Targeted Gene Delivery to Alveolar Macrophages via Fc Receptor-Mediated Endocytosis

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Alveolar macrophage (AM) plays important roles in lung homeostasis and pathogenesis of diseases. The study of macrophage gene function and regulation as well as its potential therapeutic intervention will require the development of vectors capable of safe and efficient transfer of DNA to the AM. In the present study, we report a new transfection system that utilizes Fc receptor-mediated endocytosis as a means to target DNA to the AM. This system employs molecular conjugates consisting of a cognate moiety, in this case IgG which recognizes the AM Fc receptor, covalently-linked to a DNA-binding moiety, such as a cationic polyamine. A Complex was formed between immunoglobulin G-polylysine conjugate (IgG-pL) and plasmid DNA carrying the LacZ reporter gene (pSVβ). The conjugate-DNA complex was added directly to the AMs in culture and incubated for 24 h, after which LacZ gene expression was analyzed for β-galactosidase activity by microfluorometry using a fluorogenic β-galactosidase substrate, 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG). The AMs treated with the IgG-pL/DNA complex exhibited galactosidase activity significantly augmented over background levels. Effective gene transfer was shown to require both the DNA-binding moiety and cognate moiety for the cell surface receptor. Specific internalization of the complex by the Fc receptor pathway was verified by competitive inhibition using excess IgG. Under this condition, LacZ gene expression was inhibited, suggesting complex internalization through the Fc mediated endocytosis pathway. The requirement of Fc receptors for complex internalization was further demonstrated using cells that lack Fc receptors, e.g., alveolar epithelial cells. When exposed to the IgG-pL/pSVβ complex, these epithelial cells showed no susceptibility to gene transfer. Thus, the immune conjugate system may be used to accomplish targeted gene delivery to the AMs via the endocytosis pathway. Finally, the conjugate system was found to be nontoxic at concentrations effectively enhancing gene transfer, thereby, suggesting its potential safety *in vivo*.

KEY WORDS: gene delivery; receptor-mediated endocytosis; alveolar macrophages; IgG; Fc receptor.

INTRODUCTION

The use of DNA as a therapeutic agent against genetic and metabolic disorders is of recent interest (1,2). Successful gene therapy will require knowledge of the structure, function, and regulation of the gene to be introduced into the defective cell, as well as an efficient and specific means of delivering the gene to the target cell. Despite its therapeutic potential, gene therapy has not yet been widely used, mainly because of the lack of efficient and site-specific delivery systems. Current methods of gene delivery *in vitro* include calcium phosphate/DNA coprecipitation, DEAE-dextran, microinjection, and electroporation (3–5). However, these methods are generally not applicable for use *in vivo* due to problems associated with cellular toxicity or low efficiency. Liposomes, which offer the potential advantage of improved efficiency (6) and lower toxicity (7,8) suffer from the lack of cellular targetability. Retroviral vectors, although more efficient (9), suffer from limitations related to the size and design of DNA that can be incorporated, and potential safety hazards derived from the obligatory co-transfer of elements of the parent virus genome.

As an alternative strategy to circumvent these problems, the present study investigates the feasibility of delivering DNA to AMs[4] by means of receptor-mediated endocytosis. To accomplish DNA delivery, the study utilizes a molecular conjugate consisting of the cognate moiety IgG, which recognizes the AM Fc receptor (10), covalently-linked to the DNA-binding moiety polylysine via a reversible disulfide linkage. Complex was formed between IgG-polylysine conjugate and plasmid pSVβ carrying LacZ reporter gene and SV40 early promoter. When recognized by the receptor, the conjugate is internalized by the efficient receptor-mediated pathway, co-transporting the plasmid DNA. Because the method accomplishes gene delivery by capitalizing on a normal physiologic cellular process, it is potentially nontoxic. Moreover, this strategy offers cell-specific targeting of gene delivery by virtue of specific recognition of the cognate vector by the Fc receptor bearing AMs.

The transfection efficiency of the method was evaluated by measuring the level of LacZ gene expression using a fluorogenic β-galactosidase substrate, 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG). Enzymatic hydrolysis of this membrane permeant substrate by the LacZ gene product β-galactosidase yields a highly fluorescent derivative, 5-dodecanoylaminofuorescein, which can be measured by a fluorescence method (11). Our results indicated that the conjugation technique is highly effective and selective in delivering LacZ reporter gene to the AMs. In addition, the lack of any derivative cytotoxic effects makes this method highly attractive for *in vivo* gene delivery.

