PAMAM Dendrimers as Delivery Agents for Antisense Oligonucleotides

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Purpose. To investigate the potential use of PAMAM dendrimers for the delivery of antisense oligonucleotides into cells under conditions that mimic the *in vivo* environment.

Methods. We used HeLa cells stably transfected with plasmid pLuc/705 which has a luciferase gene interrupted by a human β-globin intron mutated at nucleotide 705, thus causing incorrect splicing. An antisense oligonucleotide overlapping the 705 splice site, when delivered effectively, corrects splicing and allows luciferase expression. The ability of dendrimers to deliver oligonucleotides to HeLa Luc/705 cells was evaluated in the absence or presence of serum.

Results. PAMAM dendrimers formed stable complexes with oligonucleotides that had modest cytotoxicity and showed substantial delivery activity. The dose of the oligonucleotide, the charge ratio of oligonucleotide to dendrimer, and the size (generation) of the dendrimers were all critical variables for the antisense effect. The physical properties of dendrimer/oligonucleotide complexes were further investigated using sedimentation and gel electrophoresis methods. Effective oligonucleotide/generation 5 dendrimer complexes were macromolecular rather than particulate in nature, and were not sedimented at 100,000 RPM. Compared to other types of delivery agents, PAMAM dendrimers were more effective in delivering oligonucleotides into the nucleus of cells in the presence of serum proteins.

Conclusions. Our results suggest that PAMAM dendrimers form nonparticulate delivery complexes that function in the presence of serum proteins and thus may be suited for *in vivo* therapeutic applications.

KEY WORDS: dendrimers; antisense; drug delivery; splicing; luciferase.

INTRODUCTION

One of the major hurdles in the antisense field is the inefficient delivery of oligonucleotides to their target sites in the cytoplasm and nucleus of cells (1). Over the past decade a variety of delivery agents have been introduced to enhance the delivery efficiency of plasmid DNA or antisense oligonucleotides at the cell culture level. This includes cationic lipid complexes (2,3), polypeptides (4–6), surfactants (7,8), liposomes (9–14) and other reagents (15–18). Unfortunately, most of these agents, including many commercially available cytofectins, are rather poor in delivering oligonucleotides to cells in the presence of serum, as compared to effects attained under serum-free cell culture conditions (19). Thus, these agents are not likely to be effective *in vivo*, where plasma proteins are abundant. In addition, in most cases, the complexes formed between nucleic acids and delivery agents are particulate in nature and are likely to be rapidly cleared from the circulation (1). Thus, poor cellular delivery of nucleic acids, including genes and oligonucleotides, remains a major obstacle to use of these agents in therapy (13,20).

In order to attain a pharmacological antisense effect, intravenously injected antisense agents need to go through a series of biological barriers before reaching their ultimate target sites in the cytoplasm or nucleus of a cell. These barriers include the capillary beds which act to sieve larger particles, the phagocytic cells of the reticuloendothelial system, the molecular level sieving characteristics of the capillary endothelium, the diffusion barrier of the extracellular matrix, and finally, the cell membrane itself (21–23). In light of these considerations, the ideal *in vivo* antisense oligonucleotide delivery agent should i) stably bind oligonucleotide, ii) be small enough (<30 KD) to allow exit from the bloodstream, and iii) promote penetration into cells (1,24).

Starburst polyamidoamine (PAMAM) dendrimers are spherical, highly ordered, dendritic polymers with positively charged primary amino groups on surface at physiolgical pH (25). Recent studies from this lab and others showed that PAMAM dendrimers form stable complexes with plasmid DNAs or oligonucleotides with limited cell toxicity, and are effective in gene transfer or oligonucleotide delivery at the cell culture level (7,26–31). However, currently little is known about the properties of dendrimer-nucleic acid complexes in the biological environment. In addition, the mechanism of dendrimer-mediated cellular uptake of oligonucleotides is not fully understood. It has been suggested for cationic lipid complexes that internalization through endocytosis is followed by oligonucleotide release from endosomes to the cytoplasm, and then rapid migration of the released oligonucleotides to the nucleus (27,32–36); the same type of mechanisms may apply to dendrimer-oligonucleotide complexes.

