1.72 \pm 0.81$  for H-2<sup>d/d</sup> mice,  $p < 0.001$ , as shown by the Wilcoxon rank sum test). Similar to percentages observed for development of proteinuria, 88% of animals with serum levels  $>2$  U were H-2<sup>d/z</sup> while 12% of the mice homozygous for H-2<sup>d</sup> demonstrated this degree of elevation (compared with 4% of NZB mice; see Fig. 2). The presence of either NZW T cell receptor allele was not predictive of circulating IgG antihistone antibody levels.

Nearly identical results were obtained for IgG anti-dsDNA antibody levels (Fig. 7). A large difference in mean serum levels was apparent when comparing H-2<sup>d/z</sup> with H-2<sup>d/d</sup> backcross mice ( $15.8 \pm 1.47$  for H-2<sup>d/z</sup> mice vs.  $5.48 \pm 1.76$  for H-2<sup>d/d</sup> mice,  $p < 0.001$ ). Using 5 U of IgG anti-dsDNA activity as a cutoff, 86% of mice with elevated levels were H-2<sup>d/z</sup>. In contrast, 16% of the mice homozygous for H-2<sup>d</sup> demonstrated elevated levels of these antibodies compared



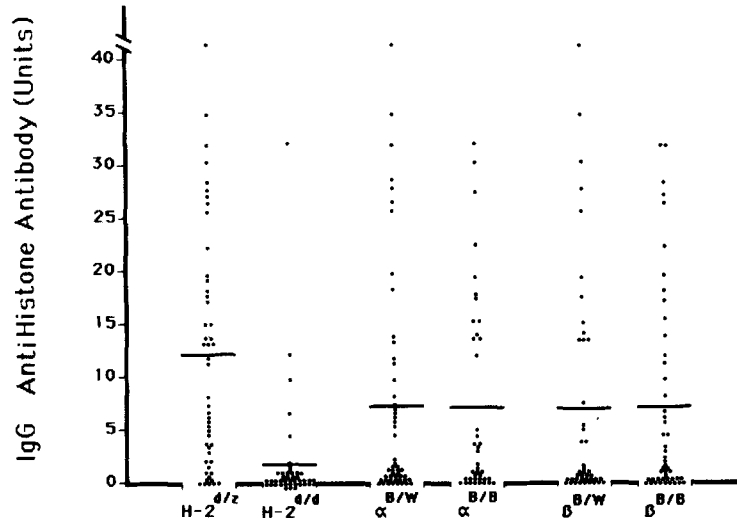


FIGURE 6. Levels of IgG antihistone antibody activity in the sera of backcross mice with different genotypes. Each point represents the peak level for an individual animal measured between 6 and 11 mo of age. The lines indicate the mean level for the different groups of mice.

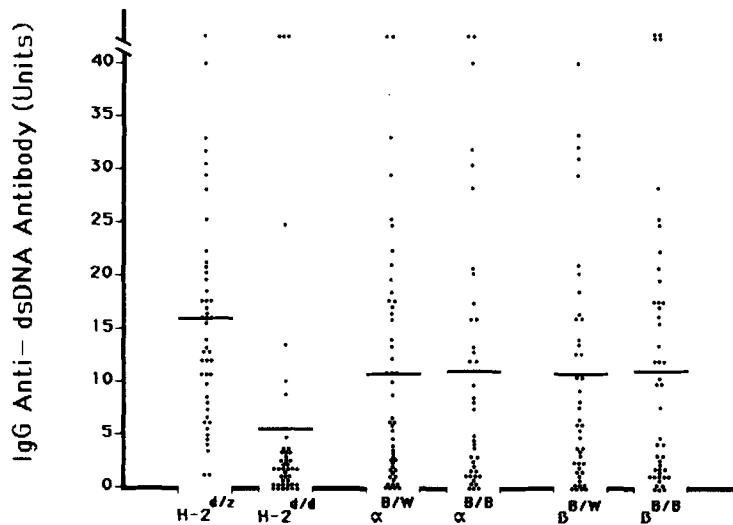


FIGURE 7. Levels of IgG anti-dsDNA antibody activity in the sera of backcross mice with different genotypes. Each point represents the peak level for an individual animal measured between 6 and 11 mo of age. The lines indicate the mean level for the different groups of mice.

with 4% of parental NZB mice (see Fig. 2). Again, the levels of these autoantibodies could not be correlated with  $\alpha$  chain or  $\beta$  chain genotypes.