EXPERIMENTAL

Alveolar Macrophage Preparation

The AMs were harvested from male Sprague-Dawley rats (200–250 gm) by bronchoalveolar lavage. Rats were

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[4] *ABBREVIATIONS:* AM, alveolar macrophage; C_{12}FDG, 5-dodecanoylaminofluorescein di-β-D-galactopyranoside; IgG, immunoglobulin G; pL, poly(L-lysine); IgG-pL, Immunoglobulin G-poly-(L-lysine) conjugate; SPDP, N-succinimidyl 3-(2-pyridyldithio) proprionate; kb, kilobase.

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anesthetized by intraperitoneal injection of sodium pentobarbital (0.2 gm/kg body weight). The trachea was cannulated and the lungs lavaged 10 times with 8 ml aliquots of Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 5.5 mM glucose, pH 7.4) (12). Lavage cell suspensions were centrifuged at 500*g* for 5 min at 4°C. The cell pellets then were washed twice by alternate resuspension and centrifugation in RPMI-1640 media (Whittaker) containing 2 mM glutamine, 100 U/ml nystatin, 100 U/ml penicillin, 10 μg/ml gentamicin, and 10% heat-activated fetal bovine serum. Cell number, purity, and mean cell volume of AM preparations were determined using a Coulter model Z_B electronic cell counter with a cell sizing attachment (Coulter Instrument, Hialeah, FL). The average values for these parameters were: yield = $5.8 \pm 0.2 \times 10^6$ AMs/rat; purity = $91.5 \pm 0.5\%$; and mean cell volume = $1{,}410 \pm 10$ μm^3. Cell viability was measured via trypan blue exclusion and was found to be > 95%. Aliquots of 1 ml containing 1×10^6 cells were added onto tissue culture cover slips (Corning, NY), placed in flat-bottomed 12 well plates (Costar, Cambridge, MA), and incubated at 37°C in a humidified atmosphere at 5% CO_2.

Alveolar Epithelial Cell Preparation

Alveolar epithelial type II cells were prepared according to the method previously described (13). Briefly, rat lungs were lavaged to remove free AMs and were then excised and filled with phosphate-buffered saline (PBS) containing elastase (40 U/ml, type I, US Biochemical) and DNase (0.006%, Sigma). After a 20-min incubation period at 37°C, the lungs were finely minced and the digestion was arrested by incubation for 5 min in PBS containing 25% fetal bovine serum and 0.006% DNase (to help prevent cell clumping). The crude extract was sequentially filtered through 160 and 45 μm screens, centrifuged, and the resulting cell pellet was spun on the Percoll density gradient. The second cell band from the surface were collected, washed, and resuspended in 1:1 F_{12} and Eagle's modified minimum essential medium, supplemented with 100 U/ml penicillin, 10 μg/ml gentamicin, 0.5 μg/ml hydrocortisone, and 10% fetal bovine serum. The cell suspension yielded $15-20 \times 10^6$ cells/rat with viability generally greater than 95%. Aliquots of 1 ml containing 1×10^6 cells were added onto tissue culture slips, placed in flat-bottomed 12 well plates, and incubated at 37°C in 5% CO_2.

Synthesis of Immunoglobulin-Polylysine Conjugate

Rat IgG and poly(L-lysine) (hydrobromide, MW 30,000) were obtained from Sigma Chemical Company (St. Louis, MO). The IgG was isolated from pooled normal rat serum by fractionation and ion-exchange chromatography. The two were coupled by ligation through disulfide bond after chemical modification with the bifunctional cross-linking reagent N-succinimidyl 3-(2-pyridyldithiol proprionate (SPDP, Sigma), according to the modified method of Carlsson *et al.* (14). The IgG (0.5 μmol) was dissolved in 1.0 ml of 0.1 M PBS, pH 7.4. An ethanol solution (100 μl) containing 10 mM SPDP was added to the stirred protein solution and the reaction proceeded for 30 min at room temperature. Excess of the reagent was removed by centrifugal filtration through a dialysis membrane filter (Durapore CL5K, Millipore Corp.) at 5,000 g for 30 min. The modified protein was then collected and reconstituted in 1 ml PBS. Modification of the polylysine was similarly performed except that the protein was reconstituted in sodium acetate buffer, pH 4.5. The polylysine-2-pyridyl disulfide derivative obtained was then converted into a thiol derivative by specific reduction of the 2-pyridyl disulfide groups with dithiothreitol (100 μmol) for 30 min, after which it was centrifuged and reconstituted in 1 ml PBS. The modified polylysine was then mixed with the IgG-2-pyridyl disulfide derivative and, after a 18 hr incubation period at 4°C, the resulting IgG-polylysine conjugate was purified by Pharmacia Superose 12 gel filtration. The conjugate fraction was collected, dialysed (Durapore CL5K), and stored at 4°C in 0.9% NaCl solution for further studies.

