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Oligonucleotide targeting to alveolar macrophages by mannose receptor-mediated endocytosis

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Abstract

Antisense oligonucleotides (ONs) have proven useful for selective inhibition of gene expression. However, their effective use is limited by inefficient cellular uptake and lack of cellular targeting. In this paper, we report a drug targeting system which utilizes mannose receptor-mediated endocytosis to enhance cellular uptake of ONs in alveolar macrophages (AMs). The system employs a molecular complex consisting of partially substituted mannosylated poly(L-lysine) (MPL), electrostatically linked to a 5′ fluorescently labeled ON. Upon recognition by the macrophage mannose receptors, the MPL was internalized by the receptor-mediated pathway, co-transporting the ON. Our results indicated that the AMs treated with the MPL:ON complex exhibited a significant increase in ON uptake (up to 17-fold) over free ON-treated controls. Effective ON uptake was shown to require the recognition of the mannose moiety since unmodified polylysine was much less effective in promoting ON uptake. Specific internalization of the ON:MPL complex by the mannose receptor pathway was verified by competitive inhibition using mannosylated albumin. Under this condition, the ON complex uptake was inhibited. The requirement of mannose receptors for complex uptake was further demonstrated using a macrophage cell line, J774.1, which expresses a low level of mannose receptors. When treated with the complex, these cells showed no susceptibility to ON uptake, thus suggesting the targeting ability of the carrier system to the AMs. Following cellular internalization, the ON complex appeared largely accumulated in endocytic vesicles. Enhanced endosomal exit of the ON was achieved using a fusogenic peptide derived from the amino terminal sequence of influenza virus hemagglutinin HA2. Cytotoxicity studies showed that at the concentrations effectively enhancing ON uptake, both MPL and the fusogenic peptide caused no toxic effects to the cells, thereby suggesting their potential safety and utilization in vivo.

Keywords: Oligonucleotide; Macrophage; Uptake; Endocytosis; Mannose receptor; Endosome

1. Introduction

Antisense oligonucleotides have been successfully used to inhibit virus or cellular gene expression by forming hybrids with mRNAs or DNAs (for review see: [1,2]). Due to their exquisite specificity, these compounds are less likely to cause undesirable side effects and therefore represent a substantial improvement over conventional drugs. A major limitation for the effective use of ONs as therapeutic agents is their low cellular uptake and lack of cellular targeting. ONs have been shown to be taken up by cells through a saturable and energy-dependent process [3,4]. However, such a process is relatively inefficient and therefore a relatively high concentration of the compounds is generally required to exert pharmacological actions.

Several methods have been proposed to increase cellular uptake of ONs. Substitution of the compounds with lipophilic molecules such as cholesterol [5,6], acridine [7], or alkyl groups [8,9], or association with liposomes [10,11] or polycations such as poly(L-lysine) [12,13] has been shown to increase cellular uptake and biological activities of ONs. However, their effective use in vivo will require a more specific method of delivery which can selectively deliver ONs to a target cell population.

Receptor-mediated endocytosis offers the potential to target selected cell types and enhance their uptake. This

Abbreviations: AM, alveolar macrophage; BSA, bovine serum albumin; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MPL, mannosylated polylysine; ON, oligonucleotide; PBS, phosphate-buffered saline; PL, poly(L-lysine).

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method has successfully been used to transfer genes [14–16] and antisense ONs in selected cells [17–20]. For example, asialoorosomucoid-polylysine conjugates have been used to target ONs to HepG2 and CAT cells [17,18]. Similarly, transferrin and folic acid conjugates have been employed to aid cellular delivery of antisense ONs in HL-60 cells [19,20]. Macrophages possess mannose-specific membrane receptors which can recognize and internalize glycoproteins bearing mannose residues [21,22]. On this basis, mannosylated glycoproteins could potentially be used to target and enhance ON uptake by the macrophages. Targeted delivery of several cytotoxic drugs and antiviral agents by glycoproteins specific to macrophages has been previously demonstrated [23,24]. Moreover, 6-phosphomannosylated glycoproteins have also been used to target antisense ONs to peritoneal macrophages [25].

