

GENETIC STUDIES OF AUTOIMMUNITY AND RETROVIRUS EXPRESSION IN CROSSES OF NEW ZEALAND BLACK MICE

I. Xenotropic Virus*

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Type C RNA viruses (retroviruses) have been implicated in the autoimmune disease of New Zealand Black (NZB)¹ mice (1, 2). However, the evidence for this is only circumstantial because the retroviruses isolated from NZB mice are xenotropic in host range; this prohibits a direct test of their pathogenicity in mice by, for example, inoculation of filtrates (3, 4). Nevertheless, the relationship between autoimmunization and xenotropic viruses can be ascertained by genetic tests because the genes of these agents are an integral part of cellular chromosomes (5) and their expression in NZB mice is governed by two autosomal dominant genes (6, 7). In this paper we describe results of genetic experiments designed to test the hypothesis that expression of xenotropic virus is required for the development of autoimmune disease. The following paper (8) deals with the relationship between autoimmune disease in NZB mice and expression of the major envelope glycoprotein of the virus, gp70.

Materials and Methods

Mice. NZB, SWR, C57BL/6, B10.A, and AKR mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Reciprocal crosses were made to produce F₁ and backcross progeny mice. Because the maternal direction of the cross did not affect the results, all data for a given cross were pooled and presented by a single nomenclature: F₁ for (SWR × NZB)F₁; (F₁ × SWR) for backcross to SWR, and (F₁ × NZB) for backcross to NZB. The mice were 11-24 mo old when tested.

Retrovirus Assays

XENOTROPIC VIRUS. Spleen cell suspensions were prepared and serially diluted as described previously (6, 7, 9). They were assayed by cocultivation on monolayers of the feline 81 cell line

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¹ *Abbreviations used in this paper:* Anti-nDNA, antibody to native DNA in serum; Anti-dDNA, antibody to denatured DNA; F₁, (SWR × NZB)F₁ and (NZB × SWR)F₁; F₂, (SWR × NZB)F₂; F₁ × SWR, first backcross F₁ × SWR and SWR × F₁; F₁ × NZB, first backcross F₁ × NZB and NZB × F₁; FCS, fetal calf serum; Log₁₀ virus titer, xenotropic virus titer expressed as log₁₀ focus-forming units/10⁷ spleen cells; NZB, New Zealand Black; PAS, periodic acid Schiff; PBS, phosphate-buffered saline. Anti-Rauscher gp70, antiserum to the envelope glycoprotein antigen of the Rauscher murine leukemia virus.

(provided by Dr. Peter Fischinger, National Institutes of Health) (10). The medium for tissue culture was McCoy's 5A (Grand Island Biological Co., Grand Island, N. Y.) supplemented with antibiotics and 15% fetal calf serum (FCS). 1 day after the 81 cells (2×10^5) were seeded into 6-cm plastic Petri dishes, the medium was withdrawn, and the cells were treated with 2 ml DEAE dextran (25 $\mu\text{g/ml}$) in McCoy's 5A medium without serum for 30 min at 37°C. Serial dilutions of spleen cells suspended in 5 ml McCoy's 5A medium were inoculated on the 81 cells after the DEAE dextran was rinsed away. On day 7 the medium was changed completely. Foci were counted on unstained dishes 13-14 days after infection. The number of foci that developed increased linearly and proportionally to the number of virus producing spleen cells inoculated (6). We define a mouse as "virus-negative" if no xenotropic or ecotropic viruses were detected by this or the following assays.

ASSAY ON MINK CELLS. Fluorescent antibody focus assays were done on mink lung cells (ATCC CCL 64) provided by Dr. J. Hartley, National Institutes of Health. Cultures on glass cover slips were done as described by Hartley and Rowe (11). Serial dilutions of spleen cells were inoculated on subconfluent monolayers of mink lung cell cultures in McCoy's 5A medium with 10% unheated FCS. 5 or 6 days after infection the cover slips were fixed in cold acetone, air dried, and stored at -20°C. They were stained with fluorescein isothiocyanate-conjugated goat antiserum prepared against Tween-ether disrupted Moloney leukemia virus (supplied by Dr. J. Gruber, National Institutes of Health, Bethesda, Md.). Foci of cells showing cytoplasmic fluorescence were counted.

ECOTROPIC VIRUS. The UV-XC procedure described by Rowe and Pincus (12) was modified for testing spleen cell suspensions as an infectious center assay. This technique has been described in detail previously (9). NIH 3T3 cells and BALB/c mouse embryo fibroblasts were used to detect N- and B-tropic viruses, respectively. For XC-negative ecotropic viruses a fluorescent focus assay was used after cocultivation of the spleen cells with NIH 3T3 and BALB/c embryo cells on glass cover slips, as described previously (11, 13).

ANTIGLOBULIN (DIRECT COOMBS') TEST. A microtiter direct Coombs' test was done as described previously (14). Erythrocytes were washed three times with warm phosphate-buffered saline (PBS) and 1 μl of the suspension was mixed with 20 μl of 1:5, 1:125, and 1:150 dilutions of preabsorbed rabbit anti-mouse immunoglobulin in a U-well microtiter plate (Cooke Laboratory Products Div. Dynatech Laboratories Inc., Engineering Co., Alexandria, Va.). After 1 h at room temperature the sedimentation pattern was read; any agglutination was verified microscopically and graded from + to 5+ (15).

ANTI-DNA ANTIBODY. Antibodies in serum to native (nDNA) and denatured (dDNA) DNA were measured by the [^{14}C]DNA/glass filter technique developed by Lewis et al. (16). Some sera were also assayed by the Farr technique in which DNA treated by a nuclease specific for single-stranded DNA was employed (17). The two methods gave comparable results.

Immunofluorescence of Kidney Sections

IMMUNOGLOBULIN DEPOSITS. Cryostat sections (4 μm) were made on snap-frozen kidney tissue. Sections were air dried, washed in PBS, fixed in ethanol:ether (1:1) for 10 min, and then in 95% ethanol for 20 min. The fixed sections were stained with fluorescein-conjugated anti-mouse IgG (Meloy Laboratories Inc., Springfield, Va.) and examined with a fluorescent microscope.

The stained sections were graded on a 0-4+ scale by estimating both the brightness and the extent of fluorescent staining (18). In addition to an overall score for the entire glomerulus, separate scores were assigned to the mesangium and capillary loops. All slides were read on coded specimens by two observers. Kidneys from 9-mo old (NZB \times NZW) F_1 mice served as positive controls and those from 3- and 12-mo old SWR or BALB/c mice were used for negative controls.

