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A Clinical Inflammatory Syndrome Attributable to Aerosolized Lipid–DNA Administration in Cystic Fibrosis

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ABSTRACT

Immunologic reactivity to lipid–DNA conjugates has traditionally been viewed as less of an issue than with viral vectors. We performed a dose escalation safety trial of aerosolized cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to the lower airways of eight adult cystic fibrosis patients, and monitored expression by RT-PCR. The cDNA was complexed to a cationic lipid amphiphile (GL-67) consisting of a cholesterol anchor linked to a spermine head group. CFTR transgene was detected in three patients at 2–7 days after gene administration. Four of the eight patients developed a pronounced clinical syndrome of fever (maximum of 103.3EF), myalgias, and arthralgia beginning within 6 hr of gene administration. Serum IL-6 but not levels of IL-8, IL-1, TNF-α, or IFN-γ became elevated within 1–3 hr of gene administration. No antibodies to the cationic liposome or plasmid DNA were detected. We found that plasmid DNA by itself elicited minimal proliferation of peripheral blood mononuclear cells taken from study patients, but led to brisk immune cell proliferation when complexed to a cationic lipid. Lipid and DNA were synergistic in causing this response. Cellular proliferation was also seen with eukaryotic DNA, suggesting that at least part of the immunologic response to lipid–DNA conjugates is independent of unmethylated (*E. coli*-derived) CpG sequences that have previously been associated with innate inflammatory changes in the lung.

OVERVIEW SUMMARY

The proinflammatory sequelae of recombinant adeno- and other viruses have limited their development for safe human gene transfer. Previous studies have indicated gene transfer and partial correction of cystic fibrosis (CF) bioelectric defects by using lipid–DNA complexes in the airways of CF patients. Although lipid–DNA conjugates are often viewed as less immunogenic, our studies point to a substantial clinical inflammatory response after administration of these vectors to humans. The inflammatory response to lipid–DNA may represent a barrier to the use of this approach in cystic fibrosis, and other plasmid–DNA-based experimental protocols.

INTRODUCTION

NONVIRAL, PLASMID-BASED STRATEGIES for airway transfer of the cystic fibrosis transmembrane conductance regulator (CFTR) gene have been supported in preclinical animal models, including nonhuman primates, and in human trials. Several laboratories (Caplen *et al.*, 1995; Gill *et al.*, 1997; Porteous *et al.*, 1997; Zabner *et al.*, 1997; Alton *et al.*, 1999) have reported successful gene transfer as judged by reverse transcription-polymerase chain reaction (RT-PCR), immunofluorescence, or functional assays of Cl^- transport after nasal or lung administration of lipid–DNA complexes in CF patients. Some (Caplen *et al.*, 1995; Gill *et al.*, 1997; Porteous *et al.*, 1997; Zabner *et al.*, 1997; Alton *et al.*, 1999), but not all (Sorscher, 1996;

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Knowles *et al.*, 1998), of these studies also noted improvements in the cystic fibrosis Cl^- transport abnormality *in vivo*. In contrast to the use of adenoviral vectors for pulmonary gene transfer of CFTR (Crystal *et al.*, 1994), little or no inflammation has been reported from cationic liposomes. One study of aerosolized lipid–DNA complexes described self-limited flulike symptoms in some patients (Alton *et al.*, 1999). In that study, although partial correction of CF ion transport defects in the trachea was reported, wild-type CFTR mRNA could not be detected in any of the patients at any time point after gene transfer. This discrepancy was attributed to technical problems concerning sample processing.

The present trial was intended to address the following questions. First, what are the clinical toxicities attributable to administration of cationic lipid–DNA complexes that could represent barriers to the use of liposomes in CF and possibly other diseases? Second, because we observed a fever, myalgia, arthralgia-type syndrome in several of the patients in our study, we examined whether serum cytokine levels were altered and whether the same cationic lipid–DNA complexes could elicit an inflammatory response in blood mononuclear cells taken from patients. Third, we tested whether CFTR gene expression could be accomplished with a nonviral vector in lower airways of CF patients. Human experiments have failed to detect wild-type CFTR mRNA in either upper (nasal) or lower airways even with an exquisitely sensitive RT-PCR technique (Knowles *et al.*, 1998; Alton *et al.*, 1999). Our results point to an acute inflammatory reaction after airway administration that could limit the usefulness of lipid–DNA conjugates in cystic fibrosis or other diseases. The experiments also provide a clinical basis for future human trials of the immune response to liposomal gene transfer.

MATERIALS AND METHODS

Study subjects

The protocol was reviewed and approved by the University of Alabama Institutional Review Board and sanctioned through the Food and Drug Administration (FDA). Eight adult cystic fibrosis patients (four male; four female), ages 16 to 37 years (median age, 26.5 ± 2.7 years), were enrolled on the basis of an FEV_1 (forced expiratory volume in 1 sec) greater than 40% predicted, willingness to omit recombinant DNase for 7 days before and 2 days after gene delivery, and no recent upper respiratory infections or significant extrapulmonary disease. The characteristics of these patients, including their CFTR genotypes, are shown in Table 1. All subjects underwent a screening evaluation that included pulmonary function tests, computed tomography (CT) scan, and blood and urine examinations. The clinical protocol is summarized in Table 2.

