The role of CpG ODN in enhancement of immune response and protection in BALB/c mice immunized with recombinant major surface glycoprotein of *Leishmania* (rgp63) encapsulated in cationic liposome

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Abstract

CpG oligodeoxynucleotides (CpG ODN) are known to be a potent immunoadjuvant for a wide range of antigens. The aim of this study was to evaluate the role of CpG ODN co-encapsulated with rgp63 antigen in cationic liposomes (Lip-rgp63-CpG ODN) in immune response enhancement and protection in BALB/c mice against leishmaniasis. Lip-rgp63-CpG ODN prepared by using dehydration–rehydration vesicle (DRV) method significantly inhibited (*P* < 0.001) *Leishmania major* infection in mice measured by footpad swelling compared to Lip-rgp63, rgp63 alone, rgp63 plus CpG ODN, PBS or control liposomes. The mice immunized with Lip-rgp63-CpG ODN also showed the lowest spleen parasite burden, highest IgG2a/IgG1 ratio and IFN-γ production and the lowest IL-4 production compared to the other groups. The results indicate that co-encapsulation of CpG ODN in liposomes improves the immunogenicity of *Leishmania* antigen.

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1. Introduction

Leishmaniasis, caused by different species of *Leishmania*, is a major health problem in some foci of 88 endemic countries [1]. Recovery and protection against further leishmaniasis infection mainly depends on induction of a Th1 type of immune response [2,3]. In susceptible BALB/c mice, immunization with *Leishmania* antigens mixed with IL-12 induced a Th1 response and protected against challenge with virulent *Leishmania major* [4].

A zinc-metalloprotease membrane glycoprotein of *Leishmania* species (gp63, MWt 63,000) is expressed in promastigotes of different species of *Leishmania* but in both promastigotes and amastigotes life stages of *L. major* and *L. mexicana* [5–7]. The gp63 acts as a receptor to mediate uptake of promastigotes by host macrophages [8]. Recombinant gp63 (rgp63) lacks the sugar molecules and has the molecular weight range of 54–58 kDa [9]. Recombinant gp63 induces a protective immune response when administered with an appropriate immunoadjuvant [10].

Liposomes, bilayer vesicles encapsulating aqueous contents, are utilized as delivery systems for drugs, peptides,
proteins and DNA. Liposomes are used as immunoadjuvants in inducing immune responses to various antigens and can be designed to preferentially induce humoral or cellular immune responses [11,12]. Liposomes are safe and well-tolerated in clinical trials, and some formulations are approved by the FDA [13].

Unmethylated CpG motifs, present at high frequency in bacterial but not vertebrate DNA, are recognized by Toll-like receptor 9 expressed by B cells and plasmacytoid dendritic cells (pDCs) [14]. The interaction of Toll-like receptor 9 with CpG motifs triggers an immune cascade, resulting in improved Ag uptake and presentation by APCs and antibodies, chemokines, and cytokines secretion by B cells, NK cells, DCs, and monocytes [14,15]. Synthetic CpG ODN mimic the immunostimulatory activity of bacterial DNA. These activities enable CpG ODN to act as an immune adjuvant, accelerating and boosting antigen-specific immune responses by 5–500-fold which might be due to maintaining close physical contact between the CpG ODN and the immunogen [14,15]. Considering the difficulties in chemical conjugation and the possibility of altering the three-dimensional structure of the antigen and thus its immunogenicity, it is of particular interest to explore other means of CpG ODN-antigen association [16]. As a co-delivery vehicle, liposomes provide close association of CpG ODN to antigen and may enhance the immune response. In this study, the extent of protection and type of immune response generated in susceptible BALB/c mice immunized with rgp63 co-administered with CpG ODN and the possibility of altering the three-dimensional structure of the antigen and thus its immunogenicity, it is of particular interest to explore other means of CpG ODN-antigen association [16]. As a co-delivery vehicle, liposomes provide close association of CpG ODN to antigen and may enhance the immune response. In this study, the extent of protection and type of immune response generated in susceptible BALB/c mice immunized with rgp63 co-administered with CpG ODN in cationic liposomes were assessed.

