

## Induction of Cross-Reactive Anti-dsDNA Antibodies in Preautoimmune NZB/NZW Mice by Immunization with Bacterial DNA

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### Abstract

To investigate the role of antigen drive in anti-double-stranded (ds) DNA production, the antibody response induced in lupus-prone NZB/NZW mice by *E. coli* (EC) dsDNA was evaluated. Preautoimmune NZB/NZW female mice were immunized with complexes of EC dsDNA with methylated bovine serum albumin (mBSA) in complete Freund's adjuvant; control mice received either mBSA complexes with calf thymus (CT) dsDNA or mBSA alone in adjuvant. IgG antibody responses were assessed by ELISA. Similar to normal mice, immunized NZB/NZW mice produced significant levels of anti-dsDNA when measured with EC dsDNA as antigen. Whereas normal mice produce antibodies which are specific for the immunizing bacterial DNA, NZB/NZW mice produced antibodies that bound crossreactively to CT dsDNA by ELISA. Furthermore, the induced antibodies resembled lupus anti-DNA in their fine specificity for polynucleotide antigens and reactivity with *Crithidia luciliae* DNA. Despite their response to EC dsDNA, NZB/NZW mice immunized with CT dsDNA failed to generate significant anti-dsDNA responses. These results provide further evidence for the enhanced immunogenicity of bacterial DNA and suggest that immune cell abnormalities in NZB/NZW mice promote the generation of crossreactive autoantibody responses when confronted with a foreign DNA. (*J. Clin. Invest.* 1995. 95:1398–1402.) Key words: autoimmunity • systemic lupus erythematosus • immunogenicity • animal models • autoantibody induction

### Introduction

Systemic lupus erythematosus (SLE) is a generalized autoimmune disease characterized by antibodies to double-stranded

(ds)<sup>1</sup> DNA (1, 2). These antibodies occur essentially only during the course of lupus in both man and mouse and serve as markers for diagnosis and prognosis (3, 4). The importance of anti-dsDNA to disease pathogenesis is substantiated by evidence that they promote glomerulonephritis either by immune complex deposition or the direct binding to crossreactive renal antigens (5–7).

Although the precise mechanisms of anti-dsDNA production remain unknown, recent studies point strongly to a role of DNA antigen drive (8). Thus, anti-dsDNA antibodies display patterns of variable (V) region gene utilization that resemble conventional responses in the content of somatic mutations and clonality (9–13). Since these mutations are associated with enhanced binding to DNA, it is likely that DNA is the selecting antigen in vivo. Among structural features associated with dsDNA binding, a high content of arginine residues in the third complementarity determining region (CDR 3) of the heavy chain appears important (9–11).

To understand the process of DNA antigen drive, our laboratory has studied the antibody response induced in normal mice by bacterial DNA. We have shown that DNA from various bacterial species elicit high titer responses in normal mice when immunized as complexes with methylated bovine serum albumin (mBSA) in adjuvant (14, 15). These responses are IgG and most likely result from stimulation by regions of DNA that differ in sequence from host DNA and therefore can be recognized as foreign (16). Unlike spontaneously produced anti-DNA in lupus mice, the induced antibodies do not bind mammalian dsDNA in the B conformation despite reactivity to bacterial dsDNA (15); these findings suggest recognition of a non-conserved dsDNA conformation which, in its immunological properties, may resemble Z-DNA (17). Immunization with bacterial DNA does, however, induce antibodies that crossreact with single-stranded (ss) bacterial and mammalian DNA (14, 15).

In a previous study, we analyzed the amino acid sequences of induced anti-DNA antibodies to assess their relationship with spontaneous anti-DNA. Our results suggested, that while induced anti-DNA use similar VH and VL genes as spontaneous anti-DNA, they differ in the content of VH CDR3 arginines as well as arginines at certain positions (18). On the basis of these findings, we postulated that B cells whose Ig product had such sequences would be autoreactive and therefore deleted or anergized in normal mice. As a result, normal mice would lack a preferred precursor population for anti-DNA and be markedly limited in their responses to dsDNA even when the DNA is presented in an immunogenic form.

A further inference from these studies is that lupus mice, because of abnormalities in B cell tolerance, would retain such

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1. Abbreviations used in this paper: CDR, complementarity determining region; CT, Calf thymus; ds, double stranded; EC, *Escherichia coli*; mBSA, methylated BSA; ss, single-stranded; V, variable.