*Correlation of Genotype with the Production of IgM Anti-dsDNA Antibodies in Backcross Mice.* We also determined whether the presence of any of these three NZW loci influenced the production of IgM anti-dsDNA antibodies in backcross mice. Fig. 8 shows that in contrast to IgG anti-dsDNA antibodies, IgM antibody

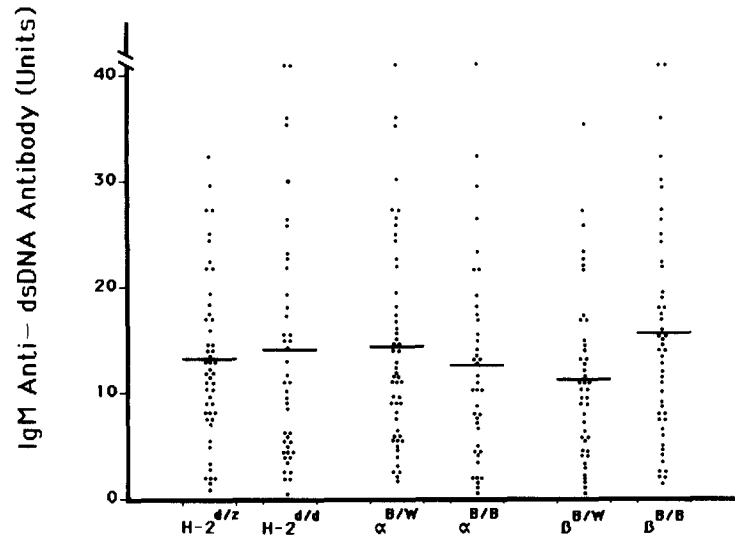


FIGURE 8. Levels of IgM anti-dsDNA antibody activity in the sera of backcross mice with different genotypes. Each point represents the peak level for an individual animal measured between 6 and 11 mo of age. The lines indicate the mean level for the different groups of mice.

levels were not influenced by the H-2 genotype. Thus, both H-2<sup>d/z</sup> and H-2<sup>d/d</sup> backcross mice had similar serum IgM anti-dsDNA antibody levels (mean  $\pm$  SE,  $13.1 \pm 1.1$  U for H-2<sup>d/z</sup> mice vs.  $13.9 \pm 1.8$  U for H-2<sup>d/d</sup> mice). No difference was detected when mice heterozygous for the NZW  $\alpha$  chain allele were compared with those homozygous for the NZB allele. Mice carrying the NZW allele for the T cell receptor  $\beta$  chain locus showed a small decrease in mean IgM anti-dsDNA activity ( $11.2 \pm 1.2$  U vs.  $15.5 \pm 1.6$  U in mice homozygous for the NZB allele,  $p = 0.05$  by Student's *t* test). This difference was much smaller than that observed for the influence of H-2 genotype on IgG antinuclear antibodies.

