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Effects of Bacterial DNA on Cytokine Production by (NZB/NZW)F₁ Mice

Gary S. Gilkeson, Jacqueline Conover, Melissa Halpern, David S. Pisetsky, Amy Feagin, and Dennis M. Klinman

Microbial DNA has multiple immune effects including the capacity to induce polyclonal B cell activation and cytokine production in normal mice. We recently described the accelerated induction of anti-DNA Abs in NZB/NZW mice immunized with Escherichia coli (EC) dsDNA; paradoxically these mice developed less renal disease than unimmunized mice or mice immunized with calf thymus DNA. We postulated that alterations in cytokine production induced by bacterial DNA may play a key role in renal protection. To determine the effect of bacterial DNA on cytokine production in NZB/NZW mice, we measured the serum cytokine levels, cell culture supernatant cytokine levels, and number of cytokine-producing splenocytes in NZB/NZW mice injected with EC DNA, calf thymus DNA, or an immune active oligonucleotide. There was a 10- to 25-fold increase in the number of cells secreting IFN-γ compared with IL-4 in mice immunized with EC DNA. IL-12-secreting cells were also increased by bacterial DNA immunization. In parallel with the increase in IFN-γ secreting cells, there was a significant rise in serum IFN-γ levels in mice receiving EC DNA. These results indicate that EC DNA modulates systemic cytokine levels in NZB/NZW mice, selectively increasing IL-12 and IFN-γ while decreasing IL-4 production. The cytokine response of NZB/NZW mice to bacterial DNA may be of significance in disease pathogenesis and relevant to the treatment of lupus-like disease. The Journal of Immunology, 1998, 161: 3890–3895.

Materials and Methods

Mice

Four-week-old female NZB/NZW mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Mice were kept under specific pathogen-free conditions at the FDA, Durham Veterans Affairs Medical Center (VAMC), and Ralph H. Johnson VAMC animal facilities. Routine serologic screening for common murine pathogens was negative in the mice used for these experiments.
DNA

EC and CT DNA were obtained from Sigma (St. Louis, MO). The DNA was purified by repeated phenol extractions followed by chloroform/isoamyl alcohol extraction with a final ethanol precipitation step. After drying, the DNA was resuspended in SSC at a concentration of 0.5 μg/ml. dsDNA was derived by treating the DNA with S1 nuclease (Sigma) as recommended by the manufacturer. DNA concentration was determined by measuring OD260 absorbance on a UV Max spectrophotometer. Purity of DNA was determined by OD260/280 ratio. All DNA used in these experiments had an OD260/280 ratio > 1.8. All DNA preparations were assayed for LPS content using the Limulus amebocyte assay (QCL-1000, BioWhitaker, Walkersville, MD). LPS content was less than 0.01 U of endotoxin/μg of DNA similar to lots of DNA used in previous studies (1–6).

To determine any bioactivity of LPS or other bacterial contaminants in the DNA preparations, the EC DNA was DNased with DNaseI (Sigma). There was no detectable proliferation by BALB/c splenocytes over baseline when the DNased EC DNA was added at a 50 μg/ml concentration (measured before DNased treatment). An aliquot of the EC DNA was treated with CpG methylase (New England Biolabs, Beverly, MA). Methylation was confirmed by differential restriction enzyme analysis. The methylated EC DNA did not stimulate B cell proliferation, induce an anti-DNA response, or stimulate IFN-γ production by BALB/c splenocytes. Finally, EC DNA derived from 30 μg/ml to splenocytes derived from C3H/HeJ and C3H/HeN mice (strains that are resistant or sensitive to LPS, respectively). EC DNA stimulated similar levels of splenocyte proliferation and IFN-γ production in the two strains. These results provide strong evidence that the EC DNA was the immune active agent in these experiments and not a contaminant such as LPS.

The sequence of the active oligonucleotide used for these experiments was GCTAGACGTAGCTAGGCT containing the immune active CpG motif. The inactive palindrome was the same sequence with reversal of the CG motifs (i.e., GCTAGACGTAGCTAGGCT). Purity of the oligonucleotides was assessed similarly to the EC DNA. The oligonucleotides all contained less than 0.01 μl of LPS/μg of oligonucleotide.

