Ability of the *xid* Gene to Prevent Autoimmunity in (NZB × NZW)F_1 Mice during the Course of their Natural History, after Polyclonal Stimulation, or following Immunization with DNA

BONNIE J. STEINBERG, PATRICIA A. SMATHERS, KIRSTEN FREDERIKSEN, and ALFRED D. STEINBERG, *Cellular Immunology Section, Arthritis and Rheumatism Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205*

ABSTRACT F_1 hybrid offspring of New Zealand Black mothers and New Zealand White fathers [(NZB × NZW)F_1] female mice develop antibodies to single-stranded (ss) and native DNA, immune complex glomerulonephritis, massive proteinuria, and premature death with renal failure. By a series of matings, congenic (NZB × NZW)F_1 · *xid/xid* mice were prepared. These mice were different from (NZB × NZW)F_1 mice in having the X chromosome-linked immune deficiency gene, *xid*, in homozygous form. Such congenic (NZB × NZW)F_1 · *xid/xid* females failed to develop antibodies to single-stranded or native DNA. They also failed to develop fatal renal disease as measured by proteinuria, glomerular histology, glomerular immunofluorescence, and survival.

To control for unknown genetic factors, studies were performed with littermates that were derived by mating NZB · *xid*/+ females with NZW · *xid*/Y males such that the resulting offspring were either (NZB × NZW)F_1 · *xid/xid* (and therefore "defective") or (NZB × NZW)F_1 · *xid*/+ [phenotypically like (NZB × NZW)F_1]. In these and in additional studies, mice were housed in the same cages and identified by ear tagging so as to avoid possible environmental variations from cage to cage. In these studies, *xid/xid* mice failed to develop the characteristic signs of autoimmunity, whereas the controls did. Similar results were also obtained with (NZW × NZB)F_1 *xid/xid* mice compared with (NZW × NZB)F_1 *xid*/+ mice.

The effect of *xid/xid* upon (NZB × NZW)F_1 mice was further investigated by assessing responses to immunization and polyclonal B cell activation in vivo. The *xid/xid* mice failed to produce anti-ssDNA following immunization with ssDNA complexed to a protein carrier in fluid form or even emulsified in adjuvant. Finally, the *xid/xid* mice failed to produce antiDNA in response to multiple injections of the polyclonal activator, bacterial lipopolysaccharide (LPS), or the polyclonal activator, polyribose inosinic acid · polyribose cytidylic acid. However, the *xid/xid* mice were neither generally hyporesponsive nor unable to recognize LPS because they made normal antibody responses following immunization with LPS to which multiple trinitrophenyl groups were chemically attached.

We conclude from these studies that *xid/xid*, which is known to cause the deletion of a B cell subset, has a profound affect upon (NZB × NZW)F_1 mice, rendering them insusceptible to the naturally occurring autoimmune disease characteristic of (NZB × NZW)F_1 mice, and preventing them from producing antibodies to DNA despite purposeful immunization and polyclonal B cell activation. These results force a reevaluation of previous concepts regarding the mechanisms by which *xid/xid* might interfere with the development of autoimmunity, and a consideration of therapeutic implications.

Received for publication 3 March 1982 and in revised form 18 May 1982.

INTRODUCTION

The effect of the X chromosome-linked recessive immune deficiency gene, *xid*,[1] upon the immune system has been under intensive study for the past 10 yr with regard to both basic immunology (1–13) and effects

[1] *Abbreviations used in this paper*: anti-μ, antibodies specific for the heavy chain portion of mouse IgM; CFA, complete Freund's adjuvant; LPS, bacterial lipopolysaccharide

upon the development of autoimmunity (14–25). It has become clear that this gene in the homozygous or hemizygous state is capable of causing the deletion of a subset of B cells, which represents approximately half of the B cells of mice not bearing the gene (1–13). Moreover, such B cells are largely responsible for immune responses to certain antigens, especially polysaccharide antigens with repeating subunits (1–10). As a result, it might be anticipated that the *xid* gene might prevent the production of antibodies to DNA.

Previous studies from our laboratory and others have attempted to assess the effect of the *xid* gene upon the natural history of New Zealand Black mice (NZB) and MRL/*lpr*/*lpr* mice. The presence of *xid* in homozygous or hemizygous form causes a marked reduction in all signs of autoimmunity in NZB mice (17–21, 23–25). However, this disease prevention is overcome by immunization or polyclonal immune stimulation (16, 19, 23–25). In contrast to the effect of *xid* upon NZB disease, MRL/*lpr*/*lpr* mice are much less markedly affected (18–22). We previously have suggested that because NZB mice have markedly hyperactive B cells that produce large amounts of immunoglobulin M (IgM) (14, 17, 20, 25–29), *xid* would be expected to markedly interfere with the disease of such mice. In contrast, MRL/*lpr*/*lpr* mice, which produce predominantly IgG antibodies, would, therefore, be much less markedly affected by *xid* (18–21). Following this line of reasoning, we anticipated that (NZB × NZW)F_1 mice, which produce large amounts of IgG antiDNA (16, 21, 30, 31), would be little altered by *xid*. We report, herein, results contrary to our expectations and discuss possible implications of those results.

METHODS

Mice. The mice used in this study were bred in our animal facility from stock colonies maintained at the National Institutes of Health (NIH). These colonies are maintained by the Small Animal Section, Division of Research Services, under the supervision of Dr. Carl Hansen. The heterozygous female NZB·*xid*/+ mice were bred by mating an NZB·*xid*/*xid* female with an NZB male, or, alternatively by mating an NZB female with an NZB·*xid*/Y male. In all these designations, the sex chromosome gene assignments are shown. The NZB·*xid*/+ females were then bred with an NZW·*xid*/Y male. The offspring of these matings were as follows: (NZB × NZW)F_1·*xid*/*xid* females, (NZB × NZW)F_1*xid*/+ females, (NZB × NZW)F_1·*xid*/Y males, and (NZB × NZW)F_1·+/Y males. The congenic NZB mice used in this study were 99% inbred. As a result, the NZB·*xid*/+ females were >99% NZB. The NZW·*xid*/Y male used was slightly >97% inbred. As a result, the (NZB × NZW)F_1·*xid*/*xid* and control (NZB × NZW)F_1·*xid*/+ females were >98% (NZB × NZW)F_1. The exact extent of inbreeding was of some concern to us. As a result, we carried out a detailed comparison of littermate (NZB × NZW)F_1·*xid*/*xid* females and (NZB × NZW)F_1·*xid*/+ females. These mice, with an identical degree of inbreeding, differed primarily in the presence or absence of *xid* in homozygous form.

