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Bacterial DNA or Oligonucleotides Containing Unmethylated CpG Motifs Can Minimize Lipopolysaccharide-Induced Inflammation in the Lower Respiratory Tract Through an IL-12-Dependent Pathway[1]

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To determine whether the systemic immune activation by CpG DNA could alter airway inflammation, we pretreated mice with either i.v. bacterial DNA (bDNA) or oligonucleotides with or without CpG motifs, exposed these mice to LPS by inhalation, and measured the inflammatory response systemically and in the lung immediately following LPS inhalation. Compared with non-CpG oligonucleotides, i.v. treatment with CpG oligonucleotides resulted in higher systemic concentrations of polymorphonuclear leukocytes, IL-10, and IL-12, but significantly reduced the concentration of total cells, polymorphonuclear leukocytes, TNF-α, and macrophage inflammatory protein-2 in the lavage fluid following LPS inhalation. The immunoprotective effect of CpG-containing oligonucleotides was dose-dependent and was most pronounced in mice pretreated between 2 and 4 h before the inhalation challenge, corresponding to the peak levels of serum cytokines. bDNA resulted in a similar immunoprotective effect, and methylation of the CpG motifs abolished the protective effect of CpG oligonucleotides. The protective effect of CpG oligonucleotides was observed in mice with either a disrupted IL-10 or IFN-γ gene, but release of cytokines in the lung was increased, especially in the mice lacking IFN-γ. In contrast, CpG DNA did not protect mice with a disrupted IL-12 gene against the LPS-induced cellular influx, even though CpG DNA reduced the release of TNF-α and macrophage inflammatory protein-2 in the lung. These findings indicate that CpG-containing oligonucleotides or bDNA are protected against LPS-induced cellular airway inflammation through an IL-12-dependent pathway, and that the pulmonary cytokine and cellular changes appear to be regulated independently. ***The Journal of Immunology,*** **1999, 163: 224–231.**

Recently, we have found that bacterial DNA (bDNA)[3] and certain oligonucleotides containing unmethylated CpG dinucleotides have stimulatory effects on murine and human lymphocytes in vitro and murine lymphocytes in vivo when compared with the effects of eukaryotic DNA or methylated oligonucleotides that are nonstimulatory (1). Unmethylated CpG motifs are ~20-fold more common in bDNA than in vertebrate DNA. CpG motifs appear to be underrepresented in vertebrate genomes; when present, they are more likely to be methylated. DNA containing unmethylated CpG motifs results in B cell proliferation and in the release of IL-6 and IL-10 (2–5), NK cell activation, secretion of IFN-γ (2, 6–8), and monocyte activation with elevated production of TNF-α and IL-12 (9, 10). Further evaluation of the immunologic response to CpG-containing oligonucleotides indicates that these specific motifs stimulate a Th1-like inflammatory response dominated by the release of IL-12 and IFN-γ (2). However, oligonucleotides containing CpG motifs also stimulate the production and secretion of IL-10 (4, 5), a potent immunosuppressive cytokine (11). Thus, in addition to the potential adjuvant effects of oligonucleotides containing CpG motifs (12–17), these agents are particularly effective in substantially modifying a Th2 cytokine-driven inflammatory response, making it more Th1-like (18). Moreover, the potent immunologic activation by unmethylated CpG motifs suggests that the vertebrate immune system uses these unique bDNA characteristics to trigger innate immune defenses against infection by microorganisms.

Endotoxin is one of the primary mediators of inflammation released by Gram-negative organisms and appears to be an important cause of environmentally induced airway disease. Inhaled endotoxin is strongly associated with the development of acute decrements in airflow among cotton workers (19–21), swine confinement workers (22), and poultry workers (23). We have shown that the concentration of endotoxin in the bioaerosol appears to be the most important occupational exposure associated with the development (24) and progression (25) of airway disease in agricultural workers. In addition, the concentration of endotoxin in the domestic environment adversely effects asthmatics, with higher concentrations of ambient endotoxin associated with greater degrees of airflow obstruction (26, 27). Physiologically, inhaled endotoxin (26, 28–31) can cause airflow obstruction in naive or previously

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[3] Abbreviations used in this paper: bDNA, bacterial DNA; PMN, polymorphonuclear leukocyte; pfw, pyrogen-free water; KO, knockout; MIP, macrophage inflammatory protein.

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unexposed subjects. Furthermore, asthmatic individuals develop airflow obstruction at lower concentrations of inhaled endotoxin than normal controls (31). Exposure-response studies have shown that inhaled LPS results in recruitment of neutrophils, activation of macrophages with production and release of proinflammatory cytokines, and damage to airway epithelia in a dose-dependent manner (32–35). In aggregate, these studies indicate that endotoxin is a potentially important cause of airway disease among exposed individuals.

Given the immune modulating effects of CpG-containing oligonucleotides and the possibility that the vertebrate immune system uses the unique unmethylated CpG motifs in bDNA to defend against the potentially adverse effects of microorganisms, we reasoned that systemic treatment with either bDNA or these oligonucleotides containing unmethylated CpG motifs may reduce the inflammatory response to inhaled LPS. Because IFN-γ is a key cytokine mediating LPS-induced inflammation (36–38) and because IL-10 inhibits IFN-γ via a macrophage-dependent step (39), we further hypothesized that these CpG-containing oligonucleotides would reduce the inflammatory response to LPS by increasing the production and release of IL-10. Although our findings demonstrate that the systemic administration of either bDNA or oligonucleotides containing unmethylated CpG motifs does indeed suppress the inflammatory response to inhaled LPS, IL-12 rather than IL-10 or IFN-γ appears to be important in mediating this process.