In vitro experiments

Spleens were removed from unmanipulated 10-wk-old female NZB/NZW mice. Single-cell suspensions were made prior to cell separation using Ficoll centrifugation. Cells were then plated at 1 × 10^5 cells/well in 96-well tissue culture plates. To individual wells, EC DNA and CT DNA were added at 30 μg/ml. After an 8-h incubation at 37°C, the cells were harvested and assayed for cytokine production via ELISPOT. In separate experiments, spleens were removed from five unmanipulated 10-wk-old NZB/NZW female mice and cells prepared as listed above. To individual wells were added EC DNA and DNased EC DNA at 10 μg/ml and 50 μg/ml and Con A at 2 μg/ml as a positive control. Supernatants were removed at 24 h and 72 h and tested for IFN-γ and TNF-α content by sandwich ELISA. This experiment was repeated to confirm results obtained.

Immunizations

Four-week-old NZB/NZW female mice were divided into immunization groups of 6 to 12 mice and studied using two different immunization protocols. The first groups of 3 to 6 mice were injected with DNA (50 μg/mouse/injection) complexed with methylated BSA (mBSA; 75 μg/mouse/injection) emulsified in incomplete adjuvant (0.3 ml total emulsion/mouse) followed 6 wk later by a second immunization in incomplete adjuvant. Groups of 3 to 6 mice were sacrificed 3 and 13 days after the immunization and bone marrow cells and peripheral blood mononuclear cells (PBMC) were harvested and utilized DNA alone without adjuvant or mBSA; thus mice were injected with EC DNA (50 μg/mouse) or CT DNA (50 μg/mouse) alone and again 6 wk later. Groups of 3 to 6 mice were then sacrificed at 3 days and 13 days after the final injection. Untreated mice were sacrificed to determine a baseline of number of cytokine-producing cells for each group (day 0).

Cytokine-specific ELISPOT assay

Ninety-six-well nitrocellulose-backed microtiter plates (Millipore, Bedford, MA) were coated with 10 μg/ml of anti-IL-4, anti-IL-6, anti-IL-12, or anti-IFN-γ in 0.1 M carbonate buffer (pH 9.6) and then blocked with PBS-5% BSA as previously described (15). Serial twofold dilutions of a single cell suspension starting with 10^6 cells/well were incubated on the plates for 6 h in a 5% CO₂ incubator. The plates were then washed with PBS-Tween and overlaid with 1 μg/ml of biotinylated anti-cytokine Ab overnight at 4°C. Washed plates were treated with a 1/2000 dilution of avidin-conjugated alkaline phosphatase (Vector Laboratories, Burlingame, CA) and then with BCIP/NBT (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Previous studies showed that cytokine ELISPOT assays detect cells secreting approximately 100 molecules/s (4).

Cytokine levels

Sera and cell culture supernatant levels of cytokines were determined by sandwich ELISA (3). Sera were tested at a 1/5 dilution for IFN-γ and IL-4. TFN-α and IFN-γ were assayed in the cell culture supernatants. Spleen cells were isolated by Ficoll and cultured in RPMI 1640/10% FCS at 10^6 cells/well for 1 to 3 days following the addition of DNA or Con A as mentioned above. Supernatants were removed and tested for cytokine content by ELISA at a 1/10 dilution.

Anti-DNA

Serum anti-DNA levels were determined by ELISA as previously described (1). Briefly, microtiter plates were coated with CT dsDNA at 5 μg/ml in SSC. Sera were added starting at a 1/100 dilution. Goat anti-mouse horseradish peroxidase conjugate was added followed by 5,5’,3’,3’-tetramethylbenzidine in 0.1 M citrate, pH 4, with 0.015% H₂O₂. OD₅₅₀ absorbance was measured on a Flow Microtiter Plate Reader. For isotype assays, isotype-specific conjugates were used that had been previously tested and titrated using isotype standards to ensure that there was equivalent detection of each isotype in the assay.

Statistics

Statistical analysis utilized the Mann-Whitney two-tailed nonparametric measure unless otherwise indicated.

