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The genetic contribution of the NZB mouse to the renal disease of the NZB × NZW hybrid

J. G. KNIGHT, D. D. ADAMS & H. D. PURVES *Immunopathology Research Unit of the Medical Research Council of New Zealand, University of Otago Medical School, Dunedin, New Zealand*

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SUMMARY

The occurrence of lupus nephritis in (NZB × NZW)F_1 mice appears to depend on the action of at least two dominant or co-dominant genes (at least one gene from each parent) as neither of the inbred parental strains shows the disorder. Identifying affected animals by antemortem determinations of renal function, using improved methods of measuring proteinuria and renal clearance, we have studied the incidence of the renal disease in 230 (NZB × NZW)F_1 × NZW backcross mice. The incidence was 49·6% which indicates that the NZB strain contributes only one gene, or cluster of closely linked genes, to the renal disorder of the F_1 hybrid. The gene(s) must be dominant or co-dominant, as it expresses its effect in the heterozygous state. Study of the H-2 status of the backcross mice showed a loose linkage of the NZB renal disease gene(s) to the D end of the H-2 complex, the crossover frequency being 32·6 ± 3·1%.

INTRODUCTION

In the course of developing inbred mice for cancer research, the Bielschowskys observed that their New Zealand Black (NZB) strain showed a high incidence of splenomegaly and anaemia (Bielschowsky, Helyer & Howie, 1959). The disorder was found to be an autoimmune haemolytic anaemia and could be ameliorated by treatment with adrenal steroids (Howie & Helyer, 1968). Helyer and Howie crossed the NZB mice with the New Zealand White (NZW) strain, which had been developed by W.H. Hall (Hall & Simpson, 1975), and discovered that (NZB × NZW)F_1 animals show a very high incidence of a renal disorder resembling lupus nephritis (Howie & Helyer, 1968). The hybrid animals die of renal failure at the early age of about 280 days, but survive much longer if treated with cyclophosphamide (Russell, Hicks & Burnet, 1966). The beneficial effect of this immunosuppressant, the occurrence of autoantibodies to cell nuclei (Howie & Helyer, 1968), and the accumulation of immunoglobulin in the glomeruli (Aarons, 1964; Lambert & Dixon, 1968) indicate that the renal failure of the NZB × NZW mice is caused by an autoimmune process. Its occurrence in the hybrids of two in-bred strains which do not themselves show the disorder is of particular interest. The NZB × NZW renal disease would appear to depend on the action of at least two genes, one from each strain, and because the parental animals can be assumed to be homozygous at every locus, these genes must be dominant or co-dominant in that they express their effect in the heterozygous state.

In this paper we report the beginning of a study of the genetic basis of the autoimmune renal disease of NZB × NZW mice. To enable precise monitoring of the renal disease, we have developed improved methods of measuring proteinuria and renal clearance in the mice. We have mated NZB × NZW hybrids with NZW mice and determined the incidence of renal disease in the back-cross animals to ascertain the number of dominant or co-dominant genes contributed to the renal disease by the NZB mice. We have also determined the histocompatibility type (H-2) of the backcross mice to see if a renal disease gene is linked to the H-2 complex.

Correspondence: Mr J. G. Knight, Immunopathology Research Unit of the Medical Research Council of New Zealand, University of Otago Medical School, Dunedin, New Zealand.

MATERIALS AND METHODS

Mice. All mice were derived from the Otago University NZB and NZW inbred colonies. Female (NZB × NZW)F_1 hybrid mice were back-crossed to NZW mice. Two hundred and thirty virgin female offspring were H-2 typed and monitored for the onset of renal disease using the criteria described below. A control group of thirty virgin female NZB × NZW mice were studied concurrently.

H-2 typing. The H-2 phenotype of each (NZB × NZW) F_1 × NZW mouse was determined using a modification of Stimpfling's PVP haemagglutination technique (Stimpfling, 1961). Anti-H-2.4 and anti-H-2·5 antisera, kindly supplied by Dr J.G. Ray*, and CBA anti-NZB and CBA anti-NZW antisera, absorbed against NZW and NZB spleen cells respectively, were used to differentiate between $H\text{-}2^d$ and $H\text{-}2^z$ haplotypes. Erythrocytes were collected in citrate phosphate dextrose (CPD) solution (Gibson *et al.*, 1957), washed four times in CPD and made up to a 2% suspension in 2% bovine serum albumin (Sigma) in CPD. Antisera were diluted appropriately in 1·5% polyvinylpyrrolidone (GAF Corporation, New York) in phosphate-buffered saline. Equal parts of diluted antiserum and erythrocyte suspension were mixed in plain microhaematocrit tubes by twirling vigorously between thumb and forefinger, and left 2 hr at room temperature (Heslop, 1968). Duplicate blood samples were tested and the results were read blind.