Gene Transfer Studies

Prior to studies, the IgG-pL conjugate (10 μg/ml) was complexed with purified pSVβ DNA (1 μg/ml) (Clontech Lab., Palo Alto, CA) in culture media at 37°C for 10 min. The plasmid pSVβ contains LacZ β-galactosidase gene under the control of SV40 early promoter. The conjugate/DNA complex was then added directly to the AMs or alveolar epithelial cells and incubated for 24 h at 37°C, under 5% CO_2. In some studies where the effect of DNA concentration was studied, varying amounts of DNA (0.1–1.0 μg/ml) were used. To evaluate structural requirement of the IgG-pL conjugate on gene transfer, control studies using DNA (1 μg/ml) alone or in combination with IgG (10 μg/ml) or pL (10 μg/ml) were also conducted. For competition experiments to test for the specificity of the conjugate for the Fc receptor, cells were treated with IgG-pL/pSVβ complex in the presence of increasing amounts of IgG (0.1–1.0 mg/ml) as a specific competitor or bovine serum albumin (1.0 mg/ml) as a nonspecific competitor. In experiments employing chloroquine as an inhibitor for lysosomal degradation, cells were treated with IgG-pL/pSVβ complex in the presence of chloroquine (100 μM) (Sigma). After a 4-h incubation period, cells were washed and incubated for an additional 24 h in culture medium (37°C, 5% CO_2). Analysis of gene transfer was accomplished by incubating the cells for 1 h with C_{12}FDG substrate (33 μM) (Molecular Probes, Eugene, OR) and then evaluating for β-galactosidase activity using the Spex ARCM microfluorometer at the excitation and emission of 490 nm and 530 nm respectively (Spex Ind., Edison, NJ). The C_{12}FDG substrate was used in this study because it provides a rapid, highly sensitive, and noninvasive means of analyzing β-galactosidase activity in living cells (11). For cytotoxicity studies, fluorescence propidium iodide assay was used (13). Cells were incubated for 30 min with culture medium containing 1 μg/ml propidium iodide and their fluorescence intensity was measured at the excitation and emission of 490 nm and 600 nm respectively. As a control, triton X-100 (1%) was added to the cells in order to establish maximum cytotoxic fluorescence response. Cell damage was determined from the maximum and measured fluorescence signals after corrected for background levels.

RESULTS

Gene Delivery to Alveolar Macrophages Mediated by IgG-pL Conjugate

To test the feasibility of gene delivery to the AMs via receptor-mediated endocytosis, cells were incubated for 24 h with the IgG-pL/pSVβ complex consisting of 1 μg/ml pSVβ and 10 μg/ml IgG-pL conjugate, after which the level of β-galactosidase gene expression was measured. As controls, cells were also treated with pSVβ (1 μg/ml) or IgG-pL (10 μ/ml) or blank medium (background), and the level of gene expression were similarly analyzed. The results, shown in Fig. 1, indicated that exposure of the cells to the conjugate/DNA complex containing the β-galactosidase reporter gene resulted in the level of reporter gene activity significantly augmented over the control levels ($p < 0.01$). The DNA- or conjugate-treated controls exhibited no significant increase in β-galactosidase activity over the background level. Thus, our results demonstrated that the conjugate system can be used to facilitate gene transfer and expression in the AMs and that this enhancing effect mediated by the conjugate was not due to an induction of the endogenous β-galactosidase expression. The failure of the plasmid DNA to induce gene expression in the absence of the conjugate suggested its inability to penetrate the cells, consistent with its molecular properties, i.e., large molecular size (7 kb) and high charge density, which disallow cellular penetration. To further confirm that the enhanced gene expression mediated by the conjugate was caused by the administered pSVβ, concentration-dependent studies of the plasmid DNA on gene expression were conducted (Fig. 2). Varying the concentration of the DNA (0.1–1.0 μg/ml) caused a proportional change in the level of β-galactosidase expression, thereby, supporting the causation effect of the administered DNA pSVβ.