Because alveolar macrophages (AMs) play a key role in the pathogenesis of several lung diseases and are the host of many inhaled pathogens, we decided to target ONs using the MPL complex system. To achieve ON delivery, the ON-binding polylysine (PL) was partially substituted with mannose residues and allowed to form a complex with ON. Our results indicated that such complex was efficiently and selectively taken up by the AMs, although the internalized complex was found mainly associated with endocytic vesicles. Since effective antisense activity will depend on the ability of the ON to exit these vesicles in order to reach its target site in the cytosol, we also tested the effect of an endosomal destabilizing peptide derived from the influenza hemagglutinin HA2 [26], on uptake and intracellular distribution of ON. The HA2 peptide has been shown to mediate fusion of viral and endosomal membranes [27]. This peptide changes conformation at acidic pH and destabilizes endosomal membranes, allowing leakage of endosomal contents into the cytosol. Synthetic HA2 peptide has also been reported to facilitate cytoplasmic delivery of genes and ONs [28]. In this study, we also found that this peptide can facilitate endosomal exit and cytoplasmic delivery of ON in AMs.

2. Materials and methods

2.1. Chemicals

4-Isothiocyanatophenyl-α-D-mannoside, poly(L-lysine) (hydrobromide, $M_r \approx 50\,000$), rhodamine-labeled poly(L-lysine), bovine serum albumin (BSA), and mannosylated BSA were obtained from Sigma (St. Louis, MO). 5′-BODIPY FL labeled, 18-mer, phosphodiester ON (TGTAAAACGACGGCCAGT) and propidium iodide were obtained from Molecular Probes (Eugene, OR). The labeled ON was purified by HPLC and was > 98% pure. The BODIPY label was used in this study because of its high fluorescence intensity and pH-insensitive property. The fusogenic peptide with the sequence GLFEAIAGFIENGWEGMIDGGGYC was synthesized by Synpep (Dublin, CA) using the Fmoc procedure. The peptide was purified by HPLC and characterized by amino acid analysis and mass spectrometry ($M + H^+$: 2506).

2.2. Synthesis of mannosylated polylysine

Mannosylated polylysine was prepared according to the method previously described [29]. Briefly, polylysine (0.25 μmol) was dissolved in 0.15 M NaCl solution. The pH of the solution was adjusted to 9 using 0.1 M NaOH. 4-Isothiocyanatophenyl-α-D-mannoside (12.5 μmol) was added in small portions to the magnetically stirred protein solution. After a 6-h reaction, the resulting solution was refrigerated overnight, and on the following day the pH was adjusted to 7.0. Unreacted mannoside and polylysine were removed by centrifugal filtration through a dialysis membrane filter (Durapore™ CL3 K, Millipore) at $5000 \times g$ for 30 min.

2.3. Determination of sugar content

Sugar content was determined by the rescorcinol sulfuric method [30] and the protein content was determined by micro Lowry assay. Percentage of sugar in mannosylated polylysine was determined using the equation:

$$X\% = nM_o/(M_p + nM)$$

Where n is the number of mannose residues per polylysine molecule and M_o, M_p, and M are the molecular weights of mannose, polylysine, and isothiocyanatophenyl mannoside, respectively. It was determined that our mannosylated polylysine contained about 50 mannose residues per polylysine molecule.

2.4. Alveolar macrophage preparation

The AMs were harvested from male Sprague-Dawley rats (200–250 g) by broncho-alveolar lavage. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (0.2 g/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). Lavaged cell suspensions were centrifuged at $500 \times g$ for 10 min at 4°C. The cell pellets were washed twice by alternate resuspension and centrifugation in Hepes-buffered medium (136 mM NaCl, 2.2 mM Na_2HPO_4, 5.3 mM KCl, 10 mM Hepes, 5.6 mM glucose, 1.0 mM $CaCl_2$, pH 7.4). Cell number and purity of the macrophage preparations were determined using a Coulter electronic cell counter with a cell sizing attachment (Coulter Instrument, Hialeah, FL). The average values for yield and purity were $(6.2 \pm 0.3) \cdot 10^6$ cells/rat

and 92.5 ± 0.4%, respectively. Cell viability, measured via trypan blue exclusion, was > 95%. Aliquots of 0.1 ml containing 10^5 cells were added onto a 96-well plate (Costar, Cambridge, MA) and incubated at 37°C in a humidified atmosphere at 5% CO_2.

2.5. Cell culture

J774.1 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 μg/ml streptomycin. They were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Prior to use, the cells were washed and resuspended in Hepes buffer medium.

2.6. Formation of oligonucleotide-mannosylated polylysine complex

In a typical experiment, the complex was prepared by adding 25 μl of 50 μg/ml MPL to 25 μl of 50 μg/ml ON in Hepes buffer for 30 min prior to use. In studies designed to evaluate the effect of concentration of MPL on ON uptake, various concentrations of MPL (0–30 μg/ml) were used. The solubility of the complex was monitored by determining the fluorescence intensity of the solution mixture. Excessive amount of MPL (> 30 μg/ml) was found to cause precipitation of the ON complex as indicated by the reduction in fluorescence intensity of the solution mixture and by microscopic observations. Therefore, only MPL with concentrations of ≤ 30 μg/ml was used in this study. In a separate study, the stability of ON and ON:MPL complex was studied in a serum-free Hepes-buffered medium using gel electrophoresis. After a 4-h incubation at 37°C, no detectable degradative products of ONs were observed.

