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Human immunodeficiency virus type-1 (HIV-1) infection increases the sensitivity of macrophages and THP-1 cells to cytotoxicity by cationic liposomes

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Transfection of human macrophages by lipoplexes via the combined use of transferrin and pH-sensitive peptides

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Abstract: The crucial function of macrophages in a variety of biological processes and pathologies render these cells important targets for gene therapeutic interventions. Commonly used synthetic gene delivery vectors have not been successful in transfecting these non-dividing cells. A combination strategy involving cationic liposomes to condense and carry DNA, transferrin to facilitate cellular uptake, and the pH-sensitive peptide GALA to promote endosome destabilization, resulted in significant expression of a luciferase gene. Transfection of macrophages was dependent on the degree of differentiation of the cells. The quaternary complexes of cationic liposomes, DNA, transferrin, and GALA exhibited a net negative charge, which may obviate a limitation of cationic synthetic vectors *in vivo*. The lack of cytotoxicity and the expected lack of immunogenicity of these complexes may render them useful for gene delivery to macrophages *in vivo*. *J. Leukoc. Biol.* 65: 270–279; 1999.

Key Words: *gene delivery · cationic liposome · GALA · granulocyte-macrophage colony stimulating factor · luciferase*

INTRODUCTION

Gene therapy is emerging as a promising therapeutic strategy for the treatment of genetic metabolic diseases, cancer, and AIDS. Macrophages are effector cells that play a major role in the defense of the organism against diseases. Their broad activity that includes tissue maintenance, immune regulation, and pathogen control makes them crucial targets for gene therapy [1]. Through the expression of therapeutic transgenes, attempts have been made to modulate the function and dysfunction of macrophages for the treatment of genetic metabolic diseases, including Gaucher's disease [2–5], to promote adoptive cellular immunotherapy against neoplastic cells [6], to repair damaged tissues [7], or to inhibit HIV replication in these cells [8, 9].

Several strategies have been developed to transfer genes into monocytes or macrophages, most of them using viral vectors. Being non-proliferating cells, macrophages are difficult to transduce with retroviruses, and this has led to the use of adenovirus for that purpose [10, 11]. However, the size limit of DNA carried and the risk of immunogenicity are serious limitations to the use of such viruses. Consequently, it is essential to develop efficient non-viral gene delivery strategies. Recently, the use of DNA/glycosylated polylysine complexes was described as a promising strategy for receptor-mediated gene delivery to these cells. The conception of such systems was based on the fact that macrophages express several membrane lectins that mediate endocytosis [12, 13].

We investigated whether promoting receptor-mediated endocytosis of cationic liposome-DNA complexes by associating them with transferrin [14–16] would result in transfection of human peripheral blood monocyte-derived macrophages. We tested complexes composed of 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP):dioleoylphosphatidylethanolamine (DOPE) liposomes and the plasmid pCMVLuc encoding luciferase, at different lipid/DNA charge ratios. We also examined whether the association to the complexes of two different synthetic, pH-sensitive, fusogenic peptides, GALA [17] and the influenza virus hemagglutinin HA-2 amino-terminal peptide [18], would result in an enhancement of transfection. The rationale was that these peptides would facilitate the destabilization of phagosomes or endocytotic vesicles in which the complexes are internalized by macrophages, thereby mediating the release of DNA into the cytoplasm and preventing its degradation at the lysosomal level. Association of both transferrin and fusogenic peptides with cationic liposomes was also tested for different lipid/DNA charge ratios.

Morphological and functional heterogeneity is an intrinsic characteristic of macrophages and explains the recognized

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Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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versatility of these cells. Among others, granulocyte-macrophage colony-stimulating factor (GM-CSF) is a polyfunctional regulator involved in the proliferation and differentiation of stem cells into circulating monocytes and also in the maturation and activation of the mature cells finally produced [19]. The latter process involves a variety of functions, including phagocytic capacity, cytotoxicity, expression of transferrin receptors, chemotactic responses, and the production of various molecules associated with inflammatory processes [20]. These functions have all been shown to be expressed maximally at specific stages of differentiation as a result of transient expression promoted by exogenous stimulation. In this study, human blood monocytes were plated and allowed to mature into macrophages in the presence or absence of GM-CSF. The goal was to evaluate the effect of this cytokine on the transfection activity mediated by cationic lipid-DNA complexes (lipoplexes [21]), with or without transferrin and/or fusogenic peptides. With the same purpose, transfection of macrophages allowed to mature for different times was also studied.

The risk of potentially adverse interactions with the biological milieu is one of the main drawbacks associated with the use of cationic liposomes for gene delivery. In fact, the inhibitory effect of serum on transfection mediated by lipoplexes has been reported for different cell types [22–24] and represents a serious limitation for their use *in vivo* [24]. We therefore evaluated the effect of serum on transfection by the ternary and quaternary complexes. The interaction of cationic liposomes with negatively charged biological macromolecules could be potentially minimized by utilizing lipoplexes with a net negative charge. Thus, we assessed the net charge of the various complexes by electrophoretic mobility measurements. Cationic liposomes can be toxic to cells, depending on the lipid concentration, duration of treatment, the cell type, and the confluency of the culture [25, 26]. To evaluate the cytotoxicity of the different complexes, cell viability was assessed after transfection. Our results have been presented earlier in preliminary form [27].

MATERIALS AND METHODS

Liposome preparation

The cationic lipid 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP), and the zwitterionic co-lipid dioleoylphosphatidylethanolamine (DOPE), were purchased from Avanti Polar Lipids (Alabaster, AL). Cationic liposomes composed of DOTAP or DOTAP/DOPE (1:1 weight ratio) were prepared by first drying a film of lipid under argon and then in a vacuum oven at room temperature and hydrating the lipid film with 1 mL deionized water, at a final concentration of 5 mg/mL. The multilamellar vesicles obtained were then sonicated briefly under argon, extruded 21 times through polycarbonate filters of 50-nm pore diameter using a Liposofast device (Avestin, Toronto, Canada), diluted five times with deionized water, and filter-sterilized utilizing Millex 0.22-μm pore-diameter filters (Millipore, Keene, NH).

Cells

Monocytes were obtained from buffy coats by centrifugation on a Ficoll-Hypaque (Histopaque-1077; Sigma, St. Louis, MO) gradient and plastic adherence. Mononuclear cells separated by centrifugation were counted and plated in Dulbecco's modified Eagle's medium, high-glucose (DME-HG) medium without serum at a density of 1.4×10^6 cells/mL/well in 48-well plates. It was assumed that approximately 5–10% of the cells plated were recovered as macrophages. The cells were allowed to adhere overnight, then washed, and the medium was replaced with DME-HG supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma), 10% (v/v) human AB serum (Advanced Biotechnologies, Columbia, MD), penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM), as described previously [28]. The cells were left undisturbed in this medium for 6–7 days for differentiation to occur. In some experiments human GM-CSF (hGM-CSF; Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to the wells (final concentration of 100 IU/well) at the second day of differentiation. In other experiments, cells were cultured for 8 more days in medium containing 20% FBS, antibiotics, and L-glutamine, but in the absence of hGM-CSF.