Retroviral Antigens

DIRECT FLUORESCENCE. Sections were stained with a 1:40 dilution of fluorescein isothiocyanate-conjugated goat antiserum prepared against Tween-ether disrupted Moloney leukemia virus (11, 13) (supplied by Dr. J. Gruber, Office of Program Resources, National Cancer Institutes, Bethesda, Md.). A 1:50 dilution of rhodamine solution was used as a counter stain.

INDIRECT IMMUNOFLUORESCENCE. Kidney sections were overlaid with an anti-Rauscher gp70 serum made in goats (provided by Dr. J. Gruber). After incubation and washing, a fluorescein-conjugated rabbit anti-goat IgG (pre-absorbed with normal mouse spleen cells and serum) was

used to stain the slides (2). A 1:40 dilution of the anti-Moloney leukemia virus serum stained with 3-4+ intensity mink (ATCC CCL 64), rabbit (SIRC), human (RD), and rat (NRK) cell lines that were infected with xenotropic viruses derived from NZB, NIH Swiss (AT 124), and BALB/c (BALB:virus-2) mice. It also stained with equal intensity at 1:40 dilution NIH 3T3 cells infected with ecotropic (N-tropic) AKR Gross virus and BALB 3T3 cells infected with ecotropic (B-tropic) virus from B10.A mice (9). A 1:20 dilution of this serum did not stain uninfected NIH and BALB 3T3 cells or uninfected rat embryo, mink, and rabbit cells. Cell lines derived from spleens and embryos of SWR mice were repeatedly negative when stained with this serum. The goat anti-Rauscher gp70 serum had similar properties: at a 1:40 dilution it brightly stained the above cell lines infected with xenotropic or ecotropic virus and at a 1:20 dilution it failed to stain the corresponding uninfected cells. These two antisera consistently stained glomeruli in kidney sections obtained from 9-mo old (NZB × NZW)_F₁ mice and regularly failed to stain glomeruli in kidney sections from 3-mo old SWR or BALB/c and 12-mo old SWR mice.

Histopathology. Glomerular lesions were graded as 2+, 3+, or 4+ (19). A 2+ lesion corresponded to focal or focal and diffuse thickening of the capillaries of 30-60% of the glomeruli; in a 3+ lesion the capillaries of all the glomeruli were diffusely and focally thickened and hypercellularity of glomeruli was present; 4+ lesions were characterized by the preceding together with sclerosis of glomeruli, massive proliferation of epithelial cells, and numerous tubular casts. A grade of "0" was given to kidneys without glomerular lesions. All specimens were coded before they were read. Lymphomas were diagnosed and classified by the criteria of Dunn and Deringer (20).

Electron microscopy. Tissues were prepared for electron microscopy as previously described (21). A minimum of 40 glomeruli was studied from each sample and a uniform grading system was utilized to record data. All interpretations were done on coded specimens.

Results

Virus Expression and Autoantibodies. NZB mice were crossed with the virus-negative, nonautoimmune SWR strain and the F₁, F₂, and backcross progeny were tested virologically and serologically. Since autoimmune disease in NZB crosses may be time-dependent (6, 22), the crosses were tested at the age of 20-24 mo, except for those animals that were moribund from anemia, nephritis, or lymphoma. In the latter cases, the youngest animals were 11 mo old. The attributes of the parental lines are shown in Table I and Fig. 1. The findings in these strains are in accord with previous results (3, 6, 23, 24). NZB, SWR, F₁, (F₁ × NZB), (F₁ × SWR), and F₂ mice were also tested by the XC technique and in some cases by the fluorescent focus assay by using NIH 3T3 and BALB/c embryo cell indicators. In no instance (0 out of 60 mice) was ecotropic virus detected.

All F₁ mice expressed xenotropic virus, with a mean log₁₀ titer for the group of 3.4 ± 0.7. Although there was no difference in virus titers between NZB and F₁ mice ($P > 0.1$), there was a clear immunological difference: only 4% of the F₁ mice had positive antiglobulin tests and antibodies to dDNA were found in just 9 of 28 (32%). These serological differences between F₁ and NZB mice were significant ($P < 0.001$, $P < 0.01$, respectively) and indicate that if xenotropic virus is related to the production of autoantibodies, it cannot be the sole factor.

The (F₁ × NZB) mice were similar to the F₁ (Table I, Fig. 1). Virus titers were virtually identical to those in NZB (mean log₁₀ titer 3.7 ± 0.5), but serological abnormalities differed considerably in that there were lower incidences of positive antiglobulin tests (6 of 27; 22.2%; $P < 0.02$) and antibodies to dDNA (10 of 27; 37.0%; $P < 0.02$).

Virus expression in (F₁ × SWR) and F₂ mice is due to the action of two genes,

TABLE I
Incidence of Xenotropic Virus Expression and Autoantibodies in NZB and SWR Mice and in NZB Crosses

Strain	n	% Mice positive for:			
		Xenotropic virus	Antiglobulin test	Anti-nDNA	Anti-dDNA
NZB	20	100	94.0	20.0	100
SWR	20	0	0	0	0
F ₁	27	100	3.7	25.2	32.4
F ₁ × NZB	27	100	22.2	25.9	37.0
F ₁ × SWR	46	73.0	4.3	13.0	21.7
F ₂	63	93.6	11.1	23.8	15.8
(B10.A × NZB)F ₁	49	100	16.3	14.3	22.4
(C57BL/6 × NZB)F ₁	33	100	9.0	24.4	29.3
(AKR × NZB)F ₁	12	100	0	0	66.6

Anti-DNA antibodies were considered present when binding to [¹⁴C]DNA was >10%. The crosses were 20-24 mo old when tested, except for animals dying of overt disease. The parents were 12 mo old.