In a second series of studies, we performed the reciprocal cross. NZW·*xid*/+ females (99.2% inbred) were mated with NZB·*xid*/Y males (99.6% inbred) to produce 124 female offspring, 65 (NZW × NZB)F_1·*xid*/*xid* and 59 (NZW × NZB)F_1·*xid*/+. These mice were studied in a manner similar to the (NZB × NZW)F_1·*xid*/*xid* and *xid*/+ mice, only with a lag period of ~4–6 mo.

Distinction between xid/xid and xid/+ mice. Assignments of mice to the *xid*/*xid* or *xid*/+ genotype were made on the basis of serum IgM concentrations at ~8 wk of age as described (25). Briefly, serum IgM concentrations were determined by radial immunodiffusion using plates impregnated with antibodies specific for the heavy chain portion of mouse IgM (anti-μ). Those mice with IgM concentrations <0.2 mg/ml were considered to be *xid*/*xid*. Those with IgM concentrations >0.40 were considered *xid*/+. No mice had equivocal assignments. In addition, a separate group was tested for responsiveness to Ficoll to which multiple trinitrophenyl groups where chemically attached (TNP-Ficoll). There was a 100% correspondence between responsiveness to TNP-Ficoll and an IgM concentration of at least 0.4 mg/ml.

Antigens and immunizations. Calf thymus DNA was purchased from Worthington Biochemical Co., Freehold, NJ, dissolved in phosphate-buffered saline, pH 7.2, and rendered single stranded by heating to 100°C for 10 min followed by rapid cooling with stirring in an ice-water bath. The single-stranded DNA (ssDNA) was then complexed with methylated bovine gamma globulin, MBGG, (dissolved in distilled water at a concentration of 10 mg/ml) such that the final mixture contained 100 μg ssDNA complexed with 50 μg of MBGG. The MBGG was prepared by the method of Sueoka and Cheng (32) with BGG purchased from Mann Research Laboratories, New York. Mice were immunized intraperitoneally with 100 μg ssDNA-MBGG (the weight refers only to ssDNA) in buffer in a volume of 0.2 ml. A portion of the ssDNA-MBGG, before dilution, was emulsified with an equal volume of complete Freund's adjuvant (CFA) (H37Ra, Difco Laboratories, Detroit, MI) and injected intraperitoneally in a volume of 0.2 ml such that each animal received 100 μg ssDNA. Mice were bled on days 7, 14, 18, 22, and 29 following immunization.

Bleeding and preparation of sera. Mice were bled by orbital sinus puncture. The samples were allowed to clot at room temperature for ~2 h after which sera were removed

(endotoxin); Lyb 3$^-$, 5$^-$, those B cells that are not reactive with the Lyb 3 and Lyb 5 antisera, and are present in *xid*/*xid* and *xid*/Y mice; Lyb 3$^+$ and Lyb 5$^+$, B cells reactive with antisera which define a B cell subset, the B cell subset defined by Lyb 3 is largely overlapping with that defined by Lyb 5, these cells are not present in *xid*/*xid* or *xid*/Y mice; MBGG, methylated bovine gamma globulin; (NZB × NZW)F_1 mice, F_1 hybrid offspring of New Zealand Black mothers and New Zealand White fathers; poly rI·rC, polyribose inosinic acid·polyribose cytidylic acid; ssDNA-MBGG, the electrostatic complex of single-stranded DNA and methylated bovine gamma globulin; TI-2 antigens, type 2 thymic independent antigens, antigens to which *xid*/*xid* and *xid*/Y mice have impaired responses; TNP-, trinitrophenyl-; TNP-Ficoll, Ficoll to which multiple TNP groups were chemically attached; TNP-LPS, LPS to which multiple TNP groups were chemically attached; *xid*, X-chromosome linked immune deficiency gene which originated in CBA/N mice; *xid*/*xid*, females homozygous for *xid*; *xid*/+, females heterozygous for *xid*; *xid*/Y, male hemizygous for *xid*.

from the clots, centrifuged to remove cellular debris, and stored at −20°C until assayed.

Assay of antibodies to ssDNA and native DNA. A modified ammonium sulfate (Farr) assay was used throughout (16, 33). Briefly, 25 μl of serum was mixed with 25 ng ^{14}C-labeled human KB cell DNA (New England Nuclear, Boston, MA) (80,000 dpm/μg) in borate-buffered saline, pH 8.0, in a volume of 75 μl. The total volume of the reaction mixture was 100 μl. The tubes were incubated at 37°C for 30 min and overnight (~18 h) at 4°C. An equal volume, 100 μl, of 70% saturated $(NH_4)_2SO_4$ was added at 0°C for 1 h and the tubes were centrifuged at 2,000 *g* for 20 min. A 100-μl aliquot of the supernatant was then removed and counted in a liquid scintillation counter. The percentage of the ^{14}C-DNA originally added that was bound by the antiDNA in the test serum was calculated.

The ligand for the native DNA assay was almost completely double stranded in that <5% bound to a rabbit antibody to ssDNA. This antibody bound 100% of the ^{14}C-ssDNA (prepared by heating the ^{14}C-DNA to 100°C for 10 min and rapid cooling). This latter material was the ligand used for assay of antibodies to ssDNA. The independence of the ssDNA and native DNA assays was further demonstrated by the ability of individual sera to bind >80% of one ligand and <20% of the other ligand and vice versa. In every assay, known positive and negative controls were run with the test samples and gave expected results.