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precursors and produce crossreactive anti-dsDNA antibodies when stimulated either during spontaneous disease or after DNA immunization. To test this possibility, we have determined the anti-dsDNA response of young NZB/NZW female mice immunized with dsDNA from *Escherichia coli* (EC). These F1 hybrids spontaneously produce anti-DNA as they age and have been extensively studied as a model of lupus (19–21). In results presented herein, we show that NZB/NZW mice, immunized with EC dsDNA prior to the onset of disease, develop a significant anti-dsDNA response that has features of lupus anti-DNA. These results indicate that lupus-prone mice respond to dsDNA immunization differently than normal mice and suggest that this responsiveness may reflect B cell disturbances critical to autoimmunity.

## Methods

**Animals.** Female NZB/NZW F1 and MRL-*lpr/lpr* mice were purchased from the Jackson Laboratory (Bar Harbor, ME) at 6 wk of age and subsequently housed in the Durham VAMC animal facility receiving food and water ad lib.

**DNA.** DNA from EC and calf thymus (CT) were purchased from Sigma Chemical Co. (St. Louis, MO). DNA was dissolved in SSC (0.15 M NaCl, 0.015 M Na Citrate, pH8) before purification by phenol extraction. dsDNA was obtained by treating the DNA with  $S_1$  nuclease while ssDNA was obtained by boiling for 10 min before rapid immersion in ice.

**Immunizations.** Groups of NZB/NZW F1 mice were immunized with DNA beginning at 8 wk of age. Each mouse received 0.3 ml of an emulsion containing 50  $\mu$ g of either EC or CT dsDNA complexed with 75  $\mu$ g of mBSA in Freund's complete adjuvant (CFA). A control group received 75  $\mu$ g of mBSA alone in CFA. Mice received two booster immunizations at 2-wk intervals containing either CT or EC dsDNA complexed with mBSA or mBSA alone in incomplete adjuvant. This immunization protocol was repeated three separate times with five mice in each group. During two of the immunization experiments, mice were prebled and then bled one week following the final immunization. In the third immunization experiment, mice were prebled and then bled weekly until one week following the last immunization.

**ELISA.** Sera were tested for reactivity to DNA antigens by ELISA as previously described (15). Briefly, 96-well polystyrene plates were coated with DNA diluted to 5  $\mu$ g/ml in SSC. Antigens used in these assays were EC dsDNA, CT dsDNA, and EC ssDNA. After addition of DNA, plates were incubated for 16 h at 37°C for dsDNA assays and for 2 h at 37°C for ssDNA assays. Two fold serial dilutions of sera in PBS-T (phosphate buffered saline containing 0.05% Tween 20) were then added to the plates starting at a 1/100 dilution.

After incubation, peroxidase conjugated goat anti-mouse IgG (Sigma Chemical Co.) was added. 3,3',5,5' tetramethylbenzidine (TMB) in 0.1 M citrate (pH4) with 0.015% H<sub>2</sub>O<sub>2</sub> was added for color development. OD 380 absorbance was determined by a microtiter plate reader (Molecular Dynamics, Menlo Park, CA).

**Inhibition assays.** In this assay, the serum dilution yielding an OD 380 of 1 in the anti-CT dsDNA assay was incubated for 45 minutes with dilutions of the inhibiting DNA (EC dsDNA or CT dsDNA) beginning at 50  $\mu$ g/ml. After incubation, the mixture was added to microtiter plates coated with CT dsDNA or EC dsDNA. Conjugate and substrate were added as described above. Percent of inhibition was determined by subtracting the inhibited value from the uninhibited value and dividing by the uninhibited value.

**Crithidia assay.** *Crithidia luciliae* assays for anti-dsDNA were performed as suggested by the manufacturer (Kallestad, Austin, TX). Sera were tested at a 1:20 and 1:50 dilution.

**Specificity assays.** Specificity of the induced antibodies was determined by the binding of sera to ss and ds synthetic polynucleotides (22). The ss polynucleotides pDA, pDC, pDI, and pDT and ds polynucleotides

Table I. Binding of Sera from Immunized NZB/NZW Mice to EC dsDNA

Immunization group	OD380 absorbance
dsEC DNA immunized	0.955 $\pm$ 0.274
dsCT DNA immunized	0.063 $\pm$ 0.060
mBSA immunized	0.068 $\pm$ 0.043

Sera obtained 1 wk after the last immunization were tested by ELISA for binding to EC dsDNA. Data presented are the mean absorbance $\pm$ SD of 5 sera in each group. The difference between the EC dsDNA group and the other two groups is significant at  $P < 0.05$ .

p(dGdC)-p(dGdC) and p(dAdT)-p(dAdT) were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). The polynucleotides were dissolved in PBS and coated to microtiter plates at 5  $\mu$ g/ml. Binding to these antigens was then tested by ELISA as described in the anti-DNA assay.

