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This information is current as of March 26, 2019.

J Immunol 1998; 160:2757-2766; ;
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Contributions of Ea^z and Eb^z MHC Genes to Lupus Susceptibility in New Zealand Mice¹

Timothy J. Vyse,* Stephen J. Rozzo,* Charles G. Drake,* Virginia B. Appel,* Marianne Lemeur,[†] Shozo Izui,[‡] Ed Palmer,[§] and Brian L. Kotzin^{2*¶}

Unlike parental New Zealand Black (NZB) or New Zealand White (NZW) mice, (NZB × NZW)F₁ mice exhibit a lupus-like disease characterized by IgG autoantibody production and severe immune complex-mediated nephritis. In studies of the genetic susceptibility to disease in this F₁ model, the NZW MHC ($H2^z$) has been strongly linked with the development of disease, and it was hypothesized that class II MHC genes, particularly E^z genes, may underlie this genetic contribution. In the present study, we bred transgenic B6 mice expressing I-E^z or congenic B6 mice carrying $H2^z$ with NZB mice and used a backcross analysis to test the hypothesis that Ea^z and/or Eb^z genes account for the effect of $H2^z$ on disease. The genetic analysis of different backcross combinations showed that unlike mice carrying $H2^z$, mice inheriting E^z transgenes do not demonstrate increased IgG autoantibody production or increased incidence of nephritis. Surprisingly, in the same transgenic backcross mice, inheritance of the endogenous $H2^b$ from the B6 strain was strongly linked with the production of IgG autoantibodies, but not with disease. Additional experiments suggested that the level of IgG3 autoantibody production, which is controlled by $H2$, may be important in the pathogenesis of renal disease. Contributions to autoantibody production were also detected from an NZB locus on distal chromosome 1 (previously named *Nba2*). Together, these studies provide new insight into the role of MHC in lupus-like autoimmunity. *The Journal of Immunology*, 1998, 160: 2757–2766.

New Zealand hybrid mice are among the best-studied models of human systemic lupus erythematosus (SLE).³ The female F₁ progeny of New Zealand Black (NZB) and New Zealand White (NZW) mice spontaneously develop a severe immune complex glomerulonephritis and usually die from renal failure before 1 yr of age (1). The production of particular IgG antinuclear Abs, such as autoantibodies to dsDNA and to chromatin, and the production of IgG autoantibodies to the endogenous retroviral glycoprotein gp70 are believed to be most important in the pathogenesis of this lupus nephritis (1–3). Genes from both the NZB and NZW strains are important for disease (reviewed in Ref. 4). In studies of the genetic contributions to disease in the F₁ model, genes encoded within or closely linked to the MHC have been shown to be pivotal for the development of pathogenic autoantibody production and the development of severe lupus nephritis (5–8). For example, in an analysis of (NZB ×

NZW)F₁ × NZB backcross mice, the most important contribution from the NZW strain was closely linked to the MHC ($H2^z$ in NZW) (6, 9). Furthermore, in a recent analysis of (B6.H2^z × NZB)F₁ × NZB backcross mice, inheritance of $H2^z$ derived from the congenic B6.H2^z strain was strongly linked to the development of severe nephritis and the production of pathogenic IgG autoantibodies (10, 11). Studies of NZB and NZW strains congenic for $H2^z$ and $H2^d$, respectively, and their F₁ hybrids have additionally supported the importance of heterozygous $H2^z$ expression in the development of severe lupus-like disease (7, 8).

The genes encoded within $H2^z$ that contribute to lupus in New Zealand hybrid mice are not known. Since the production of pathogenic IgG autoantibodies and the development of lupus nephritis in this model are CD4⁺ T cell dependent (12), it was hypothesized that class II MHC genes, either A^z or E^z , are likely candidates. This hypothesis is further supported by studies of NZB mice congenic for either $H2^b$ or $H2^{bm12}$ (13). Although the difference in the MHCs of these strains is limited to three amino acids in the I-Aβ molecules, studies showed that NZB.H2^{bm12} mice developed severe disease similar to (NZB × NZW)F₁ mice, whereas NZB.H2^b mice were similar to NZB ($H2^d$) and did not develop severe lupus nephritis. Based on particular sequence homologies between I-Aβ^{bm12} and I-Eβ, these (13) and other investigators (14) postulated that expression of I-E^z or of mixed class II molecules such as I-Eα^d/I-Eβ^z was most likely to determine the contribution of $H2^z$ genes to lupus in the (NZB × NZW)F₁ model.

In the present study, we used transgenic mice expressing I-E^z to test the hypothesis that $H2$ - Ea^z (Ea^z) and/or $H2$ - $Eb1^z$ (Eb^z) genes account for the $H2^z$ genetic contribution to lupus in the New Zealand hybrid model. Our analysis of backcross mice showed that unlike mice carrying $H2^z$, mice inheriting the Ea^z and Eb^z transgenes do not demonstrate increased IgG autoantibody production or increased incidence of nephritis. In contrast, in the same transgenic backcross mice, inheritance of the endogenous $H2^b$ from the B6 strain and inheritance of a NZB locus on distal chromosome 1

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Received for publication September 5, 1997. Accepted for publication November 25, 1997.

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¹ This work was supported by Grant AR37070 from the National Institutes of Health, a grant from the Swiss National Foundation for Scientific Research, and a grant from the Ministry of Health and Welfare, Japan.

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³ Abbreviations used in this paper: SLE, systemic lupus erythematosus; NZB, New Zealand Black; NZW, New Zealand White; SLP, simple sequence length polymorphisms; gp70 IC, gp70-anti-gp70 immune complexes; QTL, quantitative trait locus.

(previously named *Nba2*) were strongly linked with the production of IgG autoantibodies.

Materials and Methods

Mice

Parental NZB/BINJ and C57BL/6J (designated B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained in the animal care facility at the National Jewish Medical and Research Center (Denver, CO). All congenic, transgenic, F₁, and backcross mice were bred and maintained at the National Jewish Medical and Research Center. Only female mice were studied for expression of disease.

B6 mice were made congenic for *H2^Z* by mating these mice with NZW mice and backcrossing the progeny to B6. Inheritance of *H2^Z* was monitored by immunofluorescence analysis of I-A^Z expression and by screening for a simple sequence length polymorphism in the TNF- α gene (*Tnfa*) (15). The congenic strain (designated B6.H2^Z) was made homozygous for *H2^Z* after 12 generations. Congenic mice were analyzed for the length of the NZW chromosome 17 interval bred onto the recipient B6 strain. In relation to the MHC, analysis of markers approximately 1 cM proximal to MHC on chromosome 17 (*D17Mit16*; ~18.2 cM from the centromere), within the MHC (*Tnfa* (15) or *H2^Z Alu* repeat; ~19 cM from the centromere), and about 4 cM distal to MHC (*D17Mit49* or *D17Mit50*; ~23.2 cM from the centromere) showed alleles inherited from NZW in the congenic animals.

