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Inhibition of endotoxin-induced lung inflammation by interleukin-10 gene transfer in mice

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Dokka, Sujatha, Carl J. Malanga, Xianglin Shi, Fei Chen, Vincent Castranova, and Yon Rojanasakul. Inhibition of endotoxin-induced lung inflammation by interleukin-10 gene transfer in mice. *Am J Physiol Lung Cell Mol Physiol* 279: L872–L877, 2000.—Interleukin (IL)-10 is an anti-inflammatory cytokine that has great potential for use in the treatment of inflammatory and immune illnesses. In this study, gene transfer was used to induce IL-10 transgene expression in murine lungs for treatment of endotoxin-induced lung inflammation. Gene transfer was performed with a cytomegalovirus (CMV)-IL-10 plasmid with the aid of the liposomal agents LipofectAMINE and *N*-[1-(2,3-dioleoyl)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP). Administration of the endotoxin caused a marked increase in lung inflammation as indicated by increased tumor necrosis factor (TNF)-α release and neutrophil count. Pretreatment of the mice with IL-10 plasmid with and without LipofectAMINE had no inhibitory effect on lung inflammation and IL-10 transgene expression. LipofectAMINE by itself induced lung inflammation, an effect that was not observed with DOTAP. IL-10 plasmid when codelivered with DOTAP expressed biologically active IL-10 protein and caused a reduction in endotoxin-induced inflammation. Transgene expression was observed as early as 3 h after administration, peaked at 12 h, and declined thereafter. We conclude that IL-10 gene transfer is a feasible approach for the treatment of lung inflammation.

tumor necrosis factor-α; gene transfer; liposome

INTERLEUKIN (IL)-10 is an anti-inflammatory cytokine that is regularly produced during inflammatory processes in vivo, usually by the same cells that release inflammatory mediators (e.g., monocytes, macrophages, T cells). It inhibits the production of inflammatory cytokines, e.g., IL-1, IL-6, and tumor necrosis factor (TNF)-α, by inflammatory cells stimulated with endotoxin and interferon (8). IL-10 has been implicated in a number of inflammatory disorders such as sepsis, chronic arthritis, and inflammatory bowel diseases (11, 22). In animal models of sepsis, IL-10 given before or soon after gram-negative bacterial endotoxin reduces TNF-α production, hypothermia, and death (13, 15). It was found that mice are more sensitive to lipopolysaccharide (LPS)-induced shock by treatment with anti-IL-10 antibodies (14). It was also found that IL-10-deficient mice developed severe enterocolitis and that IL-10 is an essential immunoregulator in the intestinal tract (15). In an experimental model of lung inflammation, IL-10 has been shown to inhibit lung inflammation induced by IgG immune complexes (17, 23). Because of its potent anti-inflammatory effects, IL-10 has been investigated as a potential therapeutic agent for inflammatory disorders.

Most IL-10 studies to date (13, 15, 23, 25) have been carried out with recombinant IL-10 protein. However, recombinant protein has the disadvantage of being short-lived, poorly accessible to tissue interstitium, and expensive. In the present study, we used a gene transfer strategy to deliver the therapeutic IL-10 gene to the lung for the possible treatment of lung inflammation. Gene transfer technology offers the potential advantage of prolonged expression. In addition, because of the amplification process associated with gene expression, i.e., a single-gene copy can express numerous copies of protein, this method is therefore potentially more effective than the protein delivery method. However, the use of a gene transfer method has been hindered by inefficient gene expression in vivo. The present study investigated the effect of naked DNA and DNA-liposome complexes on IL-10 gene expression. The specific aims of this study were *1*) to determine whether IL-10 gene transfer can be used to produce therapeutically relevant levels of IL-10 protein in mouse lungs, *2*) to determine whether transgene expression can result in reduced lung inflammation caused by endotoxin, and *3*) to evaluate the efficacy and safety of gene delivery systems in transferring the IL-10 gene to lung cells.