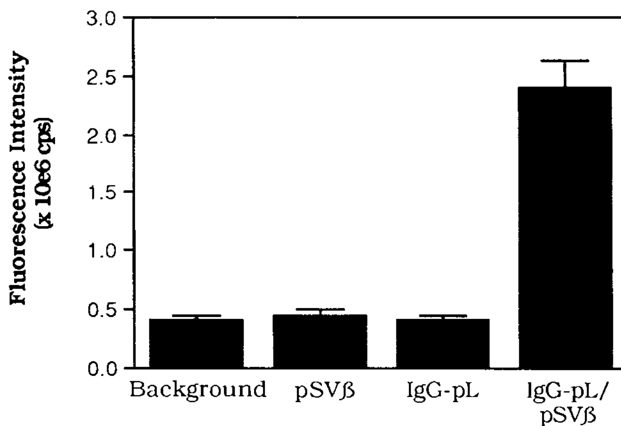

Fig. 1. Gene delivery to alveolar macrophage mediated by IgG-pL conjugate. Alveolar macrophages (1×10^6 cells/ml) were transfected with the reporter plasmid pSVβ (1 μg/ml) or IgG-pL conjugate (10 μg/ml) or IgG-pl/pSVβ complex (10:1 μg/ml). After 24 hr of incubation (37°C, 5% CO_2), the cells were washed and treated with C_{12}FDG β-galactosidase substrate (33 μM) and their fluorescence intensity was measured at the excitation and emission of 490 nm and 530 nm. Background indicates β-galactosidase activity in untreated controls. The data represent mean ± SE of four measurements obtained from different cell preparations.

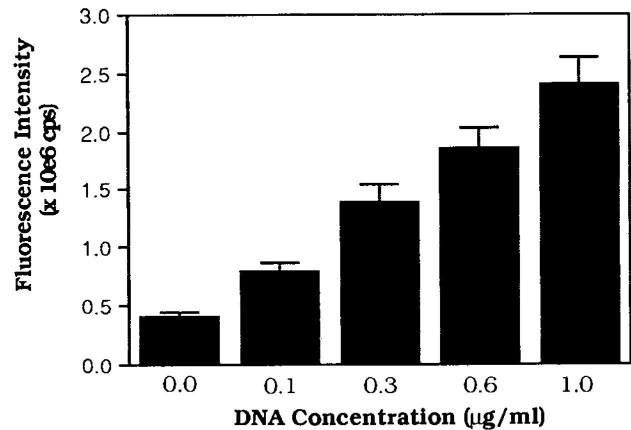

Fig. 2. Effect of DNA concentration on LacZ gene expression mediated by IgG-pL conjugate. Alveolar macrophages were transfected with IgG-pL/DNA complexes consisting of IgG-pL conjugate (10 μg/ml) and varying concentrations of plasmid pSVβ (0-1 μg/ml). After 24 h of incubation (37°C, 5% CO_2), the cells were washed and analyzed for β-galactosidase activity as previously described. The data represent mean ± SE, n = 4.

Mechanism of Gene Transfer Mediated by IgG-pL Conjugate

To determine the mechanism of gene transfer mediated by the IgG-pL conjugate, the contribution of each functional domain of the conjugate molecule in affecting gene transfer was evaluated. Reporter plasmid pSVβ alone (1 μg/ml), or in combination with IgG (10 μg/ml) or pL (10 μg/ml), or in combination with IgG-pL conjugate (10 μg/ml), was evaluated for their ability to accomplish gene delivery to the AMs (Fig. 3). In this analysis, complex consisting of the plasmid DNA and the IgG-pL conjugate exhibited a level of reporter gene expression significantly higher than did the complexes consisting of pSVβ in combination with individual conjugate components, pL or IgG ($p < 0.01$). However, the pSVβ/pL complex mediated gene transfer significantly higher than did the pSVβ/IgG complex or pSVβ alone ($p < 0.01$). These

