

Reduced Inflammatory Response to Plasmid DNA Vectors by Elimination and Inhibition of Immunostimulatory CpG Motifs

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An inflammatory response is invariably associated with administration of gene transfer complexes composed of cationic lipids and plasmid DNA (pDNA). In the lung, an influx of neutrophils and elevated levels of several proinflammatory cytokines such as TNF- α , IFN- γ , IL-6, and IL-12 characterize this dose-dependent response. The induction of these cytokines was shown previously to be due in part to the presence of unmethylated CpG dinucleotides in the bacterially derived pDNA. We have eliminated 270 of 526 CpG dinucleotides in a reporter plasmid (pCFA-CAT) and tested the inflammatory response to cationic lipid:pDNA complexes containing the modified vector (pGZA-CAT) after intravenous (iv) or intranasal (i.n.) delivery into BALB/c mice. Compared to the unmodified vector, the CpG-reduced pGZA-CAT was found to be significantly less immunostimulatory, as the levels of IL-12, IFN- γ , and IL-6 in the serum 24 h after iv delivery were reduced by 40 to 75%. Similar reductions in cytokine levels were also observed in the bronchoalveolar lavage fluids (BALF) after i.n. administration, while the levels of reporter gene expression were not affected by the modifications. We have also investigated known inhibitors of the CpG signaling pathways in order to decrease the inflammatory response. Two such inhibitors, chloroquine and quinacrine, greatly reduced the induction of IL-12 from mouse spleen cells *in vitro* and inhibited cytokine production in the lung by approximately 50% without affecting gene expression. These results illustrate that use of a less immunostimulatory pDNA vector or inhibitors of CpG immunostimulation can reduce significantly the toxicity associated with cationic lipid:pDNA complexes thereby increasing the therapeutic index of this synthetic gene transfer vector.

Key Words: CpG; immunostimulatory activity; cationic lipid; plasmid DNA; gene transfer; intravenous delivery; intranasal delivery.

INTRODUCTION

The discovery that bacterial DNA is recognized as foreign in vertebrate cells has profound implications for gene therapy approaches employing plasmid DNA (pDNA) vectors (1–3). Bacterial DNA activates several immune cell types, including B cells, macrophages, dendritic cells, and natural killer cells (4–8). Likewise, bacterially derived pDNA elicits a potent immune response, inducing the secretion of several proinflammatory cytokines when delivered into mouse tissues either intranasally (i.n.) or intravenously (iv) using cationic lipids (9–12). Plasmid DNA also induces widespread inflammation when injected directly into skeletal muscle (13). These effects have ramifications for use in humans as highlighted by the recent

observation of a transient but not insignificant inflammatory response, which included fever, myalgia, and a reduction in pulmonary function in cystic fibrosis (CF) subjects that received aerosolized cationic lipid:pDNA complexes (14). These symptoms were more severe and occurred at a greater frequency in CF subjects that received aerosolized cationic lipid:pDNA complexes than in the group that received cationic liposomes alone, suggesting an involvement of the pDNA component. Such an adverse response is clearly undesirable and the development of strategies to abate this inflammatory response is warranted, particularly for the long-term treatment of chronic diseases such as CF.

The immunostimulatory response observed in mammalian cells has been shown to arise in part from the recognition of the unmethylated CpG dinucleotides present in bacterial DNA or pDNA (5). Mammalian DNA differs from bacterial DNA in that the frequency of CpG dinucleotides

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is severely suppressed compared to that of bacterial DNA and most of the CpG sequences are methylated (15). Nucleotides immediately flanking the CpG dinucleotides strongly influence this immunostimulatory activity; e.g., GACGTT is particularly immunostimulatory in mouse cells (1). Furthermore, these effects of bacterial DNA and immunostimulatory CpG oligonucleotides are evident not only in murine but also in human immune cells (7, 16, 17).

Several strategies could be employed to decrease the immunostimulatory properties of pDNA. One approach is to make the DNA more mammalian-like by either methylating (5) or eliminating immunostimulatory CpG sequences. While the *in vitro* methylation of all the CpG dinucleotides within a given pDNA has been shown to significantly decrease cytokine induction, it also severely inhibits transgene expression and so would not be a viable solution (12, 18). For this reason, our primary approach here is directed at reducing the frequency of these inflammatory motifs in the pDNA either by eliminating nonessential regions within the plasmid backbone (e.g., sequences flanking the origin of replication) or by site-directed mutagenesis (particularly of the coding sequences). Where possible, we also generated synthetic fragments that were devoid of CpG sequences to reduce the overall immunostimulatory profile of the pDNA. Modified pDNA vectors harboring reduced numbers of immunostimulatory CpG motifs were then evaluated following pulmonary and systemic delivery into mice.