In preparation for the generation of transgenic mice, genomic fragments encoding the *Ea^Z*- and *Eb^Z*-coding regions were isolated from an NZW splenic DNA cosmid library (see below). Transgenic mice were generated in the laboratories of Diane Mathis and Christophe Benoist (Strasbourg, France) using methods previously described (16). B6 eggs were coinjected with *Ea^Z* and *Eb^Z* genomic DNA and reimplanted into foster mothers, and tails from the resulting offspring were analyzed by Southern blotting for integration of the injected DNA. Three founders were initially identified, of which one was found to have both *Ea^Z* and *Eb^Z* genes. This line was perpetuated by repeated backcrossing with B6 mice. Inheritance of the transgenes was determined by PCR analysis of genomic DNA. Primer sequences (5'-3') to detect the *Eb^Z* transgene were CTACAACCGGGAG GAGTGGG (forward) and TCCACCGCGGCCCGCTTTG (reverse), and primer sequences to detect the *Ea^Z* transgene were AAGTGAACTCA GATACTAA (forward) and CCAGGGCTCCAATTGTGGCC (reverse). Occasional offspring were also analyzed by immunofluorescence staining for expression of I-E on peripheral blood cells (see below). In the process of backcrossing, two sites of integration were identified, and these were separated during breeding to generate two transgenic lines, each with *Ea^Z* and *Eb^Z* genes. One of the lines was designated B6.E^Z, and the other had lower copy numbers and relatively lower levels of I-E expression and was designated B6.E^Zlo. Integration sites for each of these lines were not linked to MHC or to loci on distal chromosome 1 and were not studied further. Integration of the E^Z genes had no noticeable effect on the health of the B6 recipients, and there was no evidence of autoimmune disease or autoantibody production in the transgenic strains.

Evaluation of renal disease and collection of tissue

Mice were studied from 4 to 12 mo of age and were evaluated for proteinuria at bimonthly intervals using tetrachlorophenol-tetrabromosulfophthalein paper (Chemstrip, Boehringer Mannheim, Indianapolis, IN) as previously described (17). A scoring system of 0 to 3+ was used, as follows: 0/trace, <30 mg/dl; 1+, ~30 mg/dl; 2+, ~100 mg/dl; and 3+, >300 mg/dl. A score of 2+ or greater was considered indicative of severe proteinuria, and mice exhibiting severe proteinuria on two or more successive occasions or at the final evaluation before death were considered positive for renal disease. A negative phenotype was ascribed to mice that did not exhibit proteinuria during the 12 mo of follow-up, and these mice appeared healthy at the time of death. A correlation between severe proteinuria and death from renal failure was demonstrated previously (6), and a strong correlation with histologic severity of glomerulonephritis has been more recently confirmed (T. J. Vyse and B. L. Kotzin, unpublished observations), supporting the validity of using high levels of proteinuria as an indicator of severe and progressive glomerulonephritis. Similar to past studies (3, 6, 17, 18), the development of proteinuria also predicted early mortality in the current study. For example, of the 14 (B6.H2^Z × NZB)F₁ × NZB backcross mice that developed proteinuria by 10 mo of age, 13 (93%) died by 12 mo of age. In contrast, none of the 69 mice in this cohort without proteinuria died during the 1-yr observation period.

The tip of the tail from all backcross mice was excised at 4 mo of age. The liver and kidneys were collected at the time of death or elective sacrifice at 12 mo of age. All tissues were stored at -70°C, and DNA was extracted as previously described (19). The study mice were also bled

(from the tail) at monthly intervals from the age of 5 mo. The blood was allowed to clot at room temperature, and the serum was stored at -20°C until analyzed for autoantibody levels.

Generation of NZW splenic DNA cosmid library

DNA extracted from NZW spleen cells was used to generate a cosmid library as previously described (20). Splenic DNA was partially digested with *Mbo*I to generate 35- to 45-kb fragments, ligated into *Bam*HI-digested pCV 107 cosmid vector, packaged (Gigapack Gold, Stratagene, La Jolla, CA), and grown in *Escherichia coli*. The library was plated at about 10,000 colonies/filter, and 4.1 × 10⁵ total colonies were screened. Probes were generated from mRNA expressed by LPS-stimulated B cell blasts from NZW mice, PCR amplification of segments of the *Ea^Z* and *Eb^Z* genes, and cloning of PCR fragments into pEMBL. Before generation of the transgenic mice, the selected cosmid clones (see Fig. 1) were shown to mediate expression of I-E after transfection into A20 cells.

Analysis of B cell surface I-A and I-E expression

Spleen cells from the different parental strains and backcross mice were prepared and stained as previously described (21). Fluoresceinated mAbs used included 10-2-16 (anti-I-A^Z (22); hybridoma cells obtained from American Type Culture Collection, Rockville, MD), D3 (anti-I-A^b; provided by Dr. John Cambier, Denver, CO), and 14-4-4s (anti-I-E α (23); hybridoma cells obtained from American Type Culture Collection). B cells were also double stained using a biotinylated mAb to B220 (RA3-6B2; PharMingen, San Diego, CA) followed by avidin-phycoerythrin (PharMingen). Fluorescence intensity was analyzed on an EPICS C flow cytometer (Coulter Electronics, Inc., Hialeah, FL). Viable mononuclear cells were gated by scatter analysis, and 1 × 10⁴ cells were collected for each Ab combination.

Genomic mapping using simple sequence length polymorphisms (SSLP)

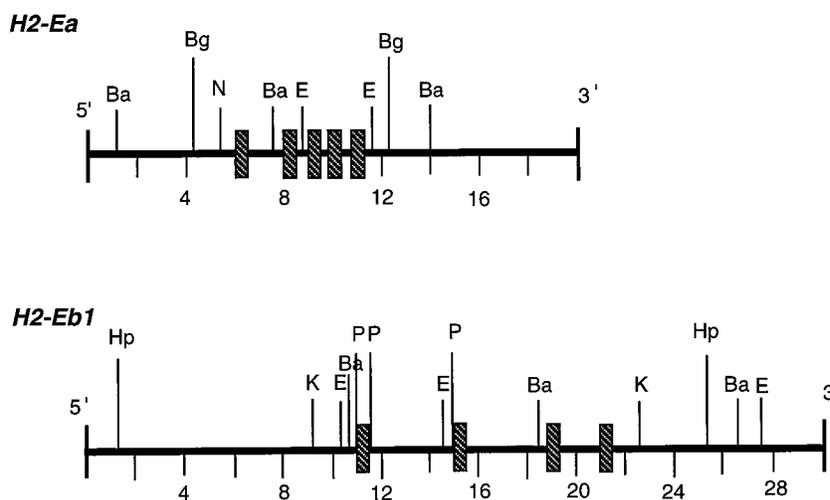
SSLP mapping was used to analyze the linkage of NZB loci on distal chromosome 1 and the MHC with the development of nephritis and IgG autoantibody production. Oligonucleotide primers for *D1Mit111* mapped 92 centiMorgans (cM) from the centromere on chromosome 1 (designated 1; 92) and *D1Mit221* (1; 102 cM) were purchased from Research Genetics (Huntsville, AL), and primers for *Crp* (1; 94 cM) and *Tnfa* (17; ~19 cM) were synthesized by the Molecular Resource Center at the National Jewish Medical and Research Center using an Applied Biosystems model 392 DNA synthesizer (Foster City, CA). Primer nucleotide sequences (internet: <http://www-genome.wi.mit.edu/>), and the methods for SSLP screening and mapping have been previously described (3). The positions of SSLP markers (and genetic loci) are given in accordance with the Mouse Chromosome Committee Reports obtained through the Encyclopedia of the Mouse Genome, Mouse Genome Database, The Jackson Library (Bar Harbor, ME); URL: <http://www.informatics.jax.org/>.