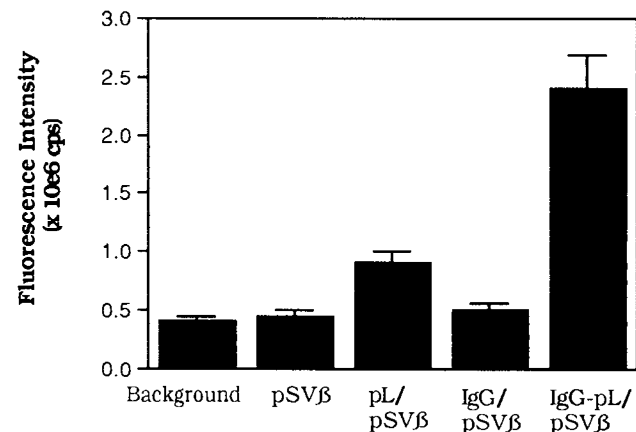

Fig. 3. Mechanism of gene transfer to alveolar macrophages mediated by IgG-pL conjugate. Cells were incubated with 1 μg/ml pSVβ, alone or in combination with 10 μg/ml of IgG or pL or IgG-pL conjugate. After a 24-hr incubation period (37°C, 5% CO_2), the cells were washed and analyzed for β-galactosidase activity. Background indicates galactosidase activity in untreated controls. The values represent mean ± SE, n = 4.

results are consistent with the concept that effective gene transfer mediated by conjugate vectors requires functional domains capable of both DNA binding and cellular surface recognition. The observation of the partial mediating effect of the pL on gene expression may be attributed to enhanced DNA uptake, presumably caused by nonspecific adsorptive endocytosis of the polycationic pL-DNA complex. Polylysine has been used to enhance endocytic uptake of a variety of macromolecules in mammalian cells (15,16).

To test whether the cellular internalization of the pSVβ/IgG-pL complex was mediated by the Fc receptor endocytosis pathway, gene transfer employing the complex in the presence of competition for the Fc receptor was carried out (Fig. 4). In these experiments, increasing competition for the Fc receptor by exogenously administered IgG (0.1–1.0 mg/ml) resulted in a concentration-dependent decrease in the level of gene transfer ($p < 0.01$). Competition with a comparable amount of a non-specific competitor, bovine serum albumin (1 mg/ml), had no effect on the gene transfer level. Thus, our results indicated that gene transfer mediated by the IgG-pL conjugate occurred via the Fc receptor-mediated endocytosis pathway.

In addition to promoting gene transfer, the specificity of the receptor-mediated pathway may also be utilized to achieve gene targeting to selected cells. Cells bearing no Fc receptor should exhibit no gene transfer mediated by the IgG-pL conjugate. Indeed, in experiments in which alveolar epithelial cells, which do not possess Fc receptors, were exposed to the pSVβ/IgG-pL complex, no significant gene transfer was observed over the control level (Fig. 5). This finding demonstrated the usefulness of the immune conjugate as a potential gene targeting system for specific lung cells such as the AMs.

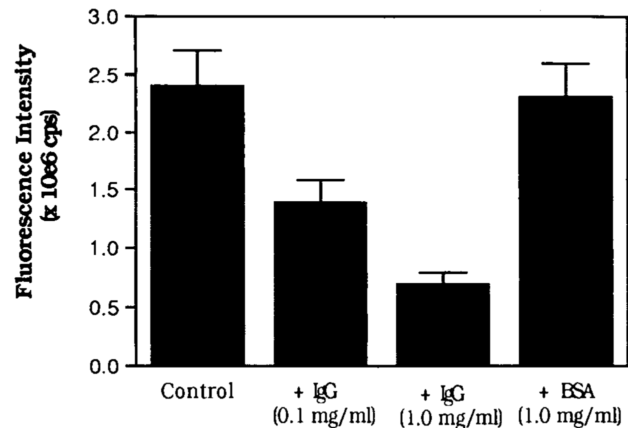

Fig. 4. Effect of specific and nonspecific inhibition for Fc receptor on gene transfer mediated by IgG-pL conjugate. Conjugate-DNA complex consisting of pSVβ DNA (1 μg/ml) and IgG-pL conjugate (10 μg/ml) wee added to the alveolar macrophages in the absence (control) or presence of IgG (0.1 and 1.0 mg/ml), as a specific competitor for the Fc receptor, or bovine serum albumin (BSA) (1.0 mg/ml), as a nonspecific competitor. After 24 h incubation (37°C, 5% CO_2), the cells were washed and analyzed for β-galactosidase activity. The values indicate mean ± SE, n = 4.