Yet another strategy to reduce the immunostimulatory properties of the pDNA vector is to use specific inhibitors of the CpG signaling pathway. Endocytic uptake of DNA into an acidified intracellular compartment is the first required step in the pathway. Inhibitors of endosomal acidification such as monensin, bafilomycin, chloroquine, and quinacrine have been shown to block CpG-induced cytokine induction by leukocytes *in vitro* (19, 20). Here we evaluated the ability of chloroquine and quinacrine to inhibit the induction of cytokines that result from instilling cationic lipid:pDNA complexes into the mouse lung.

MATERIALS AND METHODS

Mouse spleen cells. Six- to eight-week-old female BALB/c mice were sacrificed by cervical dislocation. Spleens were excised and placed in sterile phosphate-buffered saline (PBS) on ice. To prepare single-cell suspensions, the spleens were placed in a tissue culture dish in PBS and crushed between the frosted edges of two sterile microscope slides. Cells were pelleted by centrifugation at 1200 rpm for 8 min and then washed three times in PBS. After the final wash, the cells were resuspended in RPMI 1640 medium supplemented with 25 mM HEPES buffer (Gibco BRL, Gaithersburg, MD), 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 50 μ M 2-mercaptoethanol. The resuspended cells were then plated at 6×10^6 cells/ml/well in 24-well culture plates and incubated at 37°C. Supernatants were collected 24 h after adding DNA fragments or pDNA to measure cytokine production.

Site-directed mutagenesis and DNA fragment amplification. Site-directed mutagenesis was performed using the Quick-Change mutagenesis kit (Stratagene, La Jolla, CA) according to the protocol supplied by the manufacturer. Either the cytosine or guanine within the CpG dinucleotide was mutated, avoiding alterations to the coding sequence. All mutations were verified by DNA sequencing.

Plasmid DNA fragments were amplified by polymerase chain reaction (PCR) from 10 ng of pDNA with *Taq* I polymerase (94°C for 30 s, 55°C for 30 s, 72°C for 30 s, 35 cycles) using primers specific for the different regions of the plasmid. The reactions were then centrifuged through an Ultra-free Probind column (Millipore Corp., Bedford, MA) for 1 min at 14,000 rpm. DNA concentration was quantitated by UV spectrophotometry and agarose gel electrophoresis.

Plasmid vector construction. The plasmid vector pCFA-CAT has been described previously (12). A 2596-bp *Sph*I fragment from pCFA-CAT containing the aminoglycoside 3'-phosphotransferase gene (kanamycin resistance gene) and the origin of replication was isolated and ligated to itself to form pOri-K. To construct the CpG-reduced plasmids, a 957-bp fragment encoding the kanamycin resistance gene and a 703-bp fragment encoding the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene were synthesized by Operon Technologies (Alameda, CA), by assembly of overlapping oligonucleotides using a protocol proprietary to the manufacturer. The synthetic kanamycin resistance gene was designed such that the codons containing CpG dinucleotides were changed to contain a non-CpG sequence but without altering the encoded amino acid. In instances where there was a choice of alternate codons, the most frequently used of the alternate codons was chosen based on a codon usage table for gram-negative bacteria (21). A similar procedure was followed to design the synthetic CAT reporter gene, except that when choosing alternate codons a codon usage table for highly expressed human genes was used (22). A 740-bp fragment encompassing a minimal origin of replication was amplified by PCR from pCFA-CAT. This region extends 128 bp 5' and 613 bp 3' relative to the replication origin [nucleotides 738 to 1477 of pUC19 (23)]. The synthetic kanamycin resistance gene and the origin of replication were ligated to form pOri-K-syn. To construct pGZA-CAT, the CAT gene from pCFA-CAT was first replaced with the synthetic CAT gene. From this construct a 2.1-kb *Sph*I fragment containing the CMV promoter, intron, synthetic CAT, and bovine growth hormone polyadenylation signal sequence was isolated and ligated to pOri-K-syn to form pGZA-CAT.

Cationic lipids and plasmid DNA. The cationic lipid GL-67 has been described previously (24). Cationic lipid GL-62, a cholesteryl carbamoyl spermine, is the free base form of GL-37 (24). The cationic lipids GL-67 and GL-62 were formulated with the neutral lipid dioleoylphosphatidylethanolamine (DOPE) at molar ratios of 1:2 and 1:1, respectively (24). Following fermentation, pDNAs were purified by ultrafiltration and sequential column chromatography (12) or by using Endo-free plasmid purification kits (Qiagen Inc., Valencia, CA) according to the protocol supplied by the manufacturer. The purified preparations contained less than 5 endotoxin units/mg of pDNA as determined by a chromogenic *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, MD). They were also determined to be essentially free of contaminating RNA and to contain less than 10 μ g protein/mg pDNA and less than 10 μ g bacterial chromosomal DNA/mg pDNA.