### Discussion

The data from this backcross strongly suggest that one dominant gene or closely linked group of genes accounts for the NZW contribution to (NZB  $\times$  NZW)<sub>F1</sub> lupus-like disease. Approximately half of the backcross mice expressed severe renal disease at an age when 95% of the F<sub>1</sub> mice and <5% of parental mice were similarly affected. Although the distinction between positive and negative is not as clear-cut, a similar percentage of backcross mice produced IgG anti-dsDNA and antihistone antibodies, autoantibodies that are characteristic of F<sub>1</sub> but not parental mice. ~40–50% of backcross mice demonstrated elevated levels for both autoantibodies compared with <5% for NZB mice. The correlation of genotype with expression of disease also supports a single-dominant-gene hypothesis. Thus, 86% of H-2<sup>d/z</sup> mice expressed early and severe proteinuria. If two or more unlinked NZW genes are required, one would expect <50% of mice with any particular allele and <25% of the total backcross mice to express disease. Similarly, 90–95% and 75–80% of H-2<sup>d/z</sup> mice had elevated levels of IgG anti-dsDNA and antihistone antibodies, respectively. The high expression

of disease in H-2<sup>d/z</sup> mice is even more impressive when one considers the incomplete expression of disease in F<sub>1</sub> mice (95%) and the inexact correlation of the H-2<sup>z</sup> allele with disease (see below).

Previous studies (6, 11, 16) of (NZB × NZW)F<sub>1</sub> × NZB backcross mice have suggested that one or two dominant NZW genes contribute to the incidence and severity of lupus-like renal disease. Interestingly, in studies (6, 11) suggesting a single NZW genetic contribution to expression of proteinuria, two genes appeared to be involved in anti-dsDNA antibody production. In contrast, we found an extremely high correlation between expression of renal disease, IgG anti-dsDNA, and IgG antihistone antibody production. Thus, in the present study, one NZW gene appears to influence all of these traits. We cannot exclude, however, that each trait is controlled by different but closely linked loci. The reason for the discrepancy among these analyses is unclear. It is known that environmental influences such as viral infections, stress, and diet can influence the incidence and severity of disease in (NZB × NZW)F<sub>1</sub> mice (30–35). It is conceivable that these factors can override the requirement for a particular gene. Serologic evidence of murine hepatitis virus has been detected intermittently in our animal facility as it has recently in many facilities around the country. However, the kinetics of F<sub>1</sub> disease expression and autoantibody production in our breeding facility have been fairly constant for the last 5 yr and are similar to that previously observed in another facility (21).

We and others previously described (18, 19) a distinctly unusual T cell receptor  $\beta$  chain allele in NZW mice, characterized by the deletion of C $\beta$ 1, D $\beta$ 2, and J $\beta$ 2 gene segments. No other mammal studied thus far has been noted to have lost the duplication of  $\beta$  chain gene segments. Despite the unusual nature of this gene complex in an animal known to contribute genes to autoimmune disease, we found no association of disease expression with the presence of this allele in the backcross mice. The incidence of proteinuria as well as serum levels of autoantibodies were actually slightly higher in mice homozygous for the NZB allele. Using a similarly designed backcross study, Yanagi et al. (36) recently reported that the presence of the NZW  $\beta$  chain locus was associated with increased levels of IgG anti-DNA antibodies and gp70 immune complexes. This has not been confirmed in the present work and the reason for the difference in results is unknown. It is difficult to envision how a deletion of T cell receptor  $\beta$  chain gene segments behaves dominantly in an F<sub>1</sub> animal to account for enhanced autoantibody production. We are currently carrying out crosses to test whether the deletion of  $\beta$  chain gene segments in NZW mice offers protection in some way from certain types of autoantibody production. It is conceivable that the addition of new NZB T cell receptor gene segments (i.e., D $\beta$ 2, J $\beta$ 2) in F<sub>1</sub> mice might influence the expression of autoimmune disease.

We also found no association of disease expression with the NZW T cell receptor  $\alpha$  chain allele. NZW V $\alpha$  genes are polymorphic in that several probes specific for different V $\alpha$  families reveal restriction fragment length polymorphisms in NZW mice that are not seen in NZB or other nonautoimmune strains (Kotzin, B. L., V. Barr, and E. Palmer, unpublished observations). It is noteworthy that NZW mice carry rare T cell receptor  $\alpha$  and  $\beta$  chain alleles, as well as an unusual MHC haplotype. It should be emphasized that the NZW strain was

developed separately from other New Zealand strains (37), and therefore, the presence of unusual alleles in NZW but not in NZB mice is not surprising.