2.7. Cellular uptake studies

Cells (10^5/well) were plated on a 96-well plate. After a 1-h incubation period in Hepes buffer, the plate was washed to remove unattached cells. Solutions containing ON in the presence or absence of MPL or PL were added directly onto the cells and incubated for 1–4 h at 37°C. Subsequently, the cells were washed with cold Hepes buffer containing excess unlabeled ON and/or MPL or PL to remove surface-bound labeled ON. The cells were then measured for their fluorescence intensity using a fluorescence microplate reader at the excitation and emission wavelengths of 490 nm and 520 nm respectively. For competition studies, cells were treated with ON:MPL complex in the presence of mannosylated BSA (a specific competitor for mannose receptors) or BSA (a nonspecific competitor). In experiments employing the fusogenic peptide HA2, cells were incubated with the complex in the presence of increasing amounts of the peptide and their fluorescence intensity was similarly determined.

2.8. Fluorescence microscopy

Cells (10^5/ml) were plated on a glass cover slip. After 1-h incubation, the cells were washed with Hepes buffer to remove unattached cells. The solution containing ON:MPL complex (25:25 μg/ml) was added onto the cells and incubated for 1–2 h at 37°C. In experiments designed to examine the fate of ON and MPL during cellular internalization, BODIPY FL-labeled ON and rhodamine-labeled MPL were used to form the complex. After incubation, the cells were washed, mounted, and examined under a fluorescence microscope at the excitation/emission wavelengths of 490/520 nm and 560/600 nm, respectively.

2.9. Cytotoxicity studies

Following cellular uptake studies, the cells were incubated at 37°C for 10 min in Hepes buffer containing 1 μg/ml propidium iodide. Cellular propidium iodide intensities were then measured at the excitation and emission wavelengths of 490 nm and 600 nm respectively. Cellular damage was calculated according to the equation:

$$\%\ \text{Damaged Cells} = \frac{\text{Measured signal} - \text{Minimum signal}}{\text{Maximum signal} - \text{Minimum signal}} \times 100\%$$

The maximum signal is the fluorescence signal obtained in the presence of Triton X-100 (0.1%) which was used to permeabilize the cells. The minimum signal is the background autofluorescence signal.

3. Results

3.1. Mannosylated polylysine-mediated cellular uptake of oligonucleotide

To evaluate the effectiveness of MPL as a drug carrier for ON delivery to the AMs, cells were incubated for 1, 2, and 4 h with the ON:MPL complex consisting of ON (25 μg/ml) and MPL (25 μg/ml), after which their fluorescence intensity was measured. As controls, cells were also treated with ON alone (25 μg/ml) or complexed with PL (25 μg/ml), and their fluorescence intensities were similarly determined. The results indicated that exposure of the cells to ON:MPL complex resulted in a significantly higher fluorescence intensity over the control levels (Fig. 1). The enhancing effect was found to be time-dependent, i.e., a 11-, 14-, and 17-fold increase in fluorescence intensity over the ON control after 1, 2, and 4 h respectively. Thus, our results demonstrated that the MPL can greatly promote cellular uptake of ON and that ON alone was poorly taken up by the cells. As can be seen from Fig. 1, PL can also increase cellular uptake of ON, however, this effect was much less pronounced than that caused by MPL. These

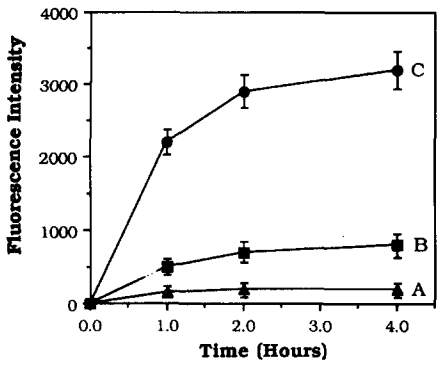

Fig. 1. Comparison of cellular uptake of free oligonucleotide or oligonucleotide complexed with polylysine or mannosylated polylysine. Macrophages (10^5 cells per well) were incubated with free ON (25 μg/ml) (A), ON:PL complex (25:25 μg/ml) (B), or ON:MPL complex (25:25 μg/ml) (C) for 1, 2, and 4 h in Hepes-buffered medium at 37°C . After incubation, the cells were washed and analyzed for their fluorescence intensity. The data represent mean ± S.E. of four measurements obtained from different cell preparations.

results indicated that effective ON uptake mediated by the complex requires a functional domain capable of effective ON cellular recognition and internalization. The observation of the increased cellular uptake of ON by PL may be attributed to non-specific adsorptive endocytosis of the ON:PL complex. In this regard, it should be noted that PL has been used to aid cellular uptake of a number of macromolecules including antisense ONs [12,13].