Administration of cationic lipid:pDNA complexes into mice. For systemic delivery, cationic lipid:pDNA complexes were formed by mixing equal volumes of GL-62:DOPE (molar ratio of 1:1) with pDNA as described previously (24) to a final concentration of 0.5:0.5 mM (GL-62:pDNA). The DNA concentration is expressed in terms of nucleotides, using an average nucleotide molecular weight of 330 Daltons. BALB/c mice were injected via the tail vein with 100 μ l of complex and serum was collected 24 h postinjection for analysis.

For delivery into the lung, 100 μ l of a complex of GL-67:pDNA (0.3:1.8 mM) was instilled intranasally into anesthetized BALB/c mice as described (9, 24). The animals were euthanized and their lungs were lavaged with PBS 24 h postinstillation. The recovered bronchoalveolar lavage fluid (BALF) was centrifuged at 1500 rpm for 4 min, and the resulting supernatants were removed and frozen at -80°C for subsequent cytokine analysis.

Cytokine and CAT activity assays. Cytokine levels (IL-6, IL-12, IFN- γ , and TNF- α) were quantitated using enzyme-linked immunosorbent assay (ELISA) kits as specified by the manufacturer (Genzyme Corp., Cambridge, MA; and R & D Systems, Minneapolis, MN). Our procedures for processing the lung tissues and assay of CAT enzymatic activity have been described elsewhere (24).

For all the *in vitro* and *in vivo* studies, each experiment was repeated independently from two to four times, using different preparations of PCR fragments, cationic lipid, and/or DNA, with results similar to those depicted in the figures.

TABLE 1
Number of CpG Motifs in Unmodified and Mutated DNA Fragments

Plasmid sequences ^a	Number of CpGs	Length (bp)
ori	160	1276
ori-mut	152	1276
ori-min	96	740
kan	116	1252
kan-mut	84	1252
kan-syn	0	957
CAT	80	809
CAT-syn	2	703
Intron	80	734
CMV	74	607
pOri-K	288	2596
pOri-K-syn	96	1735
pCFA-CAT	526	4739
pGZA-CAT	256	3788

^a Descriptions of the sequences are in the legends to Figs. 1 and 2.

RESULTS

Reducing the Number of CpG Motifs in the pDNA Vectors

The plasmid expression vector pCFA-CAT contains the enhancer and promoter from the immediate early gene of cytomegalovirus, an intron, the *E. coli* CAT reporter gene, the bovine growth hormone polyadenylation signal, a pMB1 replication origin region, and a kanamycin resistance gene. This vector contains 526 CpG dinucleotides (Table 1), counting the sites in both strands of the plasmid. To reduce the frequency of these motifs in pCFA-CAT, site-directed mutagenesis was first performed on the CpG sites within the replication origin region, which contains the largest number of these sites. Initially only 8 of 160 CpG sites were successfully eliminated within the replication origin region, as many of the attempted mutations apparently destroyed replicative function. To compare their relative immunostimulatory activity, fragments encompassing the unmodified (ori) and mutated (ori-mut) replication origin region were amplified by PCR, and then equal amounts of each purified fragment were added to mouse spleen cells. The levels of IL-12 secreted into the cell culture supernatant were measured 24 h later. The unmodified replication origin region (ori) induced high levels of IL-12 (Fig. 1), consistent with the known immunostimulatory activity of *E. coli*-derived DNA. Interestingly, despite the small number of changes, the mutated replication origin region (ori-mut) containing eight fewer CpG motifs induced less IL-12 compared to the unmodified origin (Fig. 1). Similar results also were observed for the cytokines IL-6 and IFN- γ (data not shown).

As an alternate approach to site-directed mutagenesis of the CpG sites within the replication origin region, we sought to define the minimal fragment that could still

retain replication function. Previous studies have shown that the minimal region required for ColE1 pDNA replication encompasses the origin and the upstream region encoding the RNA II primer (25). A 740-bp fragment of pCFA-CAT containing this minimal region was amplified by PCR, and when inserted into a plasmid was found to be fully competent for replication (data not shown). This approach decreased the size of the replication origin region by 536 bp and eliminated 65 CpG motifs. When tested on mouse spleen cells, this shortened replication region (ori-min) induced levels of IL-12 similar to that of the mutated origin (Fig. 1).