**Statistical analysis.** All statistical values were derived using the Mann-Whitney two-tailed U test.

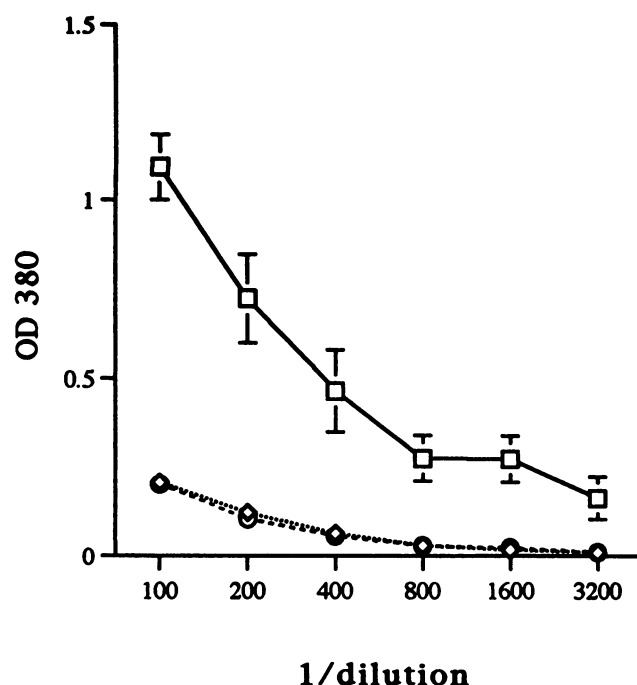
## Results

To assess the response of autoimmune-prone mice to dsDNA immunization, young NZB/NZW F1 mice were immunized with EC dsDNA or CT dsDNA as mBSA complexes in CFA and then tested for IgG anti-dsDNA activity by ELISA. As shown in Table I, NZB/NZW mice immunized with EC dsDNA showed high anti-dsDNA levels using EC dsDNA as antigen. In contrast, NZB/NZW mice immunized with either CT dsDNA complexes or mBSA alone had little anti-DNA response. When tested using EC ssDNA as antigen, sera showed a similar pattern of reactivity (data not shown.) These results indicate that preautoimmune like normal mice have enhanced responsiveness to bacterial DNA and that mammalian DNA is unable to elicit a comparable anti-DNA response.

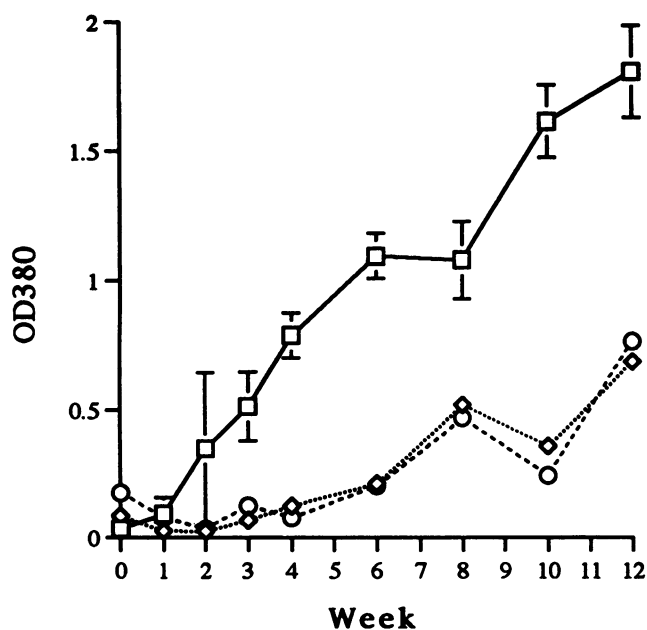
The response to mammalian dsDNA was next assessed using CT dsDNA as antigen. As shown in Figs. 1 and 2, sera from the mice immunized with EC dsDNA had significant antibody reactivity indicating the generation of anti-DNA antibodies that bind crossreactively to mammalian dsDNA. These responses differ dramatically from those of immunized BALB/c mice which produce antibodies specific for the EC dsDNA and lack crossreactivity with CT dsDNA (15).