Results

In vitro effects of bacterial DNA on cytokine production by NZB/NZW spleen cells

Initial experiments utilized spleen cells from groups of three 10-wk-old female NZB/NZW mice, as animals of that age have not yet developed clinical signs or symptoms of autoimmune disease. Spleen cells (10^7/well) were cultured in vitro for 8 h in the presence of 30 μg/ml of DNA of EC or CT origin. Previous experiments established 30 to 50 μg/ml as the maximal stimulatory dose of EC DNA. As shown in Table I, bacterial, but not mammalian, DNA stimulated a significant number of cells to secrete IL-6, IL-12, and IFN-γ in vitro. By comparison, neither type of DNA induced the production of IL-4 or IL-10 (data not shown). Similar results were obtained when the cells were cultured for 24 h (data not shown). These results are similar to the response of BALB/c spleen cells to EC DNA as previously reported (4).

Bacterial DNA contains sequence motifs known to stimulate cytokine production by spleen cells from normal mice (3, 4). These sequence motifs consist of a central unmethylated CpG dinucleotide flanked by two 5’ purines and two 3’ pyrimidines (4, 5). To examine whether such motifs contributed to the EC activation of NZB/NZW spleen cells by bacterial DNA, an oligonucleotide expressing this motif was synthesized. An oligonucleotide in which

<table>
<thead>
<tr>
<th>DNA</th>
<th>IL-6</th>
<th>IL-12</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>140 ± 38</td>
<td>236 ± 76</td>
<td>21 ± 7</td>
</tr>
<tr>
<td>EC DNA</td>
<td>592 ± 107</td>
<td>740 ± 83</td>
<td>58 ± 11</td>
</tr>
<tr>
<td>CT DNA</td>
<td>152 ± 28</td>
<td>260 ± 105</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>CpG ODN</td>
<td>736 ± 32</td>
<td>912 ± 225</td>
<td>48 ± 10</td>
</tr>
<tr>
<td>Non-CpG ODN</td>
<td>170 ± 32</td>
<td>260 ± 105</td>
<td>25 ± 6</td>
</tr>
</tbody>
</table>

Values presented are the mean ± SD of cytokine-producing cells/10⁶ spleen cells for each cytokine following incubation with the listed DNA for 8 h. *Values were derived by ELISPOT assay. n = 3 mice with three wells counted per mouse. For each cytokine tested, the differences between EC and CT DNA and CpG ODN and non-CpG ODN were significant at p < 0.05.
Table II. Cytokine levels in supernatants of spleen cell cultures of NZB/NZW mice

<table>
<thead>
<tr>
<th>Group</th>
<th>IFN-γ (U/ml)</th>
<th>TNFα (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>&lt;1</td>
<td>&lt;10</td>
</tr>
<tr>
<td>EC DNA (10 µg/ml)</td>
<td>3.4 ± 0.8</td>
<td>&lt;10</td>
</tr>
<tr>
<td>EC DNA (50 µg/ml)</td>
<td>35.6 ± 6.7</td>
<td>26.8 ± 8.3*</td>
</tr>
<tr>
<td>DNased EC DNA (10 µg/ml)</td>
<td>4.6 ± 1.5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>DNased EC DNA (50 µg/ml)</td>
<td>3.0 ± 2.9</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Values presented are the mean ± SE of cytokine levels in NZB/NZW splenocyte (10⁶/well) supernatants collected 24 or 72 h after stimulation. These results are combined from two separate experiments using five 10-wk-old female mice for each experiment. Each individual supernatant was tested in triplicate and a mean derived for that mouse. The IFN-γ levels were derived from the 24-h culture supernatant, the TNFα levels from the 72-h culture period. Asterisks represent statistically significant differences between the EC DNA (50 µg/ml) group and all other groups (p = 0.0017).

The central CpG was inverted (resulting in a loss of immunostimulatory activity) was used as a control. When spleen cells from NZB/NZW mice were cultured with 30 µg/ml of the CpG-containing oligonucleotide, increased production of IL-6, IL-12, and IFN-γ was observed (Table I). In contrast, the oligonucleotide lacking this CpG dinucleotide induced no cytokine production over background. DNase-treated CpG containing oligonucleotide did not stimulate IL-6, IL-12, or IFN-γ production above baseline (data not shown).