The region containing the second highest number of CpG sites was the kanamycin resistance gene. Thirty-two of the 116 CpG sites present within this region were eliminated by site-directed mutagenesis of the cytosine to thymine or the guanine to adenine. The mutations were designed so as not to alter the coding sequence by taking advantage of the degeneracy of amino acid codons. Fragments encompassing the unmodified (kan) and mutated (kan-mut) gene were amplified by PCR and then added to mouse spleen cells. The mutated kanamycin resistance gene (kan-mut) induced approximately 50% less IL-12 from the mouse spleen cells compared to the unmodified gene (Fig. 1). These results, along with those of the mutated and minimal origin, indicate that the strategy of reducing the CpG content within a given region can effectively reduce immunostimulatory activity *in vitro*.

It was deemed impractical to attempt to eliminate the remaining 84 CpG sites within the kanamycin resistance gene by mutagenesis, so instead the gene was synthesized by PCR-mediated assembly of several overlapping oligonucleotides. The sequence was designed to eliminate all CpG dinucleotides without altering the amino acid sequence by extensively substituting codons and pairs of neighboring codons containing CpG sites. A synthetic 957-bp fragment containing no CpG sites (kan-syn) was

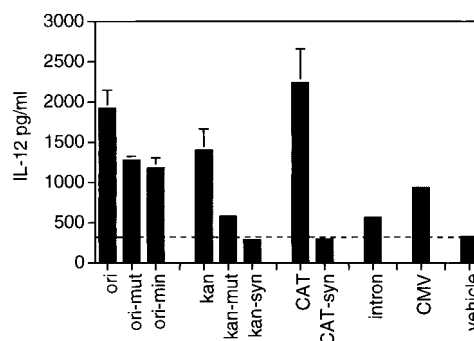


FIG. 1. Induction of IL-12 from mouse spleen cells by unmodified and mutated DNA fragments of pCFA-CAT. Fragments were amplified by PCR and then 1 μ g of each fragment was added to mouse spleen cells and the medium collected 24 h later. IL-12 levels were assayed by ELISA. Vehicle is water. ori, replication origin region; ori-mut, mutated origin; ori-min, minimal origin; kan, kanamycin resistance gene; kan-mut, mutated kanamycin resistance gene; kan-syn, synthetic kanamycin resistance gene; CMV, human cytomegalovirus enhancer-promoter; CAT, chloramphenicol acetyltransferase gene; CAT-syn, synthetic chloramphenicol acetyltransferase gene. $n = 3$ wells per fragment. Data are expressed as means \pm SEM.

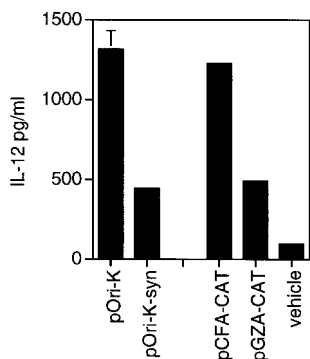


FIG. 2. IL-12 induction from mouse spleen cells by unmodified and mutated pDNA vectors. Plasmid DNA (1 μ g) was added to mouse spleen cells and the medium collected 24 h later. IL-12 levels were assayed by ELISA. Vehicle is water. pOri-K is a vector containing the origin of replication and the gene for kanamycin resistance. pOri-K-syn is a mutant form of pOri-K with a reduced number (96) of CpG dinucleotides. pCFA-CAT and pGZA-CAT are similar except that the latter contains 270 fewer CpG motifs. $n = 3$ wells per group. Data are expressed as means \pm SEM.

generated that retained the ability to confer kanamycin resistance. A 703-bp fragment encoding the CAT reporter gene was synthesized in a similar fashion, eliminating 78 of 80 CpG sites (CAT-syn). This synthetic CAT gene retained full functional enzymatic activity (data not shown). When tested on mouse spleen cells, these CpG-deficient fragments (kan-syn and CAT-syn) were essentially non-immunostimulatory, giving levels of IL-12 equal to that of the vehicle control (Fig. 1). These results illustrate that when the strategy of reducing CpG content is taken to its fullest extent, large DNA fragments can be made completely non-immunostimulatory by the elimination of all or nearly all CpG sites.

