

Modulation of Renal Disease in Autoimmune NZB/NZW Mice by Immunization with Bacterial DNA

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Summary

Preautoimmune New Zealand Black/White (NZB/NZW) mice immunized with *Escherichia coli* (EC) double stranded (ds) DNA produce antibodies that bind mammalian dsDNA and display specificities similar to spontaneous lupus anti-DNA. Since calf thymus (CT) dsDNA fails to induce these antibodies, these results suggest a special potency of foreign DNA in inducing serological manifestations of lupus in a susceptible host. To assess the effects of DNA immunization on clinical manifestations in NZB/NZW mice, we measured renal disease and survival of mice immunized with either (a) EC dsDNA as complexes with methylated bovine serum albumin (mBSA) in adjuvant; (b) CT dsDNA with mBSA in adjuvant; (c) mBSA alone in adjuvant; or (d) unimmunized. After immunization with EC dsDNA, NZB/NZW mice developed significant levels of anti-dsDNA antibodies. Nevertheless, these mice had less proteinuria, nitrate/nitrite excretion, and glomerular pathology than mice immunized with either mBSA alone, CT dsDNA/mBSA complexes, or unimmunized mice. Survival of the EC dsDNA immunized mice was significantly increased compared with the other mice. Furthermore, immunization of mice after the onset of anti-DNA production and proteinuria stabilized nephritis and prolonged survival. The improvement in renal disease occurred despite the expression of autoantibodies that bound mammalian dsDNA as well as glomerular antigens. These results suggest that bacterial DNA has immunological properties that attenuate murine lupus despite the induction of pathogenic antibodies.

SLE is a prototypic autoimmune disease characterized by the production of antibodies to DNA (anti-DNA, 1–3). These antibodies are markers for diagnosis and prognosis and, moreover, are closely linked to immunopathogenesis (4–6). A role of anti-DNA in lupus renal disease is established by its concentration within the glomerulus as well as the fluctuation of its levels with disease activity (4–6). The basis for renal localization of anti-DNA is not yet certain, however, with deposition of circulating immune complexes, in situ immune complex formation, and direct binding to cross-reactive glomerular antigens, all possible mechanisms (7, 8).

Although polyclonal B cell activation occurs prominently in SLE and can stimulate autoantibody production, anti-DNA expression appears to be the result of antigen drive (9). As demonstrated by the molecular analysis of monoclonal products from lupus mice, anti-DNA show clonal expansion as well as somatic mutation (9, 10). These

mutations are consistent with DNA as the in vivo selecting antigen since they enhance DNA binding (9, 10). Because mammalian DNA is poorly immunogenic in normal animals, this analysis further suggests that lupus involves a unique responsiveness to DNA in susceptible individuals or stimulation by a form of DNA that is much more potent than the preparations used for experimental immunization (11–13).

To gain further insight into the mechanisms of DNA antigen drive, our laboratories have studied the immune response to bacterial DNA (14). In contrast to mammalian DNA, bacterial DNA is immunologically active and can induce polyclonal B cell activation as well as cytokine production in the mouse (15, 16). These immunological effects result from sequence arrays that differ between bacterial and mammalian DNA (17–19). Although the immunological effects of bacterial DNA have been studied most extensively

in murine systems, bacterial DNA can also stimulate natural killer cell function and antibody production in humans (20, 21).

In previous studies (14, 22), we showed that bacterial DNA is highly immunogenic in normal mice under conditions in which mammalian DNA is inactive. Thus, BALB/c mice immunized with *Escherichia coli* (EC)¹ double stranded (ds) DNA as complexes with methylated bovine serum albumin (mBSA) produce antibodies that bind to EC, but not mammalian, dsDNA (14). Similarly, single stranded (ss) EC DNA can induce antibodies specific for the immunizing DNA, although this DNA form also elicits cross-reactive anti-ssDNA that have autoantibody activity (22). In contrast to results with normal mice, immunization of pre-autoimmune lupus-prone New Zealand Black/White (NZB/NZW) mice with EC dsDNA elicits true autoantibodies that bind mammalian dsDNA as well as the immunizing DNA (23). NZB/NZW mice, however, do not respond to calf thymus (CT) dsDNA immunization, indicating the unique immunogenicity of foreign DNA in mice predisposed to autoimmunity (23).

Although they lack antibodies to mammalian dsDNA, the serological hallmark of SLE, BALB/c mice immunized with EC DNA nevertheless develop mild immune complex glomerulonephritis (24). This lesion reflects the action of either antibodies to bacterial DNA or cross-reactive antibodies to mammalian ssDNA which arise during immunization. Because bacterial DNA immunization elicits anti-dsDNA autoantibodies in NZB/NZW mice, we predicted that this intervention would intensify the course of their autoimmune disease (23). As reported herein, we have studied the effects of bacterial DNA on murine lupus and found, unexpectedly, that immunization with EC dsDNA prolongs survival and reduces glomerulonephritis in NZB/NZW mice despite induction of anti-DNA. These findings thus establish a novel action of bacterial DNA and suggest that microbial products may retard specific manifestations of autoimmunity.