Monitoring renal disfunction. (*i*) *Measurement of proteinuria.* The onset of renal disease was monitored by fortnightly testing for proteinuria. Samples of urine (10μl) were spotted onto strips of chromatography paper, air dried, and fixed for 2 hr in 70% ethanol. The strips were then immersed in staining solution (0·1 g bromophenol blue, 50 g zinc sulphate to prevent leaching of stained protein, 50 ml glacial acetic acid, water to 1 litre) for at least 2 hr. Excess dye was washed out in three 20-min changes of 2% acetic acid. The strips were transferred to 2% sodium acetate in 10% acetic acid for 2 min to develop the blue colour and then air dried. A series of standard three-fold dilutions of bovine serum albumin (3000, 1000, 333, 111, 37mg%) were processed with each batch of urine samples and the degree of proteinuria in each mouse was assessed by visually comparing the colour intensity of the urine spots with that of the spots of bovine serum albumin standards.

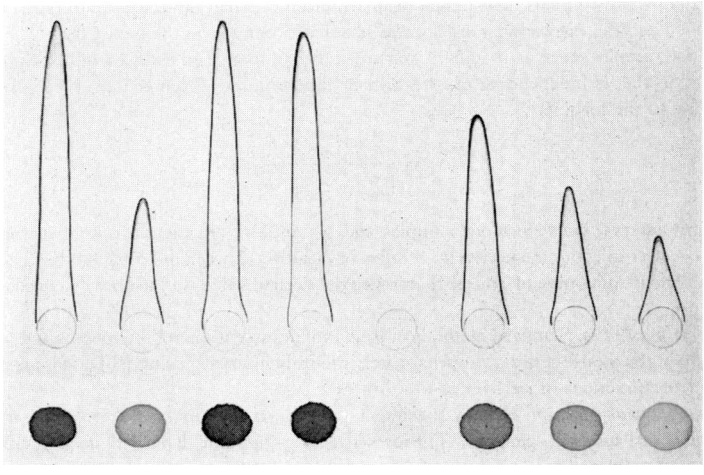

FIG. 1. Agreement of measurements of total urinary protein (intensity of bromophenol blue staining of urine spots, bottom of figure) with measurements of urinary albumin (height of precipitation lines in rocket immunoelectrophoresis, top of figure) in urine samples from eight NZB × NZW mice with varying degree of renal impairment.

(*ii*) *Validation of proteinuria measurements.* Samples of urine (diluted 1:300) were subjected to immunoelectrophoresis against 1% rabbit anti-mouse albumin antiserum (Cappel Labs.) in 1% agarose (Myles—Seravac) using standard rocket immunoelectrophoretic technique (Laurell, 1966). Fig. 1. demonstrates that urinary albumin concentration, as indicated by the height of the 'rocket' precipitation lines, correlates perfectly with the colour intensity of the corresponding stained urine spots. Therefore bromophenol blue staining of urine spots gives a valid estimate of urinary albumin concentration, which is a sensitive index of leakage of serum proteins through the kidney.

(*iii*) *Measurement of renal clearance.* Detection of marked proteinuria (> 333mg%) was followed by the assessment of glomerular function using a single injection [^{51}Cr]EDTA clearance method. Mice were injected i.v. in the tail with approximately 2 μCi[^{51}Cr]EDTA in 0·2 ml saline. Serial blood samples (20 μl) were taken from a tail vein at carefully timed intervals

* Dr J.G. Ray, Transplantation and Immunology Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, U.S.A.

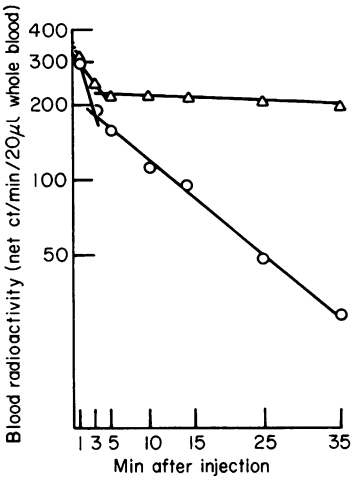

FIG. 2. Typical [^{51}Cr]EDTA disappearance curves (after a single i.v. injection) in mice with and without renal impairment. (△) Mouse with renal disease; (○) normal control mouse.