MATERIALS AND METHODS

Animals. Healthy male BALB/c mice 4–6 wk old were obtained from Jackson Laboratories (Bar Harbor, ME). They were acclimated in an American Association for Accredita-

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tion of Laboratory Animal Care-approved facility for at least 1 wk before use. The mice were fed water and food ad libitum. Intratracheal instillations into mice were performed after they were anesthetized with a mixture of ketamine and xylazine (45 and 8 mg/kg ip, respectively) and challenged by aspiration. The animals were placed on a board in a supine position. The animal's tongue was extended with lined forceps, and 50–100 μl of the test solution containing DNA complexes or endotoxin (10–30 μg/mouse) were placed on the back of the tongue.

Preparation of DNA-liposome complexes. Plasmid cytomegalovirus (CMV)-murine IL-10 (a kind gift from Dr. Leaf Huang, University of Pittsburgh, Pittsburgh, PA), which contains murine IL-10 cDNA driven by a CMV immediate-early promoter, was amplified in the DH5α strain of *Escherichia coli*, isolated, and purified according to the manufacturer's instructions (QIAGEN, Chatsworth, CA). Endotoxin-free plasmid was used for all experiments. The content of endotoxin in the plasmid DNA preparations was determined by using the chromogenic *Limulus* amebocyte lysate assay (Kinetic-QCL, BioWhittaker, Walkersville, MD) according to the manufacturer's instructions. Plasmid samples prepared by this method were found to contain <0.1 endotoxin unit/μg plasmid DNA. The cationic liposomes used in this study were LipofectAMINE (GIBCO BRL, Life Technologies, Gaithersburg, MD) and *N*-[1-(2,3-dioleoyl)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP; Boehringer Mannheim, Indianapolis, IN). For the preparation of DNA-liposome complexes, various amounts of LipofectAMINE or DOTAP in distilled water were added to the diluted DNA, and the mixture was incubated for 10 min before use.

Bronchoalveolar lavage. At selected time intervals, treated mice were euthanized with an intraperitoneal injection of 0.25 ml of pentobarbital sodium (EUTHA-6, Western Medical Supply, Arcadia, CA). A tracheal cannula was inserted, and the lungs were lavaged through the cannula with ice-cold PBS. Five lavages of 0.8 ml each were collected to obtain sufficient cells to perform differential cell counts. The first lavage was separated from the consecutive lavages and was used for lactate dehydrogenase (LDH) assay and IL-10 ELISA. Bronchoalveolar lavage (BAL) fluid (BALF) cells were isolated by centrifugation (500 *g* for 10 min at 4°C). The cell-free supernatants were collected and used for biochemical measurements. For each animal, the cell pellet was resuspended in 1 ml of HEPES buffer (10 mM HEPES, 145 mM NaCl, 5.0 mM KCl, 1.0 mM $CaCl_2$, and 5.5 mM D-glucose, pH 7.4) and centrifuged (500 *g* for 10 min at 4°C), and the supernatant was decanted and discarded. The BALF cell pellet was then resuspended in 1 ml of HEPES buffer and placed on ice. Cell counts and differentials were determined with a Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL).

LDH activity. A LDH assay was performed to assess the effect of liposomes on cellular toxicity. Mice were instilled with the test agents. After the treatments, BAL was performed, and the supernatant from the first lavage was assayed for LDH activity. LDH activity was determined by monitoring the oxidation of pyruvate coupled with the reduction of NAD at 340 nm with a LDH assay kit (Roche Diagnostic Systems, Montclair, NJ). The assay was performed on a Cobas Fara II analyzer (Roche Diagnostic Systems). One unit of LDH activity per liter is defined as the amount of enzyme that converts 1 μmol of lactate to 1 μmol of pyruvate, with the concomitant reduction of 1 μmol of NAD to 1 μmol of NADH per minute per liter of sample in the assay procedure.

