Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 7552–7555, October 1989
Immunology

Genetic analysis of the imperfect association of *H-2* haplotype with lupus-like autoimmune disease

(genetic susceptibility to autoimmune disease/major histocompatibility complex/systemic lupus erythematosus/NZB × NZW mice)

SUSAN K. BABCOCK*, VIRGINIA B. APPEL†, MARSHALL SCHIFF†, ED PALMER*†‡, AND BRIAN L. KOTZIN*†‡

†Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206; and
*Departments of Medicine and Microbiology/Immunology, University of Colorado Health Sciences Center, Denver, CO 80262

Communicated by David W. Talmage, July 3, 1989

ABSTRACT **Unlike parental New Zealand Black (NZB) or New Zealand White (NZW) mice, (NZB × NZW)F_1 mice exhibit a lupus-like disease characterized by high serum levels of IgG anti-nuclear antibodies and a fatal immune complex glomerulonephritis. Previous results from studying [(NZB × NZW)F_1 × NZB] backcross mice indicated that the NZW major histocompatibility complex (MHC) or gene(s) closely linked to this locus provides the major dominant NZW genetic contribution to the F_1 disease. A surprising feature of the results was the 12% frequency of discordance between the autoimmune phenotype and the presence of the NZW *H-2*^{z} haplotype. In the current study, we attempted to precisely define the position of the NZW gene(s) required for lupus-like renal disease by mapping genes in individual backcross mice that are both centromeric and telomeric to the MHC and then correlating genotypes for each locus with disease. The data indicate that an adjacent NZW locus does not provide a more accurate correlation with the autoimmune phenotype compared with MHC genes themselves. Thus, the imperfect association of MHC haplotype with disease in this murine model is not explained by genetic recombination with linked genes. These data may provide insight into the mechanisms by which MHC antigens increase the probability of developing autoimmune disease and may help explain the difficulty of defining MHC relationships in human systemic lupus erythematosus.**

New Zealand Black (NZB) × New Zealand White (NZW)F_1 mice develop an autoimmune disease characterized by IgG anti-nuclear antibody production and a severe immune complex glomerulonephritis (1). Features similar to these are observed in human systemic lupus erythematosus. Ninety-five percent of female F_1 animals die from renal disease by 12 months of age. In contrast, <5% of parental NZB and NZW mice develop severe glomerulonephritis in the first year of life, and parental mice rarely produce high levels of IgG antibodies to double-stranded DNA and histones (2). Since the major features of the F_1 disease are not present in the NZB or NZW strains, it has been hypothesized that each parent contributes one or more genes that act in concert to permit the development of autoimmune disease in the F_1 mice. Numerous studies have shown that the autoimmune abnormalities found in the NZB parent are multigenic (3–7). It is unclear how many of these abnormalities play a role in the development of disease in the (NZB × NZW)F_1, but it is likely that the NZB genetic contribution to renal disease in the F_1 is complex.

We recently reported the results of an (NZB × NZW)F_1 × NZB backcross in which the incidence of fatal renal disease (≈50% of backcross mice) suggested that only one dominant gene was contributed by the NZW parent (2). In addition, there was a strong correlation between the presence of the NZW haplotype of the major histocompatibility complex (MHC), *H-2*^{z}, and lupus-like renal disease in individual backcross mice. Eighty-six percent of *H-2*$^{d/z}$ backcross animals developed severe renal disease, whereas only 12% of *H-2*$^{d/d}$ backcross mice expressed this autoimmune syndrome. Thus, it appeared that either the NZW MHC or closely linked gene(s) contributed to the autoimmune disease in F_1 mice. The discordance between the presence of the NZW MHC locus and the autoimmune phenotype was surprising, and the data suggested an apparent recombination rate of ≈10% between the NZW disease-associated gene and the MHC. The current study attempts to precisely define the position of the NZW gene required for lupus-like renal disease in the F_1 by mapping genes both centromeric and telomeric to MHC. The data suggest that this genetic contribution lies within the MHC.