Amplification of simple sequence repeats was achieved using PCR in a PTC-100 thermal cycler (MJ Research, Watertown, MA). Twenty-microliter reactions were conducted using 35 cycles consisting of 30 s at 94°C, 1 min at 55°C, and 30 s at 72°C. Ten to fifteen microliters of PCR product was loaded onto a 15% polyacrylamide gel and electrophoresed at 12 V/cm for 2 to 4 h. Gels were then visualized by ethidium bromide staining after transillumination. Polymorphisms were scored in comparison to results from parental PCR products.

Serologic assays

Ab to nuclear Ags were determined by ELISA as previously described (3, 11, 24). Briefly, wells of Immulon II microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with calf thymus chromatin at 2.5 μ g/ml in PBS, pH 7.2, and postcoated with 1 mg/ml gelatin. Serum samples were diluted 1/300 in PBS with 0.5% Tween supplemented with 5 mg/ml bovine γ -globulins (Sigma Chemical Co.) and gelatin, and added to Ag-coated wells for 90 min. After washing, wells were incubated with peroxidase-conjugated Ab for mouse IgG (Kirkegaard and Perry, Gaithersburg, MD). After 90 min and washing, substrate was added, and OD was determined with an automated spectrophotometer (Dynatech Laboratories) at 405 nm. The dsDNA (plasmid pGEM dsDNA) was biotinylated and bound to streptavidin-coated microtiter plates (24). The assay was then performed as described above. All samples were also assayed in wells coated with streptavidin as a control. Previous studies have shown that this assay demonstrates minimal cross-reactivity for Ab activity in sera containing only anti-ssDNA Abs or for ssDNA-specific mAb (11). All assays were performed in duplicate and were quantified against a standard curve obtained with mAbs to the appropriate nuclear Ag as previously described (3).

FIGURE 1. Restriction maps of the *H2-Ea* (*Ea*) and *H2-Eb1* (*Eb*) genes isolated from a NZW genomic library. The sizes of the genomic fragments are shown in kilobases. Sequence analysis of the coding regions showed identity with *Ea^a* and *Eb^a* genes as previously published by others (36–40). Enzyme abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Hp, *Hpa*I; K, *Kpn*I; N, *Nci*; P, *Pvu*II.



IgG subclass anti-chromatin autoantibody levels were assayed using the same anti-chromatin ELISA, but IgG subclass-specific second-step Abs were used as detecting reagents as previously described (11). Standard curves were obtained using the same (NZB \times NZW) F_1 control sera in each assay (11).

The production of autoantibodies to gp70 was quantitated as serum levels of gp70-anti-gp70 immune complexes (gp70 IC), since the relative excess of gp70 in serum makes free anti-gp70 Abs difficult to detect (25). These complexes were measured by ELISA after precipitation of the serum with polyethylene glycol (average m.w. = 6000) as previously described (26). The results are expressed as micrograms per milliliter of gp70 complexed with anti-gp70 Abs. Although gp70 is detectable in the serum of nearly all murine strains, only lupus-prone strains produce autoantibodies to gp70 and form gp70 IC (27).

For certain comparisons, mice were separated into groups based on their serum levels of a particular autoantibody. The cut-offs used to group mice in the current study were originally determined in (NZB \times NZW) F_1 \times NZW backcross mice by dividing the frequency distribution of autoantibody levels on the basis of tertiles. This separation into autoantibody phenotypes identified one-third of mice with low/negative levels and one-third of mice with high levels for each autoantibody measured. Backcross mice with intermediate levels were defined as the middle third. The cut-offs for anti-chromatin, anti-dsDNA, and gp70 IC autoantibodies correlated well with low levels of production in NZW and nonautoimmune strains and high levels of production in (NZB \times NZW) F_1 mice (3).

Statistical analysis

The linkage of a particular locus with nephritis (categorized as positive or negative) was quantified by χ^2 analysis, using a standard (2 \times 2) contingency matrix (28). Evidence that *H2* or *Nba2* was linked with autoantibody levels as quantitative trait loci (QTL) was determined using the linkage program, MAPMAKER/QTL (29, 30). The autoantibody levels were \log_{10} transformed before analysis with MAPMAKER/QTL, because this tended to normalize their frequency distribution and improve the accuracy of MAPMAKER/QTL (30). It is emphasized that these analyses were directed at MHC genes or at *Nba2* and were not part of a genome-wide screening for linked loci. The statistical threshold used for significant linkage was $p < 0.01$, based on recommendations that this cut-off be used to confirm linkage in a new dataset (31).

In separate analyses, the frequency of nephritis was compared in B6.H2^z backcross mice vs transgenic B6.E^z and B6.E^zlo backcross mice by Fisher's exact test. The mean values for particular autoantibodies in different backcrosses were compared using Dunn's nonparametric procedure of the Kruskal-Wallis test (two-tailed).

Results

Analysis of I-E^z expression in transgenic mice

We hypothesized that class II MHC genes account for the genetic contribution of *H2^z* to the development of lupus-like disease in (NZB \times NZW) F_1 mice. To study the effect of I-E^z on disease

expression, cosmid clones encoding the *Ea^z* and *Eb^z* genes were isolated from an NZW genomic library. Restriction maps of both clones are shown in Figure 1. B6 eggs were coinjected with both clones, and transgenic mice with both *Ea^z* and *Eb^z* genes were selected for further breeding and study. Two B6 lines were subsequently generated and named B6.E^z and B6.E^zlo based on relative copy number, relative levels of *Ea^z* and *Eb^z* mRNA, and relative levels of B cell surface expression of I-E^z. Because of previous studies showing that excessive I-E expression can decrease the frequency and the severity of lupus-like autoimmune disease (32, 33), we focused on these two lines in which B cell expression was near normal and lower than normal compared with that in B6.H2^z mice.

Figure 2 shows a comparison of I-E^z expression on B cells in the different strains analyzed. As shown in Figure 2, congenic B6.H2^z mice expressed both I-A β^z and I-E on B cells, as determined by staining with mAbs 10-2.16 and 14-4-4s, respectively. In contrast, B cells from B6 mice (*H2^b*) were not stained by either of these anti-class II Abs, since their I-A β^b molecule was not detected by the 10-2.16 mAb and because they have no I-E expression due to a defect in the *Ea^b* gene (34). B cell surface expression of I-E in B6.E^z mice was nearly equivalent to that in homozygous B6.H2^z congenic mice, whereas expression in B6.E^zlo mice was decreased but clearly detectable. As expected, neither of the transgenic lines expressed I-A^z.

The mAb used to detect I-E expression recognizes the I-E α -chain and does not distinguish surface molecules with I-E β^z from I-E β^b (23). We were therefore concerned that I-E expression in the transgenic lines may be secondary to pairing of the I-E α^z (encoded by the transgene) with I-E β^b expressed in the B6 mice and that the *Eb^z* transgene might not be functionally expressed. To study this possibility, we outcrossed the B6.E^z and B6.E^zlo strains to SWR (*H2^g*) mice, which express neither *Ea* nor *Eb* gene products (35, 36). (B6.E^z \times SWR) F_1 mice were then backcrossed to SWR mice, and progeny were selected for the absence of expression of I-A^b and also for the presence of the transgenes. These mice must therefore be homozygous for *H2^g* and not have *H2*-encoded *Ea* or *Eb* genes. Staining with mAb 14-4-4s (i.e., expression of I-E) in these backcross mice was comparable to that shown in Figure 2 for the B6.E^z and B6.E^zlo parental lines (data not shown), indicating that both I-E α^z and I-E β^z proteins are functionally expressed in the transgenic strains.