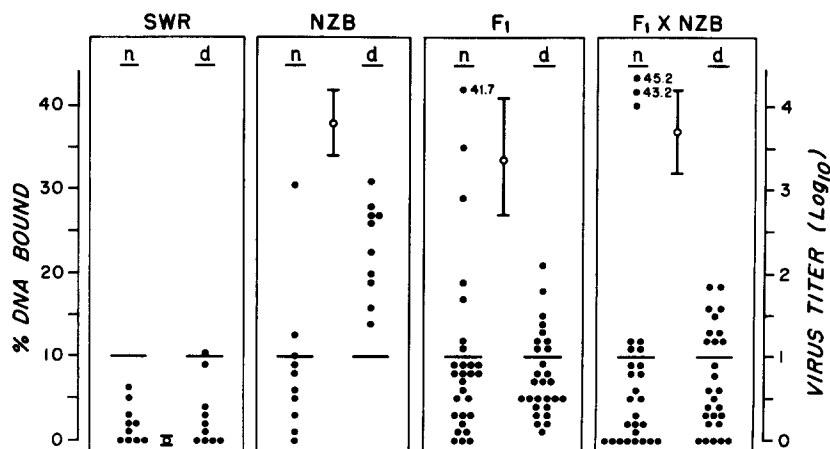


FIG. 1. Antibody to DNA in serum (% DNA bound) and infectious xenotropic virus titer (\log_{10} focus forming units/ 10^7 spleen cells) in the two parental strains, SWR and NZB, their F₁ hybrids and the backcross to NZB (F₁ × NZB). In this and subsequent figures the following will be used: serum anti-DNA antibody = % DNA bound. n = antibody to native DNA. d = antibody to denatured DNA. Horizontal bar is 2 SD above the mean for anti-DNA antibody in 10 SWR mice. Each solid circle represents an individual animal. Animals without overt disease were 20-24 mo old when tested. The mean titer of xenotropic virus for each group is represented by an open circle and \pm SD by a vertical bar. "0" titer designates virus-negative mice. See text for numerical values.

Nzu-1 and *Nzu-2* (6, 7). The former specifies high-grade expression of infectious xenotropic virus and the latter specifies low-grade expression of virus; homozygosity for recessive alleles of these two genes is associated with failure to express xenotropic virus. Fig. 2 shows the mean \log_{10} virus titers of the three phenotypes, high-virus, low-virus, and virus-negative, in (F₁ × SWR) and F₂

RETROVIRUSES AND AUTOIMMUNE DISEASE

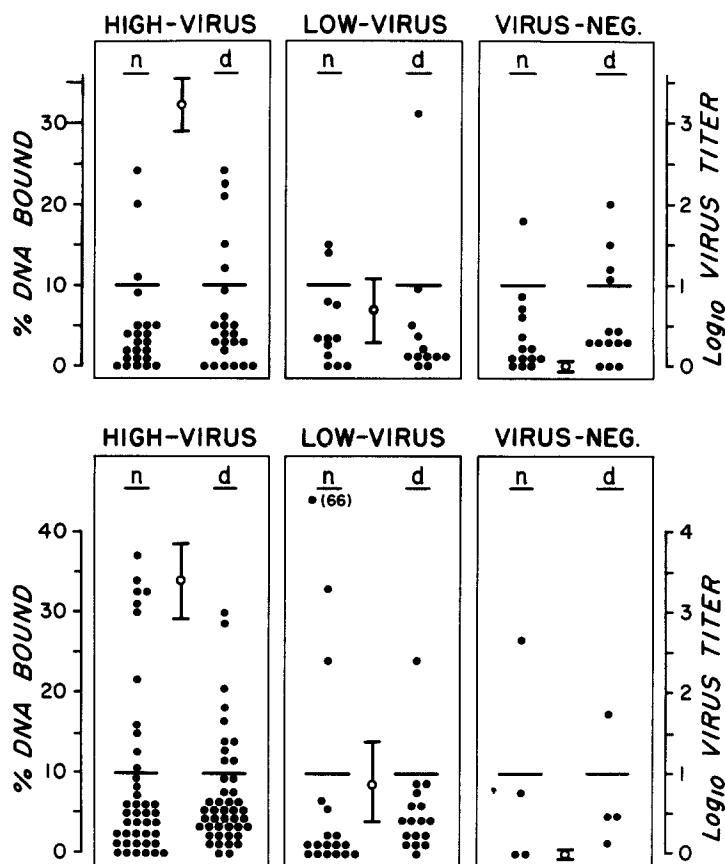


FIG. 2. Anti-DNA antibody in serum and xenotropic virus titers of the crosses that showed segregation into three virologic phenotypes: high-virus, low-virus, and virus-negative. Progeny of the first backcross to SWR ($F_1 \times$ SWR) are shown in the top panel and those of the F_2 generation are shown in the bottom panel. Note that the highest titer of antibody to native DNA among these crosses (66%) occurred in a low-virus mouse and some of the virus-negative animals developed the autoantibody.

mice. The pooled data in Fig. 3 demonstrate that the high-virus group can be separated from the low-virus group by 3 SD. In some mice the phenotype defined by virus titers on cat cells was confirmed by an independent assay on mink cells (data not shown).

Autoantibodies found in the three phenotypes of ($F_1 \times$ SWR) and F_2 mice are shown in Table II and Fig. 2. Note that the serological abnormalities in high-virus animals differed substantially from those in NZB mice (e.g., only 9 of 65 of these crosses had anti-dDNA antibodies as compared to 20 of 20 in virologically similar NZB mice). In addition, Table II shows that 7 of 30 low-virus and 6 of 18 virus-negative mice had autoantibodies. In Fig. 3 all data on anti-DNA antibodies from the three phenotypes are pooled and charted as a function of virus titer. Note the approximately equal distribution of positive and negative tests for anti-DNA antibodies in each of the three groups. There was no

TABLE II
Virologic and Serologic Findings in (F₁ × SWR) and F₂ Mice

	High-virus	Low-virus	Virus-negative
	(F ₁ × SWR)		
Mean log ₁₀ virus titer	3.22 ± 0.44	0.69 ± 0.37	0.0 ± 0.0
Number mice tested	23	12	14
Anti-nDNA	1	2	0
Anti-dDNA	3	1	3
Anti-nDNA + Anti-dDNA	2	0	1
Positive antiglobulin test	1	0	0
	F ₂		
Mean log ₁₀ virus titer	3.38 ± 0.47	0.91 ± 0.46	0.0 ± 0.0
Number mice tested	42	18	4
Anti-nDNA	7	2	0
Anti-dDNA	6	0	0
Anti-nDNA + Anti-dDNA	4	1	1
Positive antiglobulin test	5	1	1

Mean xenotropic virus titers (log₁₀ focus forming units/10⁷ spleen cells) and incidence of autoantibodies in the high-virus, low-virus, and virus-negative segregants of (F₁ × SWR) and F₂ crosses. Only four virus-negative mice in the F₂ generation could be tested since the segregation of virus-positive to virus-negative progeny was 15:1 in this cross. See Fig. 2 for the titers of anti-DNA antibody in these crosses.