3.2. Dose-dependent cellular uptake of oligonucleotide complex

In an attempt to optimize the uptake condition of the ON:MPL complex, complexes containing varying concentrations of MPL were formed and tested for their uptake efficiency. Fig. 2 showed that at a fixed concentration of ON (25 μg/ml), increasing the amount of MPL (upto 30 μg/ml) resulted in a proportional increase in cellular fluorescence intensity. These results indicated that cellular uptake of the ON mediated by MPL was concentration-dependent.

3.3. Fluorescence microscopic studies

To provide morphologic evidence of MPL-mediated ON uptake, fluorescence microscopic studies of cells treated with ON:MPL or ON alone were conducted. As shown in Fig. 3, cells treated with the complex exhibited a much stronger fluorescence intensity than those treated with free ON. In both cases, however, the internalized ONs appeared to be localized in endocytic vesicles, as indicated by the punctate fluorescence pattern. To test whether the MPL was simultaneously internalized along with the ON or become dissociated prior to ON internalization, dual-fluorescence microscopic studies were conducted. Complex was formed between the BODIPY FL-labeled ON and rhodamine-labeled MPL and cellular uptake of the complex was monitored. As shown in Fig. 4, both MPL and ON appeared to be co-internalized by the macrophages. The sites where the ON appeared in the cells corresponded well to those occupied by the MPL.

3.4. Mechanism of oligonucleotide complex uptake

To test whether the cellular internalization of the ON:MPL complex was mediated by the mannose receptor-mediated pathway, cellular uptake of the ON:MPL complex in the presence of competition for mannose receptors was carried out. In these experiments, the ON:MPL complex was incubated with AMs in the presence of a specific mannose receptor competitor, mannosylated BSA, and a non-specific competitor, BSA. The results indicated that cellular uptake of the ON:MPL complex was greatly inhibited by mannosylated BSA but not by BSA (Fig. 5), thus indicating that ON uptake mediated by MPL occurred via the mannose receptor pathway.

In further confirm the mechanism of ON complex uptake and to test the potential targeting ability of the MPL system, uptake studies were repeated in a macrophage cell line, J774.1, which expresses a low level of mannose receptors [31]. The J774.1 cells were found to exhibit a much lower uptake as compared to the AMs (Fig. 5), thus confirming the mannose receptor-mediated uptake by the AMs. The result also indicates the potential targeting ability of the mannosylated carrier system.

3.5. Cytotoxicity studies

A major concern relevant to the application of a drug carrier system is its safety. In the present study, we evaluated the potential toxicity of the carrier system in AMs using propidium iodide assay. The AMs were incubated for 4 h with ON alone or in combination with MPL,

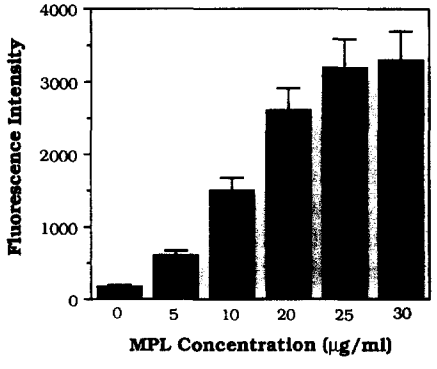

Fig. 2. Effect of mannosylated polylysine concentration on cellular uptake of oligonucleotide. Macrophages (10^5 cells per well) were incubated at 37°C with ON (25 μg/ml) complexed with various concentrations of MPL (0–30 μg/ml) for 4 h. After incubation, the cells were washed and analyzed for fluorescence intensity. The values represent mean ± S.E., $n = 4$.

or MPL or PL alone, after which they were analyzed for propidium iodide fluorescence. Propidium iodide, due to its hydrophilicity, is normally excluded from the cells, but if the membrane is damaged the probe can enter the cell and bind specifically to the cell nucleus. Upon binding, its fluorescence intensity is strongly enhanced; therefore intense nuclear fluorescence indicates cell damage and death. Our results indicated that except for the PL, none of the test agents caused apparent toxic effects to the cells (Fig. 6). PL caused a slight but significant damage to the cells as compared to the non-treated control, i.e., $\approx 3\%$ vs. 1%.