MATERIALS AND METHODS

Mice and Assessment of Renal Disease. Parental NZB and NZW mice, (NZB × NZW)F_1 mice, and the backcross animals [(NZB × NZW)F_1 × NZB] used for this analysis are identical to those described in ref. 2. Proteinuria, an indicator of renal disease, was evaluated monthly by using tetrabromphenol paper, and was graded 0–3$^+$. Trace proteinuria is <30 mg/dl, 1$^+$ detects 30 mg/dl, 2$^+$ detects 100 mg/dl, and 3$^+$ detects >500 mg/dl. Severe proteinuria was defined as >2$^+$, and animals with this level of proteinuria at 11 months of age or earlier were classified as having severe renal disease (2, 8). These mice usually died from renal failure within 4–8 weeks after development of severe proteinuria. Animals with negative or trace proteinuria at 12 months of age were classified as having no evidence of renal disease.

Southern Analysis of Genomic DNA. Liver and kidney DNA from each animal was isolated as described (9). DNA (10 μg) was digested with restriction enzymes at a concentration of 2 units per μg of DNA for 1 hr at 37°C. This procedure was repeated once. Various enzymes were utilized depending on the probe used: *Pvu* II for I-Aα, *Bam*HI for C3, *Pvu* II for TU66, and *Bgl* II for both TU169 and the α-globin pseudogene 4 (α-ψ4). Digested DNAs were subjected to electrophoresis through agarose gels (10). A 0.7% gel was used for MHC, TU66, TU169, and α-ψ4, and the gels were run for 20 hr. To separate the fragments hybridizing to the C3 probe, a 1.5% gel was run for 48 hr. The DNA was transferred to nitrocellulose filters, baked, and hybridized with a ^{32}P-labeled probe as described (10), except that the depurination step was omitted and the gel was irradiated for 7 min with shortwave UV light before denaturation to facilitate transfer of DNA to nitrocellulose (11). Hybridized filters were washed in 2× SSC/1% SDS at room temperature followed by 0.1× SSC/1% SDS at 55°C for 45 min (1× SSC = 0.15 M

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: MHC, major histocompatibility complex.

‡To whom reprint requests should be addressed.

NaCl/0.015 M sodium citrate). Autoradiography was used to detect the hybridized fragments.

A 0.7-kilobase (kb) *Pst* fragment derived from an I-Aα^d cDNA clone (12) was provided by B. Malissen (Center of Immunology, Marseille, France) and was used to identify MHC haplotypes. TU66 and TU169 represent 1.8-kb and 500-base-pair *Eco*RI fragments, respectively, and were kindly provided by L. Silver (13). A 2.4-kb fragment derived from the α-globin pseudogene 4 (α-ψ4) was provided by P. Leder (14). Polymorphism in the C3 complement locus was identified by probing Southern blots with an *Eco*RI 1.5-kb fragment, which was derived from the 5′ end of the C3 gene (provided by G. Fey and R. Maki; ref. 15).

For mapping any two genetic loci, an animal was included in the analysis only if data for both loci were known. However, data were not available for some animals because their DNA was not typed for all loci or, in a few cases, because their genotype was not clearly defined on the Southern blots. Genotyping was performed without knowledge of disease status. When correlating the genotype of a particular locus with the disease phenotype, an animal was included in the analysis only if both variables were known.

RESULTS

The imperfect association of autoimmune disease with inheritance of the NZW MHC (*H-2*^{z}) suggested that the gene controlling the expression of disease is not encoded within the MHC but is located centromeric or telomeric on chromosome 17. To address this issue, we investigated whether NZW genes linked to the MHC correlated more accurately with autoimmune disease than did the MHC locus. Genotypes of a large number of (NZB × NZW)F_1 × NZB backcross mice were determined by Southern analysis using four different chromosome 17 probes for loci with known linkage to the MHC. Each backcross animal could be categorized as heterozygous (NZW and NZB allele present) or homozygous for the NZB allele. The presence or absence of the NZW allele was then correlated with the occurrence of severe renal disease. For any particular gene, a positive association was present if an animal carried the NZW allele and developed renal disease [similar to the (NZB × NZW)F_1] or if an animal did not carry the NZW allele and failed to develop disease. The percentage of exceptions to this positive association was calculated as a frequency of discordance.