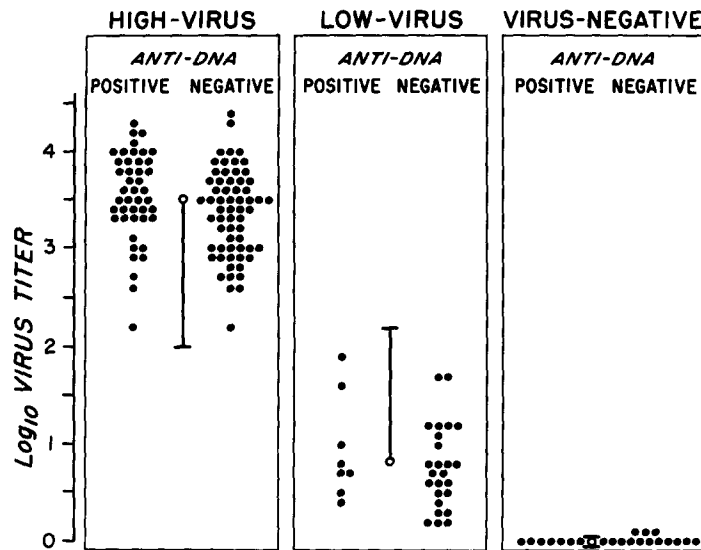


FIG. 3. Presence of antibody to DNA and virus phenotype of the SWR × NZB crosses. High-virus group = all F₁ and (F₁ × NZB) crosses plus the high-virus progeny of (F₁ × SWR) and F₂ crosses. The low-virus and virus-negative group of animals are from the (F₁ × SWR) and F₂ crosses. Mean log₁₀ xenotropic virus titers are represented for each of the three groups by an open circle with 3 SD (vertical bar) demarcating the difference between the high-virus and the low-virus group of mice. Each closed circle represents an individual animal.

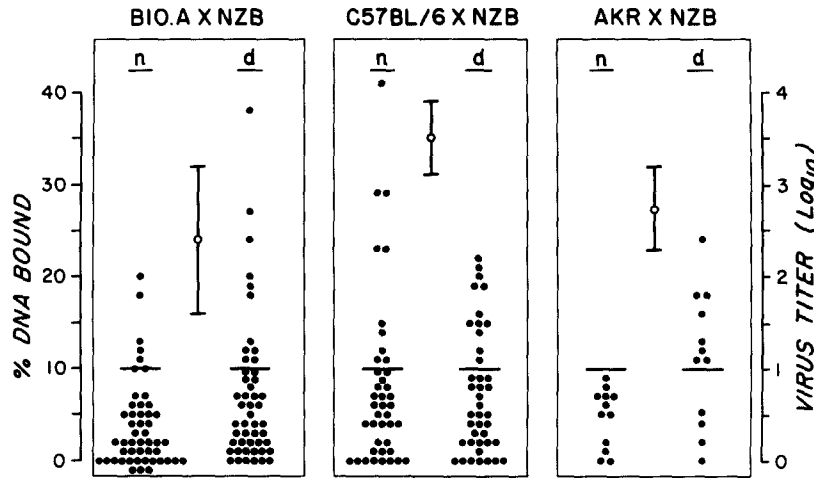


FIG. 4. Anti-DNA antibody and mean \log_{10} xenotropic virus titers in F_1 hybrids of NZB crossed with B10.A, C57BL/6, and AKR strains.

significant disproportion of antibodies to nDNA among NZB, F_1 , ($F_1 \times$ NZB), and high-virus F_2 and ($F_1 \times$ SWR) mice. However, the 100% incidence of antibodies of dDNA in NZB mice was exceptional ($P < 0.001$) relative to any of the high-virus crosses.

To determine if the dissociation between the high-virus phenotype and production of autoantibodies was peculiar to the SWR \times NZB cross we analyzed three other F_1 hybrids (Fig. 4, Table I). In each case the general pattern was similar to that found in the SWR \times NZB cross: despite titers of xenotropic virus similar to those found in the NZB mouse, autoantibodies were found in only a fraction of the animals. All (C57BL/6 \times NZB) F_1 and (B10.A \times NZB) F_1 mice were ecotropic virus-negative. Ecotropic virus (N-tropic) was detectable in all (AKR \times NZB) F_1 mice (mean \log_{10} titer/ 10^7 spleen cells was 2.6 ± 0.9).

*Immunopathology of the Kidney.*² Periodic acid Schiff (PAS)-stained sections of the kidneys of 137 NZB \times SWR crosses were examined. The incidence of glomerular lesions was: F_1 , 35%; F_2 , 41.3%; ($F_1 \times$ SWR), 54%; ($F_1 \times$ NZB), 22.7%. Female mice were somewhat more susceptible to glomerular lesions than male mice (50% versus 28.2%, $P < 0.02$). In F_2 and ($F_1 \times$ SWR) mice (Table III) there was no correlation between phenotype (high-virus, low-virus, virus-negative) and the incidence of histologically identifiable glomerular lesions ($P > 0.1$). Although 13 of 15 of the severe glomerular lesions (3+ and 4+, Fig. 5 a), were found in mice with the high virus phenotype there are two reasons why the high-virus phenotype was not in itself sufficient to cause an association with nephritis: (a) the incidence of nephritis in the high-virus progeny of ($F_1 \times$ SWR) mice was significantly higher than in high-virus ($F_1 \times$ NZB) ($P < 0.05$) or F_1 ($P < 0.01$) mice. This indicates that the "normal" SWR parent contributes to the

² Proteinuria was estimated in these animals using "Albustix" reagent strips (Ames Co. Div. of Miles Lab., Inc., Elkhart, Ind.). Although most mice with renal lesions had significant proteinuria (100->1,000 mg protein/100 ml urine), this test was found to be unreliable, particularly in male mice.