2. Materials and methods

2.1. Animals, parasites, SLA and oligodeoxynucleotide

Female BALB/c mice 6–8 weeks old were purchased from Pasteur Institute (Tehran, Iran). The mice were maintained in animal house of Biotechnology Research Center and fed with Pasteur Institute (Tehran, Iran). Animals were housed in a colony room at 21°C and had free access to water and food. Animal experiments were carried out according to Mashhad University of Medical Sciences, Ethical Committee Acts.

The L. major strain (MRHO/IR/75/ER) used in this experiment is the one which has been used for preparation of experimental Leishmania vaccine and leishmanin [17].

Soluble Leishmania antigen (SLA) was prepared from promastigotes of L. major harvested at log phase [18]. The protein concentration was determined using Lowry protein assay method [19]. The prepared antigen was stored in small aliquots at −70°C until use.

The CpG-ODN (Microsynth, Balgach, Switzerland) used in this study was a 20-mer termed 1826 (5′-TCC ATG ACG TTC CTG ACG TT-3′) with a nuclease-resistant phosphorothioate backbone, which contains two CpG motifs with known immunostimulatory effects on murine immune response [20,21].

2.2. Expression, isolation and purification of rgp63

Recombinant gp63 was expressed in E. coli BL21 (DE3) [22] and purified as described previously [23]. Briefly, cells were harvested by centrifugation and disrupted using lysis buffer; the supernatant was then purified using DEAE Sepharose Fast Flow column (16/100 mm) followed by gel filtration using Superdex 75 prep (Pharmacia LKB, Wien, Austria). Protein concentration of solubilized rgp63 was determined by Lowry protein assay [19]. The purity of rgp63 was confirmed by SDS-PAGE.

2.3. Encapsulation of rgp63 and CpG ODN in the liposomes

Liposomes containing rgp63 and CpG ODN (Lip-rgp63-CpG ODN) were prepared by the dehydration–rehydration vesicle (DRV) method [24]. The lipid phase consisting of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (16 μmol/ml; Avanti Polar lipids, USA), dimethyldodecylammonium bromide (DDAB) (Sigma, USA) and cholesterol (Avanti Polar lipids, USA) (2:1:1 molar ratio) dissolved in chloroform:methanol (2:1, v/v) in a round-bottom flask. The solvent was removed by rotary evaporation (Buchii, Switzerland) resulting in deposition of a thin lipid film on the flask’s wall. The lipid film was freeze–dried (Het0 Drywinner, Denmark) overnight to ensure total removal of the solvent. The lipid film was then hydrated and dispersed in distilled water using vortex at 45°C. The resulting empty multilamellar vesicles (MLVs) were converted to 100 nm small unilamellar vesicles (SUVs) using the Mini Extruder (Avestin, Canada). The empty MLVs were extruded repeatedly through 1000, 400 and 100 nm polycarbonate membranes at 45°C. Formulations were passed at least 11 times through the polycarbonate membrane to produce liposomes with uniform size. The rgp63 and CpG ODN were then added to empty SUV liposomes, dried with freeze-drier overnight and rehydrated as follows: one-tenth of the original SUV volume of distilled water was added, and the mixture was vortexed and incubated (30 min, 45°C); 0.1 volume of 10 mM PBS– 0.9% NaCl pH 7.4 was similarly added, and the mixture was vortexed prior to addition of 0.8 volume of PBS. Unencapsulated rgp63 and CpG ODN were separated from encapsulated materials using centrifugation at 14,000 × g for 15 min at 4°C and subsequently washed three times with PBS and the pellet was resuspended in PBS. To prepare liposomes containing only rgp63 (Lip-rgp63) and control liposomes, the same procedure was followed except the CpG ODN was omitted for Lip-rgp63; and CpG ODN and rgp63 were omitted for control liposomes.

Optical microscope (Olympus, Germany) and particle size analyzer (Klotz, Germany) were used to study the
morphological features and mean diameter of the liposomes, respectively.

The encapsulation efficiency of rgp63 and CpG ODN in the liposome was determined with Lowry protein assay and UV absorption at 260 nm, respectively. The analysis was performed on the supernatants as described previously [23].

2.4. SDS-PAGE analysis of rgp63, Lip-rgp63 and Lip-rgp63-CpG ODN

Analytical SDS-PAGE was carried out to characterize rgp63 and determine qualitatively whether Lip-rgp63-CpG ODN or Lip-rgp63 contains rgp63 after purification. The gel was consisted of running gel (12%, w/v, acrylamide) and stacking gel (3%, w/v, acrylamide). The gel thickness was 1 mm. The electrophoresis buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. Electrophoresis was carried out at 150 v constant voltage for 45 min. After electrophoresis the gels were stained with silver for protein detection.