IgG and IgM anti-DNA antibodies were measured by a solid phase radioimmunoassay as described (21).

Clinical studies. The protein concentration of freshly voided urine was determined for each mouse by assessing the color change of calibrated tetrabromphenol paper and the results of two consecutive determinations averaged. In preliminary studies, we demonstrated that this method correlated very well with protein analysis of urine collected in metabolic cages. At least five mice at each of several ages were killed for analysis of renal histology. Kidneys were fixed in 10% neutral buffered formalin and prepared for routine light microscopy according to standard techniques. Histological evaluation was carried out on thin sections stained with hematoxylin-eosin. 25 glomeruli of each specimen were graded semiquantitatively by a "blinded" observer as described (34). Immunofluorescence using fluorescein-tagged rabbit antimouse Ig was graded as described (35). Mice and spleens were weighed on a top-loading scale sensitive to increments of 5 mg. The results of individual group measurements were averaged and compared using Student's *t* test. Survival studies were compared by the Kolmogorov-Smirnov test and the test of medians.

Antibody responses to TNP-LPS and TNP-Ficoll. TNP_9-LPS was the kind gift of Dr. John Cowdery. TNP_{35}-Ficoll, prepared by the method of Inman (36), was purchased from Biosearch, San Raphael, CA. Mice were injected intraperitoneally with 2 μg TNP-LPS or 10 μg TNP-Ficoll in buffer and bled 5 d later. Serum antibody was measured in 96 well microtiter plates (Cooke Co., Alexandria, VA) by passive hemagglutination using TNP-coupled sheep erythrocytes (37) starting with 50 μl of a 1:20 dilution of serum. Known positives and negatives were included in the assays and gave expected results.

RESULTS

Preliminary comparisons between (NZB × NZW)F$_1$ and (NZB × NZW)F$_1$ · xid/xid mice. Initially, 50 female (NZB × NZW)F$_1$ and 50 female congenic *xid/xid* mice were compared with regard to the development of proteinuria, antibodies to DNA, and survival (Table I). The (NZB × NZW)F$_1$ · *xid/xid* mice failed to manifest major abnormalities of any of these measures. This was contrary to our expectations. Because (NZB × NZW)F$_1$ mice make substantial IgG antiDNA responses, we expected *xid* to offer very little protection against disease in these mice. In this study, the mice were not housed in the same cages, their weights differed somewhat, and we feared that the degree of inbreeding of congenic mice might have been inadequate. We, therefore, carried out a large experiment in which the controls were designed to be littermates of the congenic mice with equal inbreeding and environments.

Production of littermate female (NZB × NZW)F$_1$ · xid/xid and (NZB × NZW)F$_1$ · xid/+ mice. Female NZB mice were prepared such that they were heterozygous for *xid*. These NZB · *xid*/+ females were mated with an NZW · *xid*/Y male. The female offspring, theoretically, would have a 50% chance of being (NZB × NZW)F$_1$ · *xid*/+ and a 50% chance of being (NZB × NZW)F$_1$ · *xid/xid*. The results of these matings were very close to the expected: of the first 126 females produced, 62 were *xid*/+ and 64 were *xid/xid*. These mice were then kept as littermates in the same cages. Individual mice were distinguished by ear tagging. Because *xid*/+ females of other crosses do not bear the

TABLE I
Preliminary Comparison between (NZB × NZW)F$_1$ Females and Congenic (NZB × NZW)F$_1$ · xid/xid Females

Measure	(NZB × NZW)F$_1$*	(NZB × NZW)F$_1$ · *xid/xid**
Antinative DNA at 7 mo	65±6‡	6±2
Cumulative proteinuria at 9 mo (% ≥100 mg/dl)	85	0
Survival to 10 mo, %	54	100
Survival to 12 mo, %	16	100
Survival to 15 mo, %	0	100

* There were 50 mice in each group. The mice were housed in the same animal room; however, they were not housed in the same cages. Moreover, the *xid/xid* mice were housed with fewer mice per cage and seemed to be heavier than the non-*xid* bearing mice by inspection. A subgroup of 10 *xid/xid* mice were weighed at 1 yr of age; they were, indeed, an average of 9–10 g heavier than the remaining (NZB × NZW)F$_1$ mice and younger (NZB × NZW)F$_1$ females in the same room. All of the potential problems of these comparisons were eliminated by subsequent studies in which mice were housed in the same cages and identified by individual ear tagging.

‡ Solid phase radioimmunoassay indicated that between 46 and 94% of the antibody of individual mice was of the IgG class with a mean of 76%.

abnormal phenotype of *xid/xid* females or *xid*/Y males (1–13, 21, 22), we felt that they would serve as relatively good controls for the *xid/xid* females. The mice were serially studied for serum antiDNA, the development of proteinuria, and survival. An additional 137 female mice were produced. Some of these were randomly selected and analyzed for glomerular histology and immunofluorescence as indicated. The rest were periodically studied for proteinuria, antiDNA, response to immunization, and/or survival.

Spontaneous production of antiDNA by (NZB × NZW)F_1 · xid/xid mice. Antibodies to ssDNA were detected in the control *xid/+* mice when tested at 4 mo of age. The amount rose until 6 mo of age, after which there was a slight decline. In contrast, the *xid/xid* mice failed to make substantial amounts of anti-ssDNA (Fig. 1). Antibodies to native DNA were not detected in large quantity in the *xid/+* mice until after 5 mo of age (Fig. 2). However, large amounts of antinative DNA were made at all times thereafter. In individual *xid/+* mice, the amount of ssDNA and/or native DNA antibodies often declined a few weeks before dying with marked proteinuria (data not shown). In contrast to the antiDNA produced by the *xid/+* females, the littermate *xid/xid* females failed to produce anti-native DNA (Fig. 2). Data similar to those shown for the (NZB × NZW)F_1 mice were also obtained for (NZW × NZB)F_1 *xid/xid* and (NZW × NZB)F_1 *xid/+* mice (not shown).