TABLE III
Nephritis In NZB × SWR Crosses

Group	Virus phenotype	Glomerular lesion				Total
		0	2+	3+	4+	
(F ₁ × SWR)	High	7	7	1	2	10/17
	Low	3	4	0	0	4/7
	Negative	7	6	0	0	6/13
F ₂	High	19	11	5	2	18/37
	Low	13	3	2	0	5/18
	Negative	2	1	0	0	1/3
(F ₁ × NZB)	High	17	4	0	1	5/22
F ₁	High	13	5	0	2	7/20
(F ₁ × SWR) + F ₂	High	26	18	6	4	28/54
	Low	16	7	2	0	9/25
	Negative	9	7	0	0	7/16

Incidence of nephritis (glomerular lesions graded histologically on PAS-stained sections) in relation to virologic phenotype in the SWR × NZB crosses. Glomerular lesions were graded on severity of changes in glomerular capillaries and mesangium and included focal and diffuse thickening of glomerular basement membranes as well as proliferative and sclerotic changes (see Materials and Methods). For each virus phenotype the number of mice with a particular lesion is given (e.g., 5 high-virus F₂ mice had 3+ glomerular lesions and 37 F₂ mice with this phenotype, were examined). Total = number of mice with glomerular lesions of the number of mice examined.

development of nephritis in the backcross. (b) Histologically normal kidneys were found in 56 mice with high titers of xenotropic virus.

The kidneys of 102 mice were examined by both PAS and immunofluorescent stains. Since mesangial deposits of immunoglobulins are common in kidneys of old mice of various strains (25), only those specimens with immunoglobulin deposits of 3-4+ intensity in glomerular capillary loops and with staining of at least 80% of the glomeruli were read as "positive" (18). Results of immunofluorescent stains correlated with the histological findings: the two techniques were independently read on coded specimens by different observers and 90% (44 of 49) of the lesions identified by light microscopy had granular and linear deposits of immunoglobulins along the glomerular capillary walls (Fig. 5 c, d, e). Similar deposits were also found in 14 of 53 histologically normal specimens. The incidence of glomerular capillary deposits of immunoglobulins in the crosses is shown in Table IV. There was no correlation between the virus phenotypes of (F₁ × SWR) and F₂ mice and the presence or absence of such deposits (*P* > 0.05). In seven virus-negative mice there were typical immunoglobulin deposits in glomerular capillaries together with histologically identifiable lesions (Fig. 5 b, e, h).

Viral antigens, as determined by the immunofluorescent technique, were found along the glomerular capillary walls and in the mesangium of some specimens (Table IV). Staining of glomerular capillaries by the anti-viral antisera was found in 21 of 107 cases (19.5%); the deposits were typically granular and appeared similar to the immunoglobulin stains (Fig. 5 g). Table IV shows that deposits of *both* immunoglobulins and viral antigens were

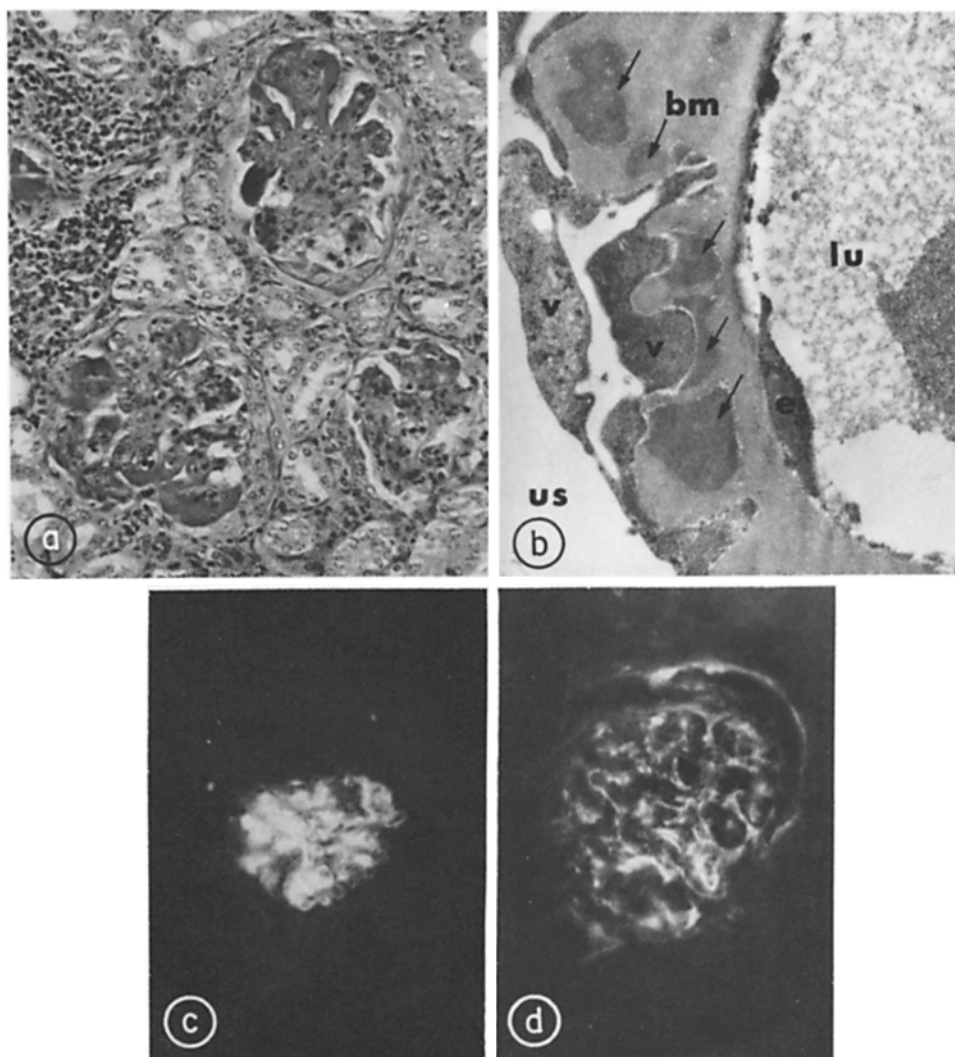


FIG. 5. Examples of renal lesions seen in NZB \times SWR crosses. (a) Severe (3+) membranous glomerulonephritis in a ($F_1 \times$ NZB) mouse. This animal had the high virus phenotype, but no viral antigen was detected in the glomerular lesions. \times 250. (b) Electron microscopic appearance of the membrane of glomerular capillaries from a virus-negative ($F_1 \times$ SWR) mouse. The basal glomerular membrane (bm) appears irregularly thickened and contains several large electron dense deposits (arrows). Its external side, facing the urinary space (us) is continuously covered by the fused foot processes of the visceral epithelial cells (v). The lumen (lu) of the capillaries is free and lined by healthy endothelial cells (e). \times 15,800. (c, d, and e) Immunoglobulin deposits in kidneys from NZB \times SWR crosses. \times 250. (c) ($F_1 \times$ NZB), high-virus phenotype. (d) F_2 with high-virus phenotype. (e) virus-negative ($F_1 \times$ SWR). (f, g, and h) Glomeruli from NZB \times SWR crosses stained for retroviral antigens. (f) Absence of detectable viral antigens in the glomeruli (arrows) of an ($F_1 \times$ NZB) high-virus mouse. The PAS stain of this kidney is shown in 5a and immunoglobulin stain in 5c. \times 250. (g) Viral antigen deposits in the mesangium and glomerular capillary loops in the kidney of an F_2 mouse with high-virus phenotype (\times 500); immunoglobulin stain shown in 5d. (h) Absence of detectable viral antigens in the glomeruli (arrows) in a virus-negative ($F_1 \times$ SWR) mouse (\times 250). This specimen had Ig deposits in glomerular capillary loops (see 5e), a 2+ lesion with the PAS stain and electron microscopic findings shown in 5b. The light areas around the glomeruli are due to tissues that stained dull orange from the rhodamine counter stain.