Preparation of the complexes

Iron-saturated, heat-inactivated human transferrin was obtained from Collaborative Biomedical Products (via Becton Dickinson, Bedford, MA). The GALA peptide [17, 29] and the peptide named HA-2, derived from the amino-terminal sequence of the influenza virus hemagglutinin subunit HA2 [18] were synthesized and high-performance liquid chromatography (HPLC)-purified by the UCSF Biomolecular Resource Center. GALA is a 30-amino acid, pH-sensitive, amphipathic peptide with the sequence WEAALAEALAEA-LAEHLAEALAEALEALAA. The sequence of HA-2 is GLFEAIAGFIENG-WEG MIDGGGC. Complexes were prepared by sequentially mixing 100 μL of a solution of 100 mM NaCl, 20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES; Sigma), pH 7.4, with or without 32-μg iron-saturated human transferrin, with 5 or 10 μL of the liposome suspension (1 mg lipid/mL) and incubated at room temperature for 15 min. One hundred microliters of buffer containing 1 μg of pCMVLuc (plasmid VR-1216, kindly provided by Dr. P. L. Felgner; Vical, Inc., San Diego, CA) were then added and gently mixed, and the mixture was further incubated for 15 min at room temperature. Peptide complexes were prepared in a similar manner, except that the 100 μL of HEPES buffer solution contained 0.6 μg of the peptides. Quaternary complexes were prepared by sequentially mixing liposomes with the transferrin solution, then adding 0.6 μg of GALA (from a stock solution) and finally 100 μL of a DNA solution containing 1 μg of the plasmid. Each addition was followed by a 15-min incubation at room temperature.

Transfection activity

Cells were rinsed twice with serum-free medium and then covered with 0.3 mL of DME-HG before lipid-DNA complexes were added. Lipid-DNA complexes were added gently to the cells in a volume of 0.2 mL/well. After an incubation of 4 h (in 5% CO_2 at 37°C) the medium was replaced with DME-HG containing 20% FBS, and the cells were further incubated for 24 or 48 h. The cells were then washed twice with phosphate-buffered saline (PBS) and 100 μL of lysis buffer (Promega, Madison, WI) were added to each well. The level of gene expression in the lysates was evaluated by measuring light production by luciferase using a scintillation counter protocol (Promega). The protein content of the lysates was measured by the DC Protein Assay reagent (Bio-Rad, Hercules, CA) using bovine serum albumin (Sigma) as the standard. The data were expressed as nanograms of luciferase (based on a standard curve for luciferase activity) per milligram of total cell protein.

Transfection efficiency

Transfection efficiency was evaluated by scoring the percentage of cells successfully transfected. For this purpose two different plasmids were used: pCMV.SPORT-β-gal (Life Technologies, Inc., Gaithersburg, MD) encoding β-galactosidase, and a modified pHooK™-2 plasmid (Invitrogen, San Diego, CA) encoding the Green Fluorescent Protein (GFP [30]). The latter plasmid (pHooK™-2-GFP) was generated by inserting the GFP gene from a pTR-UF/UF1/UF2 AAV cassette into pHooK™-2, and was kindly provided by Dr. Nancy Lee and Dr. John Rossi (City of Hope, Duarte, CA). Briefly, cells transfected with 1 μg of pCMV.SPORT-β-gal were washed with PBS, fixed in a solution of 2% formaldehyde and 0.2% glutaraldehyde, and stained with an X-gal containing solution (Invitrogen). The cells were incubated at 37°C for 24 h and then examined under a phase-contrast microscope for the development of blue color.

In the GFP assay, cells were transfected with 1 μg of pHooK™-2-GFP, washed

with PBS, and detached from plastic by adding 0.5 mL of cell dissociation buffer (Life Technologies, Inc.). They were then mixed with 0.5 mL of DME-HG medium containing 1% of FBS and 1 μg/mL propidium iodide (Sigma) to assess cell viability by flow cytometry. Green fluorescence was detected with a Becton Dickinson single argon laser FACScan flow cytometer. GFP and propidium iodide were excited at 488 nm and respective emissions were collected using a 530-nm band-pass filter (30-nm band width) for GFP and a 630-nm band-pass filter (22-nm band width) for propidium iodide. Five thousand events were recorded for each sample. Forward scatter and propidium iodide fluorescence signals were used to gate the cell subset of interest and to eliminate debris, dead cells, and cell aggregates.

Zeta potential measurements

Zeta potential measurements of the different lipid-DNA complexes, and ternary or quaternary complexes with transferrin and/or peptides, were performed using a Coulter DELSA 440 instrument (Coulter Corp., Miami, FL). The zeta potential (ζ) was calculated for each scattering angle (8.6°, 17.1°, 25.6°, and 34.2°). Data represent the mean ± standard deviation obtained for the different angles of two measurements.

Cell viability assay

After transfection under the different experimental conditions, cell viability was quantified by a modified Alamar Blue assay [26]. The assay measures the redox capacity of cells due to the production of metabolites as a result of cell growth, and allows determination of viability over the culture period without the detachment of adherent cells. Briefly, 1 mL of 10% (v/v) Alamar Blue dye (AccuMed International Companies, Westlake, OH) in complete DME medium was added to each well 45 h after the initial transfection period (4 h). After 2.5–4 h of incubation at 37°C, 200 μL of the supernatant were collected from each well and transferred to 96-well plates. The absorbance at 570 and 600 nm was measured with a microplate reader (Molecular Devices, Menlo Park, CA). Cell viability (as a percentage of control cells) was calculated according to the formula (A_{570}–A_{600}) of treated cells × 100/(A_{570}–A_{600}) of control cells.

RESULTS

Enhancement of transfection of human blood monocyte-derived macrophages by a targeting ligand and/or fusogenic peptides

In an attempt to develop viable alternatives to the use of viral vectors in gene therapy, several approaches have been tested to enhance transfection mediated by cationic liposomes. The use of ternary complexes composed of cationic liposomes, DNA, and transferrin or fusogenic peptide was previously described as a promising approach for gene delivery of proliferating cells [14–16]. The goal of this strategy was to promote receptor-mediated endocytosis of the complexes (by the presence of transferrin) and to improve cytoplasmic release of DNA from endosomes (by the inclusion of a fusogenic peptide activated by the mildly acidic pH in this compartment).