Enhancement of Gene Transfer by Inhibition of Endocytic DNA Degradation

Receptor-mediated gene transfer may be augmented by agents that enhance DNA availability in target cells. In several cell types, enhanced gene transfer by receptor-mediated endocytosis was reported in cells treated with agents that decrease intracellular degradation of the internalized DNA (16–19). In this study, a similar approach was evaluated in the Fc receptor carrying AMs. Treatment of the AMs with a lysosomatropic agent, chloroquine (100 μM), resulted in a moderate but significant increase in the level of gene transfer by the pSVβ/IgG-pL complex compared with treatment with the complex alone (1.6-fold) ($p < 0.01$, n = 4). This is consistent with the concept that increasing the availability of the internalized DNA that survives lysosomal degradation will allow an increase in the level of gene expression.

Evaluation of Cytotoxicity

A crucial consideration for the usefulness of a delivery system is its safety. In the present study, we evaluated the potential toxicity of the conjugate system in AMs using the highly sensitive fluorescence propidium iodide method. Propidium iodide, due to its hydrophilicity, is normally excluded from intact viable cells, however, if the cell is damaged, the probe can enter the cell and bind specifically to the cell nucleus. Upon binding, its fluorescence intensity is strongly enhanced and, thus, intense nuclear fluorescence indicates cell damage and death (20). In this study, the AMs were incubated for 24 h with pSVβ, alone or in combination with IgG-pL conjugate or conjugate plus chloroquine, after which they were analyzed for propidium iodide fluorescence (Fig. 6). The results indicated that, under these conditions, the AMs exhibited no significant increase in nuclear fluorescence over untreated controls, suggesting that the cells maintained their integrity and that the conjugate system did not cause any appreciable cellular damage. Thus, the conjugate system may potentially be used for gene delivery to the AMs *in vivo*.

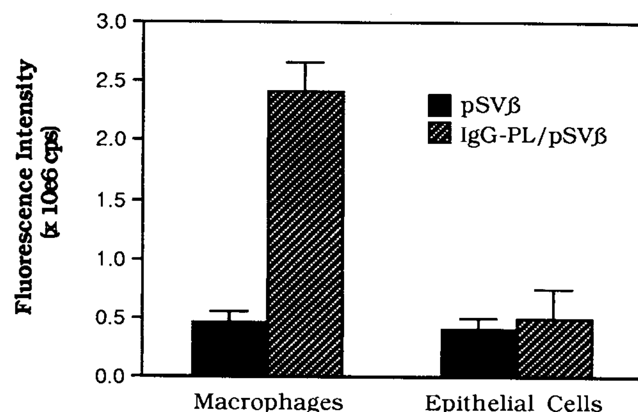

Fig. 5. Comparison of gene transfer efficiency mediated by IgG-pL conjugate in alveolar macrophages and epithelial cells. Alveolar macrophages or epithelial type II cells (1 × 10^6 cells/ml) were transfected with plasmid pSVβ (1 μg/ml) alone or complexed with IgG-pL conjugate (10 μg/ml). After 24 h incubation (37°C, 5% CO_2), the cells were washed and analyzed for β-galactosidase activity. The values represent mean ± SE, n = 4.