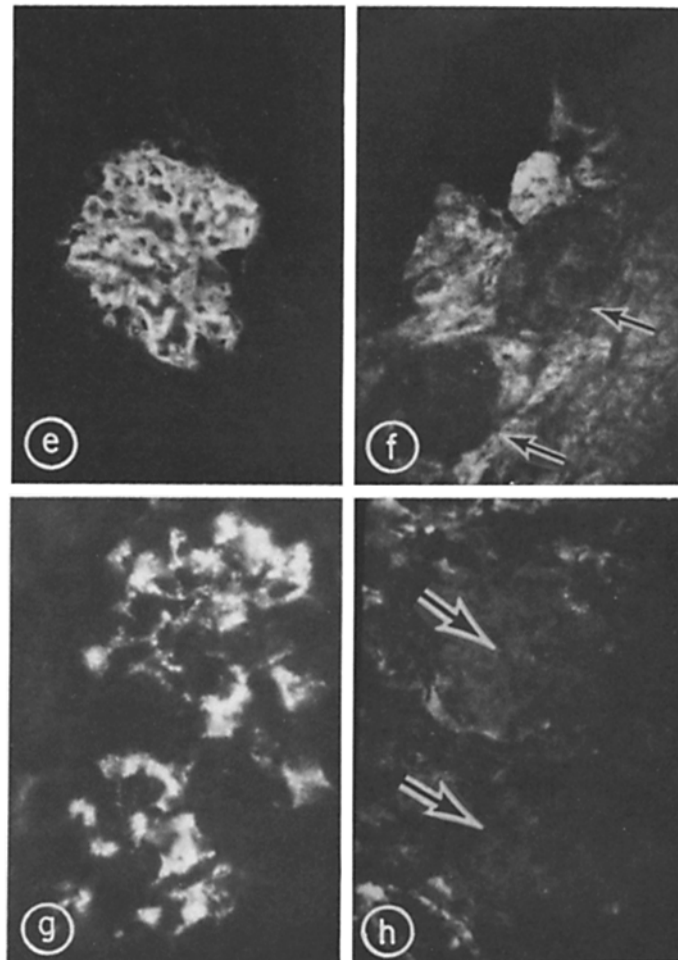


FIG. 5e-h.

present in the glomerular capillaries of 18 specimens (Fig. 5 d, and g). Of these 18 kidneys with immune deposits containing viral antigens, 15 occurred in mice with a high-virus phenotype and were distributed about equally among the four crosses. One virus-negative ($F_1 \times$ SWR) mouse out of 16 virus-negative crosses tested had deposits of immunoglobulins and viral antigens in its glomerular capillaries, but there was no lesion seen histologically. This animal had been tested virologically twice, at 3 mo of age and again at 14 mo of age, and on both occasions no virus was detected. The level of gp70 in the serum of this mouse was elevated ($15.7 \mu\text{g/ml}$); this subject will be dealt with in the succeeding paper (8).

Each of the glomerular lesions classified histologically as 3+ or 4+ contained heavy (3+ or 4+) deposits of immunoglobulins in glomerular capillaries. 13 of these 15 mice had the high-virus phenotype; however, viral antigens were present in the renal lesions in only five cases (see, for example, Fig. 5 a, c, and f). Thus, the development of moderately severe or severe glomerulonephritis

TABLE IV
Immunoglobulin and Viral Antigen Deposits in Glomeruli in Relation to Virus Phenotype in SWR × NZB Crosses

Group	Virus phenotype	Immunofluorescent strain			VM
		IgGC*	VGC	IgGC + VGC	
(F ₁ × SWR)	High	10/15	4/15	3/15	4/15
	Low	5/7	1/7	1/7	2/7
	Negative	8/13	1/13	1/13	1/13
F ₂	High	17/25	6/25	5/25	7/25
	Low	8/17	2/17	1/17	6/17
	Negative	1/3	0/3	0/3	0/3
(F ₁ × NZB)	High	10/14	3/14	3/14	7/14
F ₁	High	11/13	4/13	4/13	5/13
(F ₁ × SWR) + F ₂	High	27/40	10/40	8/40	11/40
	Low	13/24	3/24	2/24	8/24
	Negative	9/16	1/16	1/16	1/16

IgGC, presence of IgG in glomerular capillary loops with involvement of at least 80% of the glomeruli and a fluorescence intensity of 3+ to 4+. VGC, presence of viral antigen in glomerular capillaries (with or without mesangial deposition). IgGC + VGC, presence in the same specimen of deposits of immunoglobulin and viral antigen in glomerular capillaries. VM, presence of viral antigen deposits in mesangium (with or without glomerular capillary deposits). 107 kidneys from the crosses were examined for viral antigen deposits. Among these, a total of 32 stained with antisera against viral antigens. In 17 of these 32, the staining was found both in the mesangium and glomerular capillaries. Four kidneys had viral antigen deposits in glomerular capillaries only and in 11 specimens viral antigen was present in the mesangium alone. Numbers in the table refer to number of mice with the finding of the total number examined.

did not require the involvement of viral antigens. The converse was also true: of the 32 kidneys from the NZB crosses that had viral antigen deposits, histologically identifiable lesions were present only in 18 specimens. Among the remaining 14 histologically normal kidneys, viral antigen deposits were found both in the glomerular capillaries and mesangium in 10 specimens, and in 4 specimens viral antigens were present in the mesangium alone.