Development of proteinuria in congenic xid/+ and xid/xid (NZB × NZW)F_1 mice. Average proteinuria increased steadily between 6 and 9 mo of age in the *xid/+* females, but did not in the *xid/xid* female littermates (Fig. 3). This was also true of (NZW × NZB)F_1 littermates (data not shown).

Weights of the mice. In view of the effect of the weight and diet upon the natural history of (NZB × NZW)F_1 females (38–43), we checked to be certain that differences in natural history between *xid/+* and *xid/xid* mice might be due to differences in weight. They could not be due to differences in diet because the mice were housed in the same cages. We found that the mice did not differ between 2 and 7 mo of age. We did not measure weight beyond that age because effects of renal failure might be manifested in later weight determinations. The weights at 6 mo of age are shown in Table II. Also shown are spleen weights of these mice. Spleen enlargement was found in *xid/+* but not *xid/xid* mice (Table II).

Glomerular pathology in (NZB × NZW)F_1 xid/xid mice. Each kidney was graded, and the grades of the mice were averaged to provide a glomerular severity score as described (34). The *xid/xid* females had much less severe renal histological disease (Fig. 4) and much less immunoglobulin in the kidneys as determined by immunofluorescence (Fig. 5) than did the phenotypically normal *xid/+* mice.

Effect of xid upon the survival of (NZB × NZW)F_1

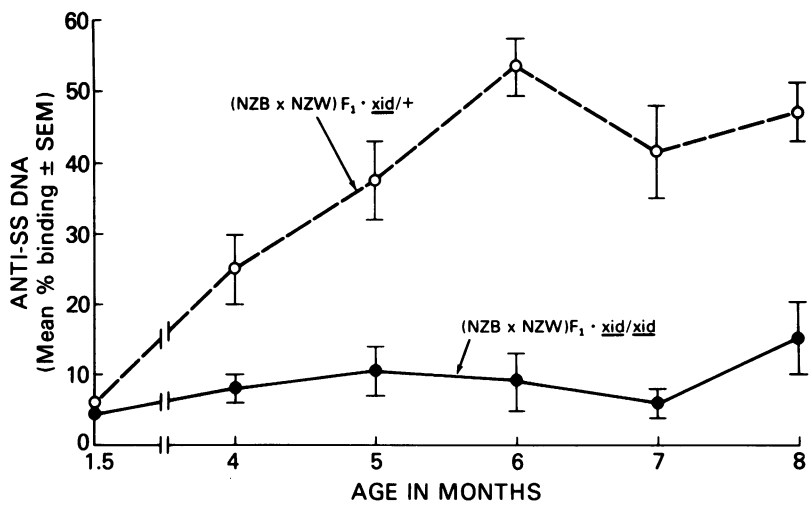

FIGURE 1 Effect of *xid/xid* upon spontaneous anti-ssDNA. Control (NZB × NZW)F_1 · *xid/+* mice (open circles, dashed line) developed anti-ssDNA by 4 mo of age. There was a progressive increase until 6 mo of age. Thereafter, the anti-ssDNA varied, but remained high. In individual mice, anti-ssDNA fell just before death. In contrast, the (NZB × NZW) · *xid/xid* mice made very little anti-ssDNA. Their values were not elevated at 1 yr of age (data not shown). Although a few *xid/xid* mice made anti-ssDNA, the groups were significantly different, $P < 0.01$, at all ages starting at 4 mo. A total of 64 *xid/xid* mice were studied at all points. A total of 62 *xid/+* mice were studied through 6 mo of age; 59 *xid/+* mice were studied at 7 mo and 50 *xid/+* mice at 8 mo of age.

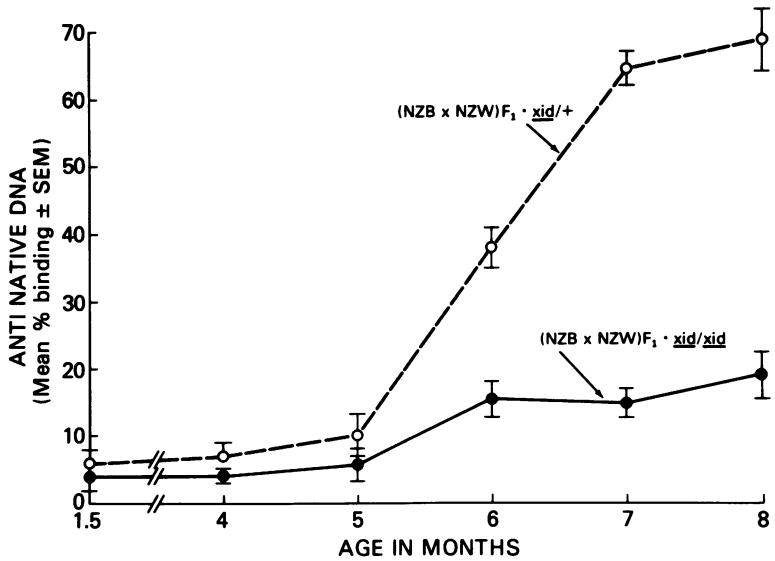

FIGURE 2 Effect of *xid/xid* upon spontaneous production of antibodies to native DNA. Antibodies to native DNA did not appear until 6 mo of age in the control (NZB × NZW)F_1 · *xid/+* mice (open circles). This was substantially later than the appearance of anti-ssDNA in these mice (compare with Fig. 1). The antibodies to native DNA increased in amount until 7–8 mo of age. Although a few *xid/xid* mice made antiDNA, the (NZB × NZW)F_1 · *xid/xid* group produced significantly less than did the *xid/+* group, $P < 0.01$, at 6, 7, and 8 mo of age. The same mice studied in Fig. 1 were also studied in Fig. 2.

mice. The failure to develop antibodies to ssDNA or native DNA, the failure to develop renal immune complex deposits in great quantity of severe renal histological abnormalities, and the failure to develop massive proteinuria all suggested strongly that the (NZB × NZW)F_1 · *xid/xid* mice were substantially protected from the usual disease of (NZB × NZW)F_1 mice. It was, therefore, no longer surprising to us that a formal survival study indicated a marked prolongation of life in (NZB × NZW)F_1 mice bearing the *xid* defect (Fig. 6). Similarly (NZW × NZB)F_1 *xid/xid* mice had prolonged survival (Fig. 6).