Materials and Methods

Mice. Female NZB/NZW mice were obtained at 6 wk of age from The Jackson Laboratory (Bar Harbor, ME) and maintained under standard conditions in the animal facility of Durham VA Medical Center.

DNA. EC and CT DNA were purchased from Sigma Chemical Co. (St. Louis, MO) and further purified by phenol extraction. For these experiments, dsDNA was obtained by digestion of the DNA with S1 nuclease (Sigma Chemical Co.). DNA concentration was determined by OD 260 absorbance whereas purity of DNA was determined by the OD 260/280 ratio. DNA used in these experiments all had a ratio of >1.9. LPS content of the DNA preparations as measured by the Limulus amoebocyte assay (BioWhittaker, Walkersville, MD) varied from 6 to 10 ng/ml for

EC DNA (1–3 mg/ml of DNA) and from 0 to 3 ng/ml for CT DNA (0.5–1 mg/ml of DNA). The LPS assays were performed according to the manufacturer's protocol using standards of known concentrations of LPS.

Immunizations. Groups of five to ten 7–8-wk-old NZB/NZW female mice were immunized with DNA as previously described (14). Briefly, mice were immunized intraperitoneally with 0.3 cc of an emulsion of Freund's adjuvant containing 50 µg of either EC or CT dsDNA complexed with 75 µg of mBSA. Mice were immunized three times at 2-wk intervals. The first immunization used CFA whereas the two booster immunizations used IFA. Control groups consisted of five mice immunized with mBSA in adjuvant alone and five unimmunized mice. The immunization experiments were performed three separate times using different groups of mice and different lots of DNA.

In one of the experiments, a cohort of NZB/NZW mice was monitored every 2 wk with 24-h urine collections. When proteinuria exceeded 2 mg/mouse/day, the mice were divided into groups and immunized with EC dsDNA/mBSA or mBSA alone in adjuvant. Another control group was unimmunized.

Urine Studies. Mice were placed in metabolic cages either weekly or biweekly for 24-h collections of urine using collection vials containing an antibiotic solution to prevent bacterial growth. After collection, urine was maintained at –70°C until testing for protein or nitrate/nitrite (N/N) content. Protein was measured using the Bio-Rad assay (Richmond, CA) according to the manufacturer's instructions. Urinary N/N, a measure of nitric oxide (NO) production, was determined spectrophotometrically using a previously described technique (25). Mice undergoing measurement of urinary N/N were placed on a N/N-free diet (Zeigler Brothers, Gardners, PA) during the study period.

ELISA. Serum was obtained before immunization and 1 wk after the last immunization in all groups. In certain immunization groups, sera were obtained monthly until the mice were 6–7 mo of age. Mice were bled from the retroorbital sinus after methoxyflurane inhalation anesthesia.

Serum anti-dsDNA activity was determined by ELISA using previously published techniques (14). Briefly, 96-well polystyrene plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 100 µl/well of dsDNA (either EC or CT) at a concentration of 5 µg/ml in SSC (0.15 M NaCl, 0.015 M Na citrate, pH 8) overnight at 37°C. Plates were blocked with 1% BSA in PBS before adding serial dilutions of sera starting at a dilution of 1/100 in PBS-Tween/1% BSA. After washing, peroxidase-conjugated goat anti-mouse IgG (γ chain specific, Sigma Chemical Co.) was added, followed by 3,3',5,5' tetramethylbenzidine diluted 1/100 in 0.1 M citrate, pH 4 with 0.015% H₂O₂. Absorbance at OD 380 was determined on a UVmax microtiter plate reader (Molecular Devices Corp., Menlo Park, CA).

The isotype of the anti-DNA response in individual sera was performed by ELISA as described above, except that isotype-specific conjugates (Southern Biotechnology Associates, Birmingham, AL) were used. The conjugates were titered to assure that equal amounts of each IgG isotype were detected in the ELISA.

Inhibition assays were performed to determine relative affinity of DNA binding as previously described (26). Briefly, sera at dilutions yielding an OD 380 absorbance of 1 were incubated for 45 min with CT dsDNA at various concentrations starting at 50 µg/ml. After the incubation period, the sera/DNA mixtures were added to microtiter plates coated with CT dsDNA as described above, and bound antibody measured. The percent inhibition was calculated by subtracting the inhibited absorbance from the uninhibited absorbance divided by the uninhibited absorbance.