Here we tested these approaches in human blood monocyte-derived macrophages. These are non-proliferating cells that are well known as being difficult to transfect by non-viral vectors, as also evidenced by our results. **Figure 1** shows the levels of luciferase expression obtained in the presence or absence of transferrin and/or fusogenic peptides. Two different lipid/DNA charge ratios were tested because previous data indicated that transfection activity may vary depending on the net charge of the complexes [15, 16]. Association of transferrin with the lipid-DNA complexes resulted in a significant enhancement of transfection. This effect was more prevalent for the 2:1 (+/−) lipid/DNA charge ratio where an enhancement of about 20-fold was observed, as compared to controls, i.e., complexes composed of only cationic lipid and DNA (lipoplexes). These results

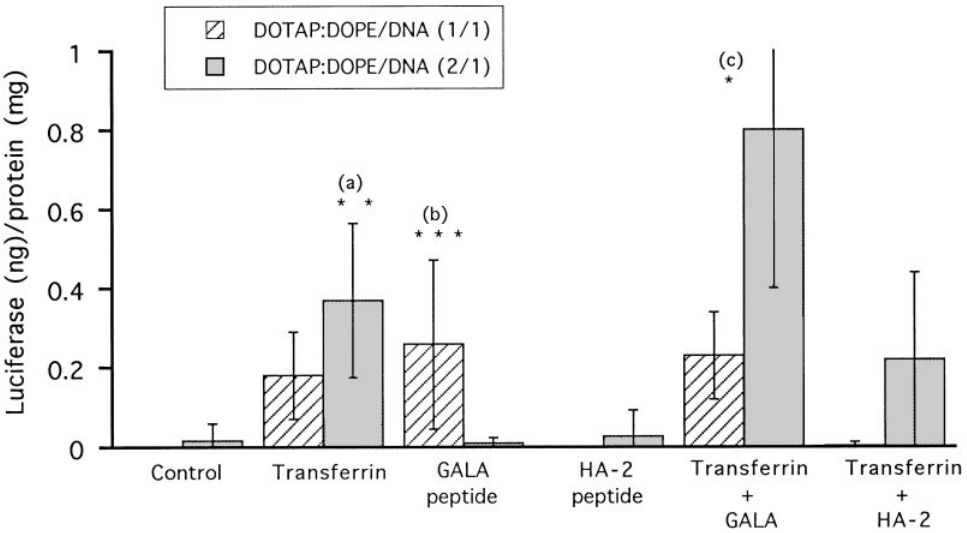

Fig. 1. Effect of the association of transferrin and/or fusogenic peptides with cationic liposome-DNA complexes on transfection of human blood monocyte-derived macrophages. DOTAP:DOPE liposomes were mixed with 32 μg of transferrin and/or 0.6 μg of fusogenic peptides (either GALA or HA-2 peptide) and then mixed with 1 μg of pCMVLuc plasmid. The amount of transferrin used reflects the optimum determined by the experiments of Cheng [14] on HeLa cells. Macrophages were obtained by plating monocytes in DME-HG supplemented with 20% FBS and 10% human AB serum for 7 days. hGM-CSF was added to the wells (final concentration of 100 IU/mL) at the 2nd day of differentiation. After rinsing the cells with serum-free DME-HG medium, complexes were added and the cells incubated for 4 h at 37°C. After removing the complexes cells were further incubated for 48 h in medium containing 20% of FBS. The level of gene expression was evaluated as described in Materials and Methods. The data are expressed as nanograms of luciferase per milligram of total cell protein (mean ± SD obtained from triplicate wells), and are representative of two independent experiments. (a) Significantly different from the corresponding control ($P < 0.01$). (b) Significantly different from the corresponding control ($P < 0.001$). (c) Significantly different from the corresponding control ($P < 0.05$).

suggest that receptor-mediated endocytosis of the ligand-bearing complexes contributes to the enhancement of the transfection levels mediated by cationic liposomes.

The association of the pH-sensitive synthetic peptide GALA with the 1:1 (+/−) charge ratio complexes resulted also in an increase of the level of luciferase expression as compared to controls. Previous studies had shown 0.6 μg of GALA to be optimal in enhancing transfection when complexed with cationic liposomes and 1 μg DNA [16]. No significant effect was observed for the 2:1 charge ratio. On the other hand, the association of the same amount of the synthetic peptide derived from the amino terminus of the HA-2 subunit of the influenza virus hemagglutinin did not result in any increase in gene expression for either charge ratio tested.

The highest levels of luciferase expression were observed when both transferrin and GALA were simultaneously combined with the lipid-DNA complexes. This effect was especially evident for the 2:1 (+/−) lipid/DNA charge ratio, where an increase of almost 50-fold was achieved compared with controls. The combined association of transferrin and the HA-2-derived peptide did not produce any significant effect compared with controls for the 1:1 (+/−) charge ratio, whereas for the 2:1 charge ratio the levels of luciferase expression did not surpass the values obtained with the transferrin-lipid-DNA complexes.

As readily noted from the standard deviations indicated by the bars shown in Figure 1, a large variability in the levels of luciferase gene expression was observed independently of the type of complex used for transfection. This variability of transfection activity was evident not only among independent experiments (i.e., using batches of macrophages obtained from the blood of different donors), but also among different wells of the same experiment (i.e., using cells obtained from the blood of the same donor). However, as noted below, the variability was largely diminished when the cells were allowed to differentiate for a longer period of time.

Effect of hGM-CSF and differentiation time on transfection activity

Myeloid hematopoietic growth factors, like hGM-CSF, are involved in the process of proliferation of monocytes and their maturation into macrophages *in vivo* and *in vitro* [31]. These cytokines also play a major role in the activation of these cells in response to external stimuli. Therefore, we evaluated the effect of hGM-CSF on the transfection activity of macrophages mediated by lipoplexes and the quaternary complexes of cationic liposomes-DNA-transferrin-GALA. For this purpose approximately 1.4×10^5 cells were allowed to mature in individual wells of 48-well plates for 7 days in the presence or absence of 100 IU/mL of hGM-CSF. The above complexes were then added to cells in a volume of 1 mL/well and incubated for 4 h at 37°C. Transfection activity was evaluated 48 h later.

Observation of the macrophages by phase contrast microscopy after this period of incubation in the presence of the cytokine and just before starting the transfection procedure, revealed some morphological differences between treated and non-treated cells. Macrophages cultured in the presence of hGM-CSF were larger, more elongated, and enriched with granules. These cells also showed a higher metabolic activity (noted by the faster color change of the medium) as compared to untreated wells.

Figure 2A illustrates the effect of hGM-CSF on transfection of human blood monocyte-derived macrophages. Promotion of maturation and activation of the cells due to the presence of the cytokine resulted in a significant enhancement of gene transfer and expression mediated by DOTAP:DOPE liposomes. Although this effect was observed under all the conditions studied (including controls), quaternary complexes composed of cationic liposomes-DNA-transferrin-GALA represented the most efficient combination. As referred to in the previous section, the highest levels of transfection for this particular type of complex were obtained for the 2:1 lipid/DNA (+/−) charge ratio.

Alerted by the importance of the stage of maturation of macrophages in transfection activity, we investigated whether the time of maturation would affect this activity. Although there is some controversy regarding the capacity of monocytes to survive and to mature into macrophages when they are cultured in the absence of myeloid hematopoietic growth factors [31, 32], it was possible, using the experimental conditions described in Materials and Methods, to maintain human blood monocyte-derived macrophages in culture for different times in the absence of any cytokine.

As shown in Figure 2A, 7-day-old macrophages kept in the absence of hGM-CSF were not transfected by the lipoplexes, and exhibited some transgene activity with the quaternary complexes. Considerably more promising results were observed when macrophages (obtained from monocytes isolated from the blood of the same donor) were maintained for 15 days in culture. Data presented in Figure 2B clearly show that transfection of cells in such conditions resulted in a significant enhancement of the levels of luciferase expression. This enhancement was observed for both the ternary complexes (containing transferrin) and the quaternary complexes (containing both transferrin and GALA), the highest levels of transfection being observed for the latter case. It should be noted that, under these conditions, the variability in the levels of luciferase expression was reduced compared to the results obtained with 7-day-old macrophages, as also illustrated by the relatively low standard deviations (compare with Fig. 1).