3.6. Enhanced cytoplasmic delivery of oligonucleotide

The successful utilization of receptor-mediated endocytosis as a means to enhance cellular uptake of ONs may be limited by ON entrapment in endocytic vesicles. As demonstrated earlier, ON uptake via the mannose receptor pathway led to the distribution of ON largely confined in vesicular compartments. As an effort to promote endosomal exit of ON, we examined the effect of the fusogenic HA2 peptide on ON uptake and its intracellular distribution in the AMs. Our results indicated that the peptide greatly facilitated cellular uptake of the ON complex in a dose-dependent manner (Fig. 7). At the peptide concentration of 10 μM, the cellular uptake of ON complex increased by over 10-fold as compared to the non-treated control. To test whether this promoting effect was due to increased endosomal exit of ON, fluorescence microscopic study was also conducted. As can be seen in Fig. 8, the peptide induced a more diffuse fluorescence pattern as compared to the non-treated control (Fig. 3B). Cell viability assays using propidium iodide also indicated that the cells maintained their integrity and that the effect of the peptide was not simply due to non-specific plasma membrane damage. Furthermore, in order to confirm the endosomal fusogenic activity of the peptide, experiment shown in Fig. 8 was repeated but with the addition of monensin (1 μM). Monensin is a weak base commonly used in endocytosis studies to neutralize the acidic pH of endosomes. Since the fusogenic activity of HA2 peptide requires conformational change at acidic pH, neutralization of the