¹ Abbreviations used in this paper: CT, calf thymus; ds, double stranded; EC, *Escherichia coli*; mBSA, methylated bovine serum albumin; N/N, nitrate/nitrite; NO, nitric oxide; NZB/NZW, New Zealand Black/New Zealand White; ss, single stranded.

Pathology. Mice were killed at varying ages ranging from 4 to 7 mo. At the time of killing, the kidneys were removed. One kidney was placed in 10% buffered formalin, fixed in paraffin, and sectioned before staining with hematoxylin and eosin. The other kidney was quick frozen in liquid N₂, fixed, and sectioned before staining with fluorescein-conjugated goat anti-mouse IgG (Sigma Chemical Co.).

Glomerular disease was graded by a pathologist (P. Ruiz) blinded as to the group of origin of the kidney sections. Scores were determined using a grading system we have previously described that assign 0–3+ scores for proliferation, necrosis, crescent formation, vasculitis, and inflammatory infiltrate (27). IgG deposition was graded 0–3+ on the fluorescent slides by the same blinded pathologist.

Glomerular Binding Assay. Sera were assessed for their ability to bind to glomeruli in an in vitro assay as previously described (28). Briefly, glomeruli were derived from saline-perfused kidneys from Lewis rats by a sieve technique. Glomeruli were permeabilized with collagenase type II and DNase in Hank's salt solution and then vacuum affixed to a nitrocellulose filter using a Bio-Dot apparatus (Bio-Rad Laboratories). Sera were added at 1/100 dilutions. Goat anti-mouse IgG (Bio-Rad Laboratories) was then added, followed by a secondary alkaline phosphatase conjugate, and standard chloronaphthol solution (Bio-Rad Laboratories). A quantitative value was obtained by determining the density of the derived dots. All assays were performed in duplicate. The absorption of a control positive serum from a MRL-*lpr/lpr* mouse was assigned a value of 100. A negative control BALB/c sera was assigned a value of 0.

Statistics. Statistical values for anti-DNA production, proteinuria, N/N excretion, and renal scores were determined using the Mann-Whitney two-tailed U test. Survival statistics were analyzed using the log-rank sum method.

Results

To determine the effects of bacterial DNA immunization on murine lupus, we first assessed serological findings of female NZB/NZW mice immunized with either EC dsDNA complexes with mBSA, CT dsDNA complexes with mBSA, or mBSA alone in adjuvant. Mice of this strain develop a genetically determined syndrome at 5–6 mo of age that resembles SLE in the occurrence of anti-DNA and immune complex-mediated renal disease (29). In confirmation of previous results, we found that 8-wk-old NZB/NZW mice immunized with EC dsDNA/mBSA complexes develop a prompt anti-dsDNA response that includes autoantibodies that bind CT as well as EC dsDNA (23). In contrast, NZB/NZW mice immunized with CT dsDNA/mBSA or mBSA alone lacked significant antibody production (Fig. 1).

To determine the effect of DNA immunization on renal disease, serial urine samples were assessed for 24-h protein excretion. Previous studies (30) have shown that NZB/NZW mice develop early renal disease when treated with polyclonal B cell activators. Consistent with these earlier observations, mice immunized with mBSA or CT dsDNA/mBSA in adjuvant had the onset of proteinuria (>2 mg/mouse/day) at 14 wk of age. Proteinuria continued in these groups until at least 17 wk of age when the mice were

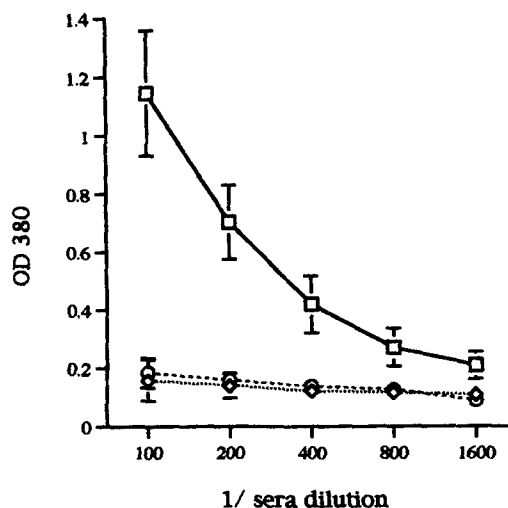


Figure 1. Anti-DNA production by immunized NZB/NZW mice. Data shown are the mean 380-nm absorbance \pm the standard deviation of five sera in each group using CT dsDNA as the antigen in an ELISA. Sera were obtained 2 wk after the final immunization (14 wk of age). The anti-DNA response is significantly greater ($P < 0.05$) in the EC dsDNA/mBSA immunized group (squares) than in the CT dsDNA/mBSA group (circles) or mBSA group (diamonds) at all sera dilutions.