Curiously, no significant differences in the levels of transfection activity were observed when macrophages were cultured for 15 days in the presence of hGM-CSF as compared to cells maintained for only 7 days in the presence of the cytokine (data not shown).

Percentage of cells expressing reporter genes

Cellular distribution of the pCMV.SPORT-β-gal expression was examined 48 h after transfection. Cells were analyzed for β-galactosidase activity by a cytochemical assay. Unfortunately, no conclusive data could be drawn from these experiments because an intense blue stain was detected on the majority of cells under all the conditions tested, including cells that were not transfected (data not shown). These results suggest that the blue staining of the macrophages occurred due

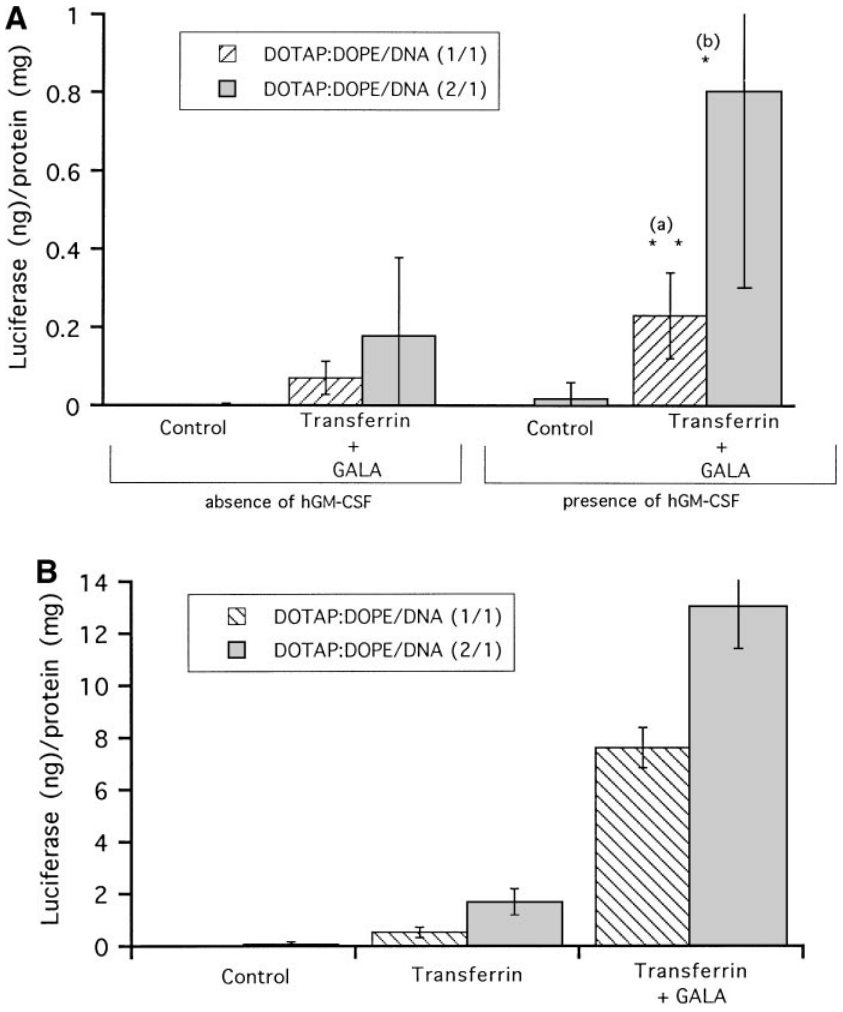

Fig. 2. Effect of the presence of hGM-CSF and of differentiation time on transfection of human blood monocyte-derived macrophages. Transferrin, or transferrin and GALA peptide, were associated with lipid-DNA complexes at the indicated theoretical charge ratios and the resulting ternary/quaternary complexes added to adherent cells cultured (A) for 7 days in the presence or absence of hGM-CSF (100 IU/mL) and (B) for 15 days in the absence of hGM-CSF. Experiments were performed as described in the legend to Figure 1. The data are expressed as nanograms of luciferase per milligram of total cell protein (mean ± SD obtained from triplicate wells), and are representative of two independent experiments. (a) Significantly different from the parallel experiment in the absence of hGM-CSF ($P < 0.01$). (b) Significantly different from the parallel experiment in the absence of hGM-CSF ($P < 0.05$).

to either an endogenous β-gal activity or to a nonspecific hydrolysis of the X-gal substrate as also suggested by Ferkol et al. [13].

To circumvent this problem, complexes containing 1 μg of pHooK™-2-GFP plasmid were prepared, as described in Materials and Methods, and used for transfection of macrophages. After 48 h of incubation at 37°C the cells were detached and analyzed by flow cytometry for the expression of Green Fluorescence Protein. Results presented in **Figure 3** illustrate the percentage of positive gated cells for fluorescent protein when different complexes were used for transfection of macrophages differentiated for 15 days in the absence of hGM-CSF. The number of cells that expressed the transgene was very low for any of the conditions tested. Nevertheless, it was evident that the quaternary complexes, cationic liposomes-DNA-transferrin-GALA, were much more effective than control complexes (complexes lacking transferrin or fusogenic peptide) in mediating gene transfer to macrophages (2% of cells gated as opposed to essentially none, Fig. 3, A and B). Figure 3C shows the flow cytometry histograms observed for each case. The enhancement of the number of cells transfected when quaternary complexes were used resulted in a shift of the observed fluorescence intensity.

Evaluation of the net charge of the complexes

In a previous study, complexes prepared at different lipid/DNA (+/−) charge ratios were characterized regarding their overall charge [15, 16]. It was shown that the association of transferrin or the fusogenic peptides with the lipid-DNA complexes results in a decrease of the zeta potential for any of the charge ratios tested. This effect was attributed to the presence of negatively charged amino acids in these compounds. We examined the effect of the simultaneous association of transferrin and GALA peptide on the overall charge of the complexes by measuring the zeta potential. As expected, the concomitant association of transferrin and the GALA peptide resulted in a significant reduction of the zeta potential of the lipid-DNA complexes (**Table 1**). Consequently, complexes with both 1:1 and 2:1 lipid/DNA charge ratios presented a net negative charge.