Failure to immunize (NZB × NZW)F_1 · xid/xid mice to ssDNA-MBGG. All of the above findings suggested that the (NZB × NZW)F_1 · *xid/xid* mice might be incapable of producing large amounts of antibody to ssDNA even if immunized. 2-mo-old females were injected with 100 μg ssDNA complexed with MBGG and bled 7, 14, 18, 22, and 29 d later. At no time did the *xid/xid* females produce anti-ssDNA (Fig. 7). In contrast, nondefective control mice made a good response. Additional mice were immunized with 100 μg ssDNA complexed with MBGG and emulsified in CFA. Again, the (NZB × NZW)F_1 · *xid/xid* mice failed to produce antibodies to this challenge immunization whereas the non*xid/xid* control (NZB × NZW)F_1 mice responded with the production of large amounts of antibody (Fig.

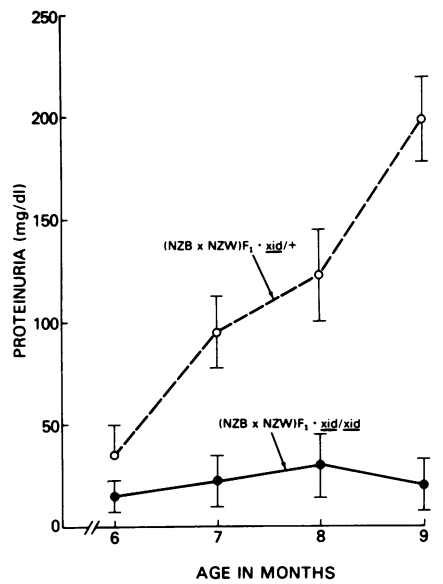

FIGURE 3 Effect of *xid/xid* upon proteinuria in (NZB × NZW)F_1 mice. Proteinuria increased steadily between 6 and 9 mo of age in the control (NZB × NZW)F_1 · *xid/+* mice. In contrast, the *xid/xid* littermates failed to demonstrate proteinuria. The differences between the groups were significant, $P < 0.01$, at 7, 8, and 9 mo of age. A total of 97 *xid/+* and 104 *xid/xid* mice were studied starting at 6 mo of age.

TABLE II

Body Weight of (NZB × NZW)F_1 · xid/xid and Control xid/+ Littermates at 6 mo of Age and Spleen Weights at 7 mo of Age

Mice	Body weight	Spleen weight
	g	*mg*
xid/xid	32.9±1.6	82.4±5.7
xid/+	32.5±1.3*	193.9±26.5‡

* Not significantly different from the *xid/xid* group, $P > 0.5$.
‡ Significantly greater than the *xid/xid* group, $P < 0.01$.

8). Immunization of 20 (NZW × NZB)F_1 · *xid/xid* and 12 (NZW × NZB)F_1 *xid/+* mice with ssDNA-MBGG in CFA resulted, 4 wk later, in high anti-ssDNA (68.2±4.1) in the *xid/+*, but not *xid/xid* mice (3.9±2.4).

Failure to stimulate (NZB × NZW)F_1 · xid/xid mice to produce anti-ssDNA with either LPS or poly rI · rC. The failure of (NZB × NZW)F_1 · *xid/xid* mice to produce much antiDNA, either during the course of their natural history or after immunization with ssDNA complexed to a protein carrier and emulsified with CFA, raised a question as to whether standard polyclonal immune stimulation might not work. Groups of (NZB × NZW)F_1 · *xid/xid* mice injected with either LPS or poly rI · rC failed to make anti-ssDNA, whereas their littermates who were *xid/+* produced large amounts (Fig. 9). It is, of course, possible that more prolonged stimulation might induce anti-ssDNA; however, the stimulation provided was quite sufficient to induce large amounts of antibody in the littermate controls.

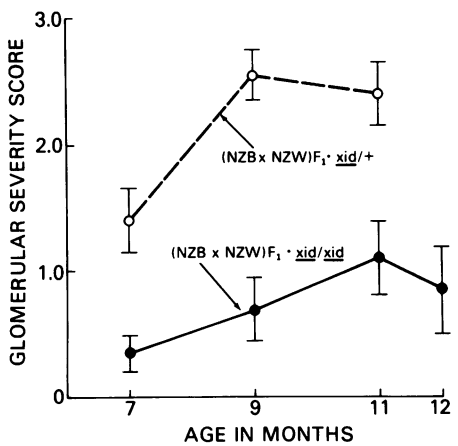

FIGURE 4 Effect of *xid/xid* upon glomerulonephritis in (NZB × NZW)F_1 females. Control (NZB × NZW)F_1 · *xid/+* mice had mild glomerular histological abnormalities at 7 mo of age, but marked glomerulonephritis at 9 and 11 mo of age. The *xid/xid* mice demonstrated only minimal glomerular changes at all ages studied. This was significantly less than that of *xid/+* mice, $P < 0.05$ at 7 and 11 mo and $P < 0.01$ at 9 mo. 8 *xid/+* and 8 *xid/xid* mice were studied at each time point except the last at which time 18 *xid/xid* mice were studied.