Materials and Methods

Experimental protocol

In the first series of experiments, mice were treated i.v. with 20 base oligonucleotides containing CpG motifs (CpG oligo) or 20 base oligonucleotides without embedded CpG motifs (non-CpG oligo) at 1, 2, 3, 4, 6, or 12 h before a 4-h inhalation challenge with *Escherichia coli* LPS (3–5 μg/m^3). Next, we determined whether bDNA was also effective in protecting against the inflammatory effects of inhaled LPS by pretreating mice with either 25 μg of *E. coli* DNA or 25 μg of calf thymus DNA 2 h before an inhalation challenge with *E. coli* LPS. To determine whether unmethylated CpG motifs were responsible for the protective effect, we subsequently pretreated mice with oligonucleotides containing either unmethylated CpG motifs or methylated CpG motifs before an inhalation challenge with *E. coli* LPS. To determine the role of IL-10, IFN-γ, or IL-12, we pretreated IL-10 (C57BL/6-*IL-10*^{tm1Cgn}), IFN-γ (C57BL/6-*IFN-*γ^{tm1Ts}), or IL-12 (C57BL/6-*IL-12b*^{tm1Jm}) knockout (KO) mice with CpG-containing oligonucleotides and then performed a similar inhalation challenge with *E. coli* LPS. Immediately after inhalation challenge, all mice were sacrificed, blood samples were obtained, whole lung lavage was performed, and lungs were harvested for mRNA analysis. In all experiments, five mice were used for each condition.

Animals

C57BL/6, C57BL/6-*IL-10*^{tm1Cgn}, C57BL/6-*IFN-*γ^{tm1Ts}, and C57BL/6-*IL-12b*^{tm1Jm} male mice (The Jackson Laboratory, Bar Harbor, ME) were obtained at 6 wk of age and used within 2 wk. All animal care and housing requirements set forth by the National Institutes of Health Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources were followed, and animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee. Mice were maintained in wood-chip bedding (Northeastern Product, Warrensberg, NY), with food (Formulab Chow 5008, PMI, Richmond, IN) and water supplied ad libitum.

Oligonucleotides

A total of 20 base oligonucleotides were synthesized with and without the embedded CpG motifs (Oligos etc., Wilsonville, OR). These oligonucleotides contained a nuclease-resistant phosphorothioate-modified backbone and were purified by two rounds of ethanol precipitation before use. The CpG oligonucleotide was selected for study because of previous studies showing it to have a strong immunostimulatory effect (40). The "nonstimulatory" oligonucleotide was identical with the stimulatory oligonucleotide, except that the two embedded CpG motifs were modified; one appeared as an ApG motif and the other appeared as a GpC motif. The two synthesized oligonucleotides had the following sequences: CpG oligonucleotide, ATA AT*CG*A*CG*TTCAAGCAAG; non-CpG oligonucleotide, ATAAT*A* *GAGC*TTCAAGCAAG. Where indicated, a methylated oligonucleotide was synthesized by replacing the two underlined Cs in the CpG oligonucleotide sequence with 5-methylcytosine.

Genomic DNA

E. coli (strain B) DNA and calf thymus DNA were purchased from Sigma (St. Louis, MO) and purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation. The LPS level in the *E. coli* and calf thymus DNA was $<$0.06 ng/mg of DNA by *Limulus* assay. Heat-denatured (single-stranded) genomic DNA was used in all experiments.

Chemicals

Endotoxin was purchased as lyophilized, purified *E. coli* 0111:B4 LPS (PN# L2630, Sigma) and had a specified activity of 1.3×10^6 ng/mg and a protein content of $<$3%.

Endotoxin assay

The endotoxin concentrations of the LPS solution, LPS aerosol, oligonucleotides, and genomic DNA were assayed using the chromogenic *Limulus* amebocyte lysate assay (QCL-1000, BioWhittaker, Walkersville, MD) with sterile, pyrogen-free labware and a temperature-controlled microplate block and microplate reader (405 nm). The LPS solution was serially diluted in pyrogen-free water (pfw) and assayed. The airborne concentration of LPS was assessed by sampling 0.30 m^3 of air drawn from the exposure chamber through 47-mm binder-free glass microfiber filters (EPM-2000, Whatman International, Maidstone, U.K.) held within a 47-mm stainless steel in-line air sampling filter holder (Gelman Sciences, Ann Arbor, MI). Air-sampling filters were extracted with 10 ml of pfw at room temperature with gentle shaking for 1 h. Next, the filters were serially diluted with pfw and assayed for endotoxin. Four to six air samples were assayed for each exposure. All standard curves (0.1–1.0 endotoxin units/ml) achieved a linear regression coefficient exceeding $r = 0.995$. Our laboratory routinely runs spiked samples and filter blanks and participates in interlaboratory validation studies.