killed. Because of the similarities in these two groups, the data are presented together in Fig. 2. In contrast, over the same time period, none of the mice immunized with EC dsDNA developed proteinuria >1 mg/mouse/day. Before

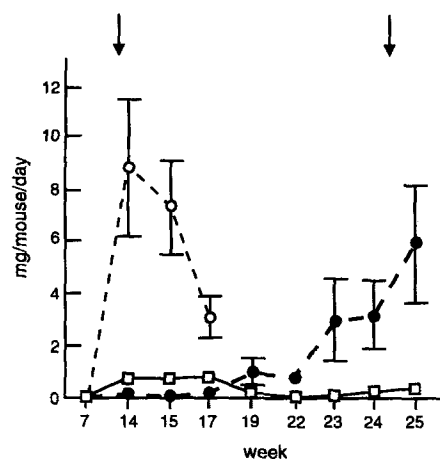


Figure 2. Urinary protein excretion of immunized NZB/NZW mice. Data presented are the mean 24-h protein excretion \pm the standard deviation plotted against the age of the mice in weeks. (Open circles) Combined data for the mBSA ($n = 5$) and the CT dsDNA/mBSA immunized mice ($n = 5$). Two mice in this group died at 15 wk and two additional mice died at 16 wk. The remaining six mice were killed at 17 wk of age. (Squares) Protein excretion of mice immunized with EC dsDNA ($n = 10$). At 17 wk of age, five mice were killed in parallel with the mBSA/CT dsDNA group for pathologic studies. The remaining five mice in this group were followed until 25 wk of age. (Closed circles) Protein excretion by unimmunized NZB/NZW mice ($n = 5$). These mice were killed along with the five remaining EC dsDNA immunized mice at 25 wk of age for pathologic studies. (Arrows) Two peaks of proteinuria.

Table 1. Urinary N/N Excretion by NZB/NZW Mice

Week	Urinary nitrate/nitrite		
	8	15	25
	μM		
EC DNA immunized	0 \pm 0	0.4 \pm 0.1	0.1 \pm 0
mBSA/CT DNA immunized	0 \pm 0	10.0 \pm 3.1	ND*
Unimmunized	0 \pm 0	0 \pm 0	13.1 \pm 3.2

Mice were fed a N/N-free diet and 24-h urine was collected in metabolic cages. Data presented are the mean 24-h urinary N/N excretion \pm standard deviation for 10 mice in the immunized groups and 5 mice in the unimmunized group. At 17 wk of age, five mice in the EC DNA group and the remaining six mice (four mice died in these groups) in the mBSA/CT DNA group were killed. The difference in urinary N/N excretion between the EC DNA immunized group and the mBSA/CT DNA group at week 15 is statistically significant ($P < 0.05$); the difference between the EC DNA group and the unimmunized group at week 25 is statistically significant ($P < 0.05$).

*Mice in this group were killed at 17 wk of age.

killing, two of five mice in each of the mBSA and CT dsDNA groups died. None of 10 mice immunized with EC dsDNA died, however.

Urinary N/N can be used as a marker of inflammation since, with animals on a N/N-free diet, levels of these molecules reflect total body NO production. As previously demonstrated, N/N levels are dramatically increased in MRL-*lpr/lpr* mice, another lupus-prone strain with glomerulonephritis (25). In the present experiments, a sharp increase in N/N followed immunization of all animals (data not shown). This production most likely reflects acute inflammation induced by adjuvant. After decline in these levels back to baseline, N/N excretion rose again in mice immunized with mBSA or mBSA/CT dsDNA. These elevations paralleled the increase in proteinuria (Table 1). In contrast, mice immunized with EC dsDNA/mBSA had low levels of urinary N/N at the same time point, consistent with the lack of proteinuria.

Kidneys obtained from mice killed at 17 wk of age were examined histologically. As shown in Table 2 and Fig. 3, glomerular disease was significantly less in the EC dsDNA immunized mice than the CT dsDNA and mBSA immunized groups. Immunofluorescence staining of the kidneys, however, revealed 3+ IgG deposition in the mesangium and glomerular loops in the kidneys of all three groups.