Cytokine determination. Supernatants from the first BAL were assayed for cytokines with ELISA kits specific for murine TNF-α and IL-10 according to the manufacturer's instructions (Endogen). The sensitivity of the assays ranged from 15 to 31 pg/ml. The coefficient of variation for all cytokine assays was <10%.

Statistical analysis. Each study group consisted of four animals. Statistical analysis between study groups was performed with paired two-tailed Student's *t*-test. The level of significance was $P < 0.05$.

RESULTS

Endotoxin-induced lung inflammation and its inhibition by IL-10 protein. Endotoxin-induced lung inflammation is associated with increased production of TNF-α and sequestered pulmonary neutrophils (3, 4). In the present study, we utilized these two indexes of inflammation as quantitative measures of lung inflammation. Lung inflammation was induced by an intratracheal instillation of LPS (30 μg/mouse) into mouse lungs. At various time points after the instillation, TNF-α level and polymorphonuclear neutrophil (PMN) cell count were measured. Figure 1*A* shows that LPS stimulation caused a rapid but transient increase in TNF-α production. This increase in cytokine level occurred within 1 h of stimulation and reached a maximum at 6 h. By 24 h, the TNF-α level had already subsided. Figure 1*B* shows that PMN cell count also increased in response to LPS stimulation. This effect was time dependent and slower in onset but more sustained than the TNF-α level.

To study the effect of IL-10 gene transfer on lung inflammation, it was first necessary to test the anti-

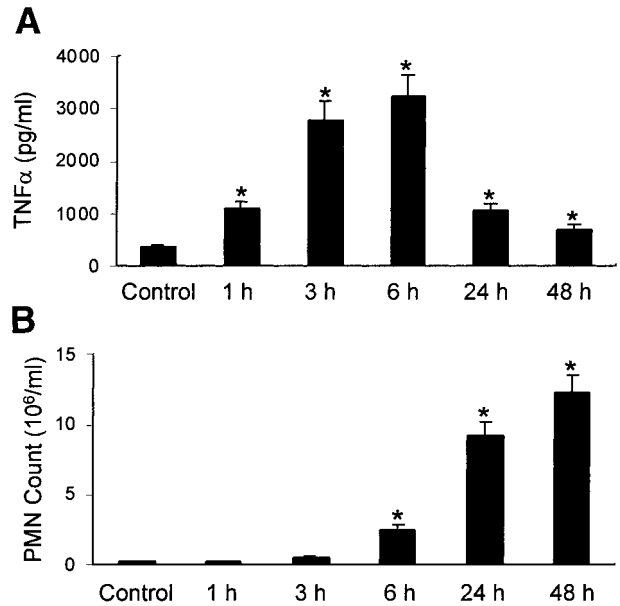

Fig. 1. Lipopolysaccharide (LPS)-induced lung inflammation in mice. Mice were instilled with LPS (0.03 mg/mouse), and bronchoalveolar lavage (BAL) was performed at indicated times after instillation. The BAL fluid was tested for tumor necrosis factor (TNF)-α concentration (*A*) and polymorphonuclear neutrophil (PMN) cell count (*B*). Values are means ± SD; $n = 4$ mice/group. *$P < 0.05$ vs. untreated control group.