Gene administration

The full-length CFTR cDNA was inserted downstream of the human cytomegalovirus immediate-early gene promoter and enhancer, and using a hybrid intron composed of a fused tripartite leader from adenovirus containing a 5′ splice donor signal, and a 3′ splice acceptor signal derived from a mouse immunoglobulin gene. A prokaryotic kanamycin resistance gene (for DNA propagation in *Escherichia coli*) was also a component of the plasmid vector. The plasmid (pCF1-CFTR) was purified from *E. coli* and validated for human use by (1) restriction digestion with five enzymes, each cutting at least twice in the vector; (2) a predominantly closed circular form of DNA; (3) no colony-forming units by a bioburden test of infectious contaminants; (4) a nonpyrogenic validation in a conventional pyrogen assay in rabbits; and (5) less than 5 endotoxin unit/mg plasmid DNA. Direct measurement of endotoxin in the lipid–DNA formulation administered to patients in our trial indicated 0.2 endotoxin unit/mg plasmid DNA. Construction and purification of the plasmid were as described in Lee *et al.* (1996). CFTR function within the plasmid was established by Cl^--dependent short-circuit current (I_{SC}) activation in polarized Fischer rat thyroid epithelial cells and by Cl^- efflux in a cystic fibrosis airway epithelial cell line (CFT1) (methods after Lee *et al.*, 1996; Wheeler *et al.*, 1996). The plasmid was then complexed to 67A (mixture of GL-67:DOPE:DMPE–PEG_{5000}; 1:2:0.05) (Lee *et al.*, 1996; Wheeler *et al.*, 1996) as summarized in Table 3 for each of three dosage groups. Aerosolization was through a Pari LC jet nebulizer with 50-psi operating

TABLE 1. CHARACTERISTICS OF PATIENTS ENROLLED IN PROTOCOL

Subject[a]	*Age (years)*	*Sex*	*Genotype*	*Amount of drug nebulized (ml)*	*FEV_1 (% predicted)*	*Pseudomonas colonization*	*Pancreatic status*
01	32	Male	ΔF508/ΔF508	6	51	+	PI
03	27	Male	ΔF508/neg	6	57	+	PI
02	27	Male	ΔF508/ΔF508	12	53	+	PI
04	17	Female	ΔF508/ΔF508	12	72	+	PI
05	28	Male	ΔF508/ΔF508	16	60	+	PI
06	28	Female	ΔF508/3849 + 10KBC-T	16	55	+	PI
07	16	Female	+Sweat	16	92	−	PS
08	37	Female	ΔF508/neg	16	102	−	PI

Abbreviations: PI, Pancreatically insufficient; PS, pancreatically sufficient.

[a]Patient 02 signed the consent before patient 03, but because of scheduling conflicts was treated on a later date and at dose level 2 (see Table 3).

TABLE 2. SUMMARY OF CLINICAL PROTOCOL

	Day relative to lung application								
	−90 to −60	*−7 to −4*	*0*	*1*	*2*	*7*	*15*	*28*	*70 to 110*
Administration of investigational material			✓						
Bronchoscopy (lung brushes/biopsies)	[a]				✓	[a]			
Medical history and physical examination	✓	✓	1 and 6[b]	✓	✓	✓	✓	✓	✓
Laboratory analysis									
Blood samples									
CBC with differential	✓	✓	✓	✓	✓	✓	✓	✓	
Clinical chemistry	✓	✓	✓	✓	✓	✓	✓	✓	
ANA	✓	✓						✓	
Serum IL-1β, IL-6, IL-8, TNF-α	✓	✓	1 and 6[b]	✓	✓	✓	✓		
Antibody to lipid–DNA	✓	✓	1 and 6[b]	✓	✓	✓	✓	✓	
Urine									
Routine urinalysis	✓	✓	✓	✓	✓	✓	✓	✓	
Pulmonary function tests:									
Full pulmonary function tests		✓			✓			✓	✓
Spirometry, oximetry	✓		1 and 6[b]	✓		✓	✓		
Radiology									
Chest X-ray (lateral and PA)								✓	
Chest CT[c]		✓			✓				

Definitions: CBC, Complete blood count; ANA, anti-nuclear antibody; IL, interleukin; TNF, tumor necrosis factor; PA, posteroanterior; CT, computed tomography.
[a]In certain patients within the trial.
[b]Hours postadministration.
[c]CT scans utilized a high-resolution sectioning format.

pressure and a flow rate of 8 liters/min, configured to a breath-activated controller with a charge time of 2 sec. This arrangement had been shown to deliver lipid–DNA particles at sizes of approximately 2.5 μm (Eastman *et al*., 1997). Patients were instructed to breathe at their baseline rate and tidal volume, and inspired lipid–DNA complexes in sets of 50 breaths, pausing between each set. Blood pressure, heart rate, and oximetry were monitored during gene administration, which required an average of 60 min (group 1) to 86 min (group 3) for completion.

Clinical endpoints

As shown in Table 2, patients underwent a bronchoscopy 2 days after gene administration in order to evaluate airway histology and to provide tissue for CFTR expression studies. Physical examination, pulmonary function testing, chest CT, and blood and urine studies comprised the primary end points. Because bronchoscopy itself, and in particular, bronchoalveolar lavage (BAL), may lead to fever and diminished pulmonary function in these patients, no bronchoscopy and BAL immediately after gene aerosolization was performed. Certain patients volunteered for repeat bronchoscopies in order to test the time course of wild-type CFTR expression in *vivo.* Some patients in the first two dosage groups also underwent a pretreatment bronchoscopy in order to standardize mRNA extraction and processing conditions. All endobronchial biopsies were reviewed in a blinded fashion by two pulmonary pathologists. In no case was CF airway histopathology altered by administration of lipid–DNA.

TABLE 3. DESCRIPTION OF THREE LIPID–DNA DOSAGE GROUPS[a]

Dose level	*Number of patients*	*Volume nebulized (ml)*	*Volume deposited[b] (ml)*	*67A (14.3 mg/ml) deposition[b] (mg)*	*pCF1-CFTR (2.64 mg/ml) deposited[b] (mg)*
1	2	6	3	42.9	7.9
2	2	12	6	85.8	15.8
3	4	16	8	114.4	21.12

[a]Endotoxin in the formulations used in these studies was 0.2 endotoxin unit/mg plasmid DNA (i.e., an estimate of 1.58, 3.16, and 4.24 endotoxin units per deposited sample in dosage levels 1, 2, and 3 respectively).
[b]Assumes 50% deposition of inhaled material.