In control experiments to rule out bacterial contaminants in our DNA preparations as stimulants of cytokine production, splenocyte cultures from 10-wk-old NZB/NZW mice were incubated with 10 and 50 µg/ml of EC DNA or DNased EC DNA. Con A was used as a positive control. Supernatants were harvested at 24 h and 72 h and tested for IFN-γ and TNFα content. As shown in Table II, EC DNA at 50 µg/ml induced significant production of IFN-γ and TNFα while DNased EC DNA did not. Con A stimulated similar levels of IFN-γ production as EC DNA (50 µg/ml). For IFN-γ, supernatant levels were similar at 24 and 72 h (data not shown at 72 h); TNF-α was undetectable at 24 h in all of the supernatants. These results indicate that it is the EC DNA, not non-DNA bacterial contaminants, that stimulated IFN-γ and TNF-α production by preimmune NZB/NZW splenocytes.

Effect of bacterial DNA on NZB/NZW mice: in vivo studies of cytokine production

To examine the in vivo effect of bacterial DNA on cytokine-producing cells, NZB/NZW mice were treated two times at a 6-week interval with DNA/mBSA in IFA. As shown in Figure 1, the profile of cytokines induced in NZB/NZW mice treated with EC DNA differed from those receiving CT DNA or mBSA.

Three days and two weeks after the final immunization, there were significantly fewer cells producing IL-4 (Fig. 1A) and significantly more cells producing IL-12 (Fig. 1B) and IFN-α (Fig. 1C) in the EC DNA-treated group than the CT DNA or mBSA-treated groups. The time frame and dynamics of individual cytokine production varied. Decreased IL-4 secretion was present at day 3, while increases in IL-12 and IFN-γ producing cells were most evident at day 13. We were unable to detect TNF-α-secreting cells due to technical limitations of that assay. The net effect of the observed changes in the cytokine profile was to skew the ratio of IFN-γ:IL-4-secreting cells from 1:1 in CT DNA-treated mice to 9:1 in EC DNA-treated animals at day 13 after the final immunization. In general, the number of cells secreting each of these cytokines was similar in the CT- and mBSA-treated groups. The fall in IFN-γ-secreting cells between day 3 and day 13 in the mBSA and CT DNA groups suggests an adjuvant effect that decreased over time. The number of IFN-γ and IL-12-secreting cells was statistically greater in the EC DNA-treated mice at 13 days and the number of IL-4-producing cells significantly less in the EC DNA group compared with the other two groups at both 3 and 13 days. No differences between groups were observed among cells secreting IL-2, IL-5, IL-6, or IL-10 (data not shown).

To separate DNA effects from adjuvant effects on cytokine production using these assays, a different set of NZB/NZW mice was injected with DNA alone without adjuvant or mBSA. As above, cytokine production by spleen cells was analyzed 3 days and 2 wk after boost of DNA. None of the DNA treatments stimulated cells to secrete IL-6 or IL-10 (data not shown). Yet at both 3 days and 2 wk after boost, mice injected with EC DNA had fewer cells secreting IL-4 and more cells secreting IL-12 and IFN-γ than did CT DNA- or mBSA-treated animals (Fig. 2, A and B). Thus, in the

**FIGURE 1.** Number of cytokine-producing cells per 10⁶ splenocytes from NZB/NZW mice 3 and 13 days following immunizations with DNA/adjuvant emulsions. Values presented are the mean cell number ± SD of cells secreting IL-4 (A), IL-12 (B), and IFN-γ (C) as determined by ELISPOT (n = 3–6 mice/group). The difference between the EC DNA group and the other two groups is significant at p < 0.05 for all three cytokines at day 13. At day 3, only the number of IL-4-secreting cells is statistically significantly different between the EC DNA and the other two groups.
absence of adjuvant or carrier, EC DNA had significant and pro-
longed in vivo effects on cytokine production in NZB/NZW mice. As was found in mice immunized in the presence of adjuvant, EC DNA alone caused a profound increase in the ratio of cells secreting IFN-γ:IL-4 when compared with the CT DNA-injected group at day 13 (25:1 IFN-γ/IL-4 cells in the EC DNA group vs 1:1 in the CT DNA group). A similar effect on cytokine-secreting cell profiles was induced by treating NZB/NZW mice with oligo-
nucleotides containing immunostimulatory CpG motifs (Fig. 2). Thus, these motifs may account (at least in part) for the ability of bacterial DNA to activate IFN-γ and IL-12 production while re-
ducing IL-4 production in vivo.