Fig. 1 shows the restriction fragment length polymorphisms used to distinguish NZB, NZW, and F_1 genotypes at each locus. The TU66 probe hybridizes to a 3.5-kb *Pvu* II fragment present in NZB and NZW DNA and to a 4.4-kb *Pvu* II fragment present only in NZW DNA. α-ψ4 hybridizes to a 3.5-kb *Bgl* II fragment present in NZB DNA and to a 4.0-kb *Bgl* II fragment present in NZW DNA. The I-Aα probe hybridizes to 2.9-kb and 5.0-kb *Pvu* II fragments present in NZB and NZW DNA, respectively. Similarly, the TU169 probe detects 2.8-kb and 3.3-kb *Bgl* II fragments present in NZB and NZW DNA, respectively. Finally, the C3 probe hybridizes to a 4.0-kb *Bam*HI fragment in NZB DNA but to a 3.8-kb *Bam*HI fragment in NZW DNA. As expected, (NZB × NZW)F_1 animals carry both polymorphic forms of the genes examined.

Table 1 summarizes the recombination rates between loci and the correlations with autoimmune disease for the different genetic loci adjacent to the MHC. Based on the recom-

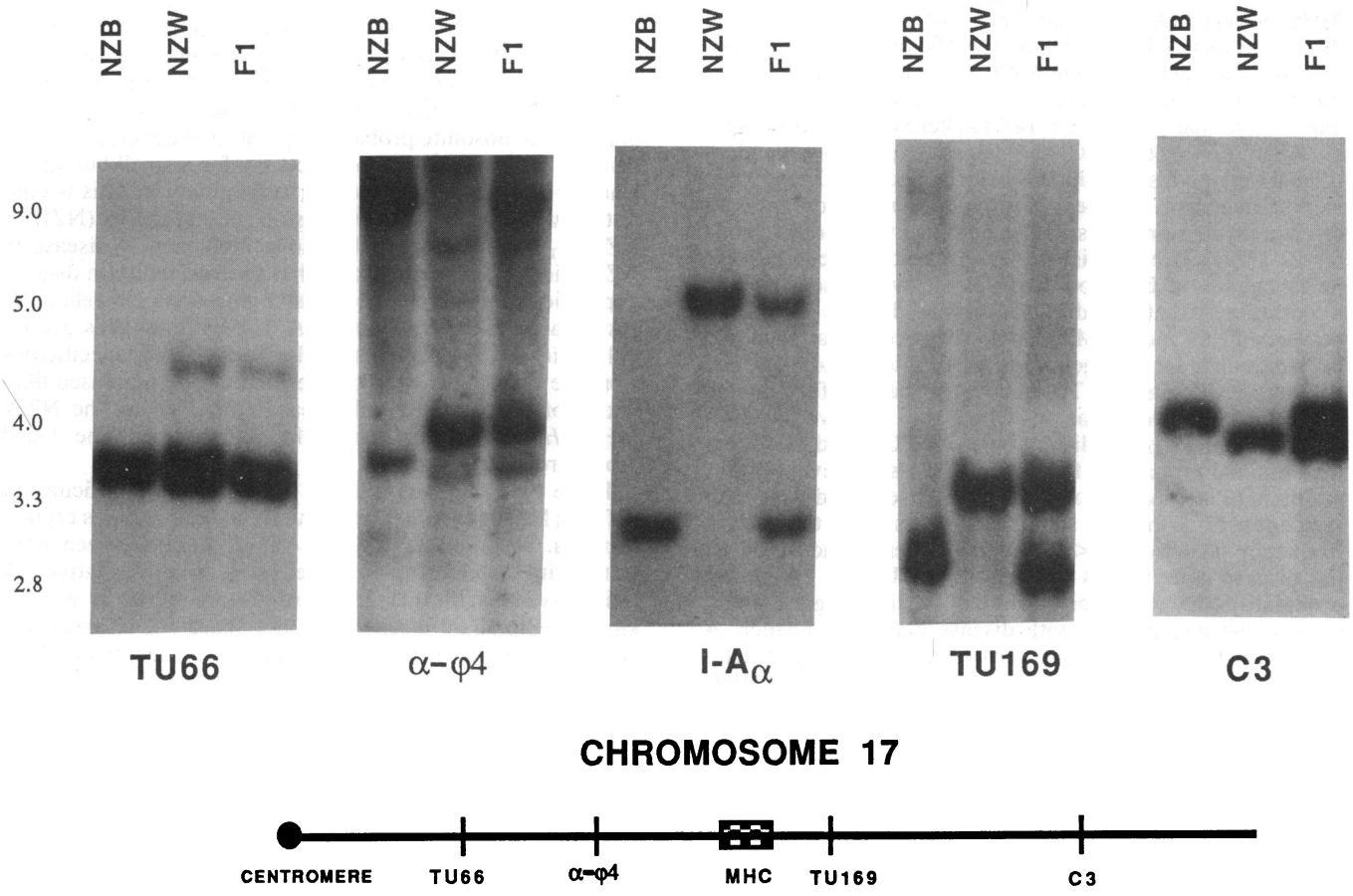

FIG. 1. Restriction fragment length polymorphisms used to distinguish NZB, NZW, and (NZB × NZW)F_1 genotypes for the different gene loci indicated. Liver DNA was digested with different enzymes and hybridized with the probes indicated. The relative positions of the different genes on chromosome 17 are also shown and were determined by recombination rates between loci (see Table 1). Numbers on left are kb.

Table 1. Recombination rates between different loci adjacent to the MHC, and exception rate for correlation of genotype with autoimmune disease

	TU66	α-φ4	I-A	TU169	C3	Disease
TU66	—	4/82 (4.9%)	13/148 (8.8%)	15/148 (10.1%)	19/117 (16.2%)	25/141 (17.7%)