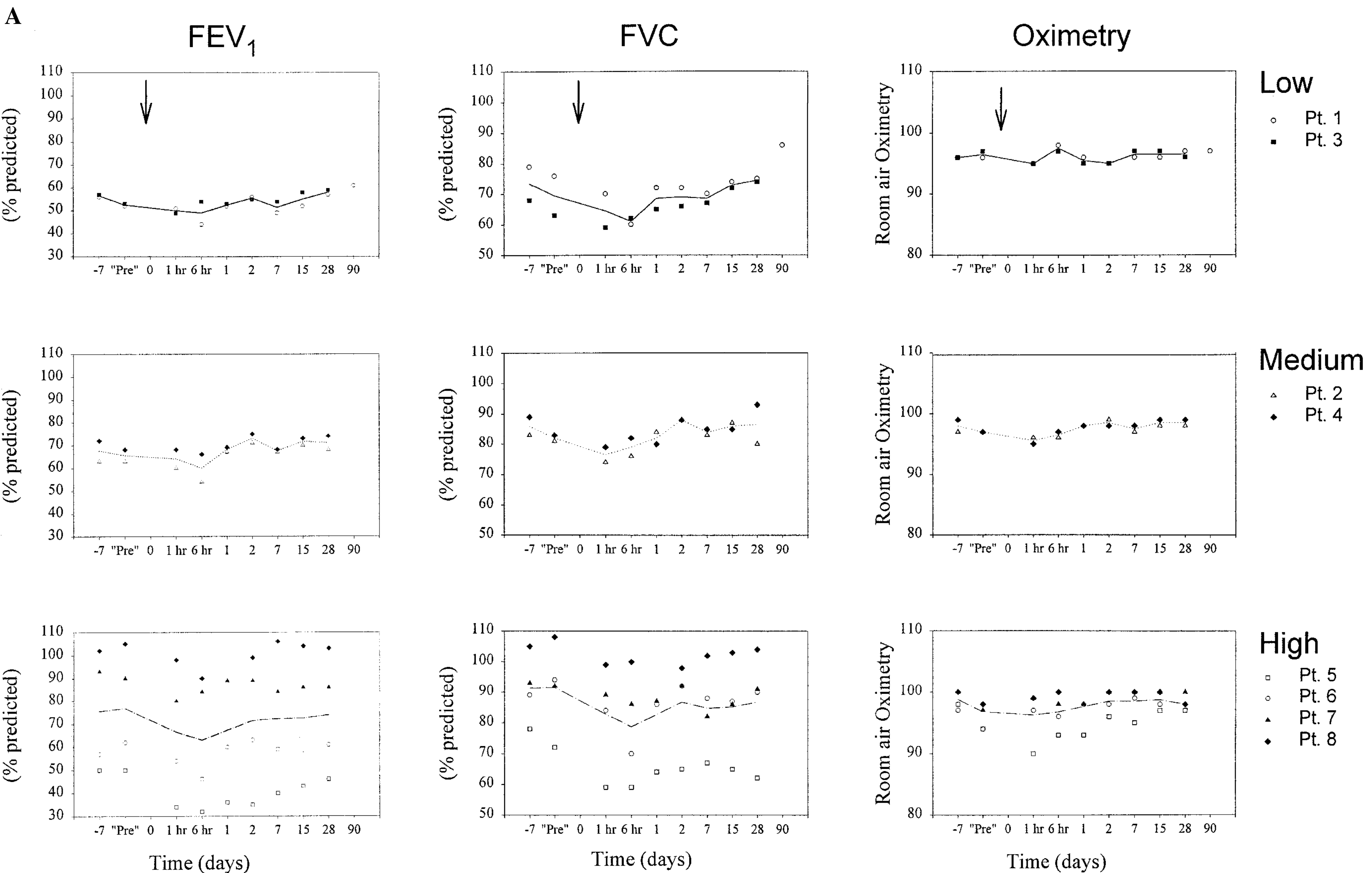


FIG. 1. (**A**) Changes in FEV_1 FVC, and oximetry as a result of lipid–DNA administration. (**B**) Elevated temperature in study patients after lipid–DNA administration: mean temperatures for the three treatment groups are shown; low, medium, and high refer to the three dosage groups (Table 3). Fever was observed in each of the dosing groups (patients 1, 2, 4, 5, and 8). Arrow indicates the time of gene administration.

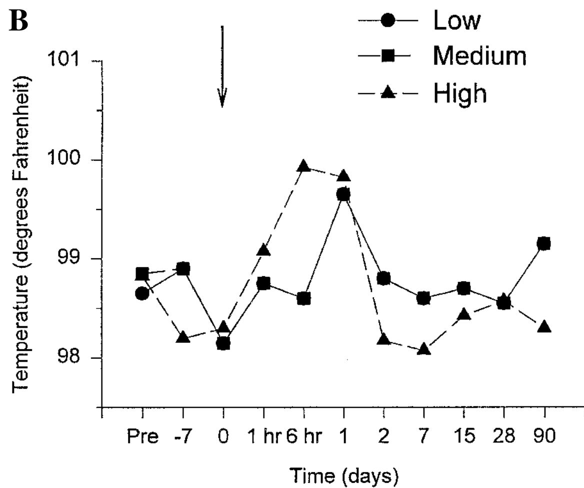


FIG. 1. *Continued.*

At each bronchoscopy, two or three brushings were taken in the region of the tracheal bifurcation and one or two endobronchial biopsies were also obtained. Both brushings and biopsies were taken at every bronchoscopy. RT-PCR was performed only on brushing samples (biopsies were processed for histopathology). At the time of initial consent, all patients agreed to at least two bronchoscopies, one before and one after lipid–DNA administration. However, in patients that developed pronounced fevers, myalgias, arthralgias, or substantially decreased FEV_1, we elected not to perform a bronchoscopy after gene administration. This decision was based on patient safety as a primary concern, but also allowed us to monitor fevers, myalgias, and arthralgias without the possible confounding effects of additional fever or further decrease in lung function that could result from an intercurrent bronchoscopy.