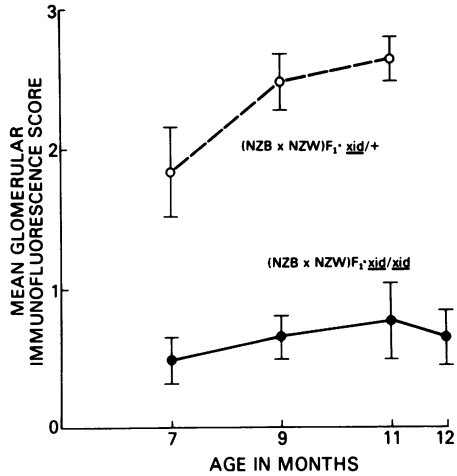

FIGURE 5 Effect of *xid/xid* upon glomerular immunoglobulin deposition. Glomerular immunoglobulin deposition was measured by immunofluorescence using an antimouse immunoglobulin reagent. Substantial fluorescence was found by 7 mo of age in the control (NZB × NZW)F_1 · *xid/+* mice. In contrast, very little immunofluorescent staining was observed in the (NZB × NZW)F_1 · *xid/xid* mice at any age. The *xid/xid* group was significantly different from the *xid/+* group at 7 mo, $P < 0.05$ and at 9 and 11 mo, $P < 0.01$. The mice studied in Fig. 4 were also studied here.

Immunization with TNP-LPS and TNP-Ficoll. The failure of (NZB × NZW)F_1 · *xid/xid* mice to spontaneously produce antiDNA or to be polyclonally or specifically stimulated to make antiDNA forced us to determine that they could, in fact, respond to immunization. Mice were injected intraperitoneally with TNP_9-LPS or TNP_{-35}-Ficoll and bled 5 d later. Both *xid/xid* and *xid/+* mice responded to TNP-LPS (Table III). Thus, *xid/xid* mice were capable of a normal immune response. The *xid/+* mice responded to TNP-Ficoll; *xid/xid* mice, consistent with the known effects of *xid* in homozygous or hemizygous form, failed to respond to TNP-Ficoll (Table III).

DISCUSSION

Although NZB mice were first discovered to spontaneously develop an autoimmune syndrome and new strains of autoimmune mice have been developed, the (NZB × NZW)F_1 model has been regarded as most closely resembling the human condition, systemic lupus erythematosus (SLE). As a result, we were most anxious to determine the effect of the *xid* gene upon

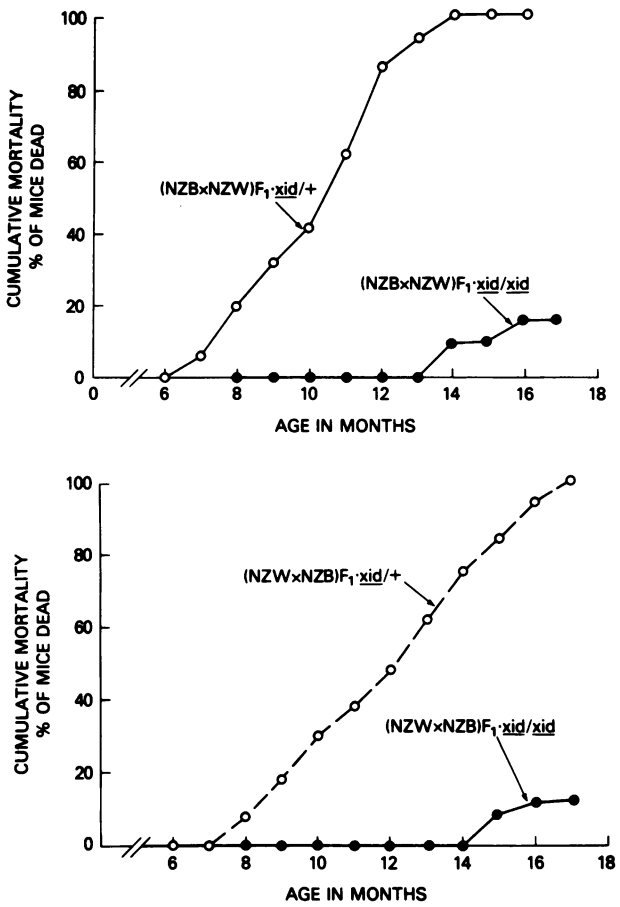

FIGURE 6 Effect of *xid/xid* upon survival in (NZB × NZW)F_1 and (NZW × NZB)F_1 females. Consistent with the reduced antiDNA production, reduced proteinuria, reduced renal immunoglobulin deposition, and reduced renal histological abnormalities, survival in the (NZB × NZW)F_1 · *xid/xid* mice was markedly prolonged in comparison with control mice, and, in fact, was similar to that of some strains of nonautoimmune females housed in our animal facilities (data not shown). Similar results were observed in (NZW × NZB)F_1 mice. The long-lived *xid/xid* mice showed no evidence of lymphoid malignancies, but some individuals did tend to gain weight during year 2 of life (which was largely fat) resulting in as much as 15 or 20 extra grams (47–60 g, total) body weight. The differences in survival curves between *xid/xid* and *xid/+* mice of each cross were significantly different, $P < 0.001$, by the Kolmogorov-Smirnov test and the test of medians.

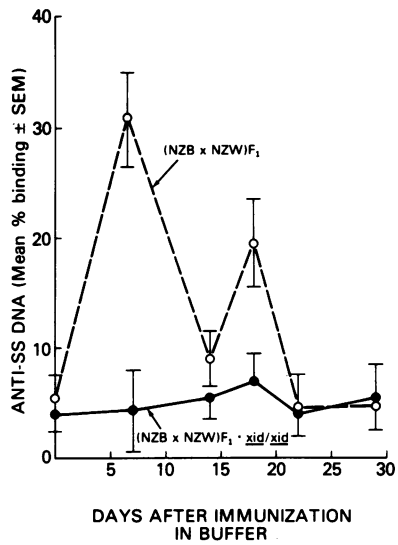

FIGURE 7 Effect of *xid/xid* upon immunization to ssDNA-MBGG in buffer. Control mice responded to immunization with 100 mg ssDNA complexed to MBGG with a 7-d antibody response and a second smaller peak at 18 d. In contrast, the *xid/xid* mice failed to produce anti-ssDNA in response to immunization. The groups differed at day 7, $P < 0.01$ and day 18, $P < 0.05$. There were 10 mice in the *xid/xid* group and 10 mice in the control group.