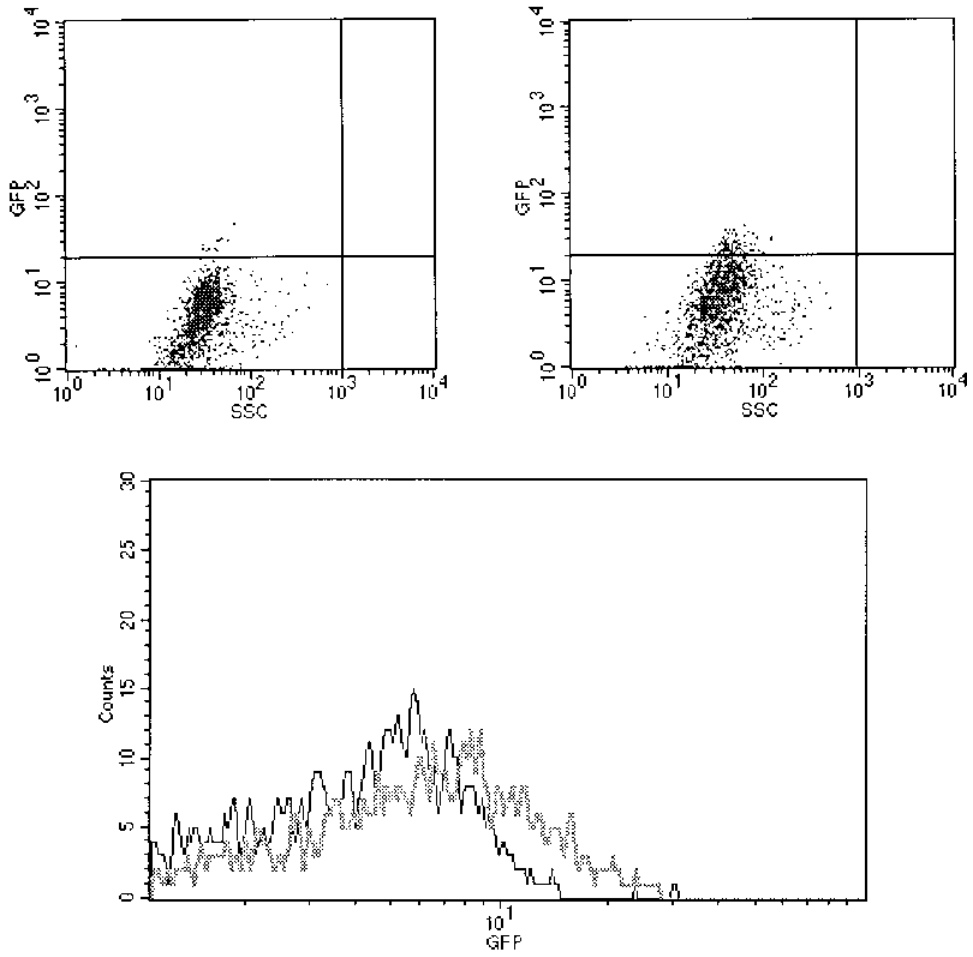

Fig. 3. Expression of Green Fluorescent Protein in human blood monocyte-derived macrophages. Scoring of the percentage of cells transfected when transfection was mediated by either DOTAP:DOPE/DNA complexes (control formulation; top left panel) or quaternary complexes composed of transferrin-lipid-GALA-DNA (top right panel). Bottom panel illustrates the overlay of fluorescence histograms obtained for both cases (thin solid line, control complexes; thick dotted line, quaternary complexes). Transfection of cells cultured for 15 days in the absence of hGM-CSF took place for 4 h and then cells were incubated for 48 h at 37°C. After washing with PBS, cells were detached from plastic with 0.5 mL of dissociation buffer. After the addition of 0.5 mL DME-HG medium containing 1% FBS and 1 μg/mL propidium iodide, cells were analyzed for fluorescence with the use of a flow cytometer.

Effect of serum on transfection activity

As mentioned above, association of transferrin or both transferrin and the GALA peptide with the lipid-DNA complexes resulted in net negatively charged complexes. Such complexes would be expected not to interact as avidly with net negatively charged macromolecules present in biological fluids as would net positively charged lipoplexes. To test this hypothesis, complexes were added to cells in the presence of cell culture medium containing 20% FBS and incubated for 4 h at 37°C. **Figure 4** illustrates the levels of transfection observed when control complexes, transferrin complexes, or transferrin + GALA peptide-associated complexes at different lipid/DNA charge ratios were used under these conditions. The presence of 20% serum during the transfection procedure resulted in a reduction of the levels of luciferase gene expression for the optimal tested condition, i.e. the combined association of transferrin and GALA with the lipid-DNA complexes (compare with Fig. 1). However, the effect of serum was not very significant in the case of transferrin complexes.

TABLE 1. Zeta Potential (mV) of Liposome-DNA Complexes (Lipoplexes) at Various Charge Ratios (+/−) and the Effect of Transferrin or Fusogenic Peptides

	Zeta potential (mV)	
Lipoplex composition	Charge ratio 1:1	(Lipid/DNA) (+/−) 2:1
DOTAP:DOPE/DNA	2.4 ± 5.1	42.8 ± 0.5
DOTAP:DOPE/DNA + Transferrin	−38.6 ± 5.2	25.0 ± 2.2
DOTAP:DOPE/DNA + GALA		−35.3 ± 3.6
DOTAP:DOPE/DNA + HA-2		−11.0 ± 3.5
DOTAP:DOPE/DNA + Transferrin + GALA		−37.8 ± 4.0

Zeta potential measurements of the different DOTAP:DOPE/DNA complexes were performed using a Coulter DELSA 440 instrument. The liposomes were associated with either 32 μg of transferrin or 0.6 μg of GALA peptide, or both, and then mixed with 1 μg DNA. Data represent the mean ± SD obtained for four different angles in two measurements.

Effect of the different complexes on cell viability

We examined the effect on cell viability of transfection with the lipoplexes at two different charge ratios, as well as their

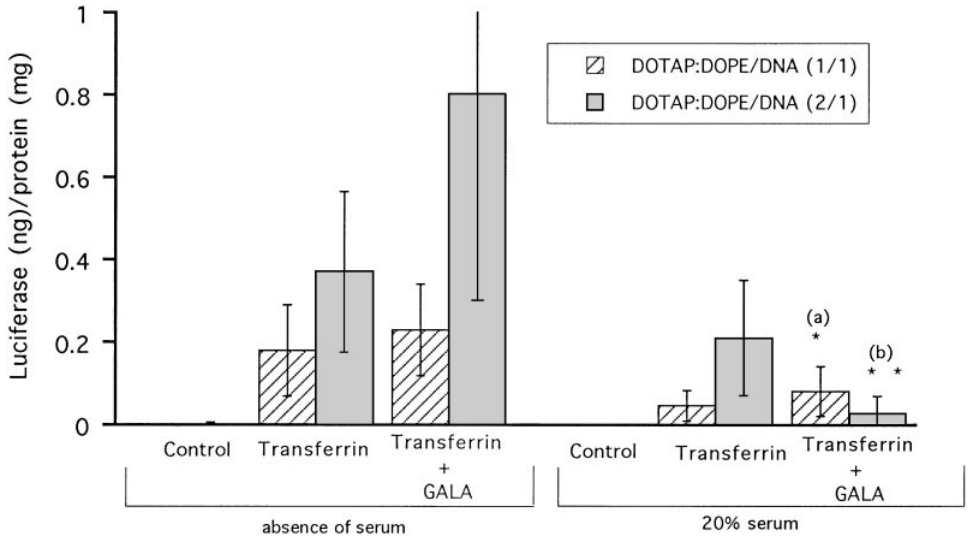

Fig. 4. Effect of the presence of serum on luciferase gene expression in human blood monocyte-derived macrophages. Transferrin and/or GALA peptide were associated with lipid-DNA complexes at the indicated theoretical charge ratios and the resulting complexes added to adherent cells cultured for 7 days in the presence of hGM-CSF (100 IU/mL) and 20% of FBS, and the cells incubated for 4 h at 37°C. After removing the complexes cells were further incubated for 48 h in medium containing 20% FBS. The level of gene expression was evaluated as described in Materials and Methods. Data are expressed as nanograms of luciferase per milligram of total cell protein (mean ± SD obtained from triplicate wells), and are representative of two independent experiments. (a) Significantly different from the parallel experiment in the absence of serum ($P < 0.05$). (b) Significantly different from the parallel experiment in the absence of serum ($P < 0.01$).

complexes with transferrin or the combination of transferrin and GALA, 45 h after the initial 4-h incubation of the cells with the complexes (**Fig. 5**). No significant effect on cell viability was observed. Cell metabolic activity was unaltered even for the highest lipid/DNA charge ratio tested (2:1), where about 8 μg of total lipid were used. The addition of transferrin, or both transferrin and fusogenic peptides, also did not result in any toxicity to the cells. These results were confirmed by both the total cell protein quantification and morphological observations of treated and untreated cells.