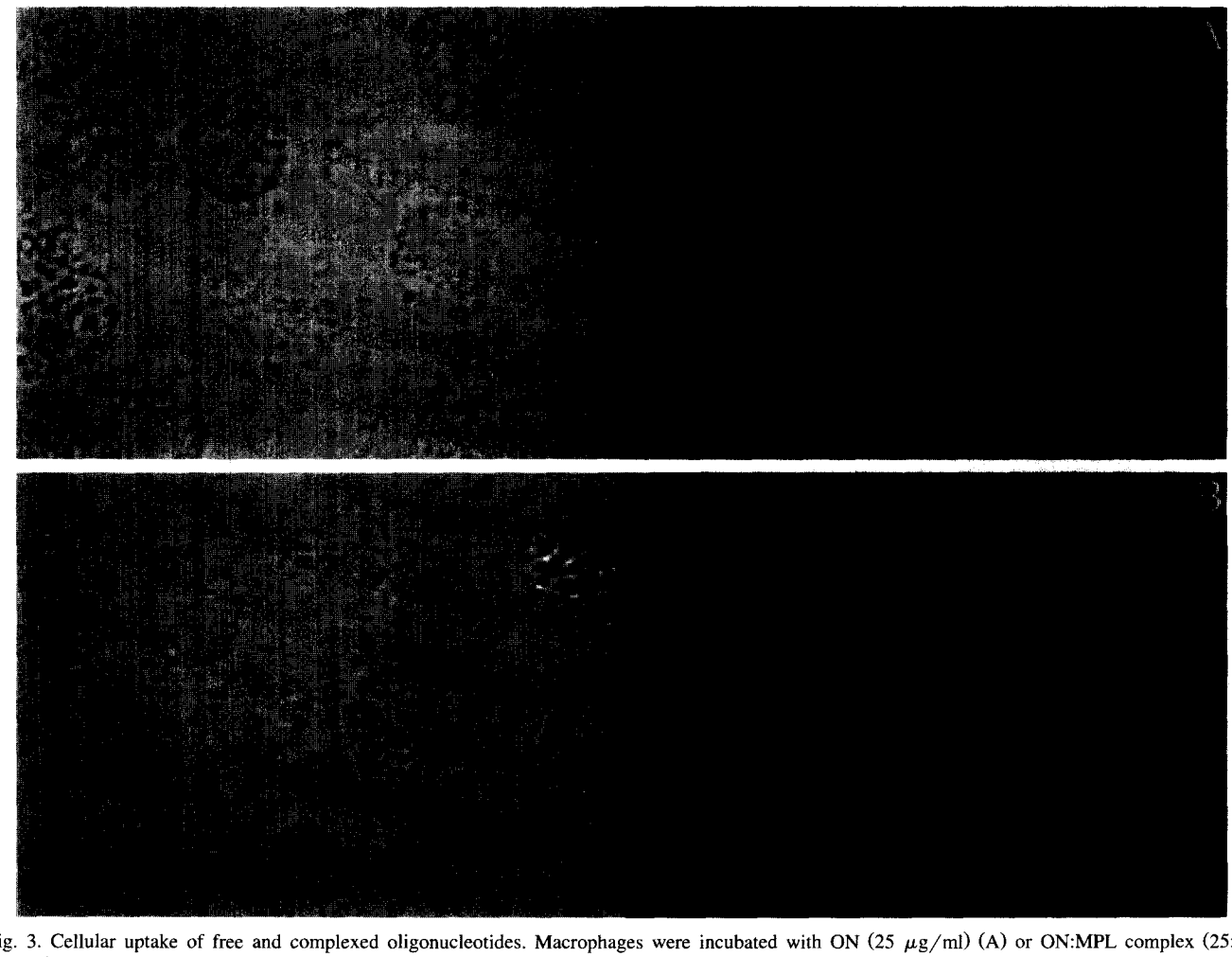

Fig. 3. Cellular uptake of free and complexed oligonucleotides. Macrophages were incubated with ON (25 μg/ml) (A) or ON:MPL complex (25:25 μg/ml) (B) at 37°C for 1 h. Images were taken at identical settings. On the right cells viewed under fluorescence microscopy, on the left phase-contrast images.

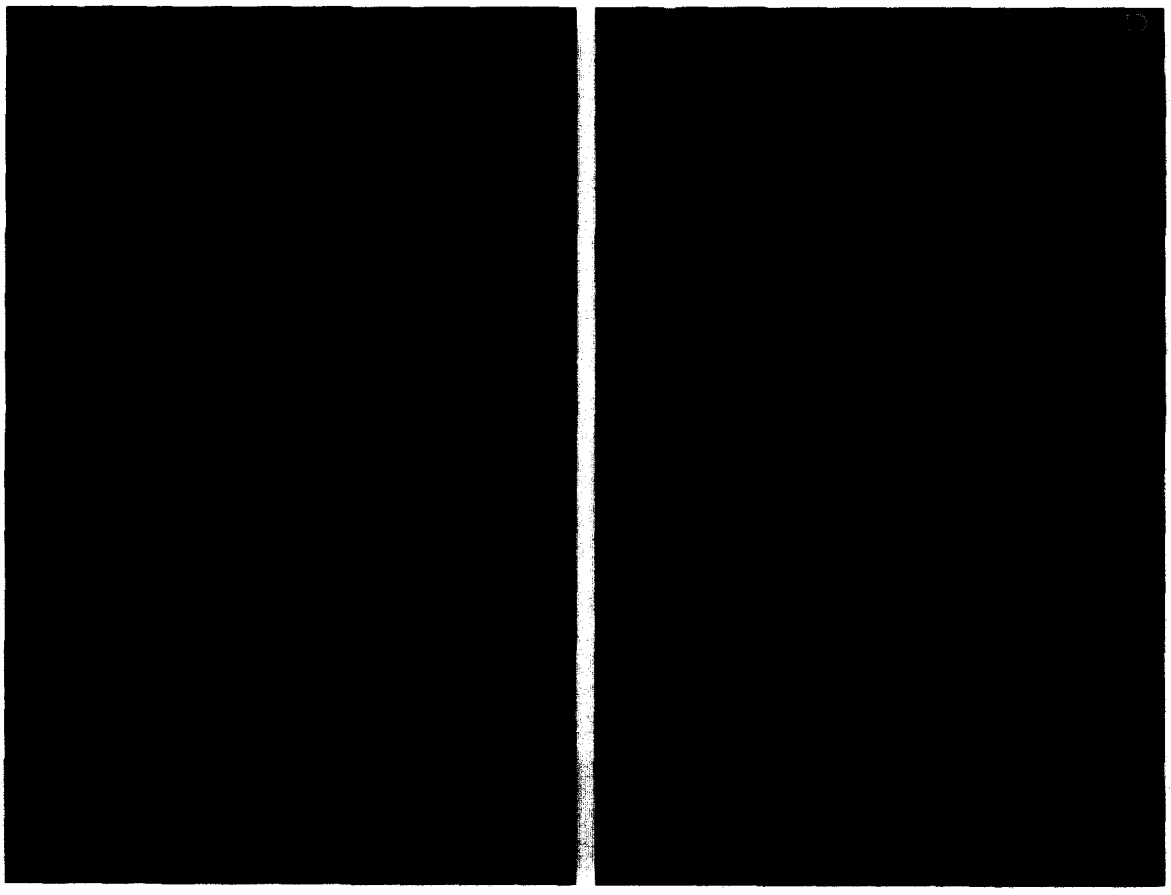

Fig. 4. Fluorescence micrographs showing intracellular distribution of oligonucleotide (A) and mannosylated polylysine (B) in alveolar macrophages. Cells were treated with ON:MPL complex consisting of BODIPY FL-labeled ON (25 μg/ml) and rhodamine-labeled MPL (25 μg/ml) at 37°C for 2 h. Images were taken from the same view but at different fluorescence settings as described in Section 2.