An additional group of five mice immunized with EC dsDNA/mBSA was followed after 17 wk of age along with unimmunized control mice. As shown in Fig. 2, urinary protein excretion in the unimmunized mice began after 19 wk of age and continued until 25 wk of age when the animals were killed. In the EC dsDNA group, proteinuria never exceeded 1 mg/mouse/day. Urinary N/N excretion was also significantly higher in the unimmunized group than in the mice immunized with EC dsDNA (Table 1). As shown in Table 2, mice immunized with EC dsDNA/mBSA had significantly lower glomerular scores than the

Table 2. Effects of DNA Immunization on Renal Disease in NZB/NZW Mice

Group (17-wk-old)	EC dsDNA	mBSA	CT dsDNA
Renal score	1.6 \pm 0.4	3.9 \pm 1.3	3.3 \pm 0.8
Glomerular IgG	3+	3+	3+
Group (25-wk-old)	EC dsDNA	Unimmunized	
Renal score	3.5 \pm 1.2	7.5 \pm 2.8	
Glomerular IgG	3+	3+	

Data presented are the mean renal score \pm standard deviation of five mice in each group. Glomerular IgG data are presented as the degree of immunofluorescence. The difference between the glomerular scores of the EC DNA group and the mBSA/CT DNA groups is statistically significant ($P < 0.05$); the difference in scores between the EC DNA and unimmunized group at 25 wk is also statistically significant ($P < 0.05$).

unimmunized group despite similarities in glomerular IgG deposition. Together, these results indicate that immunization with EC dsDNA/mBSA complexes blocks the development of early renal disease that results from adjuvant effects as well as the spontaneous disease that develops with age.

A second set of immunizations was performed using different lots of DNA, mBSA, and adjuvant. Confirming the previous experiment, the mice immunized with EC dsDNA/mBSA developed significantly less proteinuria than mice immunized with mBSA alone (Table 3). The EC dsDNA/mBSA immunized mice also had significantly decreased mortality compared with either mice immunized with mBSA alone (Fig. 4 A) or unimmunized mice (Fig. 4 B).

To determine if EC dsDNA immunization could modify established autoimmune disease, we immunized 5–6-month-old NZB/NZW mice that had proteinuria and high anti-DNA levels. In these experiments, control mice received either mBSA or were unimmunized. Although mice in all three groups had high anti-dsDNA levels, proteinuria in the EC dsDNA group stabilized after immunization whereas proteinuria in the other groups steadily increased; there was, however, no statistically significant difference in proteinuria between the groups after immunization. Survival was prolonged, however, in the EC dsDNA group compared with the other two groups (Table 4).

We next investigated whether EC dsDNA immunization altered the pathogenicity of the expressed anti-DNA antibodies. Avidity of the induced anti-DNA was similar to spontaneous anti-DNA as shown by inhibition binding studies. Thus, for the induced antibodies, 50% binding inhibition required $12 \pm 5 \mu\text{g/ml}$ of CT dsDNA whereas antibodies arising spontaneously required $11 \pm 4 \mu\text{g/ml}$ ($n = 5$ sera for each group). The induced and spontaneous antibodies also had a similar isotype distribution, since both displayed a predominance of IgG2a and IgG2b antibodies (Table 5). Finally, as shown in Table 6, sera from the EC dsDNA immunized mice bound to glomerular antigens