Exposure protocol and monitoring equipment

LPS aerosols were generated into a glass 20-liter exposure chamber using a PITT#1 nebulizer supplied with extract by a syringe pump. Liquid feed rates ranged from 0.0027 to 0.21 ml/min. High-efficiency particulate air-filtered air was supplied to the nebulizer at flow rates ranging from 10 to 17 L/min. Mixing within the chamber was aided by a magnetically coupled rotor. The chamber atmosphere was exchanged at 1 change/min. LPS concentrations were determined by sampling the total chamber outflow. Particle size distributions were determined with an aerodynamic particle sizer (TSI, St. Paul, MN) and gravimetrically with a Marble personal cascade impactor and Mylar media (41) by sampling within the exposure chamber.

Lung lavage

Immediately following the inhalation challenge, mice were euthanized, the chest was opened, and lungs were lavaged in situ via PE-90 tubing inserted into the exposed trachea. A pressure of 25 cm H_2O was used to wash the lungs with 6.0 ml of sterile pyrogen-free saline. Following whole lung lavage, the lungs were isolated, frozen in liquid nitrogen, and stored at -70°C.

Treatment of bronchoalveolar lavage fluid

Our standard method (33) of processing the sample is as follows: immediately following lavage, the volume is noted and 15-ml conical tubes are centrifuged for 5 min at $200 \times g$. The supernatant fluid is decanted and frozen at -70°C for subsequent use. The residual pellet of cells is resuspended and washed twice in HBSS (without Ca^{2+} or Mg^{2+}). After the second wash, a small aliquot of the sample is taken for cell count using a hemocytometer. Next, the cells are washed once more and resuspended in RPMI 1640 medium so that the final concentration gives a cell count of 1×10^6 cells/ml. The cells that are present in 10–12 (one of the 1×10^6 ml cell suspensions) are spun for 5 min onto a glass slide using a special filter card with a cytocentrifuge (Cytospin-2; Shanden Southern, Sewickley, PA). Staining is conducted using a Diff Quick Stain set (Harleco, Gibbstown, NY). The slide is then dried, one drop of optically clear immersion oil is put on the slide over the cells, and a coverslip is placed on top.

Cytokine analysis of lavage fluid and serum

Lavage fluid and serum were assayed for TNF-α, macrophage inflammatory protein-2 (MIP-2), IL-6, IL-10, IL-12 (p-40), and IFN-γ. In all cases, a polyclonal Ab specific for the murine recombinant cytokine (TNF-α, MIP-2, IL-6, IL-10, IL-12, or IFN-γ) was used as a capture reagent in a standard commercially available sandwich ELISA (R&D Systems, Minneapolis, MN). The limit for detection is 5.1 pg/ml for TNF-α, 1.5 pg/ml for MIP-2, 10 pg/ml for IL-6, 10 pg/ml for IL-10, 5 pg/ml for IL-12, and 10 pg/ml for IFN-γ.

Preparation of RNA and multiprobe RNase protection assay

Total RNA was extracted from lung specimens using the single-step method (42, 43), lysing flash-frozen lungs in RNA STAT-60 (Tel-Test B, Friendswood, TX). The composition of RNA STAT-60 includes phenol and guanidinium thiocyanate in a monophase solution. The lung parenchyma were homogenized in the RNA STAT-60 using a polytron homogenizer. Chloroform was added; total RNA was precipitated from the aqueous phase by addition of isopropanol, washed with ethanol, and solubilized in water. After drying the pellet in a vacuum desiccator, the yield and purity of the RNA were quantitated by measuring the ratio of absorbances at 260 and 280 nm. Minigel electrophoresis was used to confirm the integrity of the 28S and 18S rRNA bands. Gene transcripts were detected using the RNase protection assay as described previously (44). Equivalent amounts of RNA were examined, as judged by the amount of L32, which encodes an ubiquitously expressed ribosome subunit protein (45) in each sample. Commercially available probes were used to detect TNF-α, MIP-2, IL-6, IL-10, IL-12, and IFN-γ.

Statistical analysis

Four comparisons were pursued in this analysis: 1) the effect of i.v. CpG-containing oligonucleotides vs oligonucleotides without embedded CpG motifs in modulating the inflammatory response to inhaled LPS; 2) the effect of i.v. bDNA vs calf thymus DNA in modulating the inflammatory response to inhaled LPS; 3) the effect of unmethylated CpG motifs vs methylated CpG motifs in controlling the inflammatory response to LPS; and 4) the role of IL-10, IFN-γ, or IL-12 in mediating the protective effect of unmethylated CpG-containing oligonucleotides. The inflammatory response was assessed using lavage cellularity, lavage fluid cytokine concentration, serum concentration of cytokines, and the relative concentration of mRNA for specific cytokines in the lung parenchyma. Given the number of mice for each comparison (four to six per group), the Mann-Whitney *U* nonparametric statistical test was used to test all comparisons (46).

Results

CpG oligonucleotides resulted in a systemic inflammatory response. Although i.v. treatment with 25 μg of CpG oligonucleotides did not affect the total concentration of peripheral white blood cells compared with treatment with non-CpG oligonucleotides, treatment with CpG oligonucleotides resulted in a significantly higher concentration of polymorphonuclear leukocytes (PMNs) at 1, 2, 3, 4, 6, and 12 h postinjection (data not shown). As expected, i.v. treatment with CpG oligonucleotides also affected the concentration of cytokines in the serum. Compared with non-CpG oligonucleotides, CpG oligonucleotides resulted in an increase in the concentration of MIP-2, IL-6, IL-10, and IL-12 in the serum of mice at 4 h after i.v. administration (Fig. 1). These differences were most pronounced between 1 and 4 h after i.v. administration but were still present at 12 h after exposure to CpG-containing oligonucleotides. No significant differences were observed for the serum concentration of TNF-α and IFN-γ at any of the timepoints in mice pretreated with either oligonucleotide, although in some other mouse strains, these cytokines are easily detected after CpG treatment (6).