Sample collection and handling

RT-PCR. Because of difficulties in detecting expression of wild-type CFTR mRNA in more recent human gene transfer trials (Knowles *et al.*, 1998; Alton *et al.*, 1999), particular care was taken to ensure the specificity of RT-PCR after lipid–DNA aerosolization. In addition, since procedural problems may have been the reason for the failure of the RT-PCR test in the only other trial of aerosolized lipid–DNA complexes in human lungs, both sample processing and amplification conditions are described in detail below.

Cytology brush samples were collected by bronchoscopy 2 and 7 days after treatment. The cytology brush was placed in a sterile microcentrifuge tube and immediately placed on dry ice. Within 15 min, 800 μl of RNA STAT 60 (Tel-Test B, Friendswood, TX) was added to the brush sample and frozen on dry ice until the sample was transferred to a -70°C freezer for storage. The samples were transported on dry ice to Genzyme (Framingham, MA), assigned accession numbers, and then stored at -70°C. Alternatively, cytology brushes were cut and then placed into 2-ml RNase-free tubes containing 400 μl of Oligotech lysis buffer (Qiagen, Valencia, CA)–2-mercaptoethanol to inactivate RNase in the sample and immediately lyse the cells.

RNA extraction. Total RNA was isolated from the brush samples that were stored in RNA STAT 60, using a modification of the package instructions and the method of (Chomczynski and Sacchi 1987). Briefly, zirconia beads were added to the tubes containing the cytology brush and the sample was shaken by hand. Chloroform was added and the aqueous phase containing the RNA was recovered. RNA was precipitated with ethanol and the pellet was resuspended in 11 μl of diethypyrocarbonate (DEPC)-treated water.

Poly(A)-containing RNA was isolated from brushes placed in Oligotech lysis buffer–2-mercaptoethanol, using on Oligotech direct mRNA minipurification kit (Qiagen), according to the manufacturer instructions. The separation of poly(A)-containing mRNA was achieved by hybridizing the mRNA to oligo(dT) coupled to a solid-phase (Oligotex) matrix, and then eluting the bound mRNA.

PCR and RT-PCR. All amplification reactions were performed in a thermal cycler using *Taq* polymerase in a solution containing a final concentration of 10 m*M* Tris-HCl, (pH 8.3), 50 m*M* KCl, 1.75 m*M* $MgCl_2$, 0.01% gelatin (dTTP, dATP, dGTP, and dCTP (200 μM each), and primers. PCR amplification products were analyzed by electrophoresis in a 1.5% LE agarose gel (FMC, Rockland, ME). Semiquantiative reactions also contained [^{33}P]dCTP (New England Nuclear, Boston, MA). After photo documentation, the gels were dried and scanned onto a phosphoimager (Molecular Dynamics, Sunnyvale, CA). In addition to the study samples, a water negative control and a positive control were run in each assay.

pCF1-CFTR-specific mRNA was detected by a nested RT-PCR protocol. Primer 977RT was used to prime the cDNA synthesis reaction with either avian myeloblastosis virus (AMV; InVitrogen, Carlsbad, CA) or Moloney murine leukemia virus (Mo-MuLV; Pharmacia, San Diego, CA) reverse transcriptase. mRNA from 293 cells transfected with the pCF1-CFTR plasmid was used as a positive control. DNase treatment before analysis was not performed since an intron in the vector is spliced from the mRNA and the resulting RT-PCR products are distinguishable from the vector DNA amplification products by virtue of differences in their size. RT reactions were performed in duplicate, with the second tube serving as a no-RT negative control. The nested amplification reaction was performed as described above, using a 250 n*M* concentration each of primers 701 and 1338 in the first round and a 250 n*M* concentration each of primers 750 and 1211 in the nested reaction.

The sequences of the primers used in this study are as follows: 977RT, 5′-AGCGTTCCTCCTTCTTA-3′; 701, 5′-CCTCACTCTCTTCCGCATCGCTGTC-3′; 1338, 5′-GCCAGCTCTCTATCCCATTCTCTTTCCAAT-3′; 750, 5′-CGGTTGAGGACAAACTCTTC-3′; 1211, 5′-AAAAAGTTTGGATACAACGC-3′.

Mononuclear cell and serologic responses to cationic lipid–DNA complexes

To test for a possible role of immune effector cells in the clinical symptoms we observed after lipid–DNA administration, peripheral blood mononuclear cells were isolated by Hypaque-Ficoll density gradient from three subjects (patients 06–08) receiving the highest level of lipid–DNA, and cultured in 24-well

