Cationic liposome-encapsulated antisense oligonucleotide mediates efficient killing of intracellular *Leishmania*

Rila CHAKRABORTY*, Dalia DASGUPTA*, Samit ADHYA† and Mukul K. BASU*[1]

*Division of Biomembrane and †Genetic Engineering, Indian Institute of Chemical Biology, 4 Raja S.C. Mullick Road, Calcutta 700 032, India

Antisense oligonucleotides have been considered as inhibitors of growth of intracellular parasites such as *Leishmania*, but only limited inhibition has been observed *in vitro*. We have encapsulated an antisense oligonucleotide, complementary to the *Leishmania* universal miniexon sequence, in cationic liposomes. Low concentrations (4 μM) of encapsulated oligonucleotides specifically reduced the amastigote burden within cultured macrophages by 80 %. This result illustrates the importance of effective delivery for efficient antiparasitic activity of antisense oligonucleotides.

Key words: antisense oligonucleotides, cationic liposomes, *Leishmania donovani*.

INTRODUCTION

Leishmania donovani is a parasitic protozoan having two developmentally distinct stages. Amastigotes are non-motile forms that live within mammalian host macrophages, whereas promastigotes are motile, flagellated forms that develop from amastigotes after ingestion by sandflies [1]. The success of these parasites as intramacrophage pathogens is dependent on their ability to evade host defence mechanisms. Conversely, their degradation inside the intracellular compartment depends on targeting of antileishmanial drugs into the phagocytic vacuole [2].

Antimonials, which are toxic, have remained the mainstay for the treatment of leishmaniasis, and second-line drugs are too toxic to be used as first-line therapy on a large scale [3]. Thus keeping in view the present clinical scenario, it is highly desirable to identify new drugs and efficient delivery systems to combat leishmaniasis [4].

Antisense oligonucleotides, because of their low toxicity and high specificity for kinetoplastid mRNA, could be used as potential drugs against leishmaniasis. An attractive target for antisense inhibition in *Leishmania* and other kinetoplastid protozoa is the 39-nucleotide miniexon sequence universally present at the 5′-ends of cytoplasmic mRNA [1,5]. The miniexon is transferred to the mRNA from a precursor RNA (miniexon-derived RNA or medRNA) by the process of *trans*-splicing. The universal occurrence of the miniexon in cellular mRNAs, and its absence from the host system, make it an effective target for antisense inhibition through inhibition of mRNA translation or degradation of mRNA by cleavage of the hybrid by cellular ribonuclease H. Oligonucleotides complementary to the miniexon sequence of mRNA of *L. amazonensis* were shown to inhibit the amastigotes in infected macrophages, but only to a limited extent [6]. These results indicate the feasibility of the antisense approach through the use of appropriate antisense sequence and effective carrier systems.

The use of liposomes as a drug delivery system is well documented [7]. Cationic liposomes have been used previously to transfer DNA into cells through fusion with the cell membrane [8,9]. In keeping with this idea, we used positively-charged liposomes for efficient cellular delivery of antisense oligonucleotides into *Leishmania*-infected macrophages and investigated the leishmanicidal effect of the antisense-oligonucleotide-encapsulated liposomes.

MATERIALS AND METHODS

Dipalmitoylphosphatidylethanolamine and cetyltrimethyl ammonium bromide were purchased from Sigma. RPMI-1640 and heat-inactivated fetal-bovine serum were obtained from Difco; penicillin, streptomycin, gentamycin, M199 were from Gibco Laboratories. Chloroform and methanol were from Merck. All other reagents were of analytical grade.

Oligonucleotides

An antisense 20-mer (5′-AAACTGATACTTATATAGCG-3′) and its complementary sense oligonucleotide targeted to a part of the miniexon sequence [5], universally present at the 5′-ends of cytoplasmic mRNAs of *L. donovani*, were purchased from Bangalore Genei (Pvt) Ltd., Peenya, India.