and the radioactivity assessed by counting the diluted whole blood for ten minutes in a Packard auto gamma spectrometer. Initial studies revealed that the whole blood decay of [^{51}Cr]EDTA in the mouse followed a double exponential curve in agreement with Sapirstein's two compartment model of distribution of small molecules (Sapirstein et al., 1955). However mixing between the blood and the extracellular space takes less than 5 minutes in the mouse (Fig. 2.) Therefore, as a routine procedure only two blood samples taken at 10 and 40 min are sufficient to give an estimate of half-value time thus providing an index of renal function that is independent of extracellular fluid volume (Brien & Fay, 1972). The half value time ($t_{\frac{1}{2}}$) was calculated according to the formula:

$$t_{\frac{1}{2}} = \frac{(t_2 - t_1) \log 2}{\log N_1 - \log N_2}$$

where $t_2 - t_1$ is the time interval between blood samples and N_1 and N_2 are the net ct/min for the blood samples at 10 min (t_1) and 40 min (t_2) after injection respectively. Studies on healthy 12-week old mice established the normal half value time as 14·0 ± 3·1 min. A half value time of greater than twice the normal value was arbitrarily chosen as indicative of severe renal impairment.

Detection of antinuclear antibodies. Standard methods of immunofluorescent microscopy were used to monitor the presence of anti-nuclear antibody in the mouse serum at approximately monthly intervals, using FITC-conjugated rabbit anti-mouse-γ-globulin serum and frozen sections of rat liver as the substrate.

Detection of glomerular immunoglobulin. Kidney specimens were taken at autopsy and frozen sections were stained with FITC-conjugated rabbit anti-mouse γ-globulin. The presence of γ-globulin deposited in glomeruli was detected using standard methods of immunofluorescent microscopy.

RESULTS

Incidence of renal disease in (NZB × NZW)F_1 × NZW back-cross mice

Fig. 3 shows that the incidence of proteinuria in the group of 230 backcross mice reached 42% by 360 days of age, then increased only to 48% over the course of the next 160 days. The figure compares the data with that for the contemporary control group of thirty (NZB × NZW)F_1 hybrids and shows that the age incidence of proteinuria in the backcross population is almost exactly half that observed in the F_1 hybrids at any given time.

These data provide a clear indication that the NZB strain contributes only one gene, or cluster of closely linked genes, to the renal disease of the F_1 hybrid. This gene or gene cluster is dominant or co-dominant.

Age of onset of renal disease

Fig. 4 shows the distribution of the ages of onset of renal disease in the backcross mice, as measured

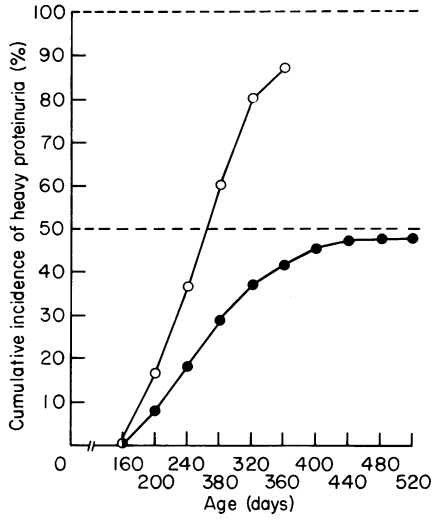

FIG. 3. A comparison of the cumulative incidence of heavy proteinuria (over 333 mg%) in thirty (NZB × NZW)F_1 (○) and in 230 (NZB × NZW)F_1 × NZW backcross mice (●).

by heavy proteinuria and decreased renal clearance. As this is not a normal distribution, showing a skew to the right, the median age is a more meaningful parameter than the mean. The median age of onset was 258 days, which closely matches the value of 250 days observed in the contemporary control group of (NZB × NZW)F_1 mice. The similarity of age of onset of renal disease in these two groups of mice provides further evidence that the backcross mice comprise two distinct populations, approximately equal in number, one population behaving like the F_1 hybrids and the other behaving like the NZW parental strain.

Correlation of parameters of renal disease

Heavy proteinuria was followed by decreased [^{51}Cr]EDTA clearance in all but three of the mice studied, although there was considerable variation in the time course and severity of the decreasing glomerular function observed. The three exceptions were mice which showed only transient proteinuria without decreased [^{51}Cr]EDTA clearance; we concluded that the proteinuria in these cases had a different cause and these three mice were omitted from the renal disease group.