DISCUSSION

Transfection of macrophages represents a significant challenge in gene therapy approaches that could utilize these cells. The

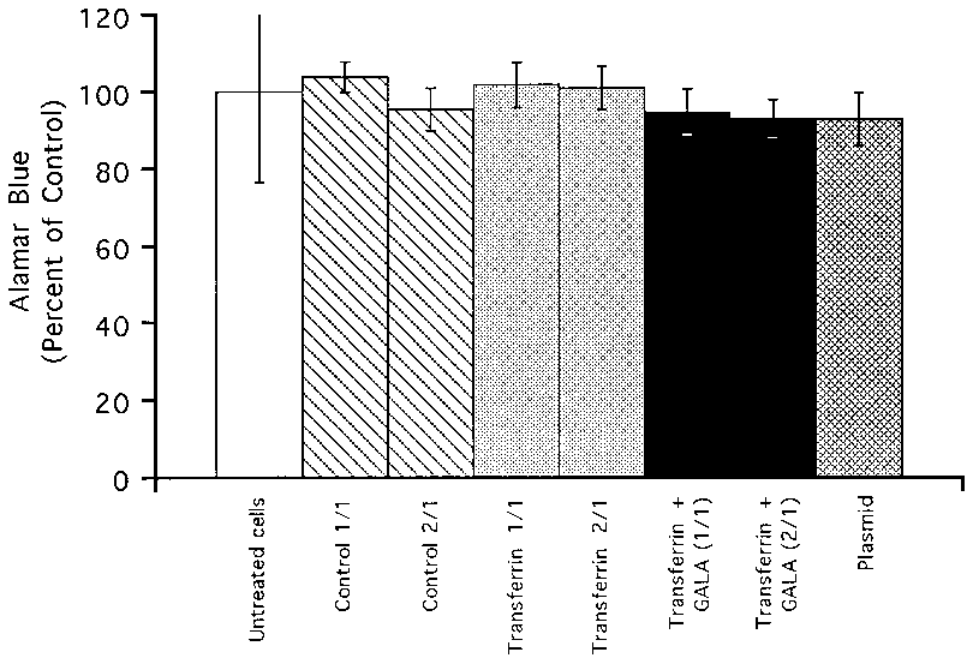

Fig. 5. Effect of DOTAP:DOPE DNA complexes, with or without transferrin or fusogenic peptides, on the viability of human blood monocyte-derived macrophages. Cells in 48-well plates were exposed to lipid-DNA complexes with or without transferrin or the fusogenic peptides at different charge ratios for 4 h at 37°C, as described in Materials and Methods. Cell viability was measured by the Alamar Blue assay 45 h after the 4-h transfection period, and was expressed as percent of the control. Data represent the mean ± SD obtained from duplicate wells.

major role played by macrophages in antigen presentation, and the control of cancer and viral infections make them crucial targets for therapeutic gene delivery. However, macrophages are non-proliferating cells that are very difficult to transfect by non-viral methods, including cationic liposomes.

In this work we tested different strategies to mediate transfection of macrophages by lipid-based complexes. Because differentiated and mature macrophages express transferrin receptors [32], and because ternary complexes composed of cationic liposomes, DNA, and transferrin are highly effective in transfecting proliferating cells [15, 16], we evaluated how the promotion of receptor-mediated endocytosis of lipoplexes via transferrin would affect transfection of macrophages. Although such a ligand would not mediate selective targeting to macrophages *in vivo*, association of transferrin with DOTAP:DOPE/DNA complexes most likely facilitates the internalization of the complexes, thus resulting in an enhancement of the levels of luciferase expression (Fig. 1). In this study we have not attempted to demonstrate whether transferrin lipoplexes are indeed internalized through the transferrin receptor, and if so, how the DNA penetrates the endosome membrane to enter the cytoplasm and reach the nucleus. Such studies are currently underway in our laboratory.

Because macrophages are professional phagocytes [33], it is important to avoid, or at least to decrease, the degradation of the internalized genetic material by lysosomes. For this purpose, low pH-activated membrane-active peptides [17, 18, 34] were also associated with the lipid-DNA complexes. The rationale was to induce the destabilization of endosomes or phagosomes and thereby mediate the entry of the DNA or the complexes into the cytoplasm before fusion with lysosomes. In our studies the synthetic GALA peptide was more effective than the influenza HA-2 peptide (Fig. 1). GALA undergoes a transition from a random coil at pH 7.5 to an amphipathic α-helix at pH 5.0, under which conditions it strongly interacts with phospholipid membranes to induce fusion, contents leakage, and phospholipid flip-flop [17, 29, 34, 35]. The covalent attachment of GALA to polyamidoamine dendrimers was found previously to enhance transfection of cell lines by 1:1 (terminal amine: phosphate) complexes by several orders of magnitude [36]. Nevertheless, it was through the combination of both strategies (i.e., promotion of internalization and facilitation of endosome destabilization) that the highest levels of transfection were obtained in our experiments. These results suggest that the association of fusogenic peptides with lipoplexes may be a promising method to facilitate the intracellular protection of DNA. This method may have advantages over other strategies, including the addition of lysosomotropic agents like chloroquine [12, 18], or the association of inactivated adenovirus [37], that may result in cytotoxicity or an immune response, respectively.

Efficient gene transfer to human blood monocyte-derived macrophages was significantly dependent on their stage of differentiation and maturation promoted by hGM-CSF. As indicated earlier, morphological and functional properties of macrophages are strongly related to the different degree of maturation of these cells. Therefore, an increase of the expression of membrane transferrin receptors may be one of the reasons why an enhancement of gene transfer was achieved under these conditions. Maturation heterogeneity of the cells may explain the variability observed in the levels of luciferase expression among wells. This variability may also be caused by the relatively low levels of transfection activity observed.