Pretreatment with 25 μg of CpG-containing oligonucleotides reduced the pulmonary response to inhaled LPS. Mice pretreated with CpG oligonucleotides at 1, 2, 3, 4, 6, or 12 h had a reduced concentration of cells and percentage of PMNs in the lavage fluid following inhalation challenge with LPS (2-h timepoint presented in Fig. 2). However, this effect appeared to be time-dependent, because pretreatment with CpG-containing oligonucleotides at 2, 3, 4, or 6 h resulted in a greater reduction in the concentration of lavage cells and in the percentage of lavage PMNs compared with pretreatment with the oligonucleotide either 1 or 12 h before the inhalation challenge. Although pretreatment with CpG-containing oligonucleotides resulted in significant changes in the concentration of cytokines in the lavage fluid, the changes in cytokine concentration were predominantly evident when mice were pretreated

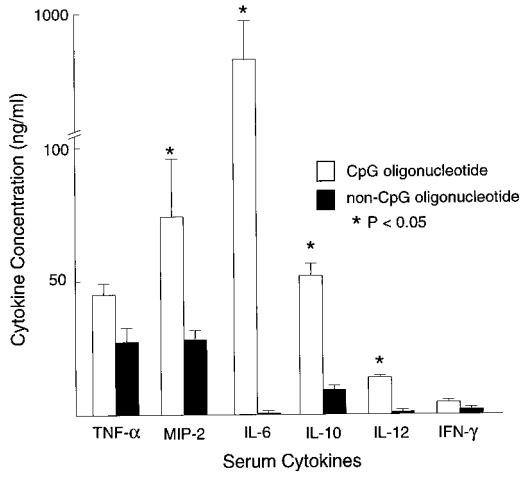

FIGURE 1. Concentration of specific cytokines (TNF-α, MIP-2, IL-6, IL-10, IL-12, and IFN-γ) in the serum at 4 h after i.v. treatment with either 25 μg of an oligonucleotide containing embedded CpG motifs or an oligonucleotide without CpG motifs. Five mice were used for each condition. Error bars, SE.

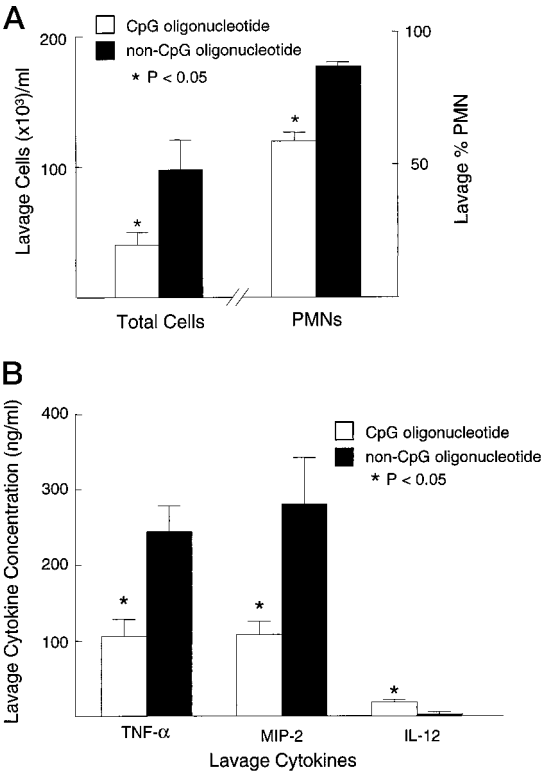

FIGURE 2. Concentration of total cells and percentage of PMNs (*A*) and concentration of cytokines (*B*) in the whole lung lavage fluid following inhalation of *E. coli* LPS. At 2 h before the inhalation challenge, mice were treated i.v. with 25 μg of an oligonucleotide containing embedded CpG motifs or with an oligonucleotide without CpG motifs. Five mice were used for each condition. Error bars, SE.

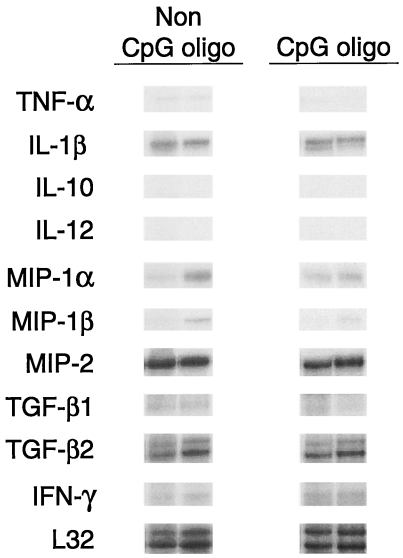

FIGURE 3. RNase protection assay of total RNA obtained from lungs of mice exposed to *E. coli* LPS by inhalation and pretreated i.v. either with 25 μg of an oligonucleotide without embedded CpG motifs or with an oligonucleotide containing embedded CpG motifs. L32 encodes a ribosomal protein and was used to assess equal RNA loading. The two lanes are from two different mice that were tested for each condition.