A hypothesis underlying this study is that dendrimers may be suitable for *in vivo* applications if they form relatively small complexes with oligonucleotides, and if they can act as delivery agents in the presence of the proteins found in blood. In the current study the physical properties of dendrimer/oligonucleotide complexes were investigated by using gel electrophoresis and sedimentation methods. The efficacy of dendrimer-mediated oligonucleotide delivery in serum-free or serum-replete conditions was evaluated by the use of a recently developed Luciferase assay system (37,38). Here we report that PAMAM dendrimers form stable complexes with oligonucleotides, and are moderately efficient in delivering oligonucleotide even in the presence of high concentrations of serum proteins. The pharmacologically active oligonucleotide/dendrimer complexes remain in solution after lengthy centrifugation at 100,000 RPM, suggesting that the complexes are macromolecules rather than large particles.

MATERIALS AND METHODS

Materials

Starburst polyamidoamine (PAMAM) dendrimers which have ethylenediamine as an initiator core were purchased from Dendritech (Dendritech Inc, MI) and used without further purification. LipofectAmine as well as DMEM and Opti-MEM, were from Life Technologies, Inc. (Gaithersburg, MD). SuperFect was obtained from Qiagen, Inc. (Valencia, CA). A

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phosphorothioate 2′-O-methyl-oligonucleotide (5′-CCUCUU-ACCUCAGUUACA-3′) directed against the beta-globin 705 splice site was purchased from the Midland Certified Reagent Company (Midland, Texas). HeLa cells stably transfected with plasmid pLuc/705 were a generous gift of Dr. R. Kole (37).

Cytotoxicity Assay

A cytotoxicity assay was performed by plating cells into 24-well plates (Nunc) at 2×10^4 cells per well. Cells were incubated with complexes of oligonucleotide (0.25 μM) and dendrimer, with oligonucleotide (0.25 μM), or with control medium, for 24 hours in serum free or 50% serum medium, rinsed twice with PBS, followed by the addition of 2 ml Opti-MEM (Gibco/BRL) and incubation for a further 24 hours. The surviving fraction was determined by the MTT dye assay; 50 μL of MTT dye solution (0.5 μg/ml) was added to each well and the plates returned to the 37°C incubator for 3 hr; after removing the Opti-MEM, 0.5 ml of DMSO was added to each well and incubation continued for another 30 min. Absorbance at 540 nm was quantitiated with an automated microplate reader (BioTech EL 340).

Antisense Splicing Correction Assay

HeLa cells transfected stably with a reporter gene construct were plated in 6-well trays at a density of 3×10^5 cells per well in 3 ml of 10% FBS/DMEM and antibiotics. Cells were maintained for 24 hr at 37°C in a humidified incubator (5% CO_2/95% air). A 100 μl aliquot of oligonucleotide at a given concentration in Opti-MEM was mixed with 100 μl of Opti-MEM containing various concentrations of PAMAM dendrimer. After being briefly mixed, the preparation was left undisturbed at room temperature for 5 min, followed by dilution to 1 ml with Opti-MEM, as Opti-MEM plus serum, before being layered on the cells. The cells were incubated for 6 hr and subsequently the medium was replaced with 10% FBS/DMEM. An additional 18 hr later, the cells were rinsed with phosphate-buffered saline (PBS) and lysed in 100 μl of lysis buffer (200 mM Tris-HCl, pH 7.8, 2 mM EDTA, 0.05% Triton X-100) on ice for 15 min. Following centrifugation (13000 rpm, 2 min), 5 μl of supernatant cell extract was mixed with 100 μl of luciferase assay buffer (3 mM ATP, 15 mM $MgSO_4$, 30 mM Tricine, 10 mM DTT, pH7.8) and 100 μl of luciferase substrate (1 mM D-luciferin). The light emission was quantified for 10 s using a Monolight®2010 luminometer (Analytical Luminescence Laboratory, USA). Luciferase activity was expressed as relative light units (RLU) per well. Light emission was normalized to the protein concentration of each sample, determined according to the bicinchonic acid assay (Pierce Chemical Co.). Relative concentrations of cation and oligonucleotide were treated as charge ratios (cationic polymer primary amines/oligonucleotide phosphates).