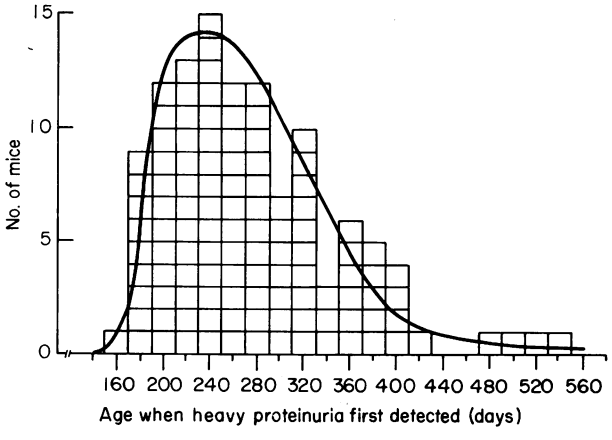

FIG. 4. Age of onset of heavy proteinuria in (NZB × NZW)F_1 × NZW back-cross mice.

Deposition of γ-globulin in the glomeruli, as detected by immunofluorescence, was universally present in mice dying with decreased glomerular function. Levels of circulating antinuclear antibody fluctuated considerably and did not appear to correlate well with severity of either proteinuria or decreased glomerular function.

Linkage between the H-2 complex and the NZB renal disease gene

The mice of this back-cross would be expected to be either homozygous ($H\text{-}2^z/H\text{-}2^z$) or heterozygous ($H\text{-}2^d/H\text{-}2^z$) with respect to the H-2 complex. The table shows that as expected, the 230 mice were nearly equally divided between the two genotypes. However a preponderance of the mice which developed renal disease were heterozygous. This imbalance is highly significant ($\chi^2 = 26{\cdot}5$, $P<0{\cdot}001$) and indicates that the gene or gene cluster contributed by the NZB strain does not segregate independently of the H-2 complex and must therefore be linked to it. The thirty-six $H\text{-}2^z/H\text{-}2^z$ mice that developed renal disease and the thirty-nine $H\text{-}2^d/H\text{-}2^z$ mice that did not presumably represent crossing over, suggesting that there is a crossover frequency of $32{\cdot}6 \pm 3{\cdot}1$ per cent between the renal disease gene(s) and the D end of H-2.

TABLE 1. Segregation of renal disease and H-2 type in (NZB × NZW)F_1 × NZW back-cross mice

	Histocompatibility type		
	$H\text{-}2^d/H\text{-}2^z$	$H\text{-}2^z/H\text{-}2^z$	Total
Mice with renal disease	78	36	114
Mice without renal disease	39	77	116
Total mice	117	113	230

DISCUSSION

Minor histological abnormalities have been reported to occur in the kidneys of young NZB mice (Braverman, 1968) but the significance of this is doubtful as although an occasional elderly animal dies of renal failure the young NZB animals do not show the early renal failure characteristic of the NZB × NZW hybrids. Howie & Helyer (1968) found no heavy proteinuria in 270 virgin female mice at 1 year of age and we observed an incidence of only 4% in a similar group of 75 NZB mice. In contrast, we observed heavy proteinuria to have occurred in 87% of a group of thirty NZB × NZW mice by the age of 1 year, in accord with Howie & Helyer's figure of 85% (Howie & Helyer, 1968). Antinuclear autoantibodies, measured by the fluorescence technique, have a high incidence in NZB × NZW mice, 77% (Braverman, 1968), but only a very low incidence, 4%, in NZB mice, in accord with the original findings with the L.E. cell test. Similarly, although minor histological abnormalities have been reported to occur in young NZW mice, early renal failure has not been observed and the incidence of antinuclear autoantibodies is very low, being found to be 4% in eighty animals studied by Braverman (1968). It seems clear that the early, florid lupus nephritis of the NZB × NZW hybrid mice does not occur in either of the inbred parental strains.

From considerations of the features and pattern of inheritance of thyroid and other human autoimmune diseases, one of us (Adams, 1977) has postulated that the genetic basis lies in the specificity of V genes, the structural genes which code for the variable portions of the light and heavy polypeptide chains which join together to form antibody molecules. There is good evidence that the specificity of an antibody combining site is determined by two, unlinked, codominant genes, one for a light chain and one for a heavy chain (Gally & Edelman, 1972). There is also good evidence that the range of antibody

specificity is increased by somatic mutations occurring in the hypervariable portions of the V genes, during antigenic stimulation (Adams, 1977; Jerne, 1971; Weigert *et al.*, 1970; Cunningham, 1974; Burch & Rowell, 1965), in accord with Burnet's (1959) original concept of the immunological clone and its somatic diversification. It is interesting to consider the genetic features of the NZB mice and their hybrids in the light of these concepts.