endosomal pH would therefore inhibit its fusogenic activity. Indeed, our results indicated that in the presence of monensin the cells exhibited a punctate fluorescence pattern similar to that observed in cells treated with the ON complex alone (see Fig. 3B). In the absence of fusogenic peptide, monensin had no effect on the fluorescence distribution of ON. Thus, our results strongly suggested that the peptide was co-internalized and exhibited fusogenic activity in the endosomes.

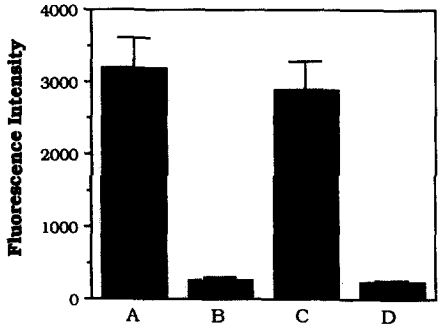

Fig. 5. Mechanisms of oligonucleotide uptake mediated by mannosylated polylysine. Macrophages were incubated with ON:MPL complex (25:25 μg/ml) at 37°C for 4 h in the absence (A) or presence of mannosylated albumin (1 mg/ml) (B), as a specific competitor for mannose receptor, or albumin (1 mg/ml) (C), as a non-specific competitor. (D) same as (A) but J774.1 cells were used. The values represent mean ± S.E., $n = 4$.

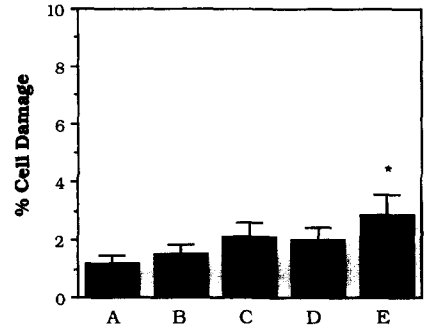

Fig. 6. Cytotoxicity of oligonucleotide and carrier systems in alveolar macrophages. Cells were incubated with (A) blank buffer, (B) free ON (25 μg/ml), (C) ON:MPL complex (25:25 μg/ml), (D) MPL alone (25 μg/ml), and (E) PL alone (25 μg/ml) at 37°C for 4 h. The values represent mean ± S.E., $n = 4$. * Significant increase over control A ($P < 0.05$).

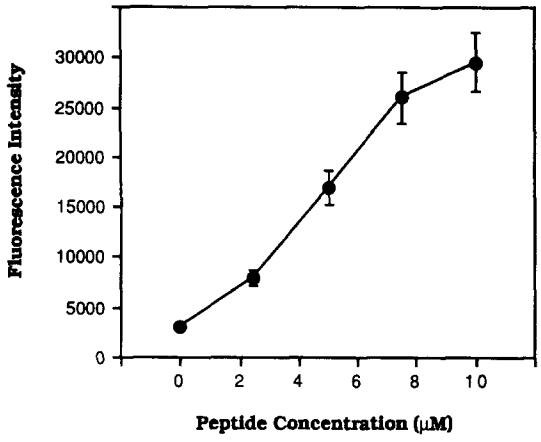

Fig. 7. Effect of fusogenic peptide concentration on cellular uptake of oligonucleotide complex. Macrophages were incubated with ON:MPL complex (25:25 μg/ml) in the presence of varying concentrations of HA2 peptide (0–10 μM) at 37°C for 4 h. The values represent mean ± S.E., $n = 4$.