Preparation of Labeled Oligonucleotide

A single stranded 18 base 2′-O-methyl phosphorothioate oligonucleotide was labeled with [γ-^{32}P]-ATP using T4 Polynucleotide kinase (USB). Labeled oligonucleotide was purified from free [γ-^{32}P]-ATP by thin layer chromatography (SurePure kit, USB). The plate was exposed to the X-ray film for 1 hr before development. The band corresponding to the labeled oligonucleotide was scraped out from the TLC plate and resuspended with water in a microcon tube (Amicon, Inc.) before centrifuged at 13000 rpm for 20 min. The concentration of the labeled oligonucleotide was determined by using an UV spectrometer (BioSpec-1601, Shimadzu).

Gel Electrophoresis

Characterization of dendrimer/oligonucleotide complexes was conducted by 1% agarose gel electrophoresis. The complexes were prepared as above and a trace amount (≤pM) of ^{32}P phosphorothioate oligonucleotide was included. The prepared complex solution was mixed with dye marker in 60% glycerol prior to electrophoresis on a 1% agarose gel. Complexes were electrophoresed for 3 hr at 46V in 1x TBE buffer and the gel was dried en vacuo on a sheet of Whatmam 3 CHR chromatography paper and exposed to Kodak X-OMAT film at −80°C overnight.

Ultracentrifugation

A 150 μl aliquot of an oligonucleotide at a given concentration in Opti-MEM was mixed with 150 μl of Opti-MEM containing PAMAM dendrimer. After being briefly mixed, the preparation was left at room temperature for 10 min, followed by dilution to 1.5 ml with Opti-MEM. The complex solution was transferred into thickwall polyallomer tubes (Beckman) and ultracentrifuged for the appropriate time at 100,000 RPM (Optima Th, Beckman, Inc). One ml of the supernatant was carefully transferred into a new tube without disturbing the bottom portion, and the supernatant was used for transfection and luciferase assay as described above.

RESULTS

Cytotoxicity

The toxic effects of PAMAM generation 5 dendrimer or oligonucleotide/dendrimer complexes on cells were assessed by a MTT dye assay (39). Figure 1 shows percent cell survival

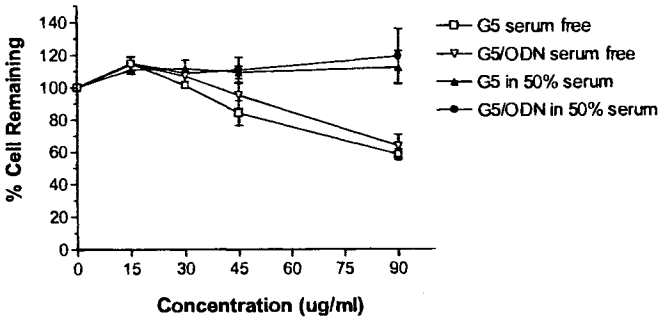


Fig. 1. Acute toxicity assay of PAMAM generation 5 dendrimer. Generation 5 dendrimer alone or dendrimer/oligonucleotide complexes were applied to cells with/without serum. Cell viability was expressed as percent cells remaining compared to untreated cells, based on the MTT assay. Open squares, Gen 5 alone in serum free medium; open inverted triangles, Gen 5/oligonucleotide complexes (1:35) in serum free medium; filled triangles, Gen 5 alone in 50% serum medium; filled circles, Gen 5/oligonucleotide in 50% serum medium. (n = 3 experiments per concentration).

versus concentration for generation 5 dendrimer, after treatment of cells in serum-free or 50% serum-containing medium. A marked decrease in the toxicity of dendrimer or dendrimer/oligonucleotide complexes was noted when cells were treated in the presence of serum, while higher toxicity was observed for dendrimer alone or the complexes in serum-free medium.

Delivery of Antisense and Correction of Splicing

The ability of PAMAM dendrimers to deliver oligonucleotides in pharmacologically active form was tested by a recently developed reporter assay that utilizes an antisense oligonucleotide to correct splicing of a mutated intron (37). This approach provides an excellent assay system for antisense activity and delivery, since only active oligonucleotide reaching nuclei of viable cells permits correct splicing, resulting in luciferase activity, which can then be used as a positive readout. In the absence of serum, oligonucleotide complexes with PAMAM dendrimer generation 5 showed only moderate delivery efficiency compared to the commercial cytofectin, Lipofectamine; increased delivery was observed as the dendrimer concentration increased (Fig. 2A). Interestingly, the dendrimer was effective even in the presence of substantial amounts of serum, unlike the case with Lipofectamine (Fig. 2B). Even in 70% serum, an oligonucleotide / dendrimer complex at a charge ratio of 1:35 resulted in modest levels of luciferase expression.