Preparation of cationic liposomes

Cationic liposomes were prepared with dipalmitoylphosphatidylethanolamine and the cationic detergent cetyltrimethyl ammonium bromide in a molar ratio of 4:1 [8]. The lipids were dissolved in chloroform/methanol mixture (2:1, v/v). A thin, dry film of these lipids was made on the surface of a round-bottomed flask by evaporating the organic solvent. The dry film of these lipids (13.4 mg) was dispersed in 1.5 ml of PBS and sonicated for 40 min (2 min pulse, followed by 3 min resting). The liposome suspension was centrifuged for 1 h at 100000 ***g***. The washed pellet was suspended in sterile 1.5 ml RPMI-1640, passed through an extruder (0.22 μm) and collected aseptically.

Purification of crude oligonucleotides

The purification mixture contained 1 μl of oligonucleotides (5 pmol), 2.5 μl of sterile water, 0.5 μl of 10 × kinase buffer (1 × kinase buffer = 70 mM Tris/HCl, pH 7.5/10 mM $MgCl_2$/5 mM dithiothreitol), 0.5 μl of [γ^{32}P]ATP (3000 Ci/mmol) and 0.5 μl of T4 polynucleotide kinase (10 units/ml) [10]. The mixture was then incubated for 45 min at 37 °C. To the kinase-treated oligonucleotide, 50 μg of crude oligonucleotide was added. The

[1] To whom correspondence should be addressed (e-mail iichbio@giascl01.vsnl.net.in).

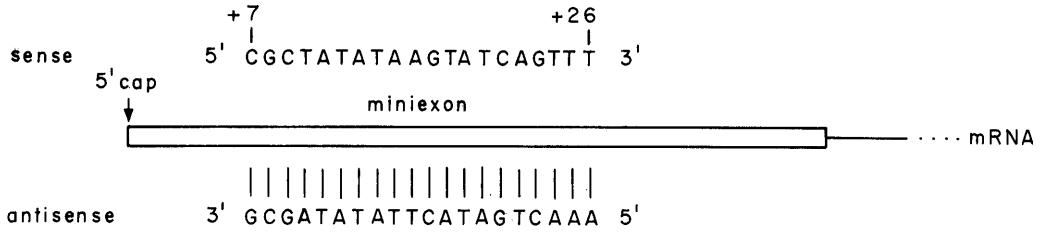

Figure 1 Oligonucleotide targeting strategy

The 39-nt universal miniexon at the 5′-end of *Leishmania* cytoplasmic mRNA is depicted as a box joined to the body of the mRNA (line). The sequences of antisense and sense oligonucleotides used for targeting are shown. Vertical lines indicate hybridization of antisense to the miniexon target.

mixture was then subjected to PAGE on a 15% denaturing polyacrylamide gel. After autoradiography, the major band was excised and eluted in 0.5 M sodium acetate at 37 °C overnight. Three volumes of ethanol were added to the eluate and the mixture was kept at −20 °C for 1 h, then centrifuged at 10000 ***g*** for 30 min at 4 °C. The pellet was finally suspended in 10 mM Tris/HCl/1 mM EDTA, pH 7.5.

γ-^{32}P-labelling of purified oligonucleotides

For labelling of oligonucleotides, the reaction mixture (10 μl) contained [γ-^{32}P]ATP (20 μCi), 10 × kinase buffer, oligonucleotides (20 pmol), T4 polynucleotide kinase (10 units/μl) and 40 μl of sterile water [10]. The mixture was incubated at 37 °C for 30 min and 40 μl of sterile water was added. To quantify the labelling, 1 μl of the reaction mixture was spotted on to DEAE (diethylaminoethyl) filter paper (Whatman DE81), air dried, washed with PBS and dried at 37 °C. Radioactivity was estimated by scintillation spectroscopy using a β-counter. The radioactivity incorporated was used to calculate the specific activity (c.p.m./pmol) of the oligonucleotide. Labelled oligonucleotides were precipitated with ethanol as described above.