α-φ4	4/82 (4.9%)	—	3/84 (3.6%)	5/85 (5.9%)	10/66 (15.2%)	13/80 (16.3%)
I-A	13/148 (8.8%)	3/84 (3.6%)	—	2/141 (1.4%)	11/125 (8.8%)	14/142 (9.9%)
TU169	15/148 (10.1%)	5/85 (5.9%)	2/141 (1.4%)	—	9/121 (7.4%)	16/135 (11.9%)
C3	19/117 (16.2%)	10/66 (15.2%)	11/125 (8.8%)	9/121 (7.4%)	—	22/125 (17.6%)

A genotype for a particular locus was considered to be discordant with disease expression if an animal carried the NZW allele but failed to develop renal disease or if an animal did not carry the NZW allele but developed (and died from) renal disease.

bination rates observed in this backcross, we found that the locus encoding TU66 maps 8.8 centimorgans (cM) centromeric to I-Aα, while α-ψ4 maps 3.6 cM centromeric to I-Aα. On the other hand, the TU169 locus is located 1.4 cM telomeric to I-Aα, while the C3 gene is situated 8.8 cM telomeric to I-Aα. The recombination rates between these loci are consistent with previous estimates of the position of these genes on chromosome 17 (14, 16–18). The MHC haplotype provided the best correlation with disease (9.9% discordance with disease phenotype). Compared with MHC, the correlation of the TU66 or C3 genotype with disease expression was significantly worse ($P = 0.05$ by χ^2 analysis). Disease correlations with the genetic loci encoding α-ψ4 and TU169 were also less accurate compared with MHC. For example, only 2 of 141 backcross mice demonstrated recombinations between the MHC and TU169, and, in both cases, expression of disease correlated with the MHC genotype. Thus, the data rule out the possibility that the NZW gene controlling autoimmunity is situated ≈10 cM away from the MHC on chromosome 17.