To assure reproducibility of these findings, this immunization protocol was performed three separate times using different lots of DNA and different litters of mice for each immunization. The pattern of responsiveness was the same in each experiment, confirming that preautoimmune NZB/W mice have a consistent ability to generate crossreactive anti-dsDNA antibodies when immunized with bacterial dsDNA. In one of these experiments, antibody levels were tested on mice bled weekly to determine the time course of the anti-DNA response. As shown in Fig. 3, the kinetics of antibody production to both EC and CT dsDNA were similar, suggesting that the immunization simultaneously stimulated the production of antibodies directed to both dsDNA.

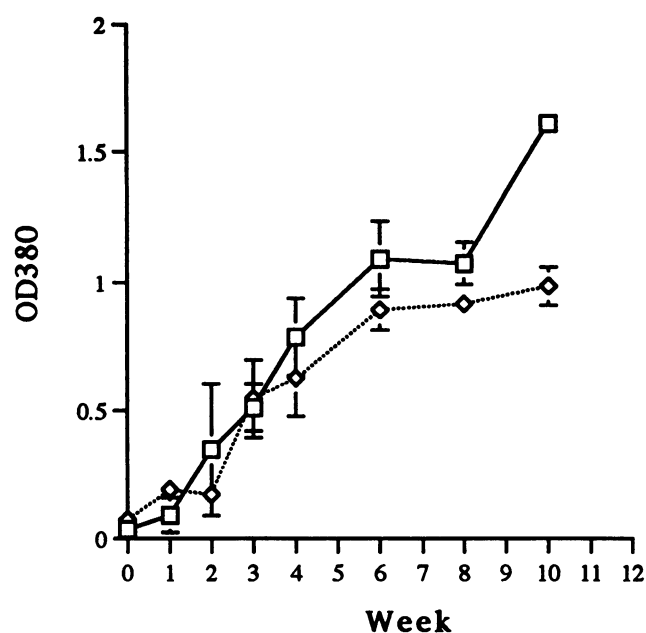
To determine whether the induced antibodies bound similarly to mammalian and bacterial DNA, inhibition ELISAs were performed. As shown in Table II, EC dsDNA and CT dsDNA showed similar inhibitory capacity when tested on either DNA.



**Figure 1.** Titration analysis of the binding of sera from immunized NZB/NZW mice to CT dsDNA. Serial dilutions of sera obtained 1 wk after the final immunization were tested by ELISA. The squares represent sera from EC dsDNA immunized mice; the circles, CT dsDNA immunized mice; the diamonds, mBSA immunized mice. Data presented are the mean absorbance  $\pm$  SD for 5 sera in each group. There is a statistically significant difference between the EC dsDNA group and the other two groups at all dilutions ( $P < 0.05$ ).



**Figure 2.** Time course of CT dsDNA binding of sera from immunized NZB/NZW mice. Sera were tested at a 1/100 dilution. Data presented are the mean absorbance  $\pm$  SD of 5 sera in each group. The squares represent sera from EC dsDNA immunized mice; the circles, CT dsDNA immunized mice; the diamonds, mBSA immunized mice. There is a statistically significant difference ( $P < 0.05$ ) between the EC dsDNA immunized group and the other two groups for weeks 3–12.



**Figure 3.** Time course of the binding of sera from EC dsDNA immunized mice to CT dsDNA ( $\square$ ) and EC dsDNA ( $\diamond$ ). Data presented are the mean  $\pm$  SD of the absorbance of 5 sera tested at a 1/100 dilution. There is no statistically significant difference at any of the time points.

These results indicate that the induced anti-dsDNA bind cross-reactively to determinants present on both dsDNA.

To confirm the specificity of the induced antibodies for dsDNA, the sera from the immunized mice were tested for reactivity by the *Crithidia luciliae* assay. This assay is commonly used for clinical testing and is highly specific for anti-dsDNA. At dilutions of 1:20 and 1:50, sera from NZB/NZW mice immunized with EC dsDNA were all positive, whereas sera from NZB/NZW mice immunized with CT dsDNA or mBSA were negative; five sera from each group were tested. Sera from unimmunized young NZB/NZW mice were also neg-

**Table II.** Inhibition of Binding of Sera from EC dsDNA Immunized Mice

DNA inhibitor	Concentration of inhibitor yielding 50% inhibition $\mu\text{g/ml}$
CT dsDNA as test antigen	
Inhibitor DNA	
CT dsDNA	$0.68 \pm 0.20$
EC dsDNA	$0.32 \pm 0.14$
EC dsDNA as test antigen	
Inhibitor DNA	
CT dsDNA	$0.13 \pm 0.06$
EC dsDNA	$0.13 \pm 0.06$

Sera from EC dsDNA immunized NZB/NZW mice were tested by inhibition ELISA for binding to CT dsDNA and EC dsDNA. Values expressed are the concentrations of DNA required to yield 50% inhibition of binding compared to uninhibited sera. Four sera were tested with each antigen.