Cytokine levels in the serum of DNA-immunized NZB/W mice
Sera obtained at the time of sacrifice were assayed for IFN-γ and IL-4 content by ELISA. The amount of sera required for testing precluded measuring other cytokines. When animals were primed and boosted in the presence of IFA, increased IFN-γ titers were detected in all groups at day 3; however at day 13, sera from mice immunized with EC DNA contained significantly more IFN-γ than sera from mice in any of the other groups (Fig. 3A). To address the adjuvant effect, we analyzed cytokine levels in the sera of mice injected with DNA alone. As seen in Figure 3B, EC DNA treatment resulted in significantly elevated IFN-γ levels 2 wk after treatment, compared with CT DNA or PBS-treated controls. Sera IFN-γ levels were not elevated in mice immunized with immunostimulatory Cpg-containing oligonucleotides, despite ELISPOT data showing that the number of cells producing IFN-γ was in-
creased in this group. IFN-γ levels were also undetectable in NZB/ NZW mice immunized with DNased EC DNA alone (data not shown). Sera IL-4 levels were not detectable by sandwich ELISA assay in any of the treatment groups (data not shown).

Effect of EC DNA on the isotype of IgG anti-DNA autoantibodies
To determine if the cytokine profile induced by EC DNA treatment affected anti-DNA Ab production or the isotype of the anti-DNA Abs produced, the concentration and isotype of IgG anti-DNA Abs present in these mice were also examined. Sera from mice treated with EC DNA/mBSA in incomplete adjuvant contained significant amounts of anti-CT dsDNA Abs as determined by ELISA (Table III). Moreover, the ratio of IgG2a/IgG1 anti-DNA in the sera of the

Table III. Serum anti-DNA Ab levels and isotype of treated NZB/NZW mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Anti-DNA^a</th>
<th>IgG2a/IgG1 Ratio^b</th>
<th>Anti-DNA Titer Units^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC DNA/IFA</td>
<td>1.13 ± 0.24</td>
<td>2.1 ± 0.1</td>
<td>340</td>
</tr>
<tr>
<td>mBSA/IFA</td>
<td>0.32 ± 0.02</td>
<td>0.8 ± 0.1</td>
<td>56</td>
</tr>
<tr>
<td>EC DNA alone</td>
<td>0.44 ± 0.18</td>
<td>ND</td>
<td>110</td>
</tr>
<tr>
<td>mBSA alone</td>
<td>0.16 ± 0.07</td>
<td>ND</td>
<td>8</td>
</tr>
</tbody>
</table>

^a Anti-DNA values represent the mean ± SD of the OD₅₉₀ absorbance of sera (n = 5) at a 1/100 dilution tested by ELISA using CT dsDNA as test Ag.
^b IgG2a/IgG1 ratios were determined by ELISA using isotype-specific conjugates that were titered on isotype standards to insure they detected equal amounts of Ig at the concentrations used in these assays.
^c Anti-DNA titer units represent the inverse of sera titer required to produce an OD₅₉₀ absorbance of 0.5 by ELISA on CT dsDNA.
EC DNA group differed from that of mice receiving mBSA. This isotype difference suggests a systemic effect of EC DNA-induced IFN-γ production leading to IgG2a isotype switching.

Similarly, the IgG anti-CT DNA titers in NZB/NZW mice treated with EC DNA in the absence of adjuvant were elevated when compared with mBSA-treated animals (Table III). Nevertheless, anti-DNA levels were manifold lower than those achieved when EC DNA is injected in an emulsion complexed with mBSA.