The results also strongly suggest that genes within the MHC or very tightly linked to this gene complex encode the disease-associated gene. Using the above data, we considered the possibility that the NZW genome contains a MHC-linked gene that correlates more accurately with disease expression than MHC. Of 141 backcross mice that were analyzed for TU66, MHC, and disease expression, we identified 13 examples for which the TU66 and MHC genotypes were concordant, yet these genotypes were discordant with disease expression. Thus, either both loci were homozygous for the NZB allele and disease was present, or the mice were heterozygous at both loci and disease was absent. If one hypothesized that the disease-associated locus is located between TU66 and the MHC, a double recombination would be necessary for the genotype at this locus to correlate accurately with disease. The greatest probability for a double recombination would occur when the putative disease-associated locus is equidistant between TU66 and MHC and the maximal probability for a double crossover event in this region is $(0.044)^2$ or 1.9×10^{-3}. Thus, one would expect an incidence of 1 in 530, significantly less than the 13/141 frequency observed ($P < 0.0001$ by Fisher's exact test). As the disease gene moves toward either TU66 or MHC, the probability that recombination with a linked gene could explain the discordance with disease decreases further. A similar analysis can also be performed for the region telomeric to MHC. We noted 12 examples in which C3 and MHC genotypes were identical, but the genotypes were discordant with disease expression. The maximal probability that a locus situated between these genes provides a more perfect correlation with disease is $(0.044)^2$ or 1.9×10^{-3}. Again, this frequency is significantly less than the 12/124 observed ($P < 0.0001$).

DISCUSSION

Our previous conclusion from studying the (NZB × NZW)F_1 × NZB backcross mice was that the NZW MHC or gene(s) closely linked to this locus provides the major, dominant NZW genetic contribution to (NZB × NZW)F_1 disease. What was most puzzling about the initial data from the backcross is that 14% of $H\text{-}2^{d/z}$ mice did not develop renal disease, while 12% of $H\text{-}2^{d/d}$ mice did develop glomerulonephritis that was indistinguishable from that seen in (NZB × NZW)F_1 animals. The present data indicate that the imperfect association of MHC haplotype with disease in this murine model is not explained by genetic recombination with linked genes. Thus, an adjacent NZW locus does not provide a significantly more accurate correlation with the autoimmune phenotype compared with MHC genes themselves. Furthermore, the data suggest that the disease-associated gene most likely lies within the MHC. This conclusion is consistent with previous studies by Hirose *et al.* (19) showing that F_1 mice produced by breeding NZB mice with a NZW congenic strain, homozygous for $H\text{-}2^d$, demonstrated a much lower incidence of renal disease and autoantibody production compared with (NZB × NZW)F_1 mice. That NZW class II MHC genes are the major contributors to F_1 disease is supported by studies showing the dependence of autoimmune disease expression on the presence of $CD4^+$ T cells (20) and by experiments showing that the development of autoimmune disease can be blocked by repeated injections of anti-$I\text{-}A^z$ antibodies (21). The present data suggest that $H\text{-}2^z$ gene products confer a high but not absolute probability (≈90%) of developing renal disease, while $H\text{-}2^d$ gene products confer a small but significant (≈10%) risk of developing autoimmunity. This is consistent with incomplete expression of disease in (NZB × NZW)F_1 mice and a low but finite incidence of disease in NZB mice (2). It seems likely that the variability in disease expression among these genetically inbred mice is related to a stochastic process. Since class II MHC antigens are involved in the selection and deletion of T-cell specificities from the repertoire, we hypothesize that the increased likelihood of developing renal disease conferred by the NZW MHC ($H\text{-}2^z$) operates during the generation of the T-cell repertoire.