We found a strong but imperfect correlation between the expression of lupus-like disease and the presence of the NZW H-2<sup>z</sup> haplotype. Of the mice that developed severe proteinuria, ~90% were heterozygous (H-2<sup>d/z</sup>). Furthermore, 12% of the homozygous H-2<sup>d</sup> mice developed severe renal disease. Similar correlations were observed for IgG but not for IgM antinuclear antibody production. This association with MHC-linked genes is consistent with previous studies in which backcross mice were phenotyped using anti-H-2 antisera (6, 11, 15). Our data were obtained using a probe that recognizes multiple class I genes that are all encoded within the MHC (reference 25 and Hood, L., personal communication). Although the chromosomal positions of the genes corresponding to the polymorphic NZW bands that were used for MHC typing are unknown, these genes must be tightly linked ( $\leq 1.5$  cM) to the MHC H-2K, I $\alpha$  region, and H-2D genes. In recent work using a probe specific for the I-A  $\alpha$  chain gene, we have confirmed (without a single exception) the H-2 genotype of the diseased backcross mice (Kotzin, B. L., and E. Palmer, unpublished observations).

A surprising feature of the present study is the frequency of discordance between the autoimmune phenotype and the presence of H-2<sup>z</sup>. Assuming that the NZW gene controlling disease expression behaves in a Mendelian fashion, there is an apparent recombination rate between this gene and the MHC of ~10%. Although it is easy to believe that the disease gene lies within H-2, our results suggest that this gene may be located ~10 cM away from the MHC. It is difficult to discount this conclusion on the basis of incomplete penetrance since 14% of the H-2<sup>d/z</sup> backcross mice failed to develop severe disease compared with only 5% of F<sub>1</sub> mice. Furthermore, a background effect seems an unlikely explanation because 12% of H-2<sup>d/d</sup> backcross mice expressed severe renal disease compared with only 2% of similarly aged NZB mice. Still, a rare NZB (H-2<sup>d/d</sup>) animal can develop severe lupus-like autoimmune disease and this low level of expression might be modified by a combination of non-H-2-linked genes from the NZW. We are currently attempting to define the position of the disease gene using genomic DNA from these backcross mice and DNA probes located 5–10 cM centromeric and telomeric to the MHC.

### Summary

Unlike parental NZB or NZW mice, (NZB  $\times$  NZW)F<sub>1</sub> mice exhibit a lupus-like disease characterized by high serum levels of IgG antinuclear antibodies and a fatal immune-complex glomerulonephritis. At least three unlinked gene loci can be distinguished in NZW mice that conceivably contribute to a T cell-dependent autoimmune disease, including the MHC (H-2<sup>z</sup>) and the T cell receptor  $\alpha$  and  $\beta$  chain gene complexes. We undertook an (NZB  $\times$  NZW)F<sub>1</sub>  $\times$  NZB backcross to determine the relative contribution of these NZW genes to lupus-like renal disease and autoantibody production in F<sub>1</sub> mice. The incidence of severe renal disease and elevated levels of IgG antibodies to dsDNA and histone in the backcross mice was approximately half of that observed in (NZB  $\times$  NZW)F<sub>1</sub> mice. Furthermore, there was a strong correlation between the presence of the NZW H-2<sup>z</sup> haplotype and lupus-like disease in backcross mice.

~90% of backcross mice with disease carried the NZW H-2<sup>z</sup> locus compared with 16% of mice without disease; nearly 90% of H-2<sup>d/z</sup> mice expressed severe autoimmune disease. In contrast, no association was apparent between the presence of the NZW T cell receptor  $\alpha$  chain gene complex or  $\beta$  chain gene complex and severe renal disease or autoantibody production. Thus, the NZW MHC or gene(s) linked to this locus appear to be the only dominant NZW genetic contribution to F<sub>1</sub> disease.

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