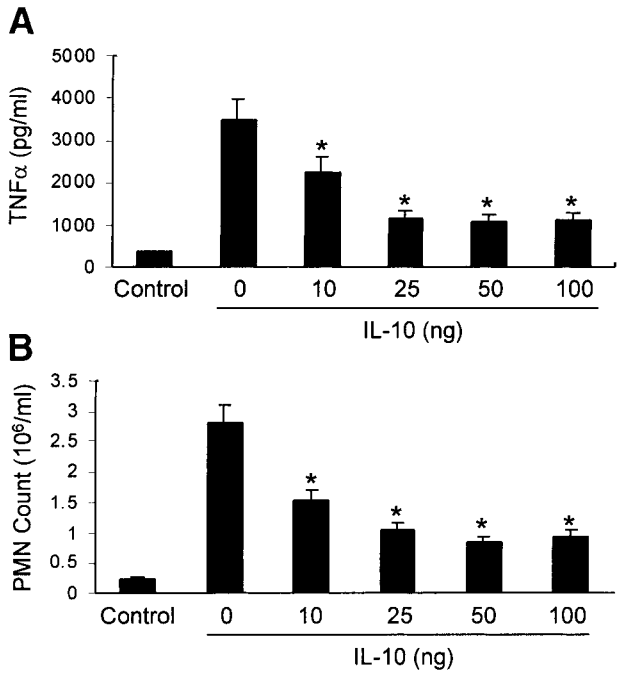

Fig. 2. Effect of pretreatment of mice with indicated amounts of recombinant interleukin (IL)-10 2 h before instillation of LPS (0.03 mg/mouse). BAL was performed 6 h after instillation. The BAL fluid was tested for TNF-α concentration (*A*) and PMN cell count (*B*). Values are means ± SD; $n = 4$ mice/group. $^*P < 0.05$ vs. LPS-treated control group.

inflammatory effect of IL-10 protein in our lung inflammation model. Mice were pretreated with varying amounts of purified recombinant IL-10 (0–100 ng/mouse) 2 h before instillation of LPS (30 μg/mouse). Six hours after LPS treatment, the mice were lavaged, and the BALF was assayed for TNF-α level and PMN cell count. Figure 2, *A* and *B*, shows that recombinant IL-10 effectively inhibited LPS-induced TNF-α production and PMN count in a dose-dependent manner. Maximum inhibitory effects were observed at the recombinant IL-10 dose of 50 ng/mouse. Increasing the dose of IL-10 beyond this point did not result in further inhibition of TNF-α level and PMN count. Thus our results indicate that IL-10 protein is indeed a potent anti-inflammatory molecule in vivo.

IL-10 gene transfer and the delivery system. A study by Meyer et al. (21) has shown that naked CMV-chloramphenicol acetyltransferase (CAT) plasmid instilled intratracheally expresses CAT protein in mouse lungs. To test if naked DNA by itself can express IL-10 in the lungs, the IL-10 plasmid was instilled, and the BALF was analyzed for IL-10 protein levels at different time points. Introduction of naked IL-10 plasmid (10–300 μg/mouse) did not lead to any detectable expression of IL-10 protein in the BALF. Because our subsequent studies showed that only 10 μg of the plasmid were sufficient to induce IL-10 protein expression when an appropriate delivery system was used, we therefore concluded that naked IL-10 DNA by itself was ineffective and that there is a need for a delivery system for efficient gene transfection in vivo.

In a separate study, we found that LipofectAMINE, a widely used transfecting agent, is one of the most effective agents in transfecting lung cells in vitro. Therefore, we attempted to use this agent to aid in the transfection of IL-10 plasmid in vivo. Varying amounts of LipofectAMINE (20–100 nmol/mouse) were instilled with the plasmid DNA (10 μg/mouse) into mouse lungs. Twelve hours after treatment, BALF was collected and analyzed for IL-10 protein, PMN cell count, and LDH activity. Our BALF analysis showed that there was no IL-10 expression (data not shown). Moreover, the PMN count was very high in mice treated with DNA-LipofectAMINE complex or LipofectAMINE alone (Fig. 3*A*). The measurement of LDH activity, which was used as an indication of lung toxicity, also showed that LipofectAMINE was very toxic at the concentrations used (Fig. 3*B*).

Because LipofectAMINE is very toxic by itself, we cannot use this liposome to enhance DNA delivery. A further study by our group (5) on the in vivo toxicity of cationic liposomes revealed that LipofectAMINE, due to its polycationic nature, caused the release of highly reactive oxygen species by lung cells that is responsible for lung toxicity. To avoid this problem, a monovalent cationic liposome, DOTAP, was used to aid in the delivery of DNA. DOTAP has been used as a gene delivery system in a number of in vivo applications (19, 20, 24). Our results show that DOTAP was relatively nontoxic and effective in promoting IL-10 gene expression in the lungs (see *Optimization of gene delivery with DOTAP*).