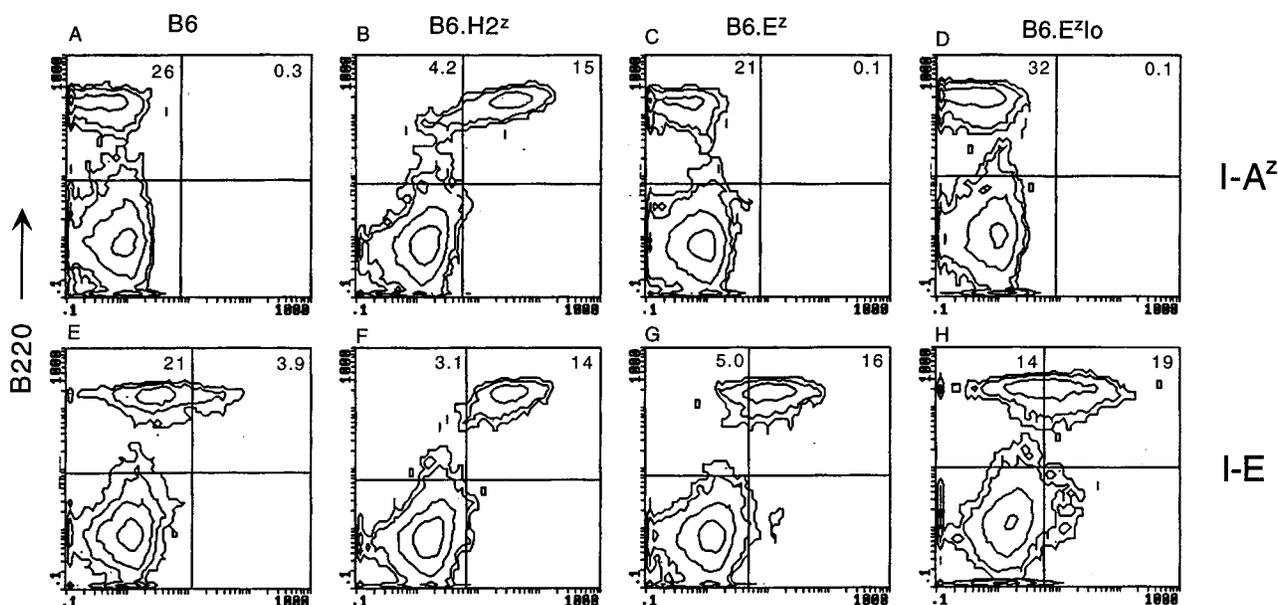


FIGURE 2. Expression of I-A^z and I-E on B cells in B6, B6.H2^z, B6.E^z, and B6.E^zlo strains. B cells were stained with biotinylated Abs to B220 (detected with avidin-phycoerythrin) and double stained with fluorescein-labeled Abs to I-A^z (top histograms) or I-E (bottom histograms). Fluorescence intensity is shown as geographic plots on a four-decade scale. The percentage of cells in the top quadrants of each histogram is also shown.

Analysis of backcross mice for the influence of I-E^z on disease expression

A backcross design was used to analyze the effect of transgenic I-E expression on the development of disease. Previous studies have shown that (B6.H2^z × NZB)F₁ mice do not develop severe renal disease (10). After backcrossing these F₁ mice to NZB, a subset of (B6.H2^z × NZB)F₁ × NZB backcross mice demonstrated high levels of proteinuria and died from renal failure within 12 mo of age. This development of severe renal disease was strongly influenced by inheritance of the congenic interval encoding H2^z (10). In the present studies, we used similar backcross combinations to analyze the effect of I-E^z expression on disease expression.

As shown in Figure 3, 88 (B6.H2^z × NZB)F₁ × NZB backcross mice were followed for the development of severe proteinuria. Of the 43 backcross mice that inherited H2^z, 33% developed proteinuria compared with 11% of the 45 H2^z-negative backcross mice ($p < 0.001$). We followed 77 (B6.E^z × NZB)F₁ × NZB and 82 (B6.E^zlo × NZB)F₁ × NZB backcross mice concomitantly. In contrast to the H2^z-positive backcross mice, none of the 27 B6.E^z transgene-positive and none of the 39 E^zlo transgene-positive backcross mice developed proteinuria ($p < 5 \times 10^{-4}$, comparing the B6.H2^z backcross to each B6.E^z backcross separately by Fisher's exact test; $p < 2 \times 10^{-8}$, compared to both B6.E^z backcrosses combined). Thus, unlike H2^z, E^z and/or E^zlo were not associated with the development of renal disease in the backcross mice. In the backcrosses of transgenic B6 mice, disease expression in the transgene-negative groups was also low and similar to that in H2^z-negative progeny in the B6.H2^z backcross (Fig. 3). Also shown for comparison in Figure 3 is the development of severe proteinuria in (NZB × NZW)F₁ × NZB backcross mice in relation to inheritance of the NZW MHC (H2^z; data taken from historical controls (6)). Disease expression was much more marked in the H2^z-positive (NZB × NZW)F₁ × NZB than in the H2^z-positive (B6.H2^z × NZB)F₁ × NZB backcross mice. Since the only difference in these crosses was the non-MHC NZW vs B6 background, these comparisons show that the NZW background contains additional

disease susceptibility genes (or lacks disease-suppressive genes) compared with the B6 background.

Analysis of IgG autoantibody production in B6.E^z backcross mice

We studied autoantibody production in the different backcrosses to better understand why the B6.E^z backcrosses failed to develop severe lupus nephritis. Figure 4 compares the serum levels of gp70 IC and IgG autoantibodies to those of chromatin and dsDNA, which have been implicated in the pathogenesis of nephritis in this murine model of lupus. The results show that a subset of B6.E^z backcross mice did produce Abs to each of these self Ags (Fig. 4 and Table I). For example, using a cut-off (>4.6 U/ml) that defined high levels of anti-chromatin Ab production in a previous analysis of (NZB × NZW)F₁ × NZW backcross mice (3), 12 (16%) of 75 B6.E^z backcross mice were positive at 7 mo of age. Thirty-three percent of the B6.E^z backcross mice produced at least intermediate levels of IgG anti-chromatin Abs, as previously defined (3). As shown in Figure 4, mean levels of IgG autoantibodies in the B6.E^z backcross mice were comparable to levels in nonnephritic B6.H2^z backcross mice, somewhat lower than those in nephritic B6.H2^z mice ($p < 0.01$ for gp70 IC), and more significantly lower than those in (NZB × NZW)F₁ mice ($p < 0.03$ for anti-chromatin, $p < 0.003$ for anti-dsDNA, and $p < 0.0001$ for gp70 IC). When mice were grouped on the basis of elevated levels of autoantibodies, B6.E^z backcross mice showed comparable and lower percentages of positive mice compared with nonnephritic and nephritic B6.H2^z backcross mice, respectively (Table I).