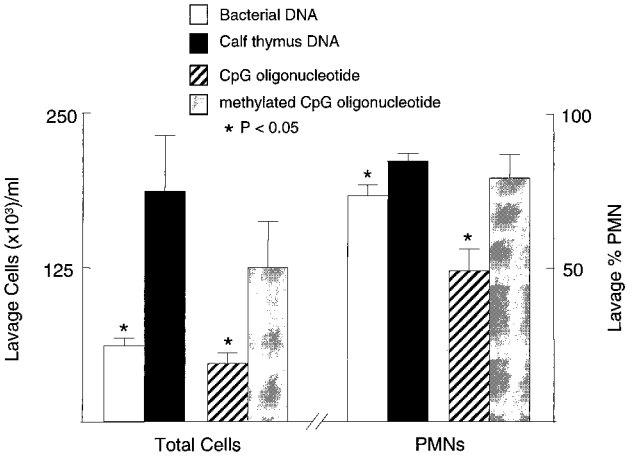

FIGURE 4. Concentration of total cells and percentage of PMNs in the whole lung lavage fluid following inhalation of *E. coli* LPS. At 2 h before the inhalation challenge, C57BL/6 mice were treated i.v. either with 25 μg of bDNA or calf thymus DNA, with 25 μg of an oligonucleotide containing embedded unmethylated CpG motifs, or with an identical oligonucleotide that contained embedded methylated CpG motifs. Five mice were used for each condition. Error bars, SE.

with CpG oligonucleotides 2 h before the inhalation challenge (differences in other timepoints not presented). Significant reductions were observed in the concentration of TNF-α and MIP-2 and in an elevation in the concentration of IL-12 in the lavage fluid following treatment with CpG oligonucleotides 2 h before the inhalation challenge (Fig. 2*B*). IL-6, IL-10, and IFN-γ were not measurable in the lavage fluid following inhalation of LPS at any of the timepoints. Interestingly, results from the RNase protection assay indicate that total lung mRNA concentrations for TNF-α, IL-1β, MIP-2, MIP-1α, MIP-1β, and IFN-γ are similar in the lung tissue of mice pretreated with CpG- or non-CpG-containing oligonucleotides 2 h before the LPS inhalation (Fig. 3), suggesting that the immunoprotective effects of CpG-containing oligonucleotides occur at a posttranscriptional level. mRNA for IL-10 and IL-12 was not evident in the lung tissue homogenate in these samples. In a dosing study, 10 μg of i.v. CpG-containing oligonucleotides was ineffective in promoting the protective effect, and 25 μg of i.v. oligonucleotides was as effective as 50 μg in minimizing the inflammatory response to inhaled LPS (data not presented). Based on these findings, all subsequent experiments were performed with 25 μg of oligonucleotides given 2 h before the inhalation challenge.

To determine whether the immunoprotective effects of CpG-containing oligonucleotides were generalizable to bDNA, we pretreated mice with 25 μg of either bDNA or calf thymus DNA; after 2 h, mice were exposed to an inhalation challenge with LPS. Although bDNA effectively decreased the cellular and neutrophilic response to inhaled LPS (Fig. 4), no changes were observed in the concentrations of lavage TNF-α, MIP-2, or IL-12 or the in serum concentration of TNF-α, IL-6, IL-10, or IFN-γ (cytokine data not presented). Interestingly, bDNA, in comparison with calf thymus DNA, resulted in a significant increase in the concentration of IL-12 in the serum (14,581.6 $\pm$ 2,067.6 vs 2,120.2 $\pm$ 747.7 ng/ml; $p = 0.009$), suggesting that the serum concentration of IL-12 might be important in the immunoprotective effect of bDNA.

To determine the role of the unmethylated CpG motifs in suppressing the inflammatory response to inhaled LPS, we synthesized an oligonucleotide with methylated CpG motifs and compared its immunoprotective effects with those of the oligonucleotide with unmethylated CpG motifs. Methylating the CpG motifs abolished the protective effect of CpG oligonucleotides in preventing the cellular inflammatory response to inhaled LPS (Fig. 4).

Because endogenous and exogenous IL-10 are known to suppress the inflammatory response to LPS (11, 47) and CpG oligonucleotides increase the production and secretion of IL-10 (4, 5), we reasoned that IL-10 might be playing a critical role in mediating the immunoprotective effects of CpG oligonucleotides. To pursue this hypothesis, we pretreated mice genetically deficient in IL-10 (C57BL/6-*IL-10*^{tm1Cgn}) with oligonucleotides with or without CpG motifs and subsequently performed an inhalation challenge with *E. coli* LPS. Compared with pretreatment with i.v. oligonucleotides not containing a CpG motif, CpG-containing oligonucleotides significantly reduced the total cellularity and the percentage of PMNs in the lavage fluid in mice with a disrupted IL-10 gene (C57BL/6-*IL-10*^{tm1Cgn}) (Fig. 5*A*). Although the concentration of TNF-α and MIP-2 was similar in the lavage fluid between these treatment groups, IL-12 was markedly elevated in the mice pretreated with CpG-containing oligonucleotides (Fig. 5*B*). Moreover, as seen with the response to i.v. bDNA, IL-12 was found to be markedly elevated in the serum in IL-10-deficient mice that were treated with CpG-containing oligonucleotides and challenged with LPS (data not presented). Importantly, the immunoprotective effects of CpG oligonucleotides in mice with a disrupted IL-10 gene were more profound than those observed among wild-type mice (Fig. 2, *A* and *B*).