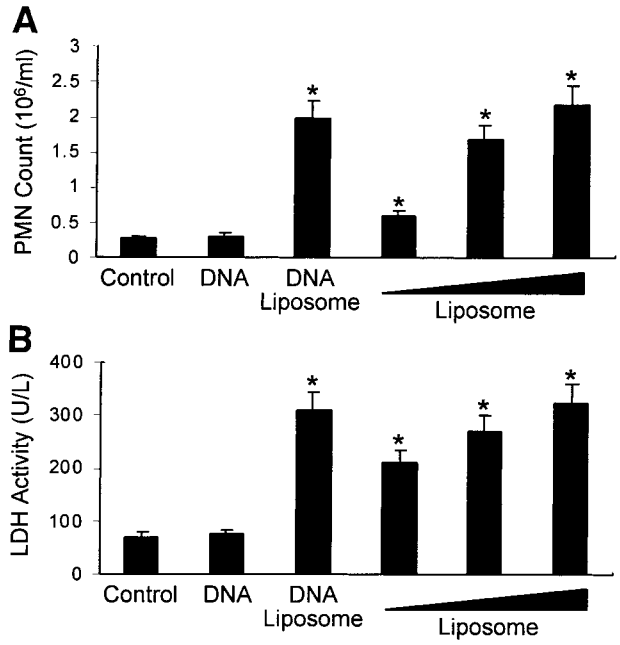

Fig. 3. Delivery of IL-10 plasmid with LipofectAMINE. Mice were instilled with DNA (10 μg/mouse), DNA (10 μg)-LipofectAMINE (60 nmol/mouse) complex, or LipofectAMINE alone (20, 60, and 100 nmol/mouse). BAL was performed 12 h after instillation. The BAL fluid was tested for PMN cell count (*A*) and lactate dehydrogenase (LDH) activity (B). Values are means ± SD; $n = 4$ mice/group. $^*P < 0.05$ vs. untreated control group.

Optimization of gene delivery with DOTAP. To optimize IL-10 gene transfer in the lung with DOTAP, we tested different DNA-to-DOTAP ratios on IL-10 gene expression (Fig. 4). As controls, animals were instilled with DOTAP alone or free DNA alone. As might be expected, no transgene expression was observed in the animals that received only DOTAP or DNA alone. Coinstillation of DNA and DOTAP resulted in a dose-dependent expression of IL-10 in the mouse lungs (Fig. 4*A*). Maximum expression was obtained when 12 nmol DOTAP/μg DNA was used. Beyond this ratio, the expression dropped significantly. PMN data showed that DOTAP, unlike LipofectAMINE, did not have any significant effect on lung inflammation (Fig. 4*B*). Similarly, LDH data indicated that DOTAP was nontoxic except when the highest concentration (150 nmol/mouse) was used (Fig. 4*C*). Because at this concentration the level of IL-10 expression also decreased, it is therefore likely that the decreased expression may be a result of toxicity associated with a high concentration of DOTAP. The kinetics of IL-10 expression after DNA-DOTAP administration was also studied (Fig. 5). IL-10 expression was seen to peak 12 h after instillation and subsided by 24 h.