Discussion

Findings in this report indicate that bacterial DNA induced significant changes in the profile of cytokines actively secreted in vitro and in vivo in NZB/NZW mice. The significant changes included an increase in the number of cells producing IFN-γ and IL-12 coupled with a decrease in the number of cells secreting IL-4. These changes culminated in a marked (10 to 25-fold) increase in the ratio of IFN-γ:IL-4 secreting cells in vivo; these alterations in the number of cytokine-secreting cells were reflected by changes in serum cytokine levels. EC DNA immunization also induced the production of anti-DNA Abs in NZB/NZW mice, and shifted the predominant isotype from IgG1 to IgG2a. The isotype shift likely resulted from increased systemic IFN-γ levels, as IFN-γ selectively promotes the production of IgG2a, while inhibiting the production of IgG1 Abs in both normal and lupus-prone mice (16).

A number of investigators have examined the effects of cytokine-based therapies on disease in lupus-prone mice. Their experiments helped elucidate the role of specific Th1 and Th2 cytokines on disease progression, and promoted understanding of the association between changes in T cell function and the development of autoimmunity. Studies to this point suggest that disease in NZB/NZW mice is caused by abnormalities in the balance between Th1 and Th2 cytokines. Evidence for a strong Th2 component include the ability of anti-IL-4 therapy to lower autoantibody production and reduce renal disease in NZB/NZW mice, and of anti-IL-10 to prevent disease. Indeed, administering IL-10 to NZB/NZW mice worsens disease (17, 18). Support for a Th1-based contribution to disease includes the ability of anti-IFN-γ treatment to prevent disease and of anti-IL-12 to lower autoantibody production (17, 19). Adding to this complexity are results from studies involving TNF-α-infected cells, which show that low doses of that cytokine prevent disease in NZB/NZW mice, moderate doses have no effect, and high doses worsen disease (20, 21).

In the context of our current findings, the decreased IL-4 production associated with bacterial DNA immunization could reduce disease in NZB/NZW mice in a manner similar to anti-IL-4 therapy (17); yet increased production of IL-12 and IFN-γ would be predicted to be harmful (17, 19). The ability of anti-IFN-γ to reduce disease does not necessarily mean that supranormal levels of IFN-γ are harmful in NZB/NZW mice. As found with TNF-α, the effects of a particular cytokine on disease may vary with the level of the cytokine (20, 21). We were able to detect changes in TNF-α only by assaying cell culture supernatants; these experiments and previous reports indicate bacterial DNA induces TNF-α production. If low levels of TNF-α were induced by EC DNA, these low levels could play a role in the beneficial effect of EC DNA on disease expression. In studies of human lupus, we found that the ratio between Th1 and Th2 cytokine-secreting cells, rather than the absolute number of cells secreting any single cytokine, correlated most closely with disease severity (22). Consistent with findings in humans, an increased ratio of Th1 vs Th2 cytokines in NZB/NZW mice was associated with improved clinical outcome.

Several technical approaches were combined in this report to study and confirm changes in cytokine production induced by bacterial DNA in a lupus-prone strain of mice. ELISPOT analyses were used to detect the effect of bacterial DNA in vivo on cells in the primary lymphoid organs. The great sensitivity of these assays (which can detect a single cell/10^6 releasing <100 molecules of cytokine/second) led to the observation that IL-4-secreting cell numbers decreased following EC DNA administration (15). The suppression of IL-4 by EC DNA has not previously been reported in either normal or lupus mice. We also analyzed serum cytokine levels, which reflect the time-averaged rates of cytokine production vs degradation on a systemic level, and in vitro levels of cytokine production by DNA-stimulated splenocytes derived from unimmunized mice. These various assays demonstrated the specificity and rapidity of the cytokine responses elicited by bacterial DNA administration.