Because IL-10 KO mice responded to the immunoprotective effects of CpG-containing oligonucleotides but had markedly elevated concentrations of IL-12 in the serum and lavage fluid, we reasoned that IL-12 may be essential to the immunoprotective effects of CpG-containing oligonucleotides. Subsequent experiments in IL-12 KO mice (C57BL/6-*IL-12b*^{tm1Jm}) indicate that CpG-containing oligonucleotides are not able to protect these mice from the inflammatory effects of inhaled LPS on lavage cellularity (Fig. 6*A*). However, CpG-containing oligonucleotides, in comparison with non-CpG-containing oligonucleotides, were able to significantly decrease the concentration of TNF-α and MIP-2 in the lavage fluid in IL-12 KO mice challenged with inhaled LPS (Fig.

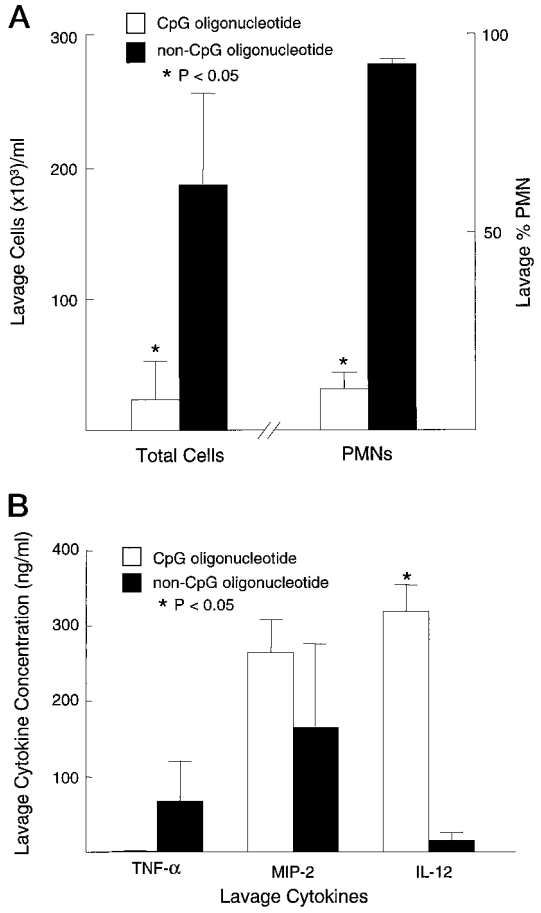

FIGURE 5. Concentration of total cells and percentage of PMNs (*A*) and concentration of cytokines (*B*) in the whole lung lavage fluid following inhalation of *E. coli* LPS. At 2 h before the inhalation challenge, IL-γ KO mice (C57BL/6-*IL-10*^{tm1Cgn}) were treated i.v. with 25 μg of an oligonucleotide containing embedded unmethylated CpG motifs or with i.v. saline. Five mice were used for each condition. Error bars, SE.

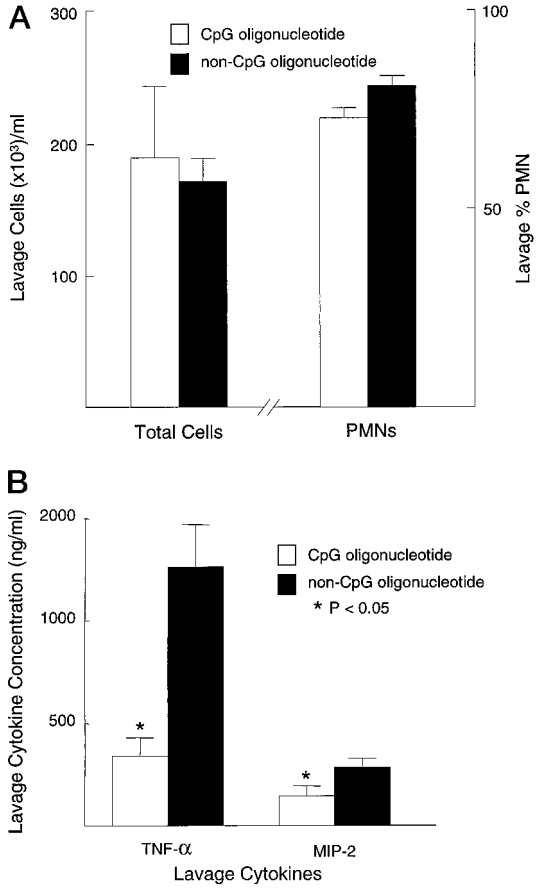

FIGURE 6. Concentration of total cells and percentage of PMNs (*A*) and concentration of cytokines (*B*) in the whole lung fluid following inhalation of *E. coli* LPS. At 2 h before the inhalation challenge, IL-12 KO mice (C57BL/6-*IL-12b*^{tm1Jm}) were treated i.v. with 25 μg of an oligonucleotide containing embedded unmethylated CpG motifs or with i.v. saline. Five mice were used for each condition. Error bars, SE.

6*B*), suggesting that TNF-α and MIP-2 may not be essential to the cellular response to inhaled LPS. Moreover, additional studies demonstrate that the IL-12-dependent immunoprotective effect of CpG-containing oligonucleotides is not dependent upon IFN-γ. CpG-containing oligonucleotides resulted in a reduced inflammatory response to inhaled LPS in IFN-γ KO mice (C57BL/6-*IFN-γ*^{tm1Ts}) (Fig. 7*A*). Although the concentration of MIP-2 in the lavage fluid was markedly elevated among IFN-γ KO mice following treatment with CpG-containing oligonucleotides, CpG-containing oligonucleotides also resulted in an increase in IL-12 in the lavage fluid.