Serum Effects on Oligonucleotide Delivery by Dendrimers

The serum dependence of oligonucleotide delivery by various dendrimers was compared at a fixed charge ratio of 1:35 (oligonucleotide/dendrimer) (Fig. 3). The commercial dendrimer Superfect showed excellent activity in the absence of serum, but in relative terms dramatically lost activity at high serum concentrations. Lipofectamine, a cationic lipid cytofectin, displayed almost no activity in the presence of serum. A generation 4 dendrimer was relatively ineffective under all circumstances. Generation 5 and 7 dendrimers showed moderate activity (compared to Lipofectamine or Superfect) in serum-free medium, but maintained a higher relative degree of effectiveness in the presence of high concentrations of serum. For example, the ratio of luciferase expression between 50% serum and serum-free was approximately 0.03 for Lipofectamine, 0.09 for Superfect and 0.28 for generation 5 dendrimer. The difference between the actual levels of luciferase induced using generation 5 dendrimers and that induced using Lipofectamine was significant at the 0.05 level. Although Superfect showed a substantial loss in relative potency, the actual levels of luciferase attained by treatments using generation 5 or 7 dendrimers or Superfect were not statistically distinct.

Effects of Oligonucleotide Concentration and of Charge Ratio

In further studies we focused on delivery effects by generation 5 dendrimer. Figure 4 illustrates a dose response relationship of luciferase activity versus oligonucleotide concentration at a constant charge ratio (terminal amines/nucleotides) of 30/1 in serum-free or 30% serum medium. Luciferase activity increased progressively with oligonucleotide concentration both in serum-free and 30% serum medium, although luciferase

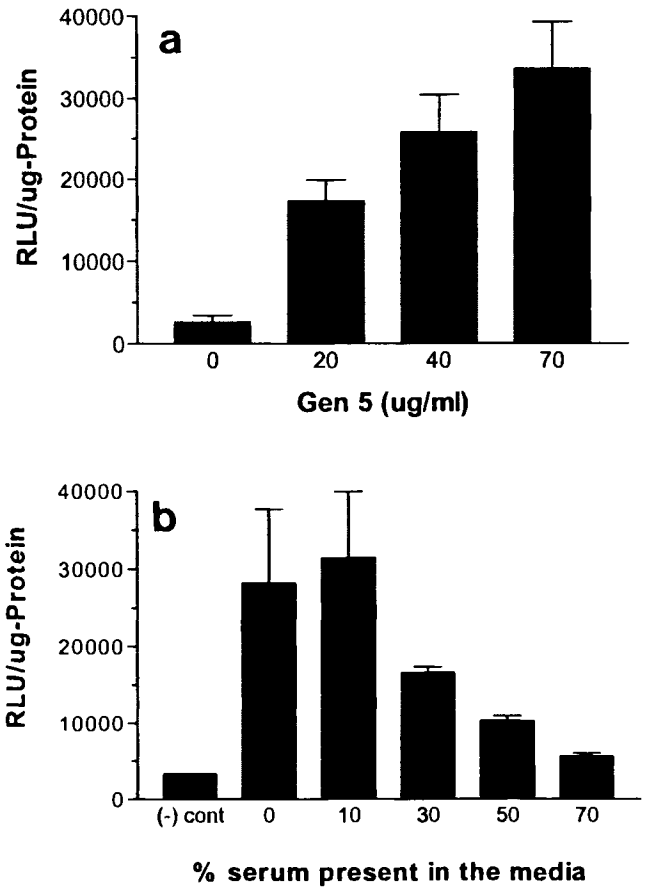


Fig. 2. Activation of a luciferase reporter using dendrimer/antisense complexes. HeLa cells stably transfected with pLuc/705 were treated with various concentrations of generation 5 dendrimer at fixed concentration (0.25 μM) of a 2′-O-Me phosporothioate oligonucleotide complementary to the β-Globin splice-site junction. (a) Luciferase expression at various concentrations of dendrimer generation 5. Numbers in abscissa also represent charge ratio of dendrimer to oligonucleotide. (b) Luciferase expression at a fixed charge ratio (1:35) of oligonucleotide to dendrimer; treatment of cells in the presence of various concentrations of serum. The activity of luciferase was measured as described in Methods and normalized on total cellular protein and is presented in relative luminescence units (RLU)/μg of protein. Vertical bars indicate means and standard errors (n = 3).