PATIENT	DOSE	DAY 2	DAY 7
001	L	-	NT
002	M	-	NT
003	L	+	-
004	M	-	NT
005	H	NT	NT
006	H	-	+
007	H	+	+
008	H	NT	NT

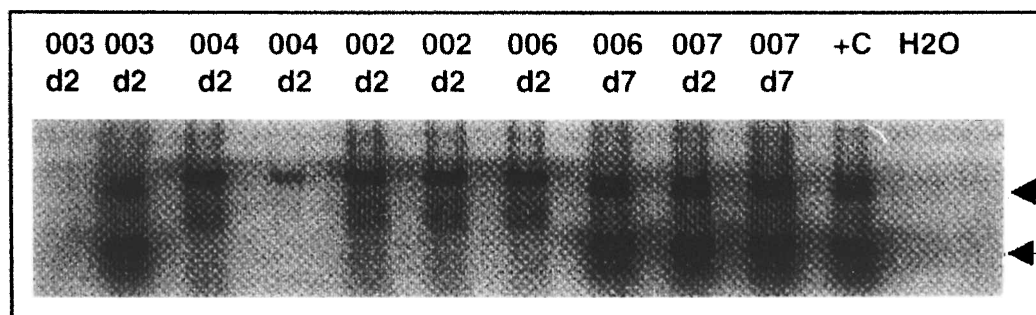
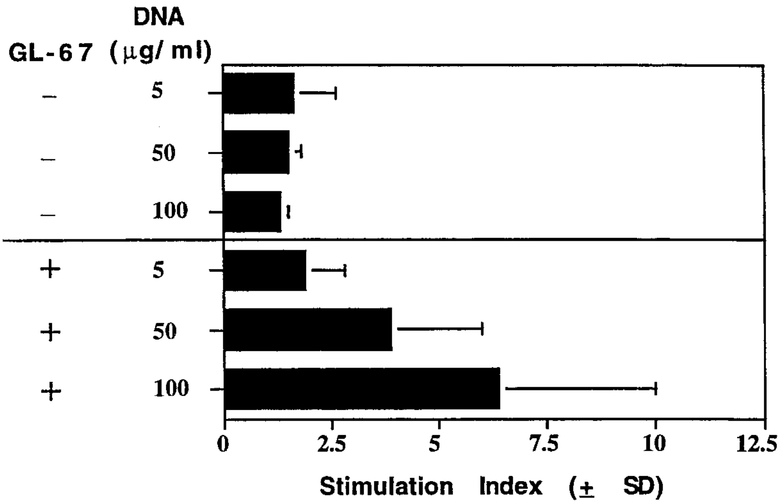


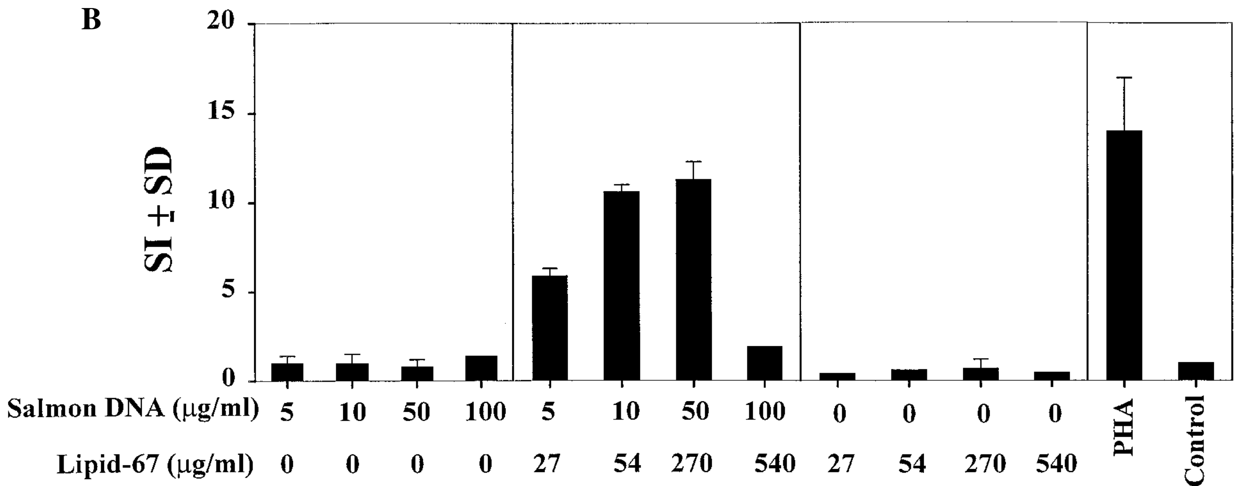
FIG. 2. RT-PCR 2 and 7 days after CFTR gene administration. *Top arrow*: PCR product derived from plasmid DNA or unspliced mRNA. *Bottom arrow*: correctly spliced CFTR mRNA expression. NT, Not taken; L, lower dosing M, medium dosing group; H, high dosing group. The observation that CFTR mRNA was detected on day 7 but not on day 2 in patient 6 suggests patchiness of distribution of CFTR expression by the aerosolization protocol, or variable recovery of CFTR mRNA when using bronchoscopic biopsies to sample airway mucosa.

plates containing RPM1 1640 and 10% human serum (type AB) as described previously (Sabbaj *et al.*, 1992). Purified salmon sperm genomic DNA, pCF1-CFTR, and 67A–DNA complexes prepared as described above were incubated with the cells for 48 hr, and cell proliferation was measured by [^{3}H]thymidine incorporation. Proliferative responses were compared with vehicle (medium)-treated controls at all doses of lipid and/or DNA, and proliferation due to lipid–DNA complexes when compared with the same dose of lipid alone or DNA alone. Statistics were by two-tailed, unpaired *t* test and analysis of vari-