By using these assays to define the full spectrum of bacterial DNA-immune activity, our understanding of the interaction of bacterial DNA with the immune system is increasing. Many basic questions remain unanswered, however, including the mechanism by which bacterial DNA enters and activates cells of the immune system (23). Previous studies established that exposure to EC DNA and CpG-based oligonucleotides triggered the production of IL-6, IL-12, IFN-γ, and TNFα in normal mice (3-5). These cytokines were produced by T cells, B cells, macrophages, and NK cells. Our results suggest that NZB/NZW cells respond similarly to microbial DNA, as a similar profile of cytokines was elicited. Increased IFN-γ and IL-12 production was detected when NZB/NZW spleen cells were stimulated with bacterial DNA either in vivo or in vitro, yet increased IL-6 production was only observed in vitro. We believe this reflects EC DNA having a rapid but short-lived effect on IL-6 transcription. Studies of normal mice indicate that IL-6 mRNA is up-regulated within minutes and returns to baseline levels within a few hours of EC DNA administration (4, 5). Secretion of IL-6 protein is maximal 10 to 12 h after stimulation, and returns to baseline after 1 day. Consistent with these findings, increased IL-6 production was detected when NZB/NZW spleen cells were treated in vitro for 8 h, but absent in mice examined 3 days later.

In contrast to the short term effect of bacterial DNA on IL-6 production, our studies indicate that some of the immunomodulatory effects of EC DNA may persist for weeks or months. Profound changes in the cytokine profile of NZB/NZW mice were present more than 2 wk postinjection in the absence of adjuvant, and an effect of bacterial DNA on disease progression was detected through the first year of life (11). This raises the possibility that naked DNA (which is rapidly cleared from the circulation of normal and autoimmune mice) may be sequestered in vivo. Alternatively, the effects of bacterial DNA on the Th1:Th2 ratio may be self-perpetuating, since the maturation of naive CD4^+ cells into Th1 cells is promoted by IFN-γ, which inhibits their maturation into Th2 cells (24).

The similar cytokine profile induced by EC DNA and the CpG-containing oligonucleotide suggests that the CpG motif is responsible for most of the cytokine stimulatory activity of the EC DNA. Although qualitatively the CpG oligonucleotide and EC DNA were similar in their cytokine effects, quantitatively they may differ. EC DNA appears to have a more profound effect on IFN-γ production than does the CpG-containing oligonucleotide (Figs. 2 and 3) and stimulates anti-DNA production while the CpG oligonucleotide does not (Table III). The DNased EC DNA experiments were performed to determine if a non-DNA bacterial contaminant was present in the EC DNA preparation that was also stimulatory for IFN-γ production. The lack of a stimulatory effect of the DNased EC DNA suggests that it is indeed the EC DNA alone that is inducing IFN-γ production. As we and others have shown, there
are other non-CpG-containing sequences in bacterial DNA that are immune stimulatory (6, 7). These additional immune active sequences may be responsible for the quantitative differences in IFN-γ stimulation between EC DNA and CpG-containing oligonucleotides.

Determining which of the many immunologic effects of bacterial DNA ameliorates renal disease in NZB/NZW mice will be difficult. Lupus disease progression is based on a complex interplay between numerous cell types and cytokines (25). EC DNA treatment alters this milieu; we are likely only detecting some, but certainly not all, of the immune effects of bacterial DNA in these experiments. Attempts to study individual elements of these changes (i.e., increased IFN-γ or decreased IL-4) fails to address the entire spectrum of immune effects of EC DNA treatment. Moreover, changes in the concentration of a single cytokine may alter other elements of the immune system, obscuring the impact of the original change.

In overview, our results provide evidence that the effects on cytokine production by bacterial DNA are systemic and long lasting and are not dependent on coadministration of adjuvant or a protein carrier. The lack of activity of DNased bacterial DNA eliminates contaminants as a possible stimulator of these effects. The stimulatory effects of EC DNA appear to be mediated, at least in part, by CpG-based sequence motifs common in bacterial, but not mammalian DNA (26). Evidence to date suggests that bacterial DNA has similar effects on spleen cells from NZB/NZW mice and normal mice. Thus, the immunomodulatory effects of bacterial DNA likely depend on the state of the animal’s immune system at the time of exposure to immune active DNA (25). This has important implications for the use of bacterial DNA, or synthetic CpG-based oligonucleotides (which can be manufactured in large quantity under controlled conditions) as immunotherapeutic agents. Ongoing efforts are directed toward further defining the protective efficacy of bacterial DNA in models of human lupus.

References