Increased levels of antibodies to DNA, either native or denatured, were found in the sera of 33 of 60 mice (55%) whose kidneys had glomerular capillary deposits of immunoglobulin and in 17 out of 46 mice (37%) whose kidneys did not have such deposits. This difference is insignificant ($P > 0.05$). There were also no differences between the two groups with regard to levels of antibodies to DNA (mean of 9.9% bound vs. mean of 13.4% bound; $P > 0.05$). There were, however, differences among the crosses. In those mice with glomerular capillary deposits of immunoglobulins, anti-DNA levels tended to be lower in the F₁ ($10.5 \pm 7.5\%$ bound) and (F₁ × SWR) ($8.5 \pm 9.1\%$ bound) than in the F₂ ($18.8 \pm 24.2\%$ bound) and (F₁ × NZB) ($24.6 \pm 15.3\%$ bound). The difference between the two backcrosses in this regard is highly significant ($P < 0.01$). This suggests that anti-DNA antibodies in serum may or may not be associated with glomerular deposits of immunoglobulin, depending on genetic factors. It is noteworthy that among the 15 mice with histological and immunohistochemical criteria of severe nephritis, 10 had elevated levels of anti-DNA antibodies.

Representative coded specimens from 10 low-virus and 15 virus-negative

TABLE V
*Incidence of Lymphomas in the SWR × NZB Crosses in
 Relation to Their Virus Phenotypes*

Group	Virus phenotype	Lymphoma
F ₁ × SWR	High	2/17
	Low	1/7
	Negative	1/13
F ₂	High	8/37
	Low	3/18
	Negative	0/3
F ₁ × NZB	High	11/22
F ₁	High	13/20
(F ₁ × SWR) + F ₂	High	10/54
	Low	4/25
	Negative	1/16

crosses were examined by electron microscopy and compared with specimens from 4 high-virus mice with nephritis. The ultrastructure of the nephritic lesions in high-virus mice was similar to that in low-virus and virus-negative mice; any variations were due to severity. In all cases the lesion was that of membranous glomerulonephritis and was invariably associated with a cellular infiltrate. Mesangial thickening was a constant finding, whereas lesions of the basement membranes of the capillaries varied. The mesangial thickening was due to increased numbers of mesangial cells as well as to a marked increase in the glomerular basement membrane-like substance of the mesangial tufts. Irregular, marked thickening of the basement membranes of the capillaries and fusion of the foot processes of visceral epithelial cells, which were swollen and often vacuolated, were found in both high-virus and virus-negative mice. The capillary endothelial cells were spared. Architectural distortion of glomeruli occurred in severe lesions. Heavy electron-dense deposits were found in the mesangium and along the capillary basement membranes in sub, intra, and supramembranous locations (Fig. 5 b). A cellular infiltrate consisting of lymphocytes and plasma cells was present around glomeruli and tubules in every specimen.

Malignant Lymphomas. Lymphomas were found in 39 out of 137 of the crosses (Table V). Most of these were reticulum cell sarcoma, type B; a few were lymphoblastic tumors or variants thereof (20). Only one of the neoplasms involved the thymus; the rest appeared to have arisen in the spleen or lymph nodes. All the lymphomas except one were found in virus-positive animals; of these 38 mice, 34 (90%) had the high-virus phenotype and only 4 had the low-virus phenotype. However, high grade expression of the virus was not the sole factor associated with development of lymphomas. The incidence of lymphomas in high virus F₂ and (F₁ × SWR) mice was 21.6% and 11.8%, whereas in F₁ and (F₁ × NZB) mice, which were all high-virus, it was 65 and 50%. Comparison of pooled data (F₁ + [F₁ × NZB] versus F₂ + [F₁ × SWR]) yielded a highly significant difference: *P* < 0.001. This indicates that genes other than those related to xenotropic virus make an important contribution to the development of lymphomas in these mice.

Discussion

The virologic aspects of NZB mice figure prominently in several current concepts of the etiology and pathogenesis of autoimmunity (1, 26). NZB mice produce the highest titers of xenotropic virus particles of any strain and the virus is present throughout the life of the animal (3, 6). This unique feature has suggested a cause and effect relationship between the virus and the autoimmune disease (1). This idea was strengthened by the detection of viral antigens and anti-viral antibodies, together with antibodies to DNA, in the renal lesions of NZB and (NZB \times NZW) F_1 mice (2, 27). The proposition has also been advanced that the xenotropic virus causes the disturbed immunoregulation of NZB mice, perhaps by instigating an immune response against thymocytes (28). Thus, the xenotropic virus has been thought both to initiate the disease and to provide antigens that generate nephritogenic immune complexes.

The presence of virus particles and the detection of viral antigens and anti-viral antibodies in lesions do not, however, constitute proof that xenotropic viruses cause the disease of NZB mice. It is not possible to test the pathogenicity of the NZB virus by inoculation of filtrates into mice because the NZB virus cannot infect mouse cells (1, 3). Thus, a traditional microbiological approach cannot be applied to the solution of this problem. However, since the xenotropic virus is represented in the chromosomes of mice by structural genes (5), the relevance of the virus to autoimmunity can be ascertained by genetic techniques.

The feasibility of a genetic approach was ensured by the identification of two autosomal dominant genes that govern the expression of xenotropic virus in NZB mice (6, 7). One of these genes, *Nzv-1*, specifies high-grade expression of the virus and the other, *Nzv-2*, specifies low-grade expression of the virus. Homozygosity for recessive alleles at both loci results in a virus-negative mouse. Matings between NZB and virus-negative SWR mice result in three kinds of progeny in F_2 and ($F_1 \times$ SWR) generations: high-virus, low-virus, and virus-negative. These phenotypes are virologically stable for many months (6, 7), and thus permit a test of the hypothesis that the development of autoimmunity requires the expression of infectious xenotropic virus.

The results we obtained were clear cut: the virological phenotype of the animal was independent of the presence of autoantibodies or glomerulonephritis. This conclusion is based on the following reasons: (a) crosses that were virologically identical to NZB mice failed to develop any signs of autoimmunization, even up to 24 mo of age. (b) Mice that were virus-negative on repeated testing were able to produce autoantibodies to erythrocytes and DNA, and in 8 out of 16 cases there were typical immune deposit glomerular lesions in the absence of deposits of viral antigens (Tables III and IV, line 11). (c) Mice that were low-virus, i.e., that had titers of xenotropic virus that were, on the average, two orders of magnitude lower than those in NZB mice, also developed typical signs of the autoimmune disease of NZB mice. (d) In high-virus mice with nephritis, viral antigens could not be detected in the lesions of 8 of 13 specimens.