expression levels in 30% serum were lower than under serum-free conditions. Experiments were also performed at 30:1 and 60:1 charge ratios (Fig. 5). Use of the higher charge ratio resulted in increased luciferase activity especially at higher serum concentration. At the higher serum concentrations, results with the 60:1 generation 5 dendrimer/oligonucleotide complex were substantially better than those attained with Lipofectamine, which was not statistically different from untreated controls when used at 10% serum or above.

Physical Characterization of the Complexes

An evaluation of the approximate size of the dendrimer/oligonucleotide complexes was performed by centrifugation of the complexes under various conditions and then testing supernatants for the ability to induce luciferase expression. Figure 6 illustrates that centrifugation at increasing speed up

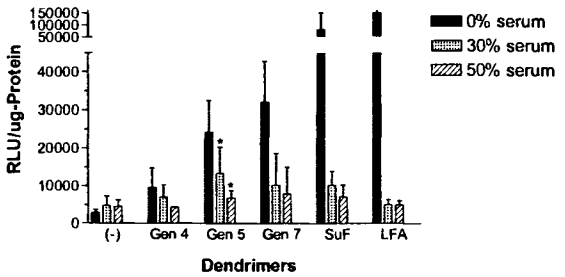


Fig. 3. Serum effects on oligonucleotide delivery by dendrimers. Serum dependence of oligonucleotide delivery by different generation dendrimers was compared. The complexes of ON705 and dendrimer were formed at constant charge ratio (1:30). Additional control samples underwent the same oligonucleotide treatment, but in the absence of delivery agent. The concentration of oligonucleotide was 0.25 μM. Ordinate:relative luminescence units (RLU)/μg of protein. LFA: Lipofectamine, SuF: SuperFect. *$p < 0.05$ versus Lipofectamine at each serum percent. ($n > 3$).

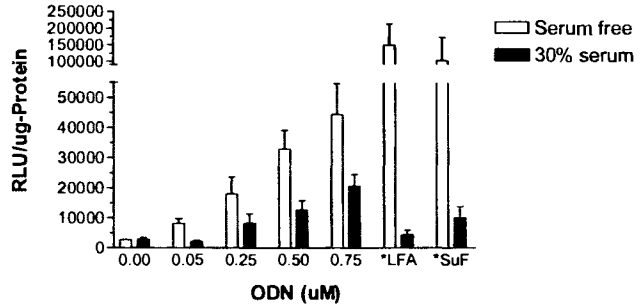


Fig. 4. Antisense dose-response at constant charge ratio (1:35) in serum free medium or 30% serum. The abscissa indicates the percent of fetal calf serum present during the incubation with oligonucleotides. Ordinate: relative luminescence units (RLU)/μg of protein. Vertical bars represent means and standard errors ($n = 3$). LFA: Lipofectamine, SuF: SuperFect (both with 0.25 μM oligonucleotide).

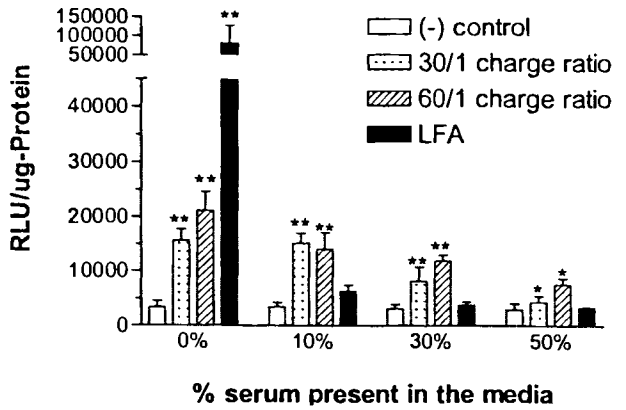
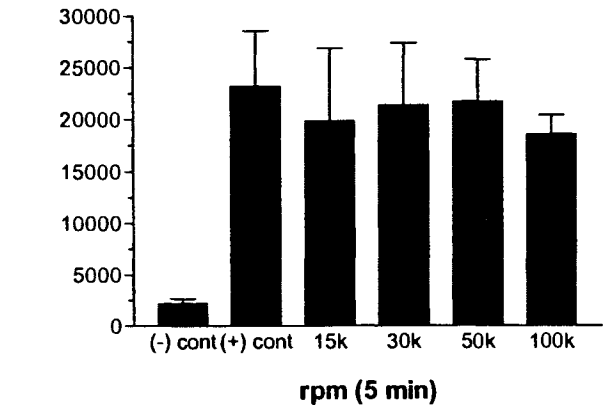