The importance of the stage of differentiation in the transfection of macrophages was also demonstrated when cells were allowed to differentiate for longer periods (15 instead of 7 days) in the absence of any cytokine (Fig. 2B). Under these conditions, a significant enhancement of the levels of luciferase expression was observed, as well as a decrease in the variability among cells, as compared to those obtained under other conditions (compare with Fig. 2A). Once again, a reasonable explanation for these results might be the increase in the number of transferrin receptors, resulting in a higher extent of internalization of the complexes. Another concurrent possibility is that, as a consequence of the higher and more uniform stage of maturation, more favorable conditions were found for the endosome-disrupting action of the peptide. This is suggested by the larger differences in the levels of transfection observed between ternary and quaternary complexes (absence or presence of GALA, respectively) as compared with results obtained in parallel experiments carried out with 7-day-old macrophages treated with hGM-CSF. In accordance with previous reports [12], we noted that luciferase gene expression was transient, a very low luciferase activity being observed 72 h after transfection (data not shown). This may be a reflection of the lack of integration of the plasmid into chromosomal DNA, as expected from non-viral vectors.

The ability of the different complexes to promote gene transfer was also evaluated by scoring the percentage of cells expressing Green Fluorescence Protein, defined as the transfection efficiency. The use of quaternary complexes resulted in an increase in the number of cells successfully transfected compared with controls, i.e. plain lipoplexes. It should be noted that the results described regarding the GFP assay were obtained under the experimental conditions that led to the highest levels of transfection activity, i.e., using macrophages differentiated for 15 days in the absence of hGM-CSF. Studies of transfection efficiency performed with 7-day-old macrophages, in the absence or presence of the cytokine, led to even lower numbers of cells successfully transfected. Although this information seems to suggest that there is a relationship between the number of cells transfected (transfection efficiency) and the overall levels of gene expression (transfection activity) obtained for the different experimental conditions, the low levels of gene expression usually observed hamper the establishment of an accurate (faithful) correlation between these two parameters. Although the low percentage of cells scored as expressing this plasmid (2%) was below our expectations, it should be noted that even lower levels of transfection efficiency have been described by Ferkol et al. [13] whose studies involved the use of mannosylated polylysine conjugates for the delivery of luciferase and β-galactosidase plasmids to macrophages, both *in vitro* and *in vivo*. The use of a modified version of the plasmid promoting not only higher expression but also brighter Green Fluorescence Protein (EGFP [38]) may enhance the sensitivity

of the assay and hence result in the detection of a larger percentage of transfected cells.

The preparation of the quaternary complexes involved the addition of transferrin and GALA to the liposomes followed by addition of DNA. For the different complexes tested, the 2:1 lipid/DNA charge ratio was the condition leading to the highest levels of luciferase expression. Because part of the positive charge of the lipid was involved in the interaction with the transferrin and the GALA peptide, both of which are net negatively charged compounds, an excess of cationic lipid seems to be required for an efficient interaction with the added DNA. Although the lipoplex itself at this lipid/DNA charge ratio is positively charged, zeta potential measurements revealed that the overall charge of the quaternary complexes was negative (Table 1). Curiously, this particular type of complex did not produce enhanced transfection of proliferating cells over that obtained with ternary complexes containing transferrin (unpublished data). On the other hand, quaternary complexes prepared with a 4:1 lipid/DNA charge ratio, exhibiting a positive zeta potential, did not show any enhancement of transfection of macrophages as compared to the 2:1 complexes (data not shown). These results suggest that the use of net negatively charged complexes may contribute to an enhancement of specificity of the targeting ligand because strong electrostatic interactions will not dominate the binding of the complex to the net negatively charged cell membrane. Previous results [15, 16] also suggested that an excess of positive charge may mask the ligand, thus decreasing the efficiency of the receptor-mediated process.

The presence of both transferrin and the GALA peptide led to the formation of net negatively charged complexes. Complexes with such characteristics are likely to alleviate some of the problems associated with the *in vivo* use of highly positively charged complexes, including the inability to efficiently transfect cells beyond the vascular endothelial cells and the avid complexation with serum or extracellular matrix proteins. The lower extent of interaction with the macromolecules in biological fluids may reinforce the target specificity and also avoid activation of the complement system, with consequent enhancement of the stability and circulation times of the complexes [39, 40].

Although it was expected that negatively charged complexes would not interact significantly with serum components, in our experiments the presence of 20% serum partially inhibited the transfection activity of the quaternary complexes. However, still higher levels of transfection were observed as compared to controls under the same conditions. The inhibitory effect of serum was essentially negligible when only transferrin was combined with the complexes. A possible explanation for these observations is the extraction of GALA from the complexes by serum components. Further studies are required to address this issue. It is interesting to note that when the ternary complexes (i.e., cationic liposomes-DNA-transferrin) were used for transfection of proliferating cells in previous studies, the presence of 10% FBS did not significantly inhibit transgene delivery and expression [15, 16].

The results presented here show clearly that the combined association of cationic liposomes with a targeting ligand and a fusogenic peptide is a valuable strategy to promote efficient *in vitro* gene transfer into macrophages. The quaternary complexes described are easy to prepare, and consequently to scale-up, are targeted to a cell surface receptor, provide sufficient protection of DNA (outside and inside the cells), and are non-cytotoxic. Furthermore, these complexes exhibit physicochemical features that may be highly advantageous for their use in intravenous gene delivery. Therefore, these systems represent the prototype of promising alternatives to the use of viral vectors for gene therapy based on the expression of therapeutic genes in macrophages.

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REFERENCES

1. Turpin, J. A., Lopez-Berestein, G. (1993) Differentiation, maturation, and activation of monocytes and macrophages: functional activity is controlled by a continuum of maturation. In *Mononuclear Phagocytes in Cell Biology* (G. Lopez-Berestein and J. Klostergaard, eds.), Boca Raton, FL: CRC Press, 71–99.
2. Ohashi, T., Boogs, S., Robbins, P., Bahnson, A., Patrene, K., Wei, F. S., Wei, J. F., Li, J., Lucht, L., Fey, Y., Clark, S., Kimak, M., He, H., Mowery-Rushton, P., Barranger, J. A. (1992) Efficient transfer and sustained high expression of the human glucocerebrosidase gene in mice and their functional macrophages following transplantation of bone marrow transduced by a retroviral vector. Proc. Natl. Acad. Sci. USA 89, 11332–11336.
3. Correll, P. H., Colilla, S., Dave, H. P., Karlsson, S. (1993) High levels of human glucocerebrosidase activity in macrophages of long term reconstituted mice after retroviral infection of hematopoietic stem cells. Blood 15, 332–336.
4. Freas, D. L., Correll, P. H., Dougherty, S. F., Karlsson, S., Pluznik, D. H. (1993) Evaluation of expression of transferred genes in differentiating myeloid cells: expression of human glucocerebrosidase in murine macrophages. Hum. Gene Ther. 4, 283–290.
5. Karlsson, S., Correll, P. H., Xu, L. (1993) Gene transfer and bone marrow transplantation with special reference to Gaucher's disease. Bone Marrow Transplant. 11 (Suppl. 1), 124–127.
6. Bartholeyns, J., Lopez, M. (1994) Immune control of neoplasia by adoptive transfer of macrophages: potentiality for antigen presentation and gene transfer. Anticancer Res. 14, 2673–2676.
7. Parrish, E. P., Cifuentes-Diaz, C., Li, Z. L, Vicart, P., Paulin, D., Dreyfus, P. A., Peschanski, M., Harris, A. J., Garcia, L. (1996) Targeting widespread site of damage in dystrophic muscle: engrafted macrophages as potential shuttles. Gene Ther. 3, 13–20.
8. Matsuda, S., Akagawa, K., Honda, M., Yokota, Y., Takebe, Y., Takebe, Y., Takemori, T. (1995) Supression of HIV replication in human monocyte-derived macrophages induced by granulocyte/macrophage colony-stimulating factor. AIDS Res. Hum. Retroviruses 11, 1031–1038.
9. Kohn, D. B., Sarver, N. (1996) Gene therapy for HIV-1 infections. Adv. Exp. Biol. Med. 395, 421–428.
10. Haddada, H., Lopez, M., Martinache, C., Ragot, T., Abina, M. A., Perricaudet, M. (1993) Efficient adenovirus-mediated gene transfer into human blood monocyte-derived macrophages. Biochem. Biophys. Res. Commun. 195, 1174–1183.
11. Xing, Z., Ohkawara, Y., Jordana, M., Graham, F. L., Gauldie, J. (1997) Adenoviral vector-mediated interleukin-10 expression in vivo: intramuscular gene transfer inhibits cytokine responses in endotoxemia. Gene Ther. 4, 140–149.