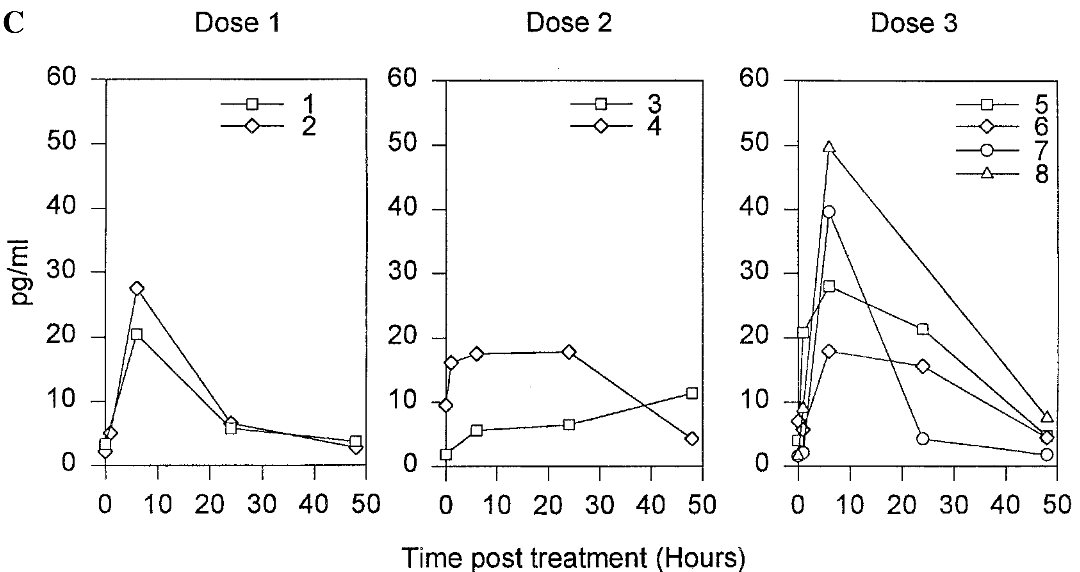
FIG. 3. Immunologic responses to lipid–DNA complexes. (**A**) Induction of proliferative responses in PBMCs of CF patients after *in vitro* stimulation with lipid–DNA complexes. PBMCs were cultured in the presence of plasmid DNA or lipid–DNA complexes at the same ratio used in the clinical trial and in the presence of 0.5 μCi of [^{3}H]thymidine per well during the last 8 hr of culture. Illustrated are the stimulation indices (SI $\pm$ SD; $n = 3$ subjects) at various DNA concentrations. The SI for unstimulated control cultures is by definition 1.0 and the SI for purified LPS (at a 10-μg/ml concentration; more than 38-fold higher than LPS in the plasmid DNA used in our studies) was 1.3 $\pm$ 0.8 (data not shown). This result indicates that LPS did not contribute significantly to observed proliferative responses. In these studies, lipid–DNA evoked significantly greater cellular proliferation compared with lipid alone ($p = 0.0007$, 0.0012, and 0.0019 for 5, 50, and 100 μg of DNA per milliliter, respectively; not shown) or compared with plasmid alone ($p = 0.1946$, 0.0001, and 0.0058 for 5, 50 and 100 μg of DNA per milliliter, respectively). Proliferation in the lipid–DNA group also was significantly greater than medium (vehicle)-treated control cells ($p < 0.0001$ at the three dosages shown). (**B**) Human PBMCs stimulated *in vitro* with various concentrations of eukaryotic DNA in the presence or absence of cationic lipid. PBMCs were cultured at 2×10^6 cells/ml and were stimulated with DNA (*left*), DNA–lipid 67 (*middle*), or lipid alone (right) for 48 hr with 0.5 μCi of [^{3}H]thymidine per well for the last 8 hr of culture. Lipid alone was added at the same concentration tested in the middle panel, but without eukaryotic DNA. Illustrated are the stimulation indices (SIs) from a representative subject performed in triplicate. The result indicates a strong, synergistic proliferative response due to the addition of 67A to eukaryotic DNA. Lipid–DNA in these experiments evoked significantly greater cellular proliferation compared with DNA alone ($p = 0.0003$, 0.0001, 0.0001, and 0.0019 for the 5, 10, 50, and 100-μg/ml DNA doses, respectively) or compared with lipid alone ($p = 0.0001$ at all lipid doses). Lipid treatment alone or DNA treatment alone did not differ from vehicle (medium)-treated control cells. However, proliferation due to lipid–DNA differed from that due to vehicle treatment (controls) in all cases ($p = 0.0001$, 0.0001, 0.0001, and 0.0386 for complexes containing 5, 10, 50, and 100 μg of DNA per milliliter, respectively). Positive proliferative control (PHA) is also shown. Growth and viability of cells were inhibited at the highest concentration of lipid–salmon sperm DNA. (**C**) Serum IL-6 levels in study patients after gene transfer to the lung. Lipid–DNA was administered at time = 0 hr. Serum IL-6 was measured by ELISA. Data from patients 1–8 are shown.



A
DNA
GL-67 (μg/ ml)
–
5
50
100
+
0
2.5
5
7.5
10
12.5
Stimulation Index (± SD)



B
SI ± SD
20
15
10
5
0
Salmon DNA (μg/ml) 5 10 50 100 5 10 50 100 0 0 0 0
Lipid-67 (μg/ml) 0 0 0 0 27 54 270 540 27 54 270 540
PHA
Control



C
Dose 1
Dose 2
Dose 3
pg/ml
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
0 10 20 30 40 50
Time post treatment (Hours)

ance (ANOVA); similar conclusions were reached by either statistical measure.

Serum interleukin 1β (IL-Iβ), IL-8, and tumor necrosis factor α (TNF-α) levels were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Genzyme, Cambridge, MA); serum IL-6 and interferon γ (IFN-γ) were detected by an ELISA kit available from R&D Systems (Minneapolis, MN). Antibodies to lipid or plasmid DNA were studied by coating microtiter cells with 67A or protamine-treated microtiter plates with plasmid DNA. After incubation with patient serum, development was with horseradish peroxidase-conjugated, anti-human IgG.

RESULTS

Safety of aerosolization of cationic lipid–DNA complexes to lungs of cystic fibrosis patients

No changes in blood, serum, or urine chemistries were observed as a result of lipid–DNA aerosolization. Chest X-rays and CT scans remained unchanged. No airway histopathology attributable to gene administration was observed. All patients exhibited a transient fall in FEV_1, forced vital capacity (FVC), and oximetry that later resolved (Fig. 1A). No changes in total lung capacity, lung diffusion capacity ($D_{L_{CO}}$), or residual volume occurred.