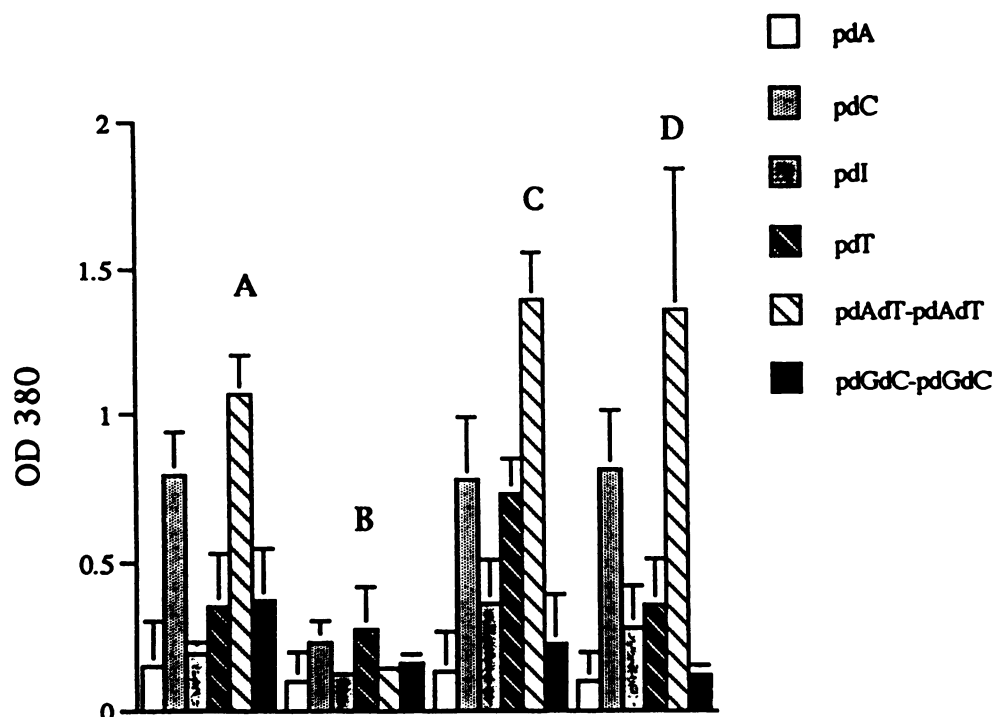


Figure 4. Synthetic polynucleotide binding by immunized and unimmunized mice. Sera from NZB/NZW mice immunized with EC dsDNA (A), NZB/NZW mice immunized with CT dsDNA (B), unimmunized 20-wk-old MRL-lpr/lpr mice (C), and unimmunized 24-wk-old NZB/NZW mice (D) were tested by ELISA at a 1/100 dilution. Data presented are the mean  $\pm$  SD absorbance of five sera in each group.

ative, while sera from unimmunized 20-wk-old MRL-lpr/lpr mice were positive at both dilutions.

The fine specificity of the induced anti-DNA was further evaluated by testing for binding to synthetic ss and ds polynucleotides. Similar to sera from immunized BALB/c mice and older NZB/NZW and MRL-lpr/lpr mice, sera from the immunized NZB/NZW mice bound significantly to pdC and pdT with minimal binding to pdI or pdA (Fig. 4). When tested on dsDNA polymers, sera from the EC dsDNA immunized mice showed greater binding to p(dAdT)-p(dAdT) than p(dGdC)-p(dGdC). This pattern is similar to that of autoimmune mice, but differs from sera from immunized BALB/c mice which bind p(dGdC)-p(dGdC), but not p(dAdT)-p(dAdT) (22). Thus, the antibodies induced in NZB/NZW mice differ from those of immunized normal mice and, in their binding to synthetic dsDNA polynucleotides, resemble spontaneous lupus antibodies.