Immunostimulatory Activity of the CpG-Reduced pDNA Vectors *In Vitro* and *In Vivo*

We next tested the immunostimulatory activity of pDNAs composed of these fragments containing reduced numbers of CpG sites. The synthetic kanamycin resistance gene and the minimal replication origin region were first ligated together to form pOri-K-syn. This plasmid contains 96 CpG sites compared to 288 CpG sites in the plasmid pOri-K which comprised the unmodified kanamycin resistance gene and unmodified origin of replication (Table 1). These plasmids were added to mouse spleen cells *in vitro* and the levels of IL-12 in the supernatant were measured 24 h later. The levels of IL-12 induced by pOri-K-syn were significantly reduced, representing only approximately one-third of the levels induced by the unmodified pOri-K (Fig. 2).

The pOri-K-syn was then used to reassemble a modified low CpG form of pCFA-CAT. The CMV enhancer-promoter was left unaltered because of concerns that mutations within this region would decrease promoter activity. The intron and polyadenylation signal sequence were also unchanged, but these regions were found to be only weakly immunostimulatory when tested on mouse spleen

cells (Fig. 1). The synthetic CAT gene was used in place of the unmodified CAT gene. The final reassembled vector pGZA-CAT contained 256 CpG sites compared to 526 sites in pCFA-CAT. When tested on mouse spleen cells the levels of IL-12 were only approximately 40% of the levels induced by the unmodified pCFA-CAT (Fig. 2). Hence, by eliminating greater than half of the CpG motifs in pCFA-CAT, a significant reduction in the *in vitro* immunostimulatory activity of the pDNA was achieved.

To assess the immunostimulatory activity of pGZA-CAT *in vivo*, the vector was complexed with cationic lipid GL-62 and then injected intravenously via the tail vein into BALB/c mice. Serum was collected 24 h postinjection and the cytokine levels were measured by ELISA. Mice that received liposomes alone did not elicit production of proinflammatory cytokines (data not shown) as was observed previously. This is congruent with the notion that most of the inflammatory response is mediated by the bacterially derived pDNA. Mice administered GL-62:pCFA-CAT complexes induced high levels of the inflammatory cytokines IFN- γ , IL-12, and IL-6 (Fig. 3). In contrast, the levels of cytokines in the serum after injection of GL-62:pGZA-CAT complexes were decreased significantly compared to the levels induced by GL-62:pCFA-CAT complexes. Figure 3 shows that the levels of IL-12, IFN- γ , and IL-6 were reduced 43, 81, and 78%, respectively. Importantly, the expression level of CAT was not decreased by the modifications. Indeed, there was an apparent twofold increase in expression from pGZA-CAT compared to pCFA-CAT. Transfection studies *in vitro* indicated that part of this increase was due to the use of codons optimal for expression in mammalian cells in the synthetic CAT

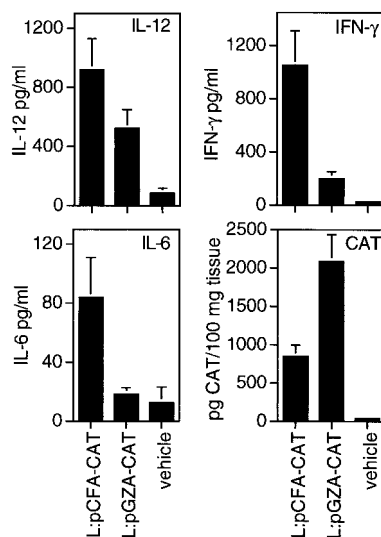


FIG. 3. Cytokine levels in serum and CAT expression in the lungs after intravenous administration of unmodified and mutated pDNA vectors. One hundred microliters of GL-62:pDNA (0.5:0.5 mM) complexes was injected via the tail vein into BALB/c mice. Serum was collected 24 h postinjection and IFN- γ , IL-12, and IL-6 levels were assayed by ELISA. CAT levels in the lung homogenates were also assayed at 24 h posttreatment. L, cationic lipid GL-62. Vehicle is water. $n = 4$ mice per group. Data are expressed as means \pm SEM.

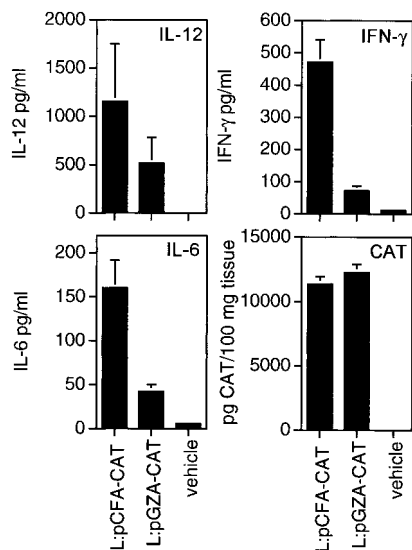


FIG. 4. Cytokine levels in bronchoalveolar lavage fluid (BALF) and CAT expression in the lungs after instilling unmodified and mutated pDNA vectors. One hundred microliters of GL-67:pDNA (0.3:1.8 mM) complexes was instilled intranasally into the lungs of BALB/c mice. BALF was collected 24 h postinstillation and IL-12, IFN- γ , and IL-6 levels were assayed by ELISA. Animals were also sacrificed at 24 h postadministration and the levels of CAT in the lung homogenates assayed. L, cationic lipid GL-67. Vehicle is water. $n = 5$ mice per group. Data are expressed as means \pm SEM.

gene (data not shown). The decreased cytokine levels may also have had a positive effect on promoter activity.