We also studied the subclass of anti-chromatin Abs produced in the different groups of mice (Fig. 5). At 7 mo of age, there were no significant differences in IgG1 anti-chromatin levels between the B6.E^z backcross and the other groups of mice. There were also no significant differences in IgG2a anti-chromatin levels when mean levels in B6.E^z backcross mice were compared with those in either nephritic or nonnephritic B6.H2^z backcross groups (the difference compared with (NZB × NZW)F₁ mice was significant at $p <$

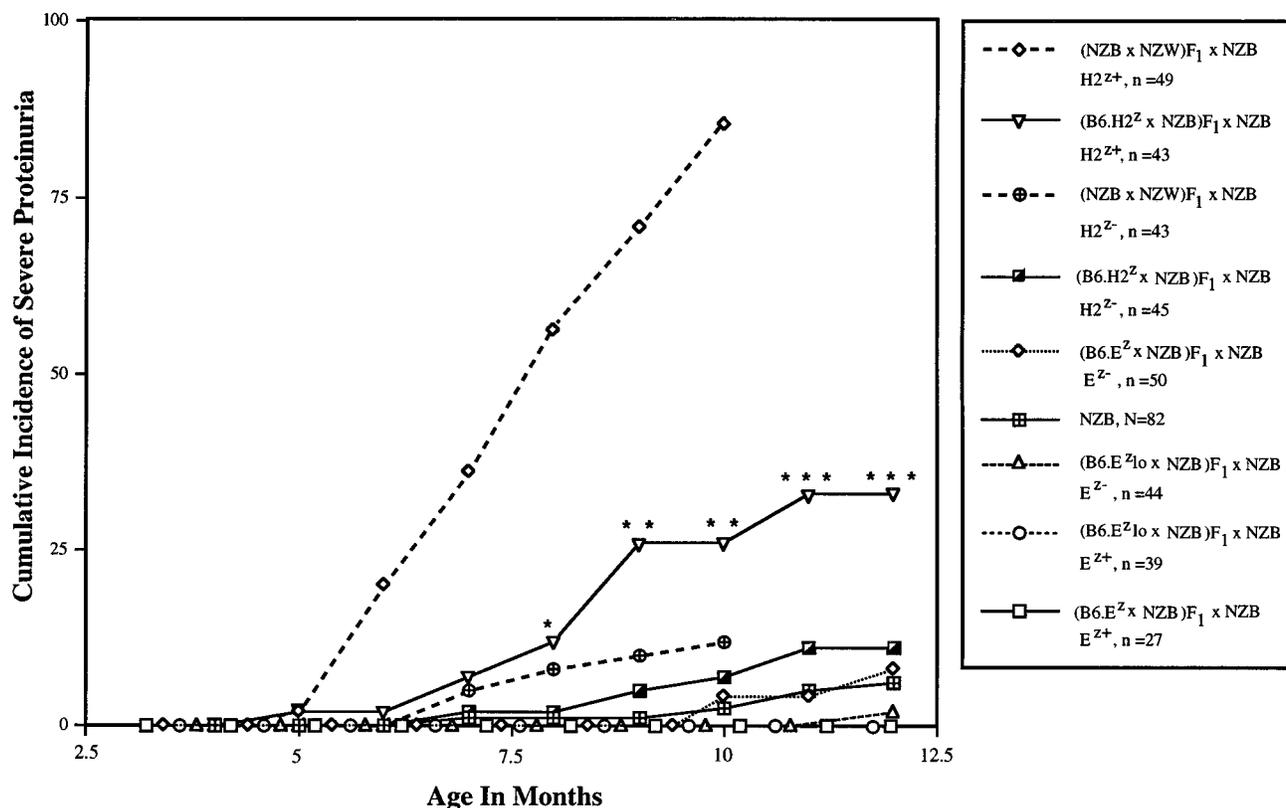


FIGURE 3. Development of lupus nephritis in backcross mice followed for >1 yr. All mice were followed concomitantly, except for the (NZB × NZW)_{F1} × NZB backcross mice (shown in dotted lines), which are historical controls, and the data for these mice have been previously reported (6). The central comparison is between $H2^z$ -positive mice in the (B6.H2^Z × NZB) × NZB backcross and E^z -transgene positive mice in both B6.E^Z and B6.E^{Z1o} backcrosses. When $H2^z$ -positive backcross mice are compared with all E^z -positive backcross mice, the differences in disease frequency are statistically significant: * indicates $p < 0.01$, ** indicates $p < 2 \times 10^{-5}$, and *** indicates $p < 2 \times 10^{-8}$.

0.002). In contrast, significantly lower ($p < 0.001$) levels of IgG3 anti-chromatin autoantibodies were apparent in the B6.E^Z backcross mice compared with the nephritic groups.

Linkage analysis of E^z , $H2^b$, and $Nba2$ with IgG autoantibody production in B6.E^Z backcross mice

We used a QTL analysis to determine whether inheritance of the Ea^z and/or Eb^z transgenes was linked with the production of IgG autoantibodies (Table II). The results revealed no significant linkage or trends toward linkage of the E^z transgenes with any of the autoantibody traits analyzed. The QTL analysis also indicated that there was no significant suppression of autoantibody production. When transgene-positive mice were compared with transgene-negative mice, no differences in mean (\pm SE) levels of IgG anti-chromatin (2.57 ± 0.80 vs 1.92 ± 0.54 U/ml), IgG anti-dsDNA (0.34 ± 0.13 vs 0.26 ± 0.12 U/ml), and gp70 IC (1.13 ± 0.28 vs 1.85 ± 0.42 μ g/ml) were apparent. Furthermore, a similar percentage of mice in each group was positive for autoantibody production. The lack of effect of E^z on autoantibody production was not influenced by inheritance of $H2^{b/d}$ vs $H2^{d/d}$ in the backcross mice (data not shown). Therefore, competition from I-E β^b chains for pairing of I-E β^z to I-E α^z chains was not responsible for the negative findings.

The design of the (B6.E^Z × NZB)_{F1} × NZB backcross also allowed an analysis of the effect of $H2^b$, inherited from the B6 background, on IgG autoantibody production. Table II shows that in contrast to E^z genes, $H2^b$ showed surprising linkage with nearly all the autoantibodies measured ($p < 1 \times 10^{-5}$ for anti-chromatin,

$p < 0.01$ for anti-dsDNA, and $p < 0.01$ for gp70 IC at 7 mo of age). The enhancing influence of $H2^b$ on IgG anti-chromatin production appeared to be selective for the IgG2a and IgG3 subclasses, as no trend for linkage was apparent for IgG1 anti-chromatin Abs (Table II).

In a previous linkage analysis of (B6.H2^Z × NZB)_{F1} × NZB backcross mice, we found that one NZB locus on distal chromosome 1 (previously named *Nba2* for *New Zealand black autoimmunity 2*) in combination with MHC accounted for >90% of the genetic contribution to disease and IgG autoantibody production (10, 11). We therefore analyzed linkage of *Nba2* with IgG autoantibody production in the current (B6.E^Z × NZB)_{F1} × NZB backcross (Table II). This directed linkage analysis showed significant linkage for *Nba2* with serum levels of IgG anti-dsDNA at 7 mo of age and with serum levels of IgG anti-chromatin, IgG anti-dsDNA, and gp70 IC at 9 mo of age. When subclasses were analyzed, only IgG1 anti-chromatin Abs showed significant linkage at $p < 0.01$.