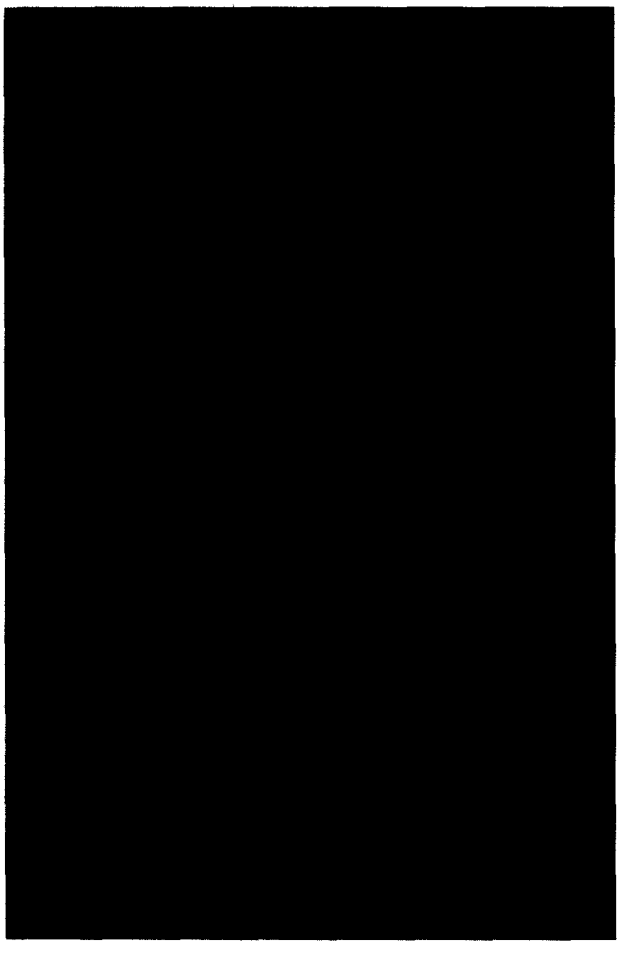

Fig. 8. Fluorescence micrograph showing effect of HA2 peptide on cellular distribution of oligonucleotide complex. Macrophages were incubated with ON:MPL complex (25:25 μg/ml) in the presence of peptide (10 μM) at 37°C for 2 h.

4. Discussion

The AM plays an important role in host defense against inhaled noxious substances and in pathogenesis of several pulmonary diseases. The study of macrophage function and regulation as well as its potential therapeutic intervention by antisense agents will require the development of drug delivery systems capable of effective and selective transfer of ONs to the AMs. In this study, we have demonstrated that receptor-mediated endocytosis of the mannose receptor can be exploited to provide enhanced and targeted delivery of ON to the AMs. Polylysine partially substituted with mannose residues, as the recognition signal, gave a soluble complex upon mixing with ON. The cellular internalization of the complex was via receptor-mediated endocytosis which offers the potential advantages of improved uptake efficiency and targeting. As demonstrated in this study, the cellular uptake of ON complexed with MPL was much more efficient than that of free ON. The intracellular fluorescence intensity was about 17-fold higher than that of cells treated with ON alone. This enhanced delivery of ON by molecular complex should improve the effectiveness of antisense ONs for the inhibition of gene expression.

The uptake of ON:MPL complex was found to be mediated by the mannose receptor since (a) the mannose receptor binding moiety was required for effective uptake of the ON complex, (b) cells bearing few receptors (J774.1 cell line) did not appreciably take up the complex, and (c) competition for mannose receptors by mannosylated BSA inhibited the cellular uptake of the complex.

After internalized by the AMs, the ON:MPL complex was localized mainly in endocytic vesicles, as revealed from microscopic studies. As biological effects have been reported with antisense ONs, it is expected that small amounts of the ONs which have been taken up into vesicles have escaped the endosomes and reached their targets in the cytosol or nucleus. The exit of antisense ONs from the lumen of endocytic vesicles to the cytosol could take place either in late endosomes, in *trans* Golgi network, or in Golgi apparatus, although the precise mechanisms of exit have not been elucidated. The observation that certain endosomal disrupting agents such as fusogenic viral peptides can dramatically improve the biological activity of ONs [28] suggests that such exit mechanisms are rather ineffective. Therefore, the ability to facilitate endosomal exit of ONs is an important consideration in their utilization as therapeutic agents. In this study, we tested the endosomal destabilizing effect of HA2 peptide and its influence on macrophage uptake and distribution of ON. Since mannose receptor-mediated endocytosis is a recycling process [22], enhanced endosomal escape by the peptide is expected to result in increased cellular distribution of ON. Accordingly, our study showed that in the presence of the peptide the cellular uptake of ON:MPL was greatly enhanced. Microscopic evidence further sug-

gested that this enhancing effect was associated with increased cytoplasmic entry of the ON complex.

The application of the complex carrier system for in vivo ON delivery remains to be established. The complex anatomy of the lung as well as the great variety of existing lung cell types may limit the effectiveness of the delivery system. However, the targeting ability provided by the system should prove beneficial to targeted delivery of ON to the AMs. The potential toxicity of the delivery system in vivo needs also to be further investigated. Although our in vitro studies indicated the relative safety of the system for the AMs, other lung cell types may be more sensitive to toxicity caused by the system.

Acknowledgements

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