Four of eight patients (subjects 1, 2, 4, and 8) experienced a pronounced syndrome of muscle and joint aches, and fevers to 103.3°F (Fig. 1B). Patient 5 had fever (101.9°F) without myalgias or arthralgias, and patient 6 had joint pain without fever. These symptoms did not correlate with baseline FEV_1, or bacterial colonization status (Table 1). Moderate to exquisite tenderness over the muscles of the upper and lower extremities, and tenderness over the ribs, elbows, wrists, and knees without clinically apparent joint effusions, were found on examination. No dose dependence of these effects was noted. Erythrocyte sedimentation rate, creatine phosphokinase, aldolase, anti-nuclear antibody, and rheumatoid factor were not altered by lipid–DNA aerosolization in patients who developed these symptoms, suggesting against an acute myositis. The symptoms partially improved after administration of acetaminophen and nonsteroidal antiinflammatory drugs. Symptoms and fevers usually resolved completely within 24–48 hr after gene administration, although one patient in dosing group 1 complained of brief periods of self-limited myalgias over the ensuing 3 weeks that resolved completely. In the setting of high fever, patient 8 developed exacerbation of an underlying superventricular tachycardia (ectopic atrial tachycardia) with brief runs to approximately 180 beats per minute, without hemodynamic compromise. The tachyarrhythmia was corrected by intravenous, followed by oral, administration of a calcium channel blocker, although the patient has experienced myalgias and arthralgias without fever intermittently after gene administration. This latter patient's daughter (patient 7) with CF was also enrolled in the study, and remained asymptomatic throughout both the gene transfer and follow-up portions of the protocol.

Wild-type CFTR expression

The specificity of RT-PCR in bronchoscopic tissue samples from the airways of CF patients (Fig. 2) was tested by (1) assaying for a CFTR mRNA splice variant that could only be plasmid derived, (2) measuring mRNA that was of a different size than PCR products derived from the administered plasmid DNA, (3) verifying the absence of any PCR product in the absence of reverse transcriptase, (4) amplification of an internal product of the correct, predicted size using internal PCR primers, and (5) correct restriction digestion patterns of PCR products after cleavage with a different restriction endonuclease.

The day 2 bronchoscopy was not performed in certain of the patients who developed the fevers, myalgias, and arthralgias syndrome (not taken, NT; Fig. 2). This was a conservative decision, but allowed our laboratory to monitor the syndrome without possible confounding effects due to the bronchoscopy itself. The plasmid used in our studies contained a synthetic intron, so that contaminating DNA (or unspliced RNA) amplified as a large band (upper arrow, Fig. 2) by RT-PCR. Plasmid taken up, processed, and spliced by airway target cells had a smaller RT-PCR product (lower arrow, Fig. 2). The lower molecular weight products were further verified by restriction digest after amplification with internal primers, and can only be from plasmid taken up, processed, and expressed in airway cells. The no-RT controls were negative in all cases for the spliced product, and a positive control from cells transfected in culture with the CFTR plasmid is also shown (lane +C; Fig. 2). Overall, four of nine bronchoscopic brushing samples taken from study patients were positive for vector-derived CFTR mRNA. Three of four samples were positive at the highest dose.

Immune responsiveness of human mononuclear cells to lipid–DNA complexes

Peripheral blood was obtained in citrated tubes from study subjects approximately 30 min prior to gene administration, and mononuclear cells were purified as described in Materials and Methods. Figure 3 indicates that highly purified plasmid DNA synthesized in *E. coli*, or eukaryotic DNA, elicited proliferation of immune mononuclear cells when added as a complex with cationic lipid molecules. Interestingly, we observed that lipid alone or DNA alone cause minimal proliferation, but that the complexes caused a marked increase in proliferation. We saw similar responses when the lipid–DNA complexes used in our study were used (Fig. 3A) or when eukaryotic DNA containing fewer immunostimulatory CpG-type sequences were complexed to lipid 67A (Fig. 3B). These results establish a proliferative response of human immune cells to lipid–DNA complexes, occurring in a fashion that is dependent on lipid and that does not occur when either lipid or DNA alone is added to the cell cultures.

Patients in the study did not develop evidence of antibodies against either 67A or plasmid DNA at any time point during the trial. Antibodies to lipid and DNA were tested at nine time points before and after CFTR administration. These included evaluation of serum 16 and 6 days before gene administration, at 1 and 6 hr after gene transfer, and at 1, 2, 7, 15, and 28 days after lipid–DNA administration. We have previously published (Scheule *et al.*, 1997) that our attempts to raise antibodies against either lipid or DNA by direct immunization (of mice and rabbits) were unsuccessful in eliciting an immune response. Serum levels of IL-1, IL-8, TNF-α, and IFN-γ did not change

as a result of gene administration. The levels of serum IL-6 in all eight patients increased after lipid–DNA administration, suggesting that pulmonary macrophages, airway epithelial cells, or other cells released this proinflammatory cytokine as part of a response to lipid–DNA conjugates (Fig. 3C; see also below).

DISCUSSION

These experiments examined the clinical safety of aerosolized lipid–DNA gene transfer to human lungs. Previous studies using lipid–DNA complexes in nasal airways have indicated gene transfer and partial correction of the CF ion transport defects *in vivo* (Caplen *et al.*, 1995; Gill *et al.*, 1997; Porteous *et al.*, 1997; Zabner *et al.*, 1997; Alton *et al.*, 1999). Additional experiments in the lower airways are therefore necessary to evaluate the overall strategy. Our results point to a clinical syndrome of myalgias, arthralgias, and fevers that occurred in more than half the subjects and was attributable to lipid–DNA administration. The clinical findings after lipid–DNA administration, together with the anticipated need for retreatment using plasmid-based CFTR gene transfer, are likely to limit the usefulness of this approach for diseases such as cystic fibrosis.

Aerosolization of the lipid–DNA decreased oxygen saturation, FVC, and FEV1 in a transient fashion. Joint and muscle symptoms were observed, including the possibility of chronic musculoskeletal complaints in one patient in the trial. These symptoms were surprising, since the same amount of cationic lipid aerosolized by the same technique in normal volunteers led to no clinical symptoms or signs (Chadwick *et al.*, 1997), and the sputum of cystic fibrosis patients contains high levels of both prokaryotic and eukaryotic DNA (up to 30 mg total DNA/ml sputum)(Shak *et al.*, 1990). Moreover, nasal administration trials have not indicated fevers, arthralgias, or other constitutional symptoms.