12. Erbacher, P., Bousser, M.-T., Raimond, J., Monsigny, M., Midoux, P., Roche, A. C. (1996) Gene transfer by DNA/glycosylated polylysine complexes into human blood monocyte-derived macrophages. Hum. Gene Ther. 7, 721–729.
13. Ferkol, T., Perales, J. C., Mularo, F., Hanson, R. W. (1996) Receptor-mediated gene transfer into macrophages. Proc. Natl. Acad. Sci. USA 93, 101–105.
14. Cheng, P. (1996) Receptor ligand-facilitated gene transfer: enhancement of liposome-mediated gene transfer and expression by transferrin. Hum. Gene Ther. 7, 275–282.
15. Simões, S., Slepushkin, V., Gaspar, R., Pedroso de Lima, M. C., Düzgüneş, N. (1997) Enhancement of cationic liposome-mediated gene delivery by transferrin and fusogenic peptides. Proc. Int. Symp. Control. Rel. Bioact. Mater. 24, 659–660.
16. Simões, S., Slepushkin, V., Gaspar, R., Pedroso de Lima, M. C., Düzgüneş, N. (1998) Gene delivery by negatively charged ternary complexes of DNA, cationic liposomes and transferrin or fusogenic peptides. Gene Ther. 5, 955–964.
17. Parente, R. A., Nir, S., Szoka, F. C., Jr. (1988) pH-dependent fusion of phosphatidylcholine small vesicles. J. Biol. Chem. 263, 4724–4730.
18. Wagner, E., Plank, C., Zatloukal, K., Cotten, M., Birnstiel, M. L. (1992) Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. Proc. Natl. Acad. Sci. USA 89, 7934–7938.
19. Metcalf, D. (1991) Control of granulocytes and macrophages: molecular, cellular, and clinical aspects. Science 254, 529–533.
20. Witsell, A. L, Schook, B. (1991) Macrophage heterogeneity occurs through a developmental mechanism. Proc. Natl. Acad. Sci. USA 88, 1963–1967.
21. Felgner, P. L., Barenholz, Y., Behr, J. P., Cheng, S. P., Cullis, P., Huang, L., Jessee, J. A., Seymour, L., Szoka, F., Thierry, A. R., Wagner, E., Wu, G. (1997) Nomenclature for synthetic gene delivery systems. Hum. Gene Ther. 8, 511–512.
22. Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., Danielsen, M. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA 84, 7413–7417.
23. Ciccarone, V., Hawley-Nelson, P., Jessee, J. (1993) Cationic liposome-mediated transfection: effect of serum on expression and efficiency. Focus 15, 80–83.
24. Li, S., Huang, L. (1996) Lipidic supramolecular assemblies for gene transfer. J. Liposome Res. 6, 589–608.
25. Malone, R. W., Felgner, P. L., Verma, I. M. (1989) Cationic liposome-mediated RNA transfection. Proc. Natl. Acad. Sci. USA 86, 6077–6081.
26. Konopka, K., Pretzer, E., Felgner, P. L., Düzgüneş, N. (1996) Human immunodeficiency virus type-1 (HIV-1) infection increases the sensitivity of macrophages and THP-1 cells to cytotoxity by cationic liposomes. Biochim. Biophys. Acta 1312, 186–196.
27. Simões, S., Slepushkin, V., Pretzer, E., Gaspar, R., Pedroso de Lima, M. C., Düzgüneş, N. (1997) Transfection of human monocyte-derived macrophages by transferrin-cationic lipid-DNA complexes. FASEB J. 11, A1090 (Abstract 1366).
28. Pretzer, E., Flasher, D., Düzgüneş, N. (1997) Inhibition of human immunodificiency virus type-1 replication in macrophages and H9 cells by free or liposome-encapsulated L-689,502, an inhibitor of the viral protease. Antiviral Res. 34, 1–15.
29. Subbarao, N. K., Parente, R. A., Szoka, F. C., Nadasdi, L., Pongracz, K. (1987) pH-dependent bilayer destabilization by an amphipathic peptide. Biochemistry 26, 2964–2972.
30. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., Prasher, D. C. (1994) Green fluorescent protein as a marker for gene expression. Science 263, 802–805.
31. Young, D., Lowe, L. D., Clark, S. C. (1990) Comparison of the effects of IL-3, granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor in supporting monocyte differentiation in culture. Analysis of macrophage antibody-dependent cellular cytotoxicity. J. Immunol. 145, 607–615.
32. Bennett, S., Por, S. B., Stanley, E. R., Breit, S. N. (1992) Monocyte proliferation in a cytokine-free, serum-free system. J. Immunol. Meth. 153, 201–212.
33. Rabinovitch, M. (1995) Professional and non-professional phagocytes: an introduction. Trends Cell Biol. 5, 85–88.
34. Parente, R. A., Nir, S., Szoka, F. C., Jr. (1990) Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. Biochemistry 29, 8720–8728.
35. Fattal, E., Nir, S., Parente, R. A., Szoka, F. C., Jr. (1994) Pore-forming peptides induce rapid phospholipid flip-flop in membranes. Biochemistry 33, 6721–6731.
36. Haensler, J., Szoka, F. C., Jr. (1993) Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. Bioconj. Chem. 4, 372–379.
37. Curiel, D. T., Agarwal, S., Wagner, E., Cotten, M. (1991) Adenovirus enhancement of transferrin-polylysine mediated gene delivery. Proc. Natl. Acad. Sci. USA 88, 8850–8854.
38. Zhang, G., Gurtu, V., Kain, R. (1996) An enhanced green fluorescence protein allows sensitive detection of gene transfer in mammalian cells. Biochem. Biophys. Res. Commun. 227, 707–721.
39. Plank, C., Mechtler, K., Szoka, F. C., Jr., Wagner, E. (1996) Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. Hum. Gene Ther. 7, 1437–1446.
40. Mahato, I. R., Rolland, A., Tomlinson, E. (1997) Cationic lipid-based gene delivery systems: pharmaceutical perspectives. Pharmaceut. Res. 14, 853–859.