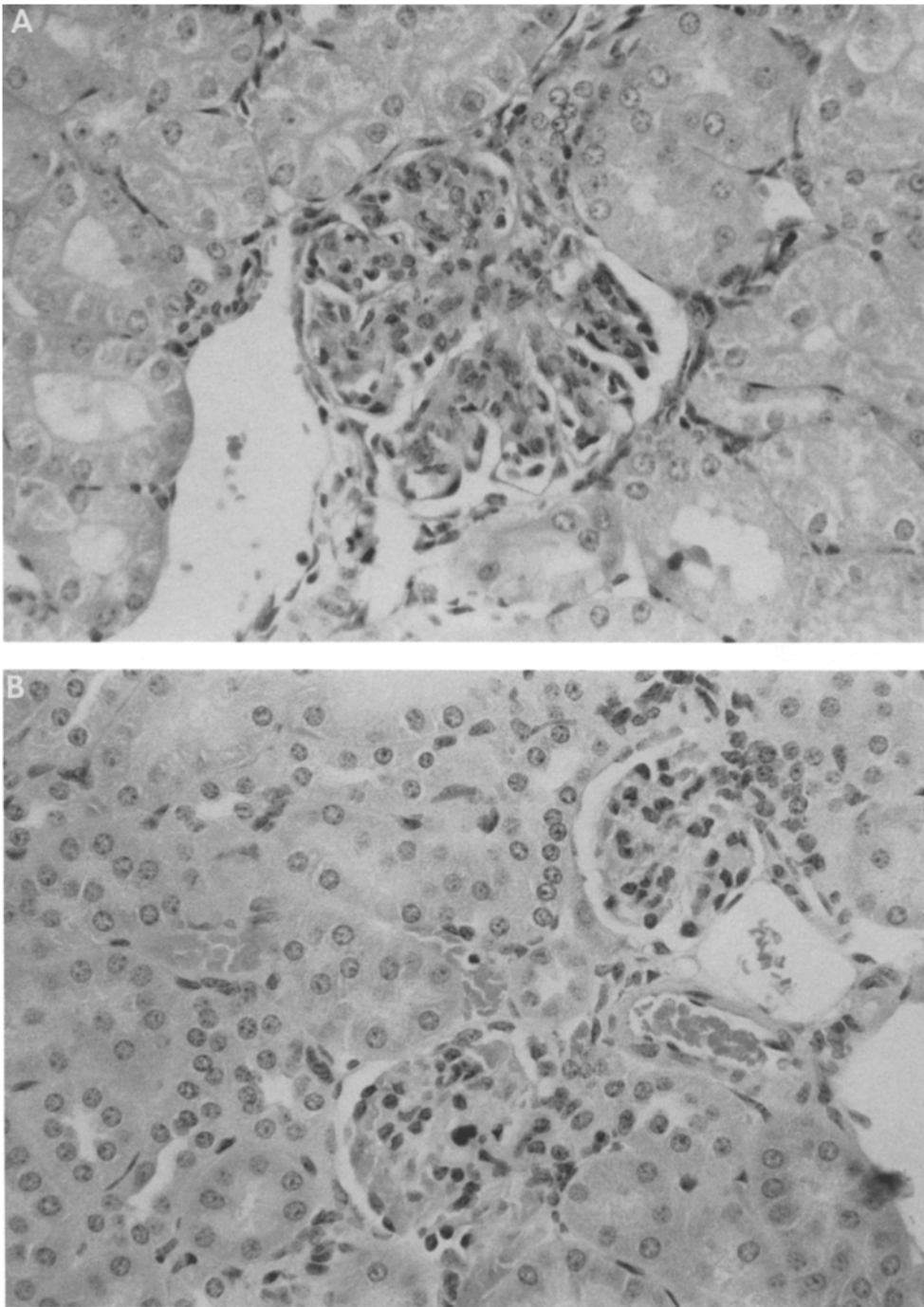


Figure 3. Glomerular pathology in NZB/NZW mice. Representative photomicrographs of glomeruli from: (A) 17-wk-old mBSA immunized NZB/NZW mouse, demonstrating marked glomerular proliferation with acute and chronic inflammation (renal score of 7); and (B) 17-wk-old EC dsDNA/mBSA immunized NZB/NZW mouse, demonstrating minimal proliferative changes without inflammation (renal score of 1.5). Photomicrographs are enlarged $\times 79$.

better than sera from the mBSA immunized or unimmunized groups immediately after immunization, whereas sera from all three groups at 5–6 mo of age bound to glomeruli similarly. Since this *in vitro* assay correlates with *in vivo* glomerular binding and pathogenicity, these findings suggest that renal protection caused by immunization with bacterial DNA does not result from a shift in the immunochemical properties of anti-DNA or elimination of pathogenic specificities.

Discussion

Results of these experiments indicate that immunization of preautoimmune NZB/NZW mice with EC dsDNA/mBSA complexes can profoundly alter the course of their disease. These changes occur despite the expression of anti-DNA that resemble spontaneous anti-DNA in their specificity, avidity, isotype, and ability to bind to glomeruli. Furthermore, immunization with EC dsDNA/mBSA after the onset of renal disease can stabilize proteinuria and prolong

Table 3. Effect of DNA Immunization on Proteinuria in NZB/NZW Mice

Group	Proteinuria
dsEC DNA (<i>n</i> = 10)	0.8 ± 0.6
mBSA immunized (<i>n</i> = 5)	5.0 ± 0.1
Unimmunized (<i>n</i> = 10)	4.2 ± 3.6

Data presented are the mean 24-h protein excretion in milligrams/day/mouse ± standard deviation of NZB/NZW mice at 25 wk of age. The difference in proteinuria between the EC DNA immunized group and the mBSA and unimmunized groups was significant at *p* < 0.05.

survival. Together, these findings suggest that foreign DNA, while provoking serological abnormalities, can nevertheless attenuate manifestations of autoimmunity.

Although DNA has generally been considered immunologically inert, recent studies (13–19) demonstrate clearly that bacterial DNA is active in mice and can induce cytokine production as well as polyclonal B cell activation. These effects result from palindromic sequence motifs that consist of an unmethylated CpG flanked by two 5' purines and two 3' pyrimidines (17–19). This motif is common in bacteria DNA and rare in mammalian DNA (19). Furthermore, methylation of cytosine in bacterial DNA eliminates mitogenicity (19). Because of differences between mammalian and bacterial DNA in their pattern of cytosine methylation, a basis for immune recognition of DNA is established.