Encapsulation of labelled oligonucleotide into liposomes and assessment of encapsulation

Cationic liposomes were prepared as described above, except that labelled oligonucleotides were added during swelling of the lipid film with PBS. The mixture was then sonicated and centrifuged at 100000 ***g***. Radioactivity present in the washed pellet was then measured. To calculate the percentage encapsulation, the liposomes were treated with pancreatic DNase I (50 unit/ml) to remove the surface-associated radioactivity. The total amount of encapsulated and surface-associated oligonucleotide was found to be 30%, whereas 10% of oligonucleotide was encapsulated.

Leishmania strain

L. donovani AG83 (MHOM/IN/1983/AG83) pathogenic strain was maintained in susceptible Balb/c mice. Before use, heavily infected spleen from 3-month-old mice was minced and added to Culture Medium 100 supplemented with 20% (v/v) heat-inactivated fetal-bovine serum, 0.15 M Hepes, 100 μg streptomycin/ml and 100 μg penicillin/ml. The culture was kept for 5 days at 22 °C, when the transformed promastigotes of AG83 emerged. The preparation was purified by culture for 2 further weeks.

Macrophage culture and *Leishmania* infection

Thioglycollate-elicited peritoneal macrophages from Swiss mice were collected into PBS and allowed to adhere to glass coverslips (10^5 macrophages/coverslip) for 2 h in RPMI medium, They were then washed and infected at a multiplicity of 20 promastigotes/macrophage for 2 h at 37 °C. After incubation, cells were washed to remove unattached promastigotes.

Treatment of infected macrophages with oligonucleotides

Infected macrophages were incubated at 37 °C with free or liposome-incorporated oligonucleotide at the indicated concentration for 24 h in RPMI medium containing 10% (v/v) fetal-bovine serum. The cultures were washed in 1 ml of PBS and placed in an equal volume of RPMI medium without oligonucleotide for 18 h. Cells were washed, fixed in methanol and stained with Giemsa. For the assessment of infection, at least 500 macrophages were scored by phase-contrast microscopy.

RESULTS

Leishmania-infected macrophages were treated with free or liposome-encapsulated 20-mer antisense oligonucleotide complementary to nucleotides +26 to +7 of the universal miniexon (Figure 1). In control experiments, the corresponding sense oligonucleotide was used; this would not hybridize to mRNA and therefore was not expected to have any effect. After treatment with oligonucleotide, infected macrophages were incubated in oligonucleotide-free medium and the number of internalized parasites was counted.

As shown in Table 1, control sense oligonucleotide had no effect on macrophage-internalized parasites up to 4 μM, ruling out non-specific toxicity. Free antisense oligonucleotide inhibited parasite load up to a maximum of 25%. By contrast, liposome-encapsulated antisense oligonucleotide showed a dose-dependent suppression of parasite load up to 78% at 4 μM. Empty liposomes had a minimal (adjuvant) effect. Free or liposomal oligonucleotides had no effect on the viability of macrophages under these conditions (results not shown). These results indicate that: (1) the effect of antisense oligonucleotide is sequence-specific and (2) encapsulation of oligonucleotide into liposome increases effectiveness by a factor of at least four.

The efficacy of liposomal antisense oligonucleotide was compared with that of established antileishmanial drugs (Table 1). Free pentamidine isethionate, at the optimal concentration of 84 μM, suppressed *Leishmania* growth within macrophages by 50% and encapsulation within liposomes increased the efficacy only marginally. A similar effect was observed for an optimal

Table 1 Leishmanicidal effect of free and liposome-encapsulated oligonucleotides

Peritoneal macrophages attached to glass coverslips (10^5 macrophages/coverslip) were infected at a macrophage to parasite ratio of 1:20 for 2 h at 37 °C. Infected macrophages were then incubated at 37 °C with free or liposomal oligonucleotides at the indicated concentrations for 24 h. The cells were incubated without oligonucleotide for 18 h, stained and the number of internalized parasites wewre counted. Similar experiments were done with other antileishmanial drugs, for comparison, at the optimal concentration. Results are shown as means ± S.D. ($n = 3$). ODN, oligonucleotide.