Discussion

The present studies were designed to determine whether Ea^z and/or Eb^z genes account for the genetic contribution of $H2^z$ to lupus-like disease in New Zealand hybrid mice. Previous studies have shown that the $\beta 1$ exon sequences of these genes are identical with those reported for Ea^u and Eb^u genes (14, 37–41). Based on various sequence homologies, it was predicted that these class II molecules would enhance the production of pathogenic IgG autoantibodies in

FIGURE 4. IgG autoantibody production in B6.E^z backcross, B6.H2^z backcross, and (NZB × NZW)F₁ mice at 7 mo of age. B6.H2^z backcross mice have also been divided into cohorts with and without nephritis. Each dot represents the value for an individual mouse, and mean levels for each group are shown. Serum samples from B6.H2^z backcross mice were taken from a larger cohort of mice (10) in addition to those followed concomitantly for disease expression and shown in Figure 3. Cut-offs previously defined (3) (see *Materials and Methods*) to identify mice with high levels of IgG autoantibody production were 4.5 U/ml for anti-chromatin, 2.8 U/ml for anti-dsDNA, and 3.7 μg/ml for gp70 IC (see Table I). The mean ± SEM for the particular autoantibodies in (B6.E^z × NZB)F₁ × NZB mice, (B6.H2^z × NZB)F₁ × NZB mice without nephritis, (B6.H2^z × NZB)F₁ × NZB mice with nephritis, and (NZB × NZW)F₁ mice, respectively, were as follows: IgG anti-chromatin, 2.24 ± 0.48, 1.50 ± 0.78, 3.44 ± 0.79, and 4.03 ± 0.71 U/ml; IgG anti-dsDNA, 0.29 ± 0.09, 0.37 ± 0.07, 1.13 ± 0.39, and 2.30 ± 0.07 U/ml; and gp70 IC, 1.59 ± 0.28, 1.04 ± 0.16, 3.17 ± 0.07, and 8.10 ± 1.80 μg/ml.

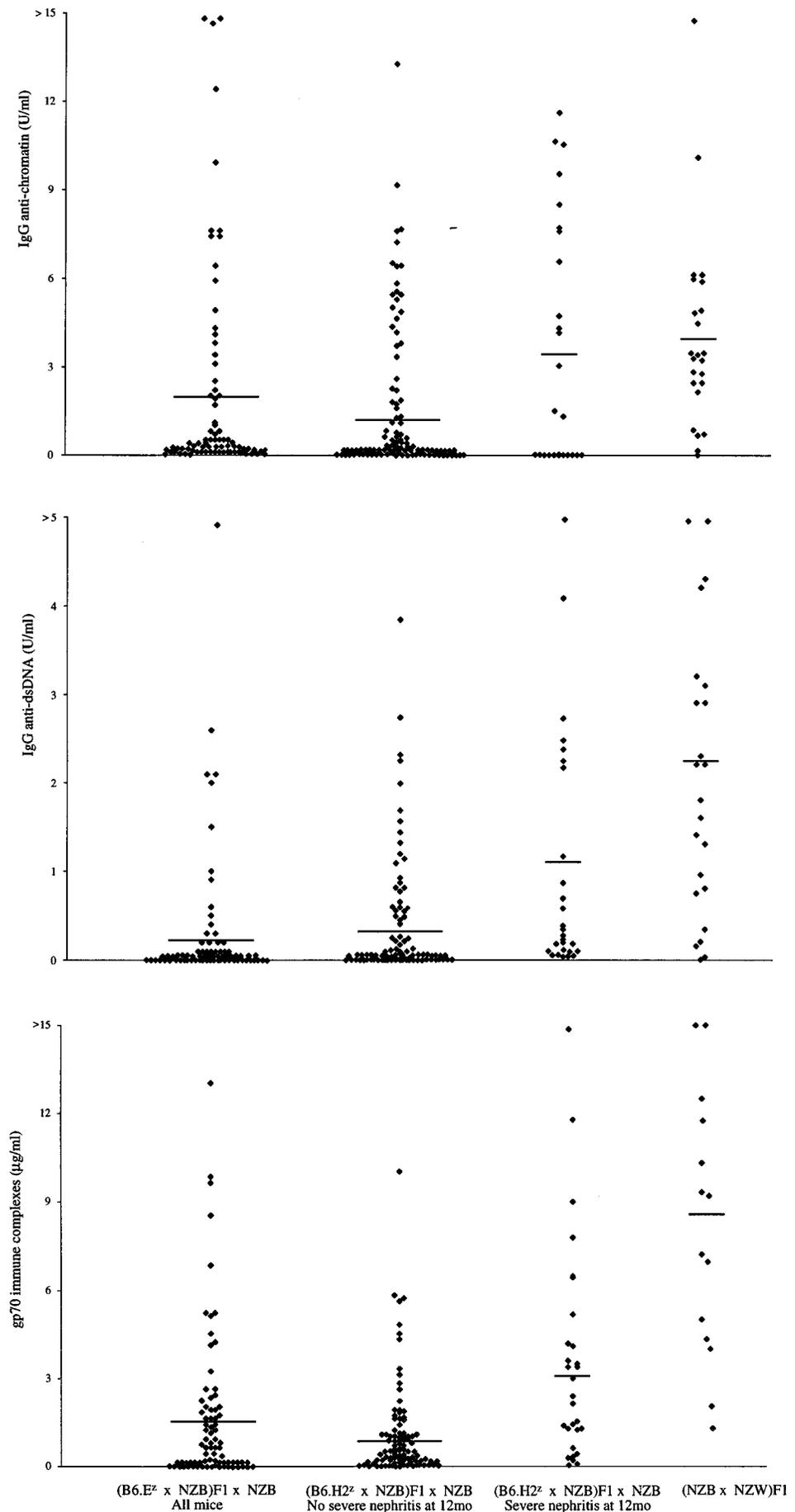


Table I. Categorization of backcross mice with respect to autoantibody production at 7 mo of age

Autoantibody ^a	Number of Mice/Total Evaluated (% of total mice)		
	(B6.E ^z × NZB)F ₁ × NZB	(B6.H2 ^z × NZB)F ₁ × NZB Without Severe Nephritis	(B6.H2 ^z × NZB)F ₁ × NZB With Severe Nephritis
Anti-chromatin (U/ml)			
High (>4.6)	12/75 (16%)	16/101 (16%)	9/25 (36%)
Intermediate (1.0–4.6)	13/75 (17%)	16/101 (16%)	5/25 (20%)
Low (<1.0)	50/75 (67%)	69/101 (68%)	11/25 (44%)
Anti-dsDNA (U/ml)			
High (>2.5)	1/72 (1%)	2/101 (2%)	3/27 (11%)
Intermediate (0.4–2.5)	10/72 (14%)	25/101 (25%)	10/27 (37%)
Low (<0.4)	61/72 (85%)	74/101 (73%)	14/27 (52%)
gp70 IC (μg/ml)			
High (>3.5)	11/72 (15%)	7/101 (7%)	8/27 (30%)
Intermediate (0.5–3.5)	30/72 (42%)	48/101 (47%)	15/27 (55%)
Low (<0.5)	31/72 (43%)	46/101 (46%)	4/27 (15%)

^a Autoantibody levels used to separate mice into different groups were defined as described in *Materials and Methods*.

the New Zealand model of lupus (13, 14, 38, 39). Our results, however, indicate that lupus-like disease in this model is not influenced by *Ea^z* and/or *Eb^z* genes.