Since polyclonal B cell activators accelerate autoimmunity in NZB/NZW mice, the reduction of renal disease by bacterial DNA is probably unrelated to this activity (30). It

is more likely that the therapeutic effects of bacterial DNA result from changes in the cytokine milieu and the balance of regulatory T cell subsets in treated animals. NZB/NZW mice produce low levels of TNF-α due to a deletion in the gene for this cytokine (31). Although genetic evidence suggests that this defect may promote autoimmunity, a role of TNF-α in glomerulonephritis is shown directly by treatment studies with recombinant cytokine (32, 33). Thus, chronic administration of TNF-α to NZB/NZW mice leads to improvement in renal disease. This effect is dose dependent since higher doses of TNF-α decrease disease severity (32, 33).

In *in vitro* studies, both EC DNA and synthetic oligonucleotides with active palindromic sequences induce TNF-α and IL-12 production by murine splenocytes (34). It is possible, therefore, that bacterial DNA immunization increases TNF-α and thereby decreases renal disease in a manner similar to exogenously administered recombinant cytokine. Alternatively, changes in other cytokines, e.g., IL-12 and IFN-γ production, may alter the Th1/Th2 ratio in these mice or otherwise modify immune responsiveness and thereby affect clinical disease.

At present, the range of cytokines induced by bacterial DNA is unknown, and it is possible that effects on other cytokines alone or together reshape the immune environment in autoimmune mice. The effects of bacterial DNA ultimately modify the generation of inflammatory mediators since, as we have shown, NZB/NZW mice immunized with bacterial DNA have reduced NO production. It is of interest in this regard that bacterial DNA immunization can block spontaneous renal disease as well as renal disease induced by administration of adjuvant, suggesting a generalized anti-inflammatory effect.

The effects of immunization with foreign DNA may vary, however, depending on the source of the DNA and the

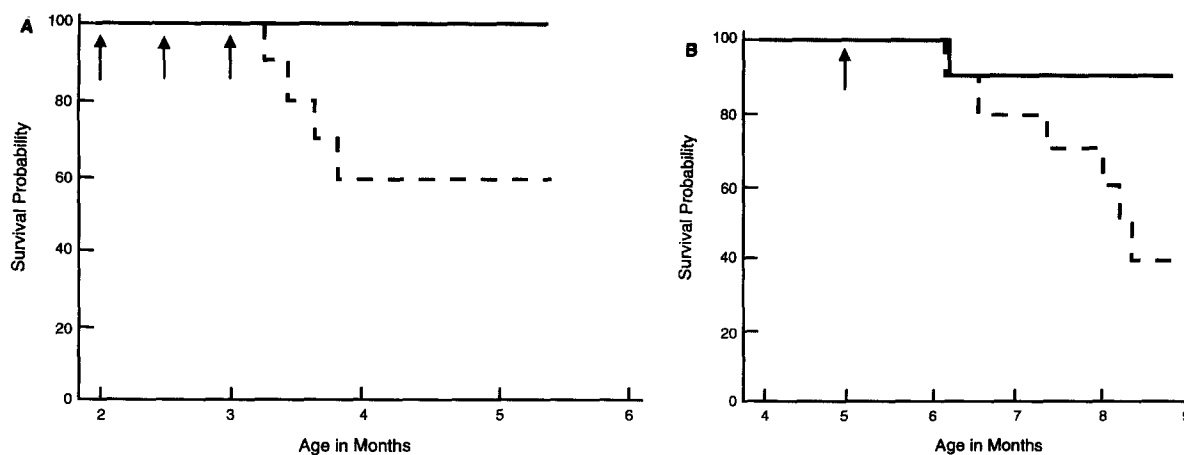


Figure 4. Survival of immunized and unimmunized NZB/NZW mice. Data presented are the number of mice surviving plotted against the age in months. (A) Survival of 10 mice immunized with EC dsDNA/mBSA (solid line) versus 10 mice immunized with mBSA alone (dashed line). Survival in the EC DNA group was significantly greater (*P* = 0.03). (Arrows) Time of immunizations. (B) Survival of 10 mice immunized with EC dsDNA/mBSA (solid line) versus 10 unimmunized mice (dashed line). Survival in the EC DNA group was significantly greater (*P* = 0.04). Statistical analysis was performed using the log-rank sum test. (Arrow) Time of the initial immunization.