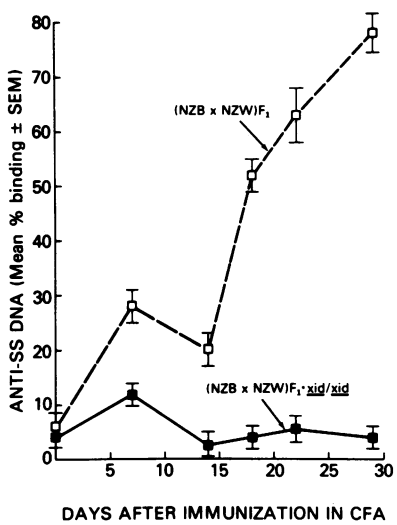

FIGURE 8 Failure of *xid/xid* mice to respond to immunization with ssDNA-MBGG in adjuvant. In a further attempt to immunize (NZB × NZW)F_1 · *xid/xid* mice to ssDNA, another group of mice was studied in parallel with those shown in Fig. 7. 16 *xid/xid* mice were injected with ssDNA-MBGG emulsified in CFA. Despite this very strong immunization, the mice failed to produce antibodies to ssDNA. The positive controls (12 mice) made very large amounts of antibody which reached an average level of 78% binding at 1 mo. The groups differed at all points, $P < 0.01$.

the natural history of (NZB × NZW)F_1 mice. We did not expect a dramatic effect because previous work had suggested that *xid* interfered primarily with IgM antibodies and IgM autoantibodies, and it is known that (NZB × NZW)F_1 mice produce large amounts of IgG and IgG autoantibodies (16, 18–21, 30, 31). In our study we found that the great majority of the antiDNA produced by (NZB × NZW)F_1 mice was IgG. We were,

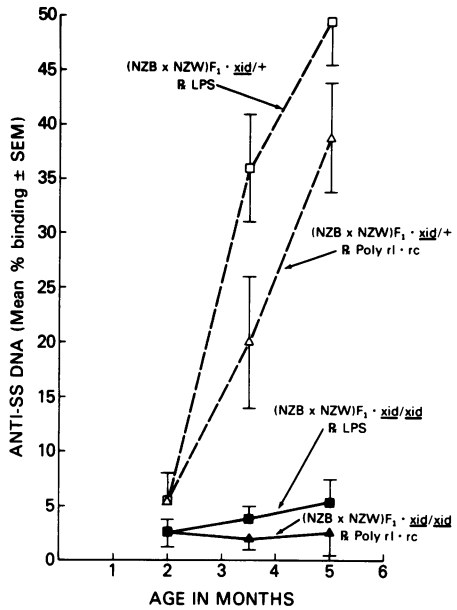

FIGURE 9 Failure of (NZB × NZW)F_1 · *xid/xid* mice to produce anti-ssDNA despite multiple injections of polyclonal B cell activators. Mice, 12–18 per group, were injected with either LPS, 20 μg twice a week or poly rI · rC, 100 μg twice a week from 2 mo of age. The *xid/xid* mice failed to produce anti-ssDNA with either LPS (closed squares) or poly rI · rC (closed triangles) stimulation. In contrast, the control mice produced large amounts of anti-ssDNA with either LPS (open squares) or poly rI · rC (open triangles). The *xid/xid* mice differed from the corresponding *xid/+* mice at 3.5 and 5 mo of age, $P < 0.01$.

therefore, very surprised by the initial series of studies that indicated that *xid* in homozygous form virtually abolished disease in most (NZB × NZW)F_1 mice. Those studies showed that antiDNA, proteinuria, and survival were markedly normalized by the *xid* gene.

In the initial studies, we compared (NZB × NZW)F_1 · *xid/xid* mice with (NZB × NZW)F_1 · +/+ mice. Because the *xid/xid* mice were not completely inbred, it remained possible that their failure to develop disease resulted not from *xid/xid*, but from a gene missing in either the NZB · *xid/xid* female parent or in the NZW · *xid*/Y male parent. This possibility was eliminated by producing female (NZB × NZW)F_1 littermates, half of which were *xid/xid* and half *xid/+*. These mice were housed together to avoid possible dietary (38–42) or environmental (43–46) influences on the development of autoimmune features. It turned out that the weights of the *xid/xid* and control *xid/+* mice were similar from 2 to 7 mo of age. As a result, we felt that the test of the effect of *xid/xid* on the natural history of (NZB × NZW)F_1 mice was a good one.

We found that most *xid/xid* mice failed to develop antibodies to either native or ssDNA or to double-stranded RNA. Moreover, the *xid/xid* mice had minimal immune complex deposition in the kidneys, little inflammatory renal disease as measured by light microscopy and proteinuria, and essentially normal survival. Thus, *xid/xid* markedly inhibited all of the cardinal features of the systemic lupus erythematosus-like illness of (NZB × NZW)F_1 females.

Additional studies indicated that *xid/xid* prevented an immune response to immunization with ssDNA complexed to a protein carrier (ssDNA-MBGG). This was true following immunization in fluid form and also following immunization in CFA. Finally, multiple injections of the polyclonal B cell activators, LPS, and poly rI · rC failed to stimulate anti-ssDNA. Thus, *xid/xid* interfered not only with spontaneous production of antiDNA in (NZB × NZW)F_1 mice, but also with induced antiDNA. The failure of (NZB × NZW)F_1 · *xid/xid* mice to produce large amounts of antiDNA in response to LPS and poly rI · rC is especially noteworthy in view of the ease with which NZB, NZW, and (NZB × NZW)F_1 mice can be so induced to produce antiDNA (47, 48). However, the (NZB × NZW)F_1 · *xid/xid* mice did respond to immunization with TNP-LPS; therefore, the mice were not generally hyporesponsive. Rather, they appeared to be specifically and profoundly hyporesponsive to a subset of antigens. This is consistent with the idea that *xid* leads to the deletion of a B cell subset (1–13), which is responsible for the production of antibody to certain type 2 thymic independent antigens, antigens to which *xid/xid* and *xid*/Y mice have impaired responses (TI-2) (1–10).