The pGZA-CAT vector was also complexed with cationic lipid GL-67 and then instilled intranasally into the lungs of BALB/c mice. Bronchoalveolar lavage fluid was collected 24 h postinjection, and the levels of cytokines were measured by ELISA. Compared to the levels induced by GL-67:pCFA-CAT complexes, there were decreased levels of IL-12, IFN- γ and IL-6 in the BALF after instilling GL-67:pGZA-CAT (Fig. 4). Repeated studies at times showed larger variations in cytokine values observed between individual animals, which was likely due to variability both in dosing and in collecting the lavage fluid from the mouse lung (data not shown). Although the degree of cytokine reduction varied, in nearly all cases cytokine levels induced by GL-67:pGZA-CAT complexes were lower than those induced by GL-67:pCFA-CAT complexes. Together, these data demonstrate that strategies that reduce the number of CpG dinucleotides in the pDNA will result in abatement of its overall immunostimulatory activity not only *in vitro* but also *in vivo*.

Inhibition of IL-12 Production from Mouse Spleen Cells with Chloroquine and Quinacrine

In addition to reducing CpG content, we evaluated a complementary strategy to reduce the immunostimulatory properties of the pDNA vector by the use of specific inhibitors of the CpG signaling pathway. Chloroquine and quinacrine have previously been shown to inhibit the immunostimulatory properties of oligonucleotides con-

taining CpG motifs *in vitro* (26). These compounds were tested first for their ability to inhibit the immunostimulatory properties of pDNA and cationic lipid:pDNA complexes *in vitro*. The unmodified pCFA-CAT was added to mouse spleen cells together with chloroquine or quinacrine and the levels of IL-12 in the culture medium were measured 24 h later. Figure 5 shows that coaddition of 10 μ M chloroquine or 1 μ M quinacrine effectively decreased the levels of IL-12 induction to near background levels. The compounds at these concentrations had no discernible effects on cell viability or on expression of the CAT reporter gene (data not shown). To determine if chloroquine and quinacrine could also inhibit IL-12 production induced by cationic lipid:pDNA complexes, chloroquine and quinacrine were added together with a complex of cationic lipid GL-67 and pCFA-CAT. Figure 5 shows that addition of 10 μ M chloroquine or 1 μ M quinacrine was sufficient to reduce the induction of IL-12 by GL-67:pCFA-CAT complexes to near background levels.

Cytokine Profiles in Bronchoalveolar Lavage Fluid after Instilling Cationic Lipid:pDNA Complexes with Chloroquine or Quinacrine

To determine whether the CpG-inhibitory effects of chloroquine and quinacrine could also be realized *in vivo*, complexes of GL-67 and pCFA-CAT were formulated with these compounds and then instilled into the lungs of BALB/c mice. Quinacrine or chloroquine was added to the formed GL-67:pDNA complexes to a final concentration of 0.01 to 100 μ M. BALF was collected 24 h postinstillation and cytokines were measured by ELISA. Adding 10 μ M chloroquine decreased the levels of IL-12, TNF- α , and IFN- γ by 54 to 79% compared to levels attained after instilling complex alone (Fig. 6). Adding 0.1 μ M quinacrine also decreased the levels of IL-12, TNF- α , and IFN- γ by 52 to 64% compared to levels observed after adminis-

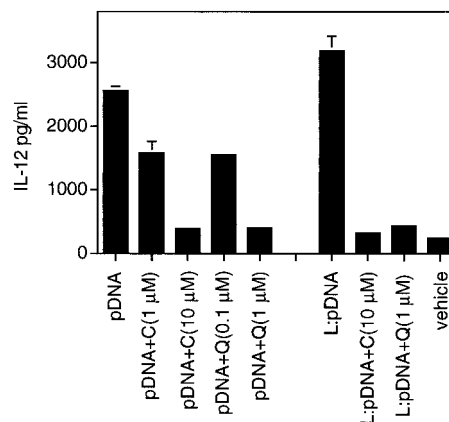


FIG. 5. Inhibition of IL-12 production from stimulated mouse spleen cells with chloroquine and quinacrine. pCFA-CAT (pDNA, 1.5 μ M final concentration in 1 ml of media) or GL-67:pCFA-CAT (L:pDNA, 0.375:1.5 μ M) plus or minus chloroquine (C) or quinacrine (Q) at the indicated concentrations was added to mouse spleen cells and the medium collected 24 h later. Vehicle is water. $n = 3$ wells per group. Data are expressed as means \pm SEM.