Table 4. *Effects of DNA Immunization after Onset of Renal Disease*

	Proteinuria		Anti-DNA	Survival
	Preimmunization	Postimmunization		
EC DNA (<i>n</i> = 5)	7.7 ± 1.3	4.3 ± 2.1	1.586 ± 0.493	4/5
mBSA (<i>n</i> = 5)	1.4 ± 0.4	3.7 ± 1.8	1.295 ± 0.489	0/5
Unimmunized (<i>n</i> = 5)	4.2 ± 2.4	5.7 ± 1.6	1.135 ± 0.311	1/5

Data presented are the mean 24-h protein excretion ± standard deviation, preimmunization, and 2 wk postimmunization. Anti-DNA data are the ELISA results in terms of absorbance ± standard deviation of sera obtained 1 wk after the final immunization. Survival data are presented in terms of mice alive at 9 mo of age. There was no statistical difference in postimmunization proteinuria and anti-DNA levels between groups. The difference in survival of the EC dsDNA/mBSA immunized group versus the other two groups approached statistical significance using the log-rank sum test (*P* = 0.06).

content of sequences with immunomodulatory potential. Frederiksen et al. (35) have shown that DNA from the BK polyomavirus can stimulate the production of antibodies specific for nonconserved sites on viral DNA in normal mice, whereas in NZB/NZW mice, immunization elicits antibodies cross-reactive with mammalian DNA. These findings are similar to our results with EC DNA. In contrast to results reported herein, however, immunization with the BK viral DNA accelerates renal disease (35). Thus, although both EC and BK DNA stimulate an early anti-DNA antibody response, they must have divergent effects on other immune cells (i.e., macrophages and T cells) resulting in disparate clinical outcomes.

Because the DNA used in these experiments was derived from bacteria, we have been concerned about the impact of any contamination by LPS on the *in vivo* responses, since this bacterial product can provoke changes in the murine immune system. We have therefore used extensively purified DNA preparations with minimal contamination by LPS as assessed by the *Limulus* amoebocyte assays (15). As we

have previously shown, in no instance can we account for any *in vivo* or *in vitro* effects of bacterial DNA by this amount of LPS (14, 15, 18, 22). Furthermore, all immunomodulatory effects of bacterial DNA have been reproduced with synthetic oligonucleotides and polynucleotides that are free of contaminating bacterial products (17–19, 34). The effects of bacterial DNA are also unaffected by polymyxin B, although fully sensitive to digestion by DNase (15).

Even if our DNA preparations had enough LPS to induce *in vivo* effects, we predicted the outcome to be the opposite of the renal protection observed. As shown in many studies, LPS causes polyclonal B cell activation and glomerulonephritis in normal mice, whereas, in autoimmune strains, it accelerates autoantibody production and glomerulonephritis (36–42). Treatment of mice with a combination of LPS and CT DNA also leads to an early onset of glomerulonephritis (43). In this regard, doses of LPS <20 µg had minimal effect on antibody production, although these levels were far greater than those present in our DNA preparations (36). Taken together, these consid-

Table 5. *Isotype of the Induced Anti-DNA Response in NZB/NZW Mice*

Isotype	IgG1	IgG2a	IgG2b	IgG3
EC DNA immunized (<i>n</i> = 10)	17.1 ± 5.5	35.2 ± 5.9	43.6 ± 11.3	3.9 ± 4.1
mBSA immunized (<i>n</i> = 7)	32.7 ± 24.1	24.6 ± 25.7	40.7 ± 22.5	4.2 ± 8.1
Unimmunized (<i>n</i> = 6)	21.2 ± 8.1	31.9 ± 10.1	36.3 ± 7.7	9.0 ± 11.1

Data presented are the mean percentage ± standard deviation of the total IgG anti-DNA for each isotype. Sera were obtained at 14 wk of age in the EC DNA and mBSA immunized groups and at 25 wk of age in the unimmunized group.

Table 6. Glomerular Binding by Sera from NZB/NZW Mice

Group	14 wk	25 wk
EC dsDNA immunized	67.3 ± 14.1	57.7 ± 3.2
mBSA immunized	28.5 ± 10.4	42
Unimmunized	14.0 ± 7.7	48.3 ± 16.7

Data presented are the sera mean glomerular binding score ± standard deviation of five sera in each group. There was only one sera available for testing at 25 wk in the mBSA immunized group. The glomerular binding by the EC dsDNA immunized group at 4 mo of age was significantly greater than that of the other two groups at $p < 0.05$.

erations suggest that it is highly unlikely that LPS in our EC DNA preparations caused the renal protective effect observed.

Infection has long been considered an etiologic factor in

the pathogenesis of SLE as well as other autoimmune diseases. Whereas most investigation has focused on the potentiating influences of microbial products on these conditions, such molecules may also have inhibitory influences, as these studies indicate. Whether bacterial DNA can play such a role is uncertain, although the consequences of this molecule likely vary, depending of the status of the host.

While pointing to novel immunomodulatory approaches in SLE, these studies also have implications for the use of nucleic acids in the therapy and prevention of disease. DNA constructs are being tested as vehicles for the introduction of genetic material to correct inherited defects, augment resistance to tumors, and provide a source of antigen for vaccination (44). Since many of these constructs have sequences from bacterial plasmids, they may promote in vivo immune alterations that could affect the result of gene therapy as well as induce the immune system to promote or restrain inflammatory disease.

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