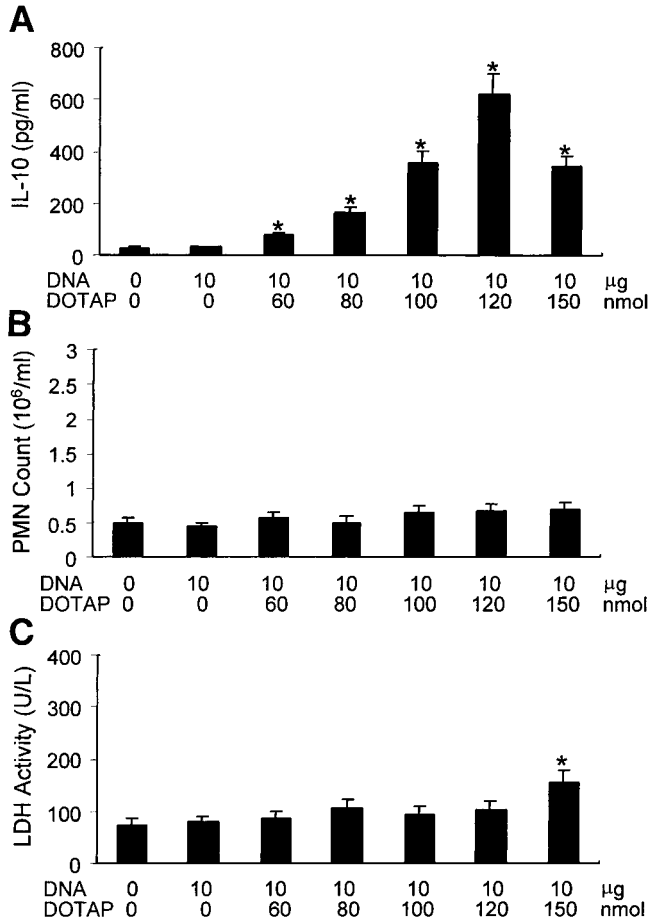

Fig. 4. Optimization of DNA-*N*-[1-(2,3-dioleoyl)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP) delivery system. Mice were instilled with indicated concentrations of DNA and DOTAP. Twelve hours after instillation, BAL was performed, and the BAL fluid was tested for IL-10 level (*A*), PMN cell count (*B*), and LDH activity (*C*). Values are means ± SD; $n = 4$ mice/group. *$P < 0.05$ vs. untreated control group.

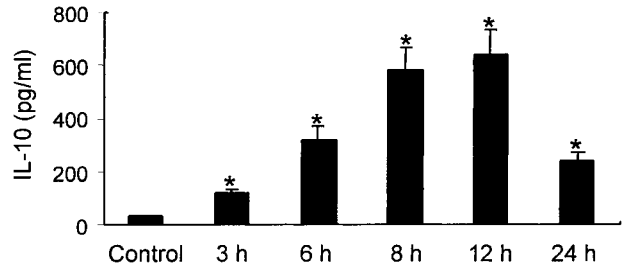

Fig. 5. Time profile of IL-10 expression after DNA-DOTAP administration. Mice were instilled with DNA (10 μg)-DOTAP (120 nmol/mouse). BAL was performed at indicated times after instillation, and the BAL fluid was tested for IL-10 level. Values are means ± SD; $n = 4$ mice/group. *$P < 0.05$ vs. untreated control group.

Effect of IL-10 gene transfer on LPS-induced lung inflammation. Because we developed a delivery system capable of expressing IL-10 in the lung, the next step was to study the functional effect of this expressed IL-10 on LPS-induced lung inflammation. Mice were instilled with IL-10-DNA-DOTAP complexes 12 h before LPS stimulation. Six hours after LPS stimulation, the mice were killed, and the BALF was tested for PMN count and IL-10 and TNF-α levels. As seen in Fig. 6*A*, DNA-DOTAP complexes delivered either with or without LPS expressed a high level of IL-10 protein. LPS by itself induced a modest but significant amount of IL-10 (Fig. 6*A*). At a dose of 30 μg/mouse, LPS caused a dramatic increase in TNF-α production that was decreased (~3-fold) by IL-10 gene expression (Fig. 6*B*). LPS (10 μg/mouse) caused a lower TNF-α response that was completely inhibited by IL-10 gene expression (Fig. 6*B*). Treatment of the mice with DNA-DOTAP complexes similarly inhibited LPS-induced PMN cell count (Fig. 6*C*). Thus our results indicate that the gene delivery system was effective in promoting IL-10 expression and a subsequent reduction in lung inflammation induced by LPS.