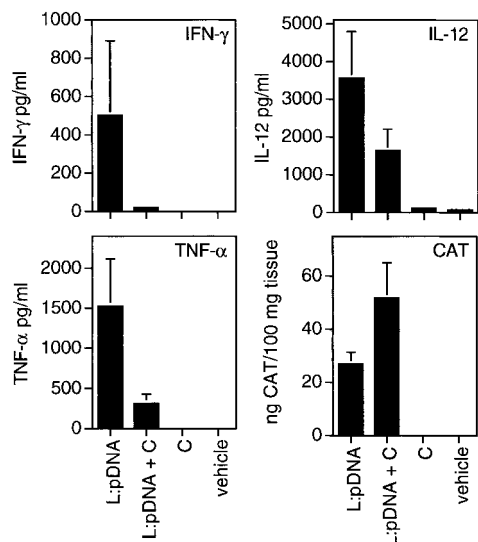


FIG. 6. Cytokine levels in bronchoalveolar lavage fluid and lung CAT expression after instilling cationic lipid:pDNA complexes containing chloroquine. pCFA-CAT (pDNA) was first complexed with GL-67 (L:pDNA, 0.3:1.8 mM), and then chloroquine (C) was added to a final concentration of 10 μ M. One hundred microliters of the GL-67:pDNA complexes plus or minus chloroquine was instilled intranasally into the lungs of BALB/c mice. BALF was collected at 24 h postadministration for cytokine analysis and lungs were harvested for CAT assay. Cytokines were assayed by ELISA. $n = 3$ mice per group. Data are expressed as means \pm SEM.

tration of complex alone (Fig. 7). Higher concentrations of either chloroquine or quinacrine did not further decrease cytokine levels (data not shown). The levels of CAT expression were the same or moderately increased by the addition of either compound (Figs. 6 and 7). These results suggest that low concentrations of chloroquine and quinacrine can effectively reduce the inflammatory response associated with instillation of cationic lipid:pDNA complexes into the lung.

DISCUSSION

In this study we have constructed a pDNA expression vector (pGZA-CAT) containing substantially fewer CpG dinucleotides within its sequence than conventional plasmids. The reduced CpG content was shown to correlate with a decreased immunostimulatory response *in vitro*, and cationic lipid:pDNA complexes containing these modified plasmids induced significantly lower levels of proinflammatory cytokines in the serum when administered intravenously, as well as decreased levels in the mouse lung BALF when administered intranasally. The critical role of CpG content was most clearly demonstrated by the observation that DNA fragments devoid of any CpG dinucleotides were essentially non-immunostimulatory.

The inflammatory properties of bacteria-derived pDNA limit many aspects of current plasmid-based gene therapy strategies. Safety is a key concern, and in the case of CF gene therapy, delivering an inflammatory compound to

an already damaged and chronically inflamed lung is to be avoided. In addition to causing inflammation, the unmethylated CpGs in pDNA have been shown to induce apoptosis of lung endothelial cells when delivered systemically (11). A second concern is the potential for augmenting an antibody response to the transgene product or to the pDNA itself. The Th1-type response to the immunostimulatory CpG motifs makes pDNA an excellent adjuvant for immunization to a variety of protein antigens (27–29). This property is useful for and indeed has been exploited for generating vaccines, but is deleterious for the treatment of genetic disorders that require repeated delivery of cationic lipid:pDNA complexes. Bacterial DNA also has been shown to be capable of inducing the production of anti-DNA antibodies in mice (30), and this finding suggests the possibility of developing an autoimmune response in patients as a result of treatment. A third limiting issue is that repeat administration of cationic lipid:pDNA complexes is ineffective at short intervals, likely the result of the inflammatory response to the initial dose. This refractory effect can be reconstituted by injecting complexes containing oligonucleotides with CpG motifs, while complexes containing oligonucleotides lacking CpG motifs permit much more effective redosing (31). Finally, expression levels and duration of expression are adversely affected by the toxicity of the complex (12, 31).