Treatment	Number of internalized parasites/100 macrophages	Suppression (%)
Untreated control	220 ± 15	–
Sense ODN (1 μM)	209 ± 10	5
Free antisense ODN (1 μM)	184 ± 10	16
Liposomal antisense ODN (1 μM)	130 ± 7	41
Untreated control	390 ± 12	–
Sense ODN (2 μM)	366 ± 10	6
Free antisense ODN (2 μM)	310 ± 20	20
Liposomal antisense ODN (2 μM)	143 ± 17	63
Untreated control	395 ± 10	–
Sense ODN (4 μM)	371 ± 10	6
Free antisense ODN (4 μM)	295 ± 25	25
Liposomal antisense ODN (4 μM)	90 ± 5	78
Untreated control	300 ± 23	–
Empty liposomes	270 ± 7	10
Untreated control	350 ± 10	–
Free primaquine (160 μM)	130 ± 10	62
Liposomal primaquine (160 μM)	85 ± 15	76
Free pentamidine isethionate (84 μM)	172 ± 12	50
Liposomal pentamidine isethionate (84 μM)	133 ± 3	62

concentration of primaquine, which has been shown to have antileishmanial activity *in vivo* [3,4]. The marginal effect of liposomal entrapment of these drugs *in vitro*, in contrast to that of oligonucleotide, is probably due to their higher stability and/or penetration.

DISCUSSION

The use of antisense oligonucleotides as therapeutic agents has been reported previously [11,12]. Receptor-mediated delivery of antisense oligonucleotides to specific cell types [13] using liposomes as a mode of delivery has also been known [14,15]. In the present study, the experiments performed *in vitro* confirm that liposome-encapsulated antisense oligonucleotides targeted to the miniexon sequence, universally present at the 5′-end of cytoplasmic mRNAs of *Leishmania*, can inhibit parasite growth or survival within macrophages. The corresponding free oligonucleotides, at all concentrations, have a much lower leishmanicidal effect. This may be due to their sensitivity to cellular nucleases [16,17]. Mole for mole, liposomal antisense oligonucleotide was much more efficient at reducing parasite load than the conventional antileishmanial drugs pentamidine or primaquine.

The increased leishmanicidal activity of liposome-encapsulated oligonucleotides might be due to the fact that cationic liposomes form a polyelectrolyte complex with the oligonucleotides, thus protecting them from nuclease degradation, enhancing their cellular uptake and improving their potency [18].

A probable mechanism for the delivery of antisense oligonucleotide by the liposomes is predominantly through the endocytic pathway [18]. First, the liposome-entrapped oligonucleotides are internalized via an endosome, which then fuses with the phagolysosome. The liposome disintegrates and releases the oligonucleotides, which then enter the parasites present in the phagosomes, probably through the flagellar pocket.

Viral vectors as gene-transfer vehicles have gained prominence because of intense research in this area [19]. The clinical efficacy, technical complexity and high cost of viral-based gene therapy has led to the development of non-viral methods. Since positively-charged liposomes or cationic liposomes can entrap negatively charged DNA, these liposomes have been used for oligonucleotide transfer [20,21]. Two potential applications of cationic lipids are the aerosol delivery of genes to the lung and in the development of cancer vaccines.

It has been demonstrated by others previously that *Plasmodium* [22], *Trypanosoma* [23] and *Leishmania* [6] are sensitive to oligonucleotides. Our results not only support the observation of Ramazeilles et al. [6] but also show the superiority of cationic liposomal oligonucleotides towards the killing of these parasites, even in the absence of specific targeting. Cheaper phosphodiester oligonucleotides encapsulated in liposomes are nearly twice as effective at half the concentration (Table 1) than the more expensive phosphorothioate oligonucleotides administered in the free form [6]. Recently, a significant leishmanicidal effect was observed using phosphorothioate oligonucleotides encapsulated in maleylated albumin-coated liposomes [24]. Therefore, liposome-entrapped oligonucleotides could serve as a better tool and a prototype of therapeutic agents against protozoan parasites.

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