## Discussion

The results presented herein establish clearly that bacterial dsDNA complexed to methylated BSA is a potent immunogen and can induce antibodies with features of lupus anti-dsDNA in animals predisposed to autoimmunity. The induced NZB/NZW responses are striking and confirm previous results indicating that bacterial dsDNA can be recognized by the immune system as foreign and has both conserved and nonconserved determinants that can be the targets of antibody reactivity. The utility of immunization with bacterial DNA as a model for autoantibody production is thus substantiated.

Although normal mice can generate antibodies specific for bacterial dsDNA as well as crossreactive antibodies to ssDNA, they nevertheless fail to produce antibodies to mammalian dsDNA after immunization with bacterial DNA (14, 15). This inability has been ascribed to the B cell compartment and the

absence of precursors whose sequences facilitate dsDNA interaction, e.g., CDR3 arginines (16, 18). We have suggested that such V regions, even without somatic mutations, bind sufficiently to DNA to cause deletion or anergy in a normal animal (18). Without such precursors, the normal animal would lack a preferred pathway for the generation of anti-dsDNA antibodies. Even when confronted with a potent dsDNA immunogen and adequate T cell help, these animals would be constrained in their ability to produce antibodies of this specificity.

According to this postulate, an important determinant of autoimmunity is the B cell compartment, with the composition of the repertoire of autoimmune mice different from that of normals because of abnormalities in B cell tolerance. Indeed, studies of NZB/NZW as well as other lupus strains indicate defects in tolerance induction, B cell responsiveness, and patterns of V gene and idiotype expression (19–21). We have suggested therefore that the preimmune repertoire of lupus mice is uniquely enriched in autoreactive B cells that can be mutated to high affinity anti-dsDNA when appropriately stimulated. The high anti-dsDNA responses we have observed is consistent with this model, although it will be necessary to determine the V region sequences of the induced antibodies to show a high content of CDR3 arginines.

These considerations, however, do not explain the failure of CT dsDNA to induce a comparable anti-dsDNA response by immunization. Indeed, it appears the B cells that can bind CT dsDNA are not triggered by this antigen, even when coupled to a carrier that should provide adequate T cell help. One explanation for this finding relates to the intrinsic mitogenicity of bacterial DNA. As demonstrated previously, bacterial DNA can directly stimulate murine B cells to proliferate and secrete antibody (23). It is possible, therefore, that EC dsDNA enhances the crossreactive response by more potently stimulating B cells than mammalian DNA.

A major unanswered question in the study of the lupus anti-

dsDNA response has been how a poorly immunogenic molecule can drive antibody production. Our results help resolve this contradiction and suggest that DNA antigen drive can occur in SLE if two conditions are met: (a) the host has an abnormal B repertoire enriched in autoreactive precursors; and (b) the DNA antigen has enhanced immunogenicity. This enhancement could result from the structure of the DNA itself and the presence of mitogenic sequences. Alternatively, the immunogenicity of DNA may be dramatically altered by coupling to a highly immunogenic protein. The induction of antibodies using the Trypanosoma DNA-binding protein Fus 1 as a carrier may be an example of the latter situation (24).

The studies with Fus 1 represent an informative contrast with our findings since immunization of normal BALB/c mice with complexes of CT dsDNA and Fus 1 led to crossreactive anti-dsDNA, some of which contained CDR3 arginines (25). These observations can be reconciled with those reported herein by postulating that the deficiency of anti-dsDNA precursors is not absolute and that prolonged stimulation with certain DNA-protein complexes can elicit the expression of these antibodies. It is conceivable that Fus 1 is a more effective carrier than mBSA because of the stability of the complexes, its capacity to stimulate T cell help or its ability to present DNA in a form that leads to better stimulation of B cells.

Although these studies demonstrate that bacterial DNA can provoke a lupus-like anti-dsDNA response, the role of foreign DNA in human lupus remains speculative. Flares of lupus often occur in association with infection and there is evidence that patients with lupus have abnormalities in the clearance of bacteria and other pathogens suggesting a possible role of microbial antigens in inducing disease activity (26). Furthermore, bacterial DNA has been isolated from the serum of lupus patients with clinically active disease in the absence of known infection, while infection with the BK virus is associated with anti-DNA production in both man and animal models (27, 28). Studies are therefore in progress to determine whether foreign DNA introduced during infection initiates or augments anti-dsDNA production during the course of lupus in both man and mouse. Additional studies will analyze the V gene sequences of induced anti-dsDNA from NZB/NZW mice to determine their similarity to those arising spontaneously in disease.

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