By contrast with the lack of correlation with virus titer, nephritic lesions were very significantly correlated with the presence in serum of antibodies to

DNA. Such antibodies (either to n or dDNA) were present in the serum of 26 of 56 mice (46.4%) with nephritis and in 20 of 81 mice (24.7%) with histologically normal kidneys ($P < 0.01$). This supports other observations that the major portion of the elutable IgG in the immune deposit lesions of (NZB \times NZW) F_1 kidneys was reactive with DNA (27).

The dissociation between expression of xenotropic virus and production of autoantibodies was not a peculiar feature of SWR \times NZB crosses; similar results were found in crosses between NZB and B10.A, C57BL/6 and AKR mice. Ecotropic viruses can be excluded as a possible influence because (AKR \times NZB) F_1 mice produced relatively high titers of both ecotropic and xenotropic viruses but they did not behave differently from the other crosses. Moreover, we previously showed that NZB, SWR, and (NZB \times SWR) F_1 , (C57BL/6 \times NZB) F_1 , and (B10.A \times NZB) F_1 mice failed to express ecotropic virus (29). In the present work, SWR \times NZB crosses were again tested, in some cases with two different methods, the XC test and a fluorescent focus assay, and ecotropic viruses could not be detected. Xenotropic viruses are the only kinds of retroviruses known to be spontaneously expressed by NZB mice (1, 3, 6); whether or not other kinds of retroviruses that are undetectable by the methods we employed are involved in the autoimmune disease of NZB mice cannot be excluded.

The endogenous xenotropic virus of NZB mice (5, 30) may be considered an "autovirus" and thus a potential autoantigen. Our data indicate that xenotropic viruses may provide autoantigens in a manner comparable to erythrocytes and DNA; i.e., they may play secondary roles, but they are not required for the development of autoimmunization. Whether an autoimmune reaction occurs against xenotropic viral antigens or other antigens probably depends on genes ("autoimmunity genes") that are distinct from viral genes. This interpretation sets the NZB model apart from examples of immune complex-mediated injury involving infection by *exogenous* viruses, in which case the infectious agent participates in both the etiology and pathogenesis of the immunological lesions (31).

Identification and enumeration of "autoimmunity genes" in NZB mice has not been achieved because the pattern of their inheritance is complex (32, 33). Howie and Helyer pointed out that (NZB \times NZC) F_1 mice had predominantly hemolytic anemia, whereas the characteristic lesion of (NZB \times NZW) F_1 hybrids was nephritis (34). This led them to the important concept that the expression of autoimmunity in NZB crosses was modified by the genetic contribution of the normal parent. The modification involved not only the time of onset of the disease but also the nature of the lesion. Braverman's study (35) of crosses between NZB and NZW mice found evidence that NZB has a dominant gene that determines the production of autoantibodies against erythrocytes, whereas NZW has a "modifying gene," which, in the presence of an NZB gene, allows the development of antinuclear antibodies. He concluded that multiple genes, independent of those related to anti-nuclear and anti-erythrocyte antibodies, were involved in the nephritis. Ghaffar and Playfair found that autoantibodies against erythrocytes appeared much earlier in NZB mice than in (NZB \times BALB/c) F_1 hybrids (22). They suggested that this might be due to gene dosage effects: a single copy of a given gene may be associated with the late appearance of a positive antiglobulin test, whereas multiple copies of the gene may correlate

with an earlier expression of this abnormality. Our experiments took this into account by aging the crosses for 20–24 mo, except for animals that had developed overt disease before then.

We also found that the expression of autoimmunity in NZB crosses was strongly influenced by genes inherited from the normal parent. For instance, the incidence of nephritis was significantly higher ($P < 0.05$) in the high-virus progeny of ($F_1 \times$ SWR) mice than in high-virus ($F_1 \times$ NZB) mice (Table III). We also found important differences among the crosses with regard to antibodies to DNA: F_2 and ($F_1 \times$ NZB) mice made significantly higher levels of anti-DNA antibodies than F_1 and ($F_1 \times$ SWR) mice ($P < 0.01$). These data were completely independent of virus expression, even in the case of lymphomas. The results suggest that "normal" mice possess genes that affect the development of autoimmunization and lymphomas by either positive or negative influences.

Finally, we suggest that the high-grade expression of xenotropic virus, although not a requirement for autoimmunization, is so unique to NZB mice that its concordance with autoimmunization *in this strain* may be more than a coincidence. Expression of genes of the xenotropic viruses seems to occur in differentiating cells. The viral glycoprotein gp70 has been found in differentiating thymocytes (36, 37), in the male genital tract, and in sperm (38). Hematopoietic cells (39), embryos and placenta (40), as well as regenerating liver cells (41) probably express these viruses or part of their genes. Lymphocytes stimulated by antigens or mitogens also produce xenotropic viruses (42–44). All this suggests that the structural genes for these viruses may be derepressed during normal differentiation. NZB mice, which express these genes continuously, may have abnormal regulatory genes, or abnormal regulatory mechanisms (45, 46), which could be involved in the premature and uniform development of autoantibodies. Thus, the two abnormalities of NZB mice—virologic and immunologic—may be manifestations of a fundamental disturbance of gene regulation.

Summary

The relationship between expression of xenotropic virus and the development of autoimmunization was studied in the progeny of crosses between New Zealand Black (NZB) and SWR mice. The ($F_1 \times$ SWR) and F_2 progeny segregated into three phenotypes: high-virus, low-virus, and virus-negative; F_1 and ($F_1 \times$ NZB) progeny were always high-virus. Autoantibodies, immune deposit nephritis and lymphomas developed in the progeny of these crosses. The virological phenotype of the animal could be dissociated from the presence of either autoantibodies or nephritis. For example, mice that expressed titers of virus as high as the NZB parent failed to develop signs of autoimmunization, even up to 24 mo of age. By contrast, some ($F_1 \times$ SWR) and F_2 mice that expressed low titers of virus developed autoimmune disease. Furthermore, a proportion of virus-negative mice produced autoantibodies and were found to have typical immune deposit nephritis. No viral antigens could be detected in the renal lesions of such virus-negative animals. By contrast with the dissociation between expression of virus and occurrence of nephritis, the presence of antibodies to DNA correlated with the development of renal lesions. We

conclude that the genes that determine the expression of infectious xenotropic virus in NZB mice segregate independently from those that are involved in the autoimmune disease of these animals.

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