The transgene-positive backcross mice analyzed in these studies appeared to express I-E^z in a manner similar to that of the *H2^z*-bearing backcross mice studied as positive controls. Thus, both *Ea^z* and *Eb^z* genes were expressed, as determined by outcrossing transgenic mice to the I-E α - and I-E β -negative SWR strain. Furthermore, the B6.E^z transgenic mice and their backcross progeny expressed quantitatively similar B cell surface levels of I-E compared with B6.H2^z mice and their backcross progeny. The B6.E^zlo backcross, which also showed no linkage of disease with inheritance of the transgene, was studied to avoid the potentially suppressive influence of I-E overexpression on autoimmunity (32, 33, 42–51). It is emphasized that the transgenic strains were derived by injection of genomic *E^z* clones with wild-type promoter and enhancer elements. The pattern of expression in different cell types and after various types of induction should therefore be similar to that in wild-type *H2^z* mice as previously shown for other *Ea* transgenes (16); however, we formally documented normal expression only on splenic B cells. The lack of effect of *E^z* genes also indicates that mixed haplotype I-E α ^d/I-E β ^z molecules do not explain the effect of *H2^z* on disease in (NZB × NZW)F₁ mice as previously suggested (14). I-E β ^z molecules in the transgenic backcross mice, which all express I-E α ^d molecules, should have been equally likely to pair with I-E α ^d as in wild-type *H2^z* backcross mice. In addition, the lack of effect of the transgene on lupus-like disease was not influenced by the inheritance of *H2^{b/d}* vs *H2^{d/d}* and therefore was not related to competition from I-E β ^b for pairing with I-E α -chains.

Other studies have suggested that increased I-E expression can suppress different types of autoimmune processes (42–48), including autoantibody production and nephritis in the New Zealand hybrid (49), BXSB (32, 33), and *lpr* murine models of lupus (50, 51). Results from studies of murine lupus suggest that the effect is not specific for one autoantibody but, instead, appears to involve down-regulation of autoantibody production in general. Competition by I-E α -derived peptides resulting in decreased self-peptide presentation on I-A molecules has been suggested as a possible mechanism (32, 33). In our current studies, the development of lupus nephritis appeared to be decreased by inheritance of the transgenes. However, the difference between transgene-positive and transgene-negative mice was not statistically significant, and there was no trend for increased or decreased autoantibody pro-

duction caused by the presence of the transgene. Compared with previous studies, the lack of any negative effect of the transgene may be related to the normal expression levels achieved in the current crosses (32, 33). Furthermore, since backcrossing was always performed with NZB mice, which are *H2^d* (I-E^d expressing), the additional expression of I-E^z in transgene-positive mice may have had little consequence. Most previous studies had investigated the effects of I-E α expression in an I-E α -negative strain.

Studies have indicated that heterozygosity for *H2^z* and *H2^d* is important for the full expression of autoimmunity in the New Zealand hybrid model (3, 6–11, 52, 53). Thus, New Zealand hybrid or backcross mice that are *H2^{d/z}* have increased IgG autoantibody production and increased incidence of nephritis compared with genetically similar mice homozygous for either *H2^d* or *H2^z* haplotypes. More recent studies have suggested that heterozygosity for *H2* haplotypes other than *H2^d* or *H2^z* may also enhance disease (18, 54, 55). For example, in an analysis of (NZM × B6)F₁ × NZM backcross mice (NZM is an *H2^z*-positive recombinant inbred of NZB and NZW mice), inheritance of *H2^b* from the B6 strain was strongly linked with the production of autoantibodies and the development of nephritis (54). Our analysis of autoantibody production in the B6.E^z backcross shows that *H2^{b/d}* compared with *H2^{d/d}* mice also have increased production of IgG autoantibodies. The linkage of *H2^b* with IgG autoantibody production was apparent in the same mice that showed no influence from the inheritance of the *E^z* transgenes. The reason why a double dose of *H2^d* or *H2^z* genes is associated with less disease compared with that in *H2* heterozygous states is unknown. Some investigators have postulated that mixed haplotype class II molecules, such as I-A α ^d/I-A β ^z, or mixed isotype class II molecules, such as I-E α ^d/I-A β ^z, may increase self recognition (14, 38, 55). However, it seems unlikely that the disease-enhancing effect of multiple different haplotype combinations are all explained by mixed class II molecules.

Although our results showed that *H2^b* is similar to *H2^z* in that both are linked with IgG autoantibody production, the two haplotypes were not comparable in the magnitude of their effect on autoantibody production or development of nephritis in these particular crosses. It is also possible that *H2^z* encodes more than one lupus susceptibility gene, and that different genes underlie the contributions from different haplotypes. Levels of IgG3 autoantibody production in particular were increased in the B6.H2^z compared with the B6.E^z backcross mice. Furthermore, since nephritis was only observed in the cross that involved *H2^z*, the results imply that IgG3 autoantibodies may have greater pathogenic importance than the other subclasses. Studies analyzing pathogenic Abs in other