DISCUSSION

IL-10 is a potent anti-inflammatory cytokine that regulates the function of various cell types of the immune system (22). Its anti-inflammatory properties have been established in a number of in vivo models including sepsis, enterocolitis, and immune complex- or allergen-induced inflammation (13, 15, 17, 23, 25). Most of the in vivo studies to date (13, 15, 17, 23, 25) have been carried out with recombinant IL-10 protein. The present study investigated the feasibility of utilizing IL-10 gene transfer for the treatment of lung inflammation. Gene transfer technology now permits directed expression of exogenous genes within specific organs (e.g., lung) of intact animals (2).

There have been conflicting reports on the capability of naked DNA to express proteins when administered to the lung (7, 18, 21). Meyer et al. (21) have shown that transgene expression from naked DNA is as high as that seen when the DNA is coadministered with liposomes. Other groups have shown either no expres-

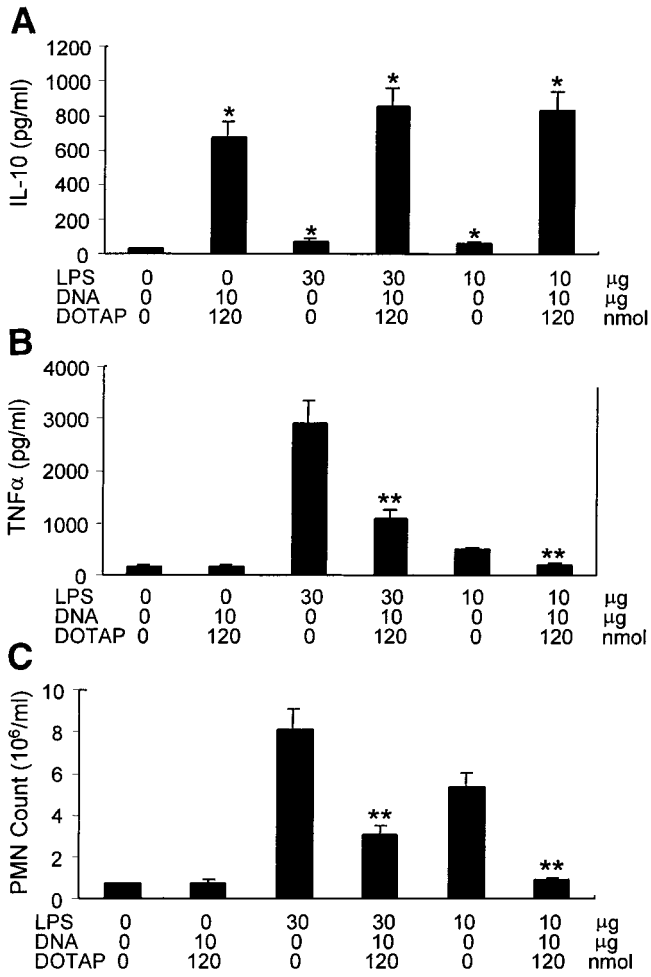

Fig. 6. Effect of IL-10 gene transfer on LPS-induced lung inflammation. Mice were instilled with indicated concentrations of DNA and DOTAP 12 h before intratracheal instillation of indicated concentrations of LPS. BAL was performed 6 h after LPS instillation. The BAL fluid was tested for IL-10 concentration (*A*), TNF-α concentration (*B*), and PMN cell count (*C*). Values are means ± SD; $n = 4$ mice/group. *$P < 0.05$ vs. untreated control group. **$P < 0.05$ vs. LPS-treated control group.

sion of naked DNA (1, 12) or expression that is much less compared with DNA-liposome complexes (7, 16). The cause of the discrepancy between the results is not known but could be due to the difference in the method of administration. To test whether naked DNA is effective in our system, we instilled naked DNA into mouse lungs and tested for IL-10 gene expression. We observed no gene expression with the naked DNA. Thus we concluded that a delivery system is required for efficient IL-10 gene expression in the lung.