Discussion

Our results indicate that systemic administration of CpG-containing oligonucleotides or bDNA substantially reduces the pulmonary inflammatory response to inhaled LPS, in terms of reducing both the cellular influx and the local levels of TNF-α and MIP-2. The immunoprotective effect of CpG-containing oligonucleotides requires unmethylated CpG motifs embedded within the oligonucleotide. Moreover, our results indicate that the protective effect of CpG oligonucleotides against the cellular influx is dependent upon IL-12 but does not require IL-10 or IFN-γ. These findings suggest that oligonucleotides containing CpG motifs may prove helpful in controlling the inflammatory response to inhaled LPS and possibly other environmental toxins.

In previous studies, we and others have shown that DNA containing CpG motifs can cause a systemic inflammatory response syndrome and prime inflammatory cells for LPS-induced toxicity (6, 48). Superficially, those results are surprising in light of the current demonstration that CpG DNA can prevent LPS-induced toxicity in the lung. However, a closer examination of these experimental systems and results sheds light on the organ-specific regulation of the inflammatory response to LPS. In the previous studies, the administration of high doses of CpG DNA caused a strong systemic immune activation that predisposed to systemic inflammatory response syndrome. However, lower doses of CpG DNA cause a less marked immune activation which can be therapeutically useful (12–18, 49, 50).

The immune activation triggered by CpG DNA can be associated with the expression of a set of cytokines, including TNF-α, IFN-αβ, IFN-γ, IL-6, IL-10, IL-12, IL-18, and MIP-2, depending upon the cell type and mouse strain studied (1, 2, 6, 7, 10, 13, 40, 51). The predominant effects of these cytokines are generally thought to be proinflammatory, although some of the cytokines, such as IL-10 and IL-12, have mutually antagonistic effects. It is not obvious that any of these cytokines should mediate the CpG-induced suppression of endotoxin toxicity. Indeed, both IL-10 and IL-12 are produced in the lung following endotoxin inhalation, and IL-12 is required for endotoxin-induced lethality (52). Alternatively, IL-12 can either promote or suppress collagen-induced inflammatory arthritis, depending upon the timing of administration

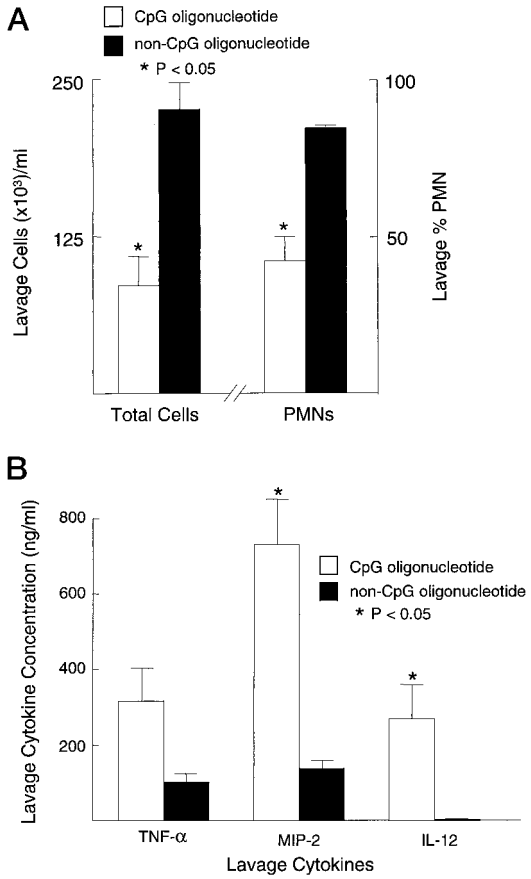

FIGURE 7. Concentration of total cells and percentage of PMNs (*A*) and concentration of cytokines (*B*) in the whole lung lavage fluid following inhalation of *E. coli* LPS. At 2 h before the inhalation challenge, IFN-10 KO mice (C57BL/6-*IFN-*γ^{tm1Ts}) were treated i.v. with 25 μg of an oligonucleotide containing embedded unmethylated CpG motifs or with i.v. saline. Five mice were used for each condition. Error bars, SE.

(53). IL-12 has also been reported to suppress angiogenesis (54) and graft vs host disease (55) and to induce NO-mediated immunosuppression following vaccination (56). Thus, although our finding that IL-12 is required for the CpG-mediated suppression of endotoxin-induced pulmonary cellular inflammation was unexpected, there is some precedent for IL-12 to antagonize inflammation. Moreover, IL-12 is down-regulated in allergic asthma and increases in asthmatics that have a beneficial response to corticosteroids (57). These findings suggest that IL-12 may play an important role in preventing or reducing airway inflammation.

In general, IL-12 could block lung inflammation either through local direct effects (mediated through the action on cells in the lung), through systemic effects possibly involving the induction of other cytokines, or through some combination of effects. We believe that a pure local effect may not explain our results, because inhaled CpG induces local inflammation (40) and does not block LPS-induced lung inflammation. The cellular and molecular mechanisms through which IL-12 exerts these effects await further investigation.