These data also may provide insight into the difficulty of defining MHC relationships in human systemic lupus erythematosus. Several different *HLA* haplotypes have been associated with systemic lupus erythematosus but the relative risk ratios have been low (22, 23). Furthermore, in family studies, kindred who share disease may not share *HLA* haplotypes and considerable discordance is present in identical twins. Certainly, genetic heterogeneity at non-MHC loci and diverse environmental factors may influence variability in human disease expression. However, the variable disease expression in these murine experimental systems in which genetically inbred mice are studied and environmental conditions are relatively uniform emphasizes that disease expression may also be related to a stochastic process greatly influenced by MHC antigens.

The authors thank Marjorie Greiner and Judy Franconi for help in the preparation of the manuscript. This work was supported by Grant AR37070 from the National Institutes of Health and a grant from the

Arthritis Foundation. S.K.B. is supported by Physician Scientist Award DK01615 from the National Institutes of Health.

1. Howie, J. B. & Helyer, B. J. (1968) *Adv. Immunol.* **9,** 215–266.
2. Kotzin, B. L. & Palmer, E. (1987) *J. Exp. Med.* **167,** 1237–1251.
3. Raveche, E. S., Novotny, E. A., Hansen, C. T., Tito, J. H. & Steinberg, A. D. (1981) *J. Exp. Med.* **153,** 1187–1197.
4. Yoshida, H., Kohno, A., Ohta, K., Hirose, S., Maruyama, N. & Shirai, T. (1981) *J. Immunol.* **127,** 433–437.
5. Datta, S. K., Owen, F. L., Womach, J. E. & Riblet, R. J. (1982) *J. Immunol.* **129,** 1539–1544.
6. Maruyama, N., Furukawa, F., Nakai, Y., Sasaki, Y., Ohta, K., Ozaki, S., Hirose, S. & Shirai, T. (1983) *J. Immunol.* **130,** 740–746.
7. Miller, M. L., Raveche, E. S., Laskin, C. A., Klinman, D. M. & Steinberg, A. D. (1984) *J. Immunol.* **133,** 1325–1331.
8. Kotzin, B. L. & Strober, S. (1979) *J. Exp. Med.* **150,** 371–378.
9. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
10. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3683–3687.
11. Kotzin, B. L., Barr, V. L. & Palmer, E. (1985) *Science* **229,** 167–171.
12. Germain, R. N., Ashwell, J. D., Lechler, R. L., Margulies, D. H., Nickerson, K. M., Suzuki, G. & Tou, J. Y. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2940–2944.
13. Röhme, D., Fox, H., Herrmann, B., Frischauf, A. M., Edstrom, J., Mains, P., Silver, L. M. & Lehrach, H. (1984) *Cell* **36,** 783–788.
14. Leder, A., Swan, D., Ruddle, F., D'Eustachio, P. & Leder, P. (1981) *Nature (London)* **293,** 196–200.
15. Wiebauer, K., Domdey, H., Diggelmann, H. & Fey, G. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7077–7081.
16. Fox, H. S., Silver, L. M. & Martin, G. R. (1984) *Immunogenetics* **19,** 125–130.
17. Silver, L. M. (1985) *Annu. Rev. Genet.* **19,** 179–208.
18. Da Silva, F. P., Hoecker, G. F., Day, N. K., Vienne, K. & Rubinstein, P. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 963–965.
19. Hirose, S., Ueda, G., Noguchi, K., Okada, T., Seikgawa, I., Sato, H. & Shirai, T. (1986) *Eur. J. Immunol.* **16,** 1631–1633.
20. Wofsy, D. & Seaman, W. E. (1985) *J. Exp. Med.* **161,** 378–391.
21. Adelman, N. E., Watling, D. L. & McDeVitt, H. O. (1983) *J. Exp. Med.* **158,** 1350–1355.
22. Winchester, R. J. & Lahita, R. G. (1987) in *Systemic Lupus Erythematosus*, ed. Lahita, R. G. (Wiley, New York), pp. 81–118.
23. Fronek, Z., Timmerman, L. A., Alper, C. A., Hahn, B. H., Kalunian, K., Peterlin, B. M. & McDeVitt, H. O. (1988) *Am. J. Med.* **85,** Suppl. 6A, 42–44.