To examine the clinical symptoms associated with lipid–DNA administration, we asked whether inflammatory cells taken from patients in our trial showed characteristic immune responsiveness to lipid or plasmid DNA. We found that DNA alone elicited minimal immune cell proliferation, but that either prokaryotic or eukaryotic DNA led to pronounced immune cell proliferation when complexed to a cationic lipid (Fig. 3). This effect does not appear to require specific immunologic memory to GL-67, since study subjects were naive to this cationic lipid, and effects were seen in cells taken prior to gene administration. Moreover, cellular proliferation was also seen with eukaryotic DNA, suggesting that at least part of the immunologic response to lipid–DNA conjugates is independent of unmethylated (*E. coli*-derived) CpG sequences, which have previously been associated with innate inflammatory changes in the lung. Cationic lipids may bring foreign DNA molecules in close proximity to the negatively charged proteins in the plasma membranes of immune effector cells, facilitating responses to DNA that depend on surface receptors or internalization. The possibility that cationic lipids other than Lipid 67 may also promote immunologic reactivity should be considered in studies of gene transfer to pulmonary and other tissues. The number of subjects studied here made it difficult to draw conclusions regarding the relationship between clinical symptoms and lymphocyte proliferation. In either case, we speculate that cationic lipids alone (as in a normal lung administered 67A; Chadwick *et al.*, 1997), or prokaryotic or eukaryotic DNA alone (e.g., in CF sputum) incites much less clinical inflammation than the synergistic effect of lipid and DNA administered together. Finally, although we did not detect antibodies to lipid or DNA in serum of study subjects, antibodies to lipid–DNA complexes (and humoral responsiveness in readministration protocols) remain important considerations regarding the overall usefulness of nonviral vectors of this type.

The existence of an underlying defect in the airway immune response of CF patients is controversial; however, increasing evidence suggests exaggerated immunologic reactivity even in the absence of bacterial infection. For example, Osika and colleagues have reported extremely low levels of IL-6 in the sputum of CF patients as compared with non-CF controls (Osika *et al.*, 1999) whereas Bonfield *et al.* have reported elevated levels of IL-6 in BAL fluid (Bonfield *et al.*, 1999). Others have demonstrated elevated IL-6 production from both CF lung macrophages and airway epithelial cells (Bonfield *et al.*, 1995; Kammouni *et al.*, 1997; Tabary *et al.*, 1998). It is therefore possible that IL-6 secretion and systemic inflammation in our experiments could reflect immune hyperresponsiveness in the CF lung. However, our studies also indicate strong proliferative responses in mononuclear cells taken from the peripheral blood of CF patients under conditions that preclude a contribution of the pulmonary epithelium. Consequently, it seems less likely that the clinical alterations observed here are primarily a result of mutations in CFTR; that is, similar effects in other individuals should also be anticipated.

The aerosolization protocol examined in this trial was designed to generate particles that would deposit in the lower airways and alveolar regions. In preclinical experiments using the same aerosolization apparatus and lipid–DNA formulation, we verified deposition to these regions of the lung in nonhuman primates. Although precise quantitation is not available in the human trial, we expect that the particle size distribution would primarily lead to deposition in the lower airways and alveoli. It is therefore possible that some of the systemic reaction observed in our experiments could be attributable to distal airway delivery; for example, if aerosolized complexes led to immediate (irritant-type) inflammatory responses.

On the basis of the known effects of IL-6, including proinflammatory symptoms and immunoregulation through hypothalamic temperature regulatory centers, IL-6 may be an important contributor to clinical sequelae after aerosolization of lipid–DNA to human lung. Although elevations of serum IL-6 do not establish causality, in other settings serum IL-6 levels similar to those reported in our trial contributed to fevers and related symptoms in human subjects (Minamishima *et al.*, 1993; Otto *et al.*, 1999), indicating that the cytokine is a plausible mediator for some of the changes observed after lipid–DNA aerosolization. Additional studies will be required in order to test this possibility.

Previous findings in the nasal airways, using the same plasmid that was studied in our trial (even without GL-67) (Zabner *et al.*, 1997), have indicated partial correction of the CF nasal bioelectric abnormality. In the lung, the same formulation of cationic lipid–DNA used in our experiments (GL-67/pCF-1) partially corrected the CF Cl^- transport defect in the trachea and lower airways *in vivo* (Alton *et al.*, 1999), although wild-

type CFTR mRNA was not detected by RT-PCR. RT-PCR provides no evidence concerning cell type distribution after gene transfer in human airways, and the technique is often considered too sensitive to provide useful information concerning physiologic levels of CFTR expression on a per-cell basis. Despite its exquisite sensitivity, the detection of wild-type CFTR after liposomal gene transfer in human airways has been problematic in more recent trials, possibly for procedural reasons (Knowles *et al.*, 1998; Alton *et al.*, 1999). The present studies establish wild-type CFTR mRNA *in vivo* and describe an RT-PCR protocol with sufficient specificity to reproducibly detect CFTR transgene in human lung samples.

In conclusion, a response of human cells to highly purified, plasmid DNA has not been described previously, and animal trials have not tested ultrapurified plasmid DNA, suitable for human administration, for a possible role in inflammatory responsiveness. Our results emphasize that even after extraction of endotoxin and other contaminants, ultrapure plasmid DNA synthesized in bacteria is still recognized by host mononuclear cells in humans, and this recognition is augmented by the presence of cationic liposomes. The findings show that highly purified plasmid DNA complexed to a cationic lipid will still evoke an inflammatory response in humans, even in the absence of endotoxin or other *E. coli*-derived products. Liposomes are under study for manifold gene therapy applications (as reviewed in Clancy and Sorscher, 1999). Clinical trials using plasmid or cationic lipid–plasmid DNA complexes for vaccines or gene complementation in diseases such as HIV, influenza, mycoplasm, melanoma, α_1-antitrypsin deficiency, or cystic fibrosis should consider the proinflammatory effects of plasmid DNA as part of experimental design and interpretation, since these vectors have significant potential for mediating a pronounced, systemic inflammatory response in human subjects.

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