Fig. 5. Effect of charge ratio on dendrimer delivery of oligonucleotides in the presence of serum. Luciferase expression was compared at two different charge ratios, 30/1 and 60/1. Cells treated with Lipofectamine according to the manufacturer's instructions were used as a positive control. The concentration of oligonucleotide was 0.25 μM. Ordinate: relative luminescence units (RLU)/μg of protein. *$p < 0.05$ and **$p < 0.01$ versus untreated control at each serum percent. ($n > 3$).



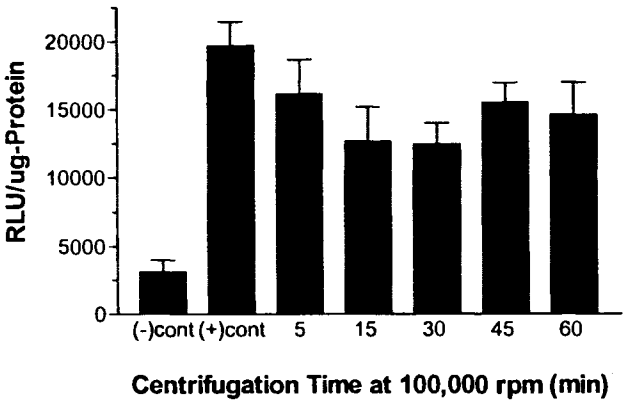


Fig. 6. Effect of centrifugation on the pharmacological activity of dendrimer/oligonucleotide complexes. Complexes prepared as described in Methods were transferred into thickwall polyallomer tube (Backman), ultracentrifuged for 5 min at the indicated speed or for various time at 100K rpm, 21°C. The supernatant solution was compared with the uncentrifuged total sample for the ability to induce luciferase expression. Ordinate: relative luminescence units (RLU)/μg of protein. ($n = 3$).

to 100,000 rpm, as well as increasing the time of centrifugation, failed to deplete the supernatant of pharmacological activity. Thus, the oligonucleotide complexes that induce luciferase expression fit a standard biochemical criterion for being soluble molecules rather than sedimentable particles.

Agarose gel electrophoresis was performed to further evaluate the size and/or charge characteristics of the complexes (Fig. 7). The oligonucleotide/dendrimer complexes formed at charge ratio 35:1 in serum free medium remained in the well, while at 1:60 charge ratio the complexes migrated toward the cathode, indicating the formation of positively charged entities. Failure of the 35:1 complex to migrate from the well indicates either that the complex is too large to penetrate the pores of the gel or that it is uncharged (or both). When complexes were electrophoresed after exposure to serum, dramatic alterations in the gel pattern were observed. There was a decrease in the material remaining in the well, and the appearance of broad bands that migrated significantly toward the anode, indicating negative charge and a sufficiently small size to enter the gel. Some of this material probably represents binding between the radiolabled oligonucleotide and serum proteins, while tripartite