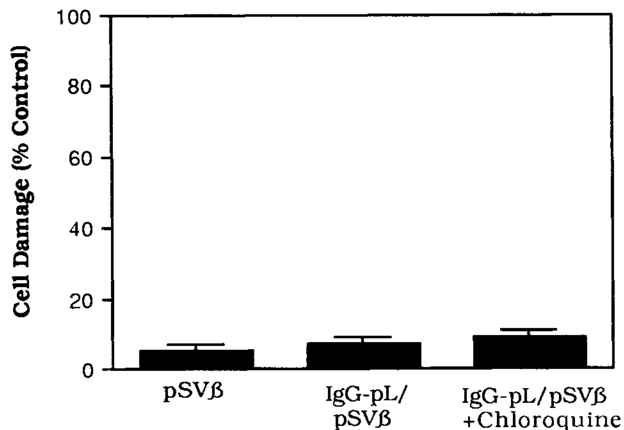

Fig. 6. Effect of immune conjugate vector on cellular toxicity of alveolar macrophages. The cells were incubated for 24 h (37°C, 5% CO_2) with a) pSVβ (1 μg/ml), b) IgG-pL/pSVβ complex (10:1 μg/ml), or c) complex (10:1 μg/ml) plus chloroquine (100 μM) for 4 h, followed by a 24 h incubation with fresh culture medium. The cells were then washed and treated with propidium iodide (1 μg/ml) for 30 min in culture medium, after which they were analyzed for propidium iodide fluorescence (excitation, 490 nm; emission, 600 nm). The data represent percent control values of untreated cells (mean ± SE, n = 4). Calculation of cellular damage was based on maximum (Triton X-100-treated) and minimum (background) fluorescence values.

DISCUSSION

The AM provides an important line of host defense against inhaled noxious substances and its role in lung homeostasis and pathogenesis of diseases is well recognized (21). The study of AM function and control mechanisms, as well as its potential treatment for metabolic or genetic disorders, can be approached utilizing methods directed at the level of gene expression. However, presently available gene transfer methods are not well suited to accomplish direct gene transfer to the AMs. The various DNA-mediated and fusion methods are of relatively low efficiency or associated with cellular toxicity or lack of specificity. Despite these drawbacks, direct gene delivery to the lung has been reported utilizing cationic liposomes (22–25). Recombinant viral vectors have been shown to possess higher gene transfection efficiency (9,26), however, they suffer the drawbacks related to safety issue in the context of direct *in vivo* delivery. Both recombinant adenovirus and retrovirus vectors mediate obligatory co-delivery of viral gene elements with transfer of the heterologous gene.

Gene delivery via the receptor-mediated endocytosis utilizing the ligand conjugates offers several advantages including high transfection efficiency (17,19) and targetability (27,28). The cellular internalization of the conjugate is via a natural endocytic process and thus is not likely to be cytotoxic. Indeed, our study as well as those using other ligand conjugates such as the transferrin-polylysine conjugate (17) indicate no apparent cytotoxic effects on the transfected cells. In addition, since the interaction between the DNA-binding moiety of the conjugate and DNA is via a sequence-nonspecific electrostatic interaction, there is no restriction on the design of the transfected DNA construct, which is essential for the viral gene transfer system. The ligand-polycation conjugate was previously found to form a stable complex with a wide variety of nucleic acid molecules, including the double-stranded DNAs, single-stranded RNAs, and short oligonucleotides (17).

Receptor-mediated gene delivery to selected lung cells utilizing ligand-polycation conjugates such as the transferrin-polylysine conjugate has been reported (19). However, the ubiquitous presence of transferrin receptors in various cell types in the lung could potentially result in non-specific targeting of the gene. In this article, we report a gene targeting system capable of delivering DNA, effectively and selectively, to the AMs. The system employs the cognate moiety IgG which recognizes the AM Fc receptor, covalently linked to the DNA-binding moiety polylysine. Gene transfer by this method was found to be Fc receptor-mediated since: (a) cells bearing no Fc receptor (alveolar epithelial cells) are not susceptible to the gene transfer, (b) the Fc binding moiety IgG is required for effective gene transfer, and (c) competition for Fc receptor by IgG, but not by the non-specific competitor BSA, greatly reduces gene transfer level. Moreover, agents that promote endosomal stability of the internalized DNA such as chloroquine significantly improve gene expression level mediated by the conjugate.

The application of the immune conjugate as a carrier system for *in vivo* gene transfer remains to be established. The complex anatomy of the lung as well as the great variety of existing lung cell types may limit targetability and efficiency of the delivery system. Nonetheless, *in vivo* gene delivery to the lung via aerosolization techniques has been reported (22). Regulators of gene expression such as corticosteroids or cAMP inducers already used to treat patients with pulmonary disorders and are given routinely by the inhaled route. Site-specific gene delivery to the alveoli could also be accomplished using inhalation devices capable of producing fine aerosols which are favorably deposited in the alveolar region (29,30). With the use of inhalation technology and cell-specific delivery systems, gene targeting to selective lung cells *in vivo* could be accomplished.

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