Creating a Less Immunostimulatory pDNA Vector

The decrease in the immunostimulatory activity of the pDNA vector was found to be roughly proportional to the

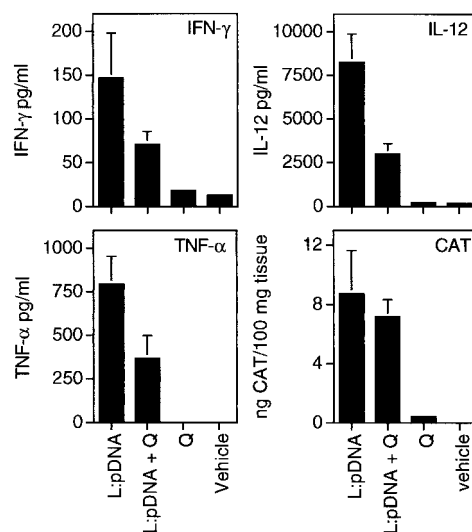


FIG. 7. Cytokine levels in bronchoalveolar lavage fluid and CAT expression in the lung after instilling cationic lipid:pDNA complexes containing quinacrine. pCFA-CAT (pDNA) was first complexed with GL-67 (L:pDNA, 0.3:1.8 mM), and then quinacrine (Q) was added to a final concentration of 0.1 μ M. One hundred microliters of the GL-67:pDNA complexes plus or minus quinacrine was instilled intranasally into the lungs of BALB/c mice. BALF was collected for cytokine analysis and lungs were harvested for CAT assay. Cytokines were assayed by ELISA. $n = 3$ mice per group. Data are expressed as means \pm SEM.

number of CpG sites. This mimics the strategy of CpG suppression found in vertebrate DNA, but vertebrate DNA also contains other features within its sequence that reduce its immunostimulatory properties. CpG motifs in particular sequence contexts have been shown to be non-immunostimulatory and have been termed neutralizing (CpG-N) motifs (32). Oligonucleotides containing certain patterns of CGG, CCG, and CGCG direct repeat motifs not only lack immunostimulatory activity but they can also inhibit immunostimulatory CpG motifs *in cis* and *in trans* (32). Although potentially useful, inserting additional CpG-N motifs into the pDNA vector has so far not altered immunostimulatory activity (data not shown). The interactions between CpG-N and CpG-S motifs are also not well understood, and at present the most effective strategy has been simply to reduce the number of CpG sites.

The remaining problematic CpG sites of our CpG-reduced plasmid, pGZA, reside within the enhancer-promoter and replication origin region. An enhancer-promoter containing fewer CpG sites than found in CMV could be used in its place. The replication origin region, along with the antibiotic resistance gene, could be deleted entirely. Site-specific recombination using a phage lambda integrase has been demonstrated to produce "minicircles" composed only of the expression cassette and a fragment of the recombined site (33). While the efficiency of recombination needs to be improved and the purification of these recombined plasmids is at present only suitable for small-scale analytical purposes, large-scale methods are certainly plausible.

Inhibitors of CpG Stimulation

While developing a completely non-immunostimulatory vector is the ultimate goal, a complementary strategy to reduce the inflammatory response is to use specific inhibitors of the CpG signaling pathway such as chloroquine and quinacrine. These compounds appear to inhibit a very early step, blocking the downstream generation of reactive oxygen species and NF κ B activation in leukocytes exposed to CpG (20). Although general immunosuppressants such as FK506, cyclosporin, and dexamethasone have been used to reduce the immune response to viral or nonviral vectors, a distinctive property of chloroquine and quinacrine is the extremely low (nanomolar to micromolar) concentrations required for the specific inhibition of CpG-mediated stimulation. These concentrations are well below the concentrations required for the antimalarial or the reported anti-inflammatory effects of these drugs (34, 35). Ideally such compounds will not be needed as the immunostimulatory activity of the pDNA is further reduced. Nevertheless, a conceivable treatment regimen could involve coadministration of low concentrations of chloroquine or quinacrine along with cationic lipid:pDNA complexes harboring significantly reduced CpG motifs.

In conclusion, we have shown that a pDNA vector containing substantially reduced numbers of CpG sites is

significantly less inflammatory compared to conventional pDNA vectors. This strategy is certainly not unique in that many viruses have evolved their genomes to contain fewer CpG sites in order to apparently evade the host immune system (32, 36). One cautionary note relevant to this strategy is the report that exogenous DNA, regardless of CpG content, can upregulate MHC I expression in nonimmune cells (37). Nevertheless, a pDNA with decreased immunostimulatory properties is a useful step toward increasing the safety profile and hence the therapeutic index of non-viral-based gene therapy vectors.

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