The Bielschowskys mated NZB mice with the New Zealand Chocolate (NZC) strain, finding the incidence of autoimmune haemolytic anaemia to be 100% in the F_1 generation and 74% in the F_2 generation, both sexes being equally affected (Bielschowsky & Bielschowsky, 1964). This indicates that the NZB strain contributes a single autosomal dominant gene (or gene cluster) towards development of the autoimmune haemolytic anaemia of the NZB × NZC hybrid. This gene(s) could be an immune response (Ir) gene (Benacerraf & McDevitt, 1972) or an immunoglobulin variable region gene (V gene) coding for part of the combining site of the anti-erythrocyte autoantibodies. In the latter case, the second V gene for the autoantibody must have been present in the NZC mice, or the incidence of anaemia in the F_2 generation would have been only 56%. In the NZB × NZY hybrids, the autoimmune anaemia does not occur (Helyer & Howie, 1961) which indicates either that occurrence of the disorder depends on recessive genes in addition to the dominant one(s) (Ghaffar & Playfair, 1971), or, alternatively, if the disorder depends on two V genes, then at least one of these is inhibited in the NZB × NZY animal. Similarly, Holmes and Burnet, crossing NZB mice with the AKR and C3H strains observed marked delay in development of positive Coombs tests in the F_1 animals (Holmes & Burnet, 1964; Holmes & Burnet, 1966; Burnet & Holmes, 1965). Again this might indicate inhibition of one of the NZB V genes contributing to one anti-erythrocyte forbidden clone, and an alternative V gene forming a 'preforbidden' clone which required more somatic mutations and therefore more time to develop its pathogenic specificity.

The present study of the genetic basis of the lupus nephritis of the NZB × NZW mice differs from previous studies in using tests of renal function to identify affected animals. Ghaffar & Playfair (1971), using fluorescence tests for the presence of glomerular immunoglobulin, were hampered by being unable to distinguish between NZB and NZB × NZW animals. Braverman (1968) saw histological changes in the kidneys of NZB and NZW animals but the changes were less severe than those occurring in the NZB × NZW hybrids, which enabled him to observe segregation of the renal disorder in back-cross and F_2 animals. This segregation showed independence from Coombs' positivity and antinuclear autoantibodies suggesting that the latter are not the sole pathogenic agents in the nephritis and that the anaemia requires at least some agents which are not involved in the nephritis. Our study, showing a 50% incidence of the nephritis in (NZB × NZW)F_1 × NZW backcross animals, indicates that the NZB strain's genetic contribution to the nephritis is a single gene, or cluster of closely-linked genes, which must be dominant or co-dominant in that the effect is expressed in the heterozygous state. The gene(s) cannot be a defective regulator gene failing to code for a genetic repressor (Jacob & Monod, 1961) or the effect would be recessively expressed. The same argument excludes DNA repair genes which could conceivably promote autoimmunity by a defective repair which permitted occurrence of unusual somatic mutations. Further evidence against the involvement of DNA repair genes is provided by a study in which exposure of NZB × NZW mice to life-long gamma irradiation, at about 200 times the normal background rate, was without effect (Croft, Adams & Purves, 1975). With evidence against a role for regulator genes or DNA repair genes, we feel that immunoglobulin V genes are the most likely candidates for the genetic basis of inherited autoimmunity and the findings in this paper are compatible with the V gene concept. At present there are believed to be at least three clusters of V genes, closely-linked to constant region (C) genes for light and heavy chains to make three 'translocons' (Gally & Edelman, 1972). The chromosomal location of these translocons is not established but in mice the heavy chain region is not closely linked to the major histocompatibility complex (H-2) (Herzenberg, Warner & Herzenberg, 1965). Gally & Edelman (1972) have suggested that Ir genes are V genes of a fourth translocon, in which case some V genes are closely linked to the H-2 complex. However, the NZB gene(s) contributing to the lupus nephritis is not closely linked to the H-2 complex, so it cannot be a histocompatibility-linked Ir gene. Interestingly, its cross-over frequency from the D end of H-2, 32·6%, is very

close to that (31·4%) for a gene observed by Lotzova & Cudkowicz (1973) to control resistance to bone marrow grafts in mice. Perhaps these two genes are members of a single cluster of V genes.

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