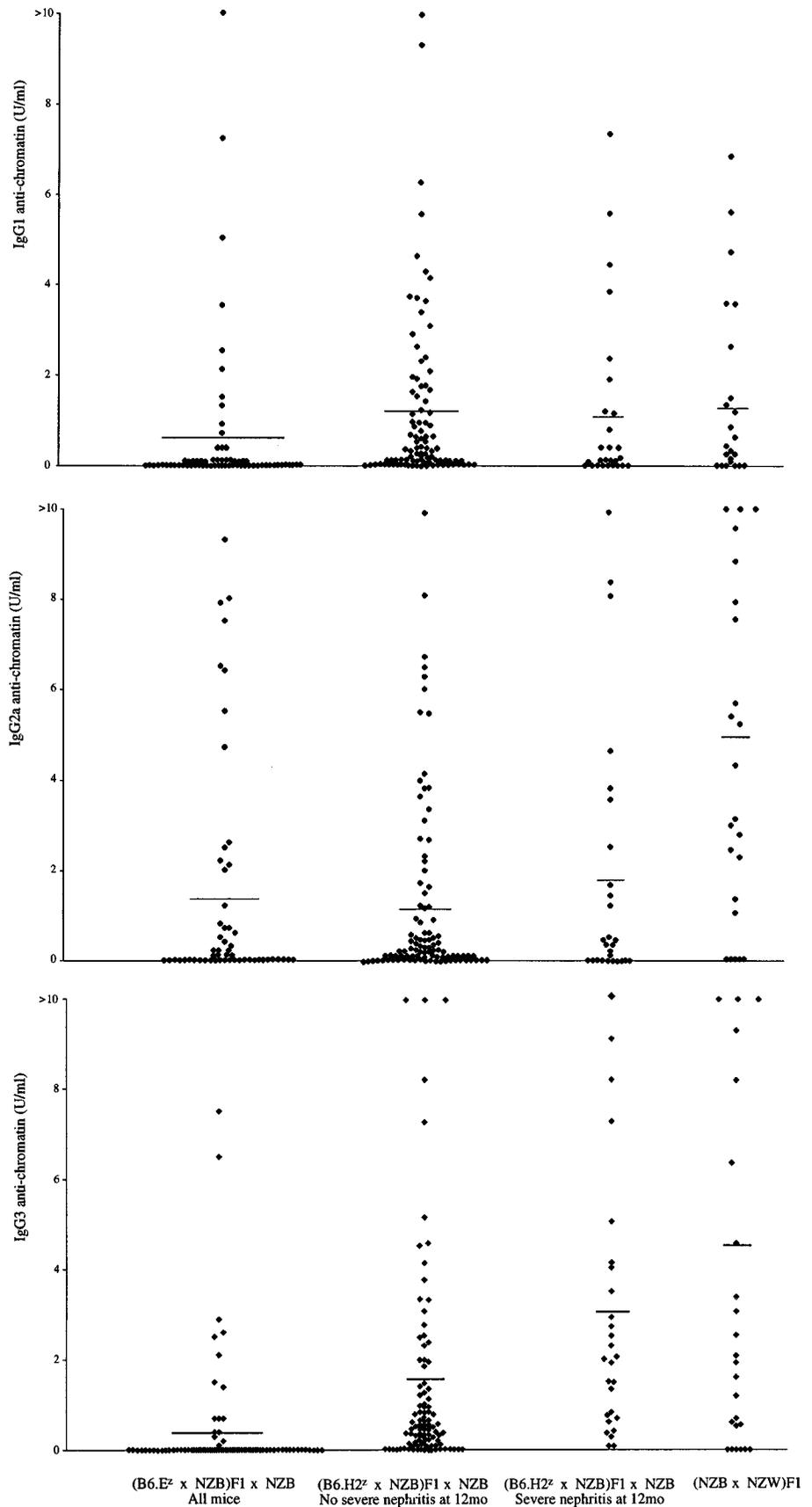


FIGURE 5. IgG1, IgG2a, and IgG3 anti-chromatin autoantibody levels in B6.E^z backcross, B6.H2^z backcross, and (NZB × NZW)F₁ mice at 7 mo of age. The groups are otherwise as described in Figure 4. The mean ± SEM for the respective serologic traits in (B6.E^z × NZB)F₁ × NZB mice, (B6.H2^z × NZB)F₁ × NZB mice without nephritis, (B6.H2^z × NZB)F₁ × NZB mice with nephritis, and (NZB × NZW)F₁ mice, respectively, were as follows: IgG1 anti-chromatin, 0.61 ± 0.23, 1.22 ± 0.26, 1.14 ± 0.37, and 1.47 ± 0.43 U/ml; IgG2a anti-chromatin, 1.39 ± 0.34, 1.22 ± 0.21, 1.82 ± 0.56, and 4.94 ± 1.05 U/ml; and IgG3 anti-chromatin, 0.39 ± 0.14, 1.62 ± 0.31, 2.92 ± 0.74, and 4.45 ± 1.54 U/ml.

Table II. Linkage of *Nba2*, *H2^b*, and *E^z* with IgG autoantibody production in (*B6.E^z* × *NZB*)*F*₁ × *NZB* backcross mice

Trait	Maximum lod score (<i>p</i> value) ^a for		
	<i>Nba2</i>	<i>H2^b</i>	<i>E^z</i>
7 mo of age			
IgG anti-chromatin	1.3	5.0 (2 × 10 ⁻⁶)	0.80
IgG1 anti-chromatin	0.70	0.02	0.55
IgG2a anti-chromatin	0.92	2.9 (3 × 10 ⁻⁴)	0.03
IgG3 anti-chromatin	0.92	2.9 (3 × 10 ⁻⁴)	0.03
IgG anti-dsDNA	1.5 (0.009)	1.5 (0.009)	0.03
gp70 IC	1.0	1.6 (0.008)	0.23
9 mo of age			
IgG anti-chromatin	1.9 (0.003)	4.4 (7 × 10 ⁻⁶)	0.90
IgG1 anti-chromatin	1.9 (0.003)	0.06	0.01
IgG2a anti-chromatin	1.1	7.5 (4 × 10 ⁻⁹)	0.09
IgG3 anti-chromatin	0.68	3.0 (2 × 10 ⁻⁴)	0.75
IgG anti-dsDNA	2.1(0.002)	4.7 (3 × 10 ⁻⁶)	0.22
gp70 IC	1.5 (0.009)	1.2	0.02

^a *p* values are shown for *p* < 0.01.

murine models of lupus support the hypothesis that IgG3 autoantibodies may be particularly nephritogenic (56–60).

The linkage of *H2^b* with IgG autoantibody production in the current backcross analysis raises questions similar to those that prompted the current studies. For example, is this effect mediated by class II genes or by other genes encoded with the MHC? Although the answer is unknown at this time, the autoantibody results provide interesting insight. Thus, the effect of *H2^b* did not appear to be specific for one type of autoantibody. Increased serum levels of IgG autoantibodies to chromatin and gp70 were both linked with *H2^b*. Furthermore, linkage was selective for the IgG2a and IgG3 subclasses of IgG anti-chromatin autoantibodies. Although class II MHC polymorphisms can alter Th subsets and therefore IgG subclass production, polymorphisms within class III genes encoded within the MHC may be more likely to account for the lack of Ag specificity and subclass effects. In this regard, the *Tnfa* gene has been previously proposed as a gene that may underlie the *H2^z* contribution to lupus in (*NZB* × *NZW*)*F*₁ mice (61). It is of interest that a restriction fragment length polymorphism in the *Tnfa* gene, which was shown to correlate with decreased TNF- α production, is present in *H2^b* and *H2^z*, but not in the *H2^d* haplotype (15, 62–64). Although cytokine genes may be involved in the *H2* contribution to disease, especially because of the heterozygous effects of each haplotype, other contributing genes seem likely.

In the analysis of *B6.E^z* backcross mice, a locus on distal chromosome 1, named *Nba2*, was also shown to be linked with the production of anti-chromatin and anti-DNA Abs, and a trend was observed for linkage with anti-gp70 autoantibodies. It is important to emphasize that the stringent statistical thresholds proposed for a genome-wide screening (31) do not apply to the directed linkage analysis of one non-MHC locus in the current study. In previous genome-wide screenings of (*B6.H2^z* × *NZB*)*F*₁ × *NZB* and (*SM/J* × *NZB*)*F*₁ × *NZW* backcrosses, *Nba2* was shown to be strongly linked with the development of nephritis and increased serum levels of IgG autoantibodies (10, 11, 18). A locus in a similar chromosomal location has been mapped in other studies analyzing *NZB* and/or *NZW* genes (18, 53, 54). In the *B6.H2^z* backcross, *Nba2* in conjunction with *H2^z* provided >90% of the genetic contribution to nephritis and autoantibody production (10, 11). *Nba2* is situated between 92 and 97 cM from the centromere, and this region encodes several candidate genes, including the low affinity Fc γ receptor genes. Because *Nba2* was linked with the coordinate production of multiple autoantibodies and total IgG and IgG sub-

classes levels (11), it was hypothesized that it functions as an immune response gene. Similar traits were linked with *Nba2* in the current study, although the level of autoantibody production was less pronounced, and the extent of linkage appeared to be less strong in the current study. The effects of *Nba2* also appear to be subject to the influence of the MHC haplotype (10, 11), and the difference between *H2^b* and *H2^z* in the *B6.E^z* and *B6.H2^z* crosses, respectively, may have altered the influence of the *Nba2* effect.

In summary, the current studies appear to exclude *Ea^z* and/or *Eb^z* genes in the contribution of *H2^z* to nephritis and IgG autoantibody production in New Zealand hybrid mice. It remains possible, however, that a contribution of I-E^z to nephritis is dependent on other molecules encoded within *H2^z*, but not *H2^b* or *H2^d*. Our results also show that *H2^b* is similar to *H2^z*, but has quantitatively less influence on disease expression in this model. Transgenic mice with *A^z* genes have been generated, and studies are in progress to address the contribution of these class II genes to disease and autoantibody production. However, the current results suggest that other MHC genes, such as genes influencing the pattern of cytokine production, may be more important in the contribution to lupus-like disease in this model.

Acknowledgments

The authors thank Drs. Diane Mathis and Christophe Benoist (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, Université Louis Pasteur, Strasbourg, France) for help with generation of the transgenic mice and for critical review of the manuscript, and Ellen Roper for technical assistance with the autoantibody assays.

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