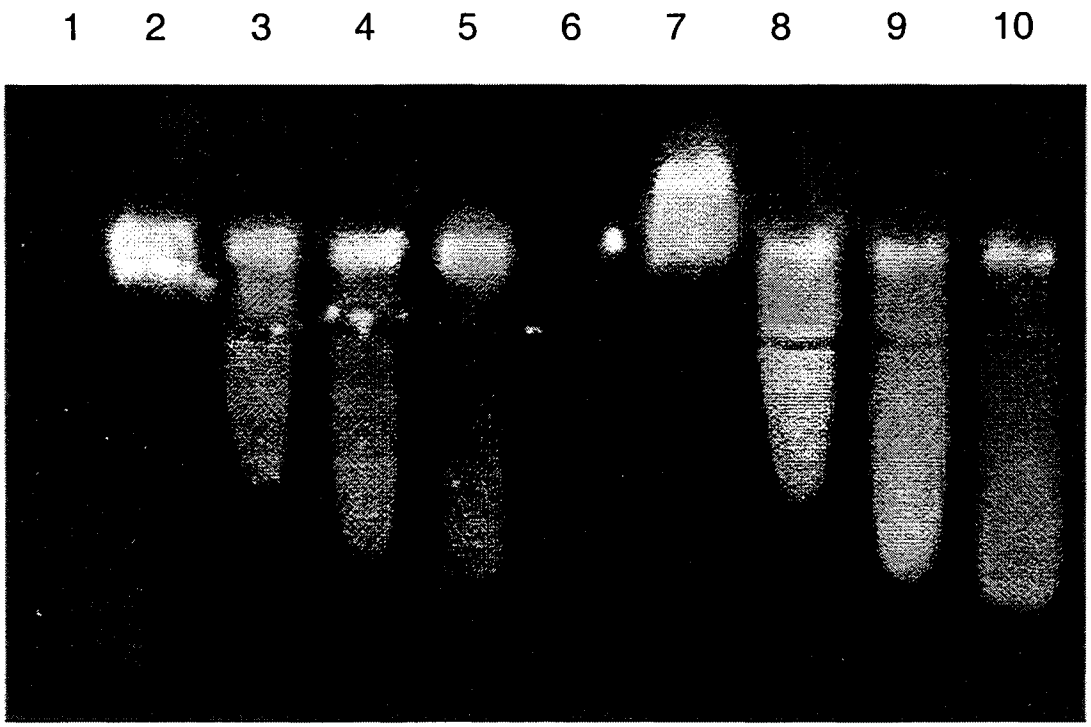


Fig. 7. Gel electrophoresis of oligonucleotide/generation 5 dendrimer complexes. Complexes of antisense oligonucleotide (0.25 μM) and dendrimer generation 5 formed at 1:35 and 1:60 charge ratio were electrophoresed in an 1% agarose gel after exposure to various percentages of serum. Tracer quantities of ^{32}P-oligo were used to visualize bands in the autoradiogram. Lane 1: oligonucleotide alone in serum free medium; lane 2–5: complexes formed at charge ratio 35:1 in 0%, 10%, 30%, 50% serum; lane 6: oligonucleotide alone in 50% serum; lane 7–10: complexes formed at charge ratio 60:1 in 0%, 10%, 30%, 50% serum.

complexes between oligonucleotide, dendrimer, and proteins are also possible.

DISCUSSION

The main goal of this research was to evaluate the potential use of PAMAM dendrimers as *in vivo* delivery agents for antisense oligonucleotides. We were particularly interested in lower generation dendrimers since they have relatively small molecular masses, and since preliminary evidence suggested (27) that they may form relatively small complexes with oligonucleotides. The delivery efficiency of dendrimers was evaluated using a rapid and sensitive luciferase reporter assay system, involving correction by antisense of an aberrant splicing reaction (37).

Of the dendrimers tested in this study, the generation 5 and 7 species seemed most promising. For example, generation 5 dendrimer displayed substantial activity for the delivery of oligonucleotides in serum-free medium, had moderate activity under standard cell culture conditions (10% serum), and maintained some activity even in the presence of 70% serum. The concentration of oligonucleotide, the charge ratio of oligonucleotide to dendrimer, and the generation of the dendrimer all influenced the antisense delivery effect. The pharmacologically active complexes formed by generation 5 dendrimer with oligonucleotides were molecular rather than particulate in nature, since high speed centrfugation failed to reduce activity. This contrasts with the situation with commercially available cationic lipid delivery agents, which form large particulate complexes with plasmids or oligonucleotides (13,40). The generation 7 dendrimers as well as Superfect, a dendrimer-type cytofectin, also were able to attain delivery effects in serum-replete medium. By contrast, cationic lipid complexes such as Lipofectamine did not.

Relatively small oligonucleotide/dendrimer complexes may provide many advantages for *in vivo* applications such as: i) extending circulation life time, ii) improving access to disease sites such as tumors or sites of inflammation, iii) increasing effective concentration at target site. This, coupled with the observation that the dendrimer/oligonucleotide complexes maintain activity in serum make these agents attractive candidates for further development in the context of *in vivo* application of antisense oligonucleotides.

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