Our finding that the IL-12-dependent immunoprotective effects of CpG-containing oligonucleotides do not require IFN-γ is very important. IFN-γ has been shown previously to mediate several of the immunologic activities of IL-12, although other effects of IL-12 are IFN-γ-independent (58–61). The local production of IFN-γ is a critical element in the initiation of cytokine-mediated inflammatory responses. IFN-γ, in turn, can direct the release of other effector molecules such as TNF-α, IL-1, and IL-6 (62). Experimental manipulations that increase or decrease the amount of IFN-γ produced result in a corresponding accentuation or inhibition of inflammatory responses. IFN-γ is produced by T cells and NK cells in response to IL-12 (60, 63), but IFN-γ production is inhibited by IL-10 (39). Recent studies have established that the magnitude of the systemic response to LPS administration, such as the Shwartzman reaction or the response to Gram-negative infection, is significantly increased by IFN-γ or IL-12 (36, 37). However, our findings demonstrate that the lung cellular inflammatory response to inhaled LPS and the IL-12-dependent immunoprotective effects of CpG-containing oligonucleotides are not dependent upon IFN-γ. Nonetheless, IL-12 is not required for the protective effect of CpG DNA on the pulmonary cytokine response to inhaled LPS (Fig. 6*B*). This finding suggests that these acute proinflammatory cytokines, which are regulated by IL-12 or IFN-γ, are neither essential to the acute cellular response to inhaled LPS nor involved in this immunoprotective effect of CpG-containing oligonucleotides. In fact, our studies with the IFN-γ KO mice demonstrate that CpG oligonucleotides inhibit PMN recruitment to the lung despite marked increases in MIP-2, suggesting that IL-12 inhibits the chemotactic effects of potent chemokines.

IL-10 opposes the inflammatory actions of Th1-like cytokines, such as those whose expression is induced by CpG DNA. Thus, the fact that IL-10 is not required for the endotoxin-neutralizing effect of CpG DNA is quite compatible with the requirement of IL-12 for this effect. IL-10 has well-established immunosuppressive effects in models of transplant rejection (64), indicating that the inflammatory mechanisms involved in transplant rejection differ from those that are involved in LPS-induced pulmonary inflammation.

In considering the immunoprotective effects of CpG-containing oligonucleotides, one must ask whether these oligonucleotides are simply tolerizing mice to LPS through mechanisms of LPS tolerance. Tolerance to the toxic effects of endotoxin was first recognized by physicians who used bacterial vaccines for fever therapy (65). Several studies have shown that LPS tolerance is an active, well-orchestrated response presumably designed to limit excessive inflammation. Repeated sublethal injections of endotoxin in humans and animals result in a diminished release of inflammatory mediators to endotoxin (33, 66, 67). The "hyporesponsive" macrophage is thought to play a key role in the development of early endotoxin tolerance for three reasons. First, early endotoxin tolerance is associated with a decreased release of arachidonic acid metabolites by macrophages (68), decreased endotoxin-induced G protein function in peritoneal macrophages (66), and diminished production of TNF-α by peritoneal macrophages (67, 69). Second, the transfer of peritoneal macrophages from endotoxin-sensitive mice to endotoxin-resistant mice will render the resistant mice sensitive to the toxic effects of endotoxin (70, 71). Third, tolerance to endotoxin is associated with an increase in the concentration of macrophage progenitors in the bone marrow (72), suggesting that the pool of available macrophages in endotoxin-tolerant animals may be immature and unable to respond to endotoxin. The specific importance of TNF-α and IL-1 in the development of endotoxin tolerance is suggested by several studies that have shown that repeated administrations of TNF-α or IL-1 can significantly decrease morbidity and mortality from endotoxin (73–76) and Gram-negative sepsis (76, 77). However, tolerance to LPS is independent of TNF-α and IL-1 (78). LPS tolerance results in an increase in the expression of several genes including p50 of NF-κB (79), TNF receptor type II (80), IL-10 (81, 82), and TGF-β_1 (82). In fact, IL-10 and TGF-β_1 may be important in mediating tolerance to LPS (82). Moreover, IL-12 (as well as IFN-γ and GM-CSF) can effectively prevent and reverse LPS desensitization (83). Because IL-12

is important in mediating the immunoprotective effects of CpG-containing oligonucleotides in our model of lung injury, the mechanisms of LPS tolerance appear to be distinct from those involved with CpG-containing oligonucleotides.

The immunoprotective effect of CpG-containing oligonucleotides suggests that this class of agents may prove effective in the prevention and treatment of LPS-mediated airway disease. Although we have shown previously in mice that either tolerance to LPS (33) or pretreatment with an LPS antagonist (84) substantially reduces the inflammatory response to inhaled grain dust, these specific approaches are not practical in humans occupationally or environmentally exposed to aerosols containing LPS. In fact, in human volunteers, we have found that pretreatment with either inhaled triamcinolone (85), i.v. hydrocortisone (85), or pentoxifylline (86) does not substantially alter the physiologic or biologic response to inhaled grain dust. Because the concentration of endotoxin in the domestic environment adversely affects asthmatics (26), with higher concentrations of ambient endotoxin associated with greater degrees of airflow obstruction (27), endotoxin may play an important role in the airway disease caused by agents other than organic dusts. In fact, among subjects sensitized to the house dust mite, the concentration of endotoxin rather than house dust in the domestic environment is associated with the severity of asthma (27). Thus, controlling the inflammatory response to inhaled endotoxin may provide a novel approach for the treatment of airway disease.

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