IL-10 gene transfer has been used with some success for the treatment of rheumatoid arthritis (10, 26) and endotoxemia (6, 27). However, in all these studies, replication-defective viral vectors were used as a delivery system. Viral vectors, although more efficient than nonviral systems, are considered to be risky due to their potential for viral infection and immunogenicity. These potential deleterious side effects have steered gene transfer research toward developing nonviral delivery systems. Cationic liposome technology has become well established for introducing DNA into cells, and these liposomes have been considered to be among the most promising carriers for gene therapy (9). Despite the effectiveness of cationic liposomes in transfecting cells in vitro, their transfection efficiency in vivo is still fairly low compared with that of the viral vectors (16). DOTAP is a cationic liposome that has been used to transfect lung cells in vivo (19, 20, 24) . In our studies, we found DOTAP to be relatively nontoxic, and hence we chose this liposomal system for IL-10 gene transfer.

We have shown that it is possible to express IL-10 in the lung when it is delivered as a DNA-liposome complex. The elevated IL-10 was due to transgene expression and not to an induction of endogenous IL-10 because the DNA complex lacking the IL-10 gene did not induce the IL-10 protein level (data not shown). The expressed IL-10 is capable of suppressing endotoxin-induced lung inflammation. Because endotoxin-induced lung inflammatory injury is a common cause of death in sepsis and respiratory distress syndrome, our findings strongly suggest the therapeutic potential of IL-10 gene transfer for the protection of endotoxic lung injury. Nonetheless, the expression of DNA plasmid from our delivery system is transitory and subsides by around 24 h. These results are consistent with those observed by Li and Huang (18). They also observed that there was a rapid decline in gene expression in vivo in the lungs. They performed Southern blot analysis on DNA in the lungs and could detect DNA until around 6 h, after which the level declined and was barely detectable at 24 h (18). Meyer et al. (21) also showed that there was a reduction in intact DNA as early as 5 min after instillation in the lungs. They concluded that DNA was rapidly eliminated from the lungs due to degradation because short DNA fragments were observed at early times in Southern blots.

Both groups (18, 21) reported that cationic liposomes increase the extent and duration of plasmid DNA in the lungs. Southern blot and PCR analysis demonstrated that rapid degradation of DNA is significantly slowed in the presence of cationic liposomes. Cationic liposomes assist in retaining DNA molecules in the lung for a time sufficient for gene transfer to be complete before they are washed out of the capillary bed by normal blood flow. Thus it appears that the retention time of DNA molecules in the lung is likely to play a critical role in determining the level of gene expression. There could be a number of potential causes for this rapid decline in DNA expression including *1*) loss of plasmid DNA from the transfected cells, *2*) loss of transfected cells due to cell death, and *3*) loss of transgene expression due to promoter shutoff.

We report here for the first time that intratracheal administration of DNA-liposome complexes results in clinically relevant concentrations of IL-10 protein. This IL-10 gene transfer can downregulate TNF-α expression and suppress lung inflammation induced by LPS. One of the goals of gene therapy is to provide a prolonged expression of therapeutic proteins over a period

of time. This would eliminate the need for repeated administration of short-lived recombinant proteins. However, in our system, we have shown that although gene delivery of IL-10 does inhibit LPS-induced inflammation, IL-10 expression does not last beyond 24 h. Although these results are encouraging, it is clear that there are still many hurdles to overcome before liposome-mediated gene transfer can be regarded as a viable therapy for lung inflammation. Significant improvements in gene transfer efficiency and persistence of gene expression need to be attained. Development of better vectors would allow gene therapy to become a clinical reality.

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