The results reported herein force a reevaluation of the idea that *xid* might interfere with NZB disease because NZB mice make enormous amounts of IgM (18–21, 23–29). However, it is consistent with the notion that the Lyb 3+, 5+ subset of B cells is responsible for the production of autoantibodies in NZB mice (14–21). The NZB mice, however, are capable of spontaneously breaking through the *xid* effect (24) and of

TABLE III
Response of (NZB × NZW)F_1 · xid/xid or xid/+ Littermates to Immunization with TNP-LPS or TNP-Ficoll

Antigen*	Anti-TNP (Mean titer, $\log_8$, ±SEM)	
	xid/xid	*xid/+*
TNP_9-LPS	7.9±1.3	9.1±1.4
TNP_{35}-Ficoll	0.3±0.2	8.4±1.6

* Mice were immunized with either antigen and bled 5 d later. There were 8–15 mice per group. The titers did not differ significantly except for the *xid/xid* response to TNP-Ficoll.

being stimulated to overcome it (16, 19–21, 23, 25). In the (NZB × NZW)F_1 mice, it appears that the effect of *xid* is even more profound than in NZB mice; in (NZB × NZW)F_1 mice, *xid* profoundly reduces the ability to produce antiDNA. Such observations suggest that all processes of immune responsiveness to DNA—natural immunization, specific exogenous immunization, and polyclonal immune activation—are inhibited. The cellular basis of this defect has not been defined herein. We found that the *xid/xid* mice responded perfectly well to immunization with TNP-LPS. Therefore, we cannot attribute their failure to make antiDNA to nonspecific immune suppression. For similar reasons, the response to TNP-LPS indicates that the *xid/xid* mice could recognize LPS even though they failed to demonstrate the polyclonal antiDNA response seen in the *xid/+* mice.

Genetic studies have suggested that there is a single gene that plays the major role in leading to spontaneous and induced antibodies to DNA in NZB mice (49, 50). Therefore, it could be argued that either *xid* itself, or a closely linked gene controlling antibody responses to nucleic acids, might have led to the observed results, and that the present observations are unrelated to the effects of *xid* upon B cell subsets. However, prior studies have demonstrated that the genes controlling such responses are not on the X chromosome and that any apparent X-linked effect is related to sex hormones (51). Therefore, we believe that it is extremely unlikely that the results obtained herein are related to an X chromosome-linked gene controlling antibody responses to nucleic acids. However, it is possible that a gene on the X chromosome near *xid* could influence the magnitude of such responses. Our studies of (NZW × NZB)F_1 · *xid/xid* mice argue against another gene unless it is very closely linked to *xid*. The parents of those mice each had over 20 backcrosses during which crossover events on the X chromosome could have occurred. In the (NZW × NZB)F_1 mice, *xid/xid* littermates failed to manifest autoimmune disease, whereas *xid/+* littermates did. As our inbreeding continues and more and more crossover events take place on the X chromosome, the continued absence of antinucleic acid antibody responses makes less and less likely an important X linked gene controlling such responses independent of *xid*.

It has been found that the Lyb 3^-5^- B cells, those present in *xid/xid* mice, cannot receive and respond to signals from T cell helper factors (52–54) or from anti-Lyb 3 signals (8). Therefore, if in (NZB × NZW)F_1 mice the immune response to DNA has a very strong requirement for such signals, and they are not possible in (NZB × NZW)F_1 · *xid/xid* mice, it might not be surprising that such mice fail to produce antiDNA antibodies despite immunization or polyclonal activation. Moreover, the inability of the *xid/xid* Lyb 5^- B cells to respond to immunization with DNA may not relate to B cell:T cell interactions so much as to macrophage:B cell interactions since it has been found that Lyb 5^- B-cells have a markedly reduced ability to receive activation signals from macrophages (13) and *xid* mice may have a structural macrophage abnormality (55). Therefore, despite the ability of polyclonal B cell activators, such as LPS and poly rI · rC, to activate both macrophages and T cells, such activation might not be expected readily to lead to stimulation of the Lyb 5^- B cells, which are the B cells present in *xid/xid* mice. Finally, the antiDNA response may be especially difficult for *xid/xid* mice because DNA is regularly released from dying cells. Such released DNA might suppress, rather than enhance, antiDNA responses since experimental administration of a related antigen, polyvinylpyrrolidone, in large amounts inhibited priming and subsequent immune responses (56).

Recent studies by Osughi et al. (57, 58) have demonstrated that old *xid*-bearing New Zealand mice occasionally produce autoantibodies, but that the majority do not. Moreover, those authors have demonstrated that the autoantibody-producing mice do not manifest polyclonal B cell activation and do not have elevated IgM or IgG_3 levels. Those studies indicate that Lyb 5^- cells of *xid*-bearing mice do not, with age, become Lyb 5^+. This finding helps to explain why *xid* bearing New Zealand mice are capable of long life despite the occasional production of autoantibodies and confirms the importance of Lyb 5^+ B cells in the early severe autoimmunity characteristic of (NZB × NZW)F_1 mice.

Regardless of the exact mechanism, the finding that *xid/xid* markedly retards the disease of (NZB × NZW)F_1 mice points to the possibility of understanding an important pathway of antiDNA production. The dissection of such a pathway could lead to an understanding of one or more critical steps in which one might successfully interfere therapeutically. Such an approach might ultimately be applied to humans with systemic lupus erythematosus. An additional possibility is that the *xid* gene could somehow be imparted to non-*xid* bearing hosts. Although this is technically possible in inbred mice with lethal irradiation and stem cell transfers, it is not immediately practical in humans. Nevertheless, advances in genetics may allow the current information to, someday, be of use in humans.

ACKNOWLEDGMENTS

The authors are indebted to Dr. John Cowdery for help with the immunizations with and measurements of antibody responses to TNP-LPS; to Dr. Carl Laskin for critical review of the manuscript; to Dr. Carl Hansen for providing stock

mice for breeding; to Mr. Robert Steinberg for helpful discussion in the initial stages of the work; to Mr. J. Patton Reeves for assistance with IgM and IgG antiDNA determinations; and to Ms. Martha McDonald for devoted secretarial assistance.

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