2.5. Immunization of BALB/c mice

Different groups of mice, 10 mice per group, were subcutaneously (SC) immunized 3 times at 3 weeks intervals with one of the following formulations: Lip-rgp63-CpG ODN (2 μg rgp63-10 μg CpG ODN/50 μl liposome/mouse), Lip-rgp63 (2 μg rgp63/50 μl liposome/mouse), rgp63 in PBS (2 μg rgp63/50 μl PBS/mouse), rgp63 in PBS (2 μg rgp63/50 μl PBS/mouse) plus CpG ODN in PBS (10 μg CpG ODN/50 μl PBS/mouse), PBS, or control empty liposomes.

2.6. Challenge with L. major promastigotes

The immunized mice (seven per group) were challenged SC in the left footpad with L. major promastigotes harvested at stationary phase (1.5 × 10⁶ in 50 μl volume), at 3 weeks after the last booster and as a control right footpads were injected with the same volume of PBS. Lesion development was recorded in each mouse by measurement of footpad swelling (or thickness) with a metric caliper (Mitutoyo Measuring Instruments, Japan). Grading of lesion size was done by subtracting the thickness of the uninfected contralateral footpad from that of the infected one.

2.7. Quantitative parasite burden after challenge

The number of viable L. major parasites in the spleen of mice was determined by a limiting dilution assay [25]. The mice were sacrificed at 14 weeks post challenge; the spleens were aseptically removed and homogenized in 2 ml RPMI-FCS with a sterile syringe piston. The homogenate was diluted with the same media in 8 serial 10-fold dilutions to a final volume of 2 ml. Then 150 μl of each dilution was placed in each well of flat-bottom 96-well microtiter plates containing solid layer of rabbit blood agar in triplicate and kept at 25 °C for 10 days. The positive (presence of motile parasite) and the negative (absence of motile parasite) wells were detected using an invert microscope. The number of viable parasite per spleen was determined by ELIDA software, a statistical method for limiting dilution assay [26].

2.8. Antibody isotype assay

Blood samples were collected from the mice before and 14 weeks after challenge and the sera were used to titrate anti-rgp63 and anti-SLA IgG total, IgG1 and IgG2a antibodies by ELISA method using mouse IgG isotyping kit (Zymed Laboratories Inc., San Francisco, USA) according to the manufacturer’s instructions. Briefly, 96-well microtiter plates (Nunc) were coated with 50 μl of 5 μg/ml of rgp63 or SLA overnight at 4 °C. Plates were washed and blocked with 1% bovine serum albumin (BSA) in PBS–Tween. Serum samples were diluted to 1:200 with PBS–Tween and applied to the plates. Optical density was determined at 450 nm by using 630 nm as the reference wavelength.

2.9. In vitro spleen cell responses

Three mice from each group were sacrificed at 3 weeks after the last booster (at the same time as challenge experiment), the spleens were aseptically removed and a single-cell suspension was obtained by homogenization of the tissue, and the erythrocytes were disrupted using ammonium chloride. The splenocytes were washed and resuspended in complete medium (RPMI 1640-FCS) and seeded at 2 × 10⁶/ml in 96-well flat-bottom plates (Nunc). The spleen cells were stimulated in vitro with either rgp63 (5 μg/ml) or SLA (10 μg/ml) or Con A (2.5 μg/ml), or medium alone at 37 °C in 5% CO₂. Supernatants were collected at 72 h of culture and the concentration of IL-4 and IFN-γ were checked using ELISA method according to the manufacturer’s instructions (Bender MedSystems GmbH, Vienna, Austria).

2.10. Statistical Analysis

One-way ANOVA statistical test was used to assess the significance of the differences among various groups. In case of significant F-value multiple comparison Tukey test was used to compare the means of different treatment groups. Results with P < 0.05 were considered to be statistically significant.

3. Results

3.1. Liposome characterization

Liposomes used in this study were morphologically multilamellar vesicles, heterogeneous in size with mean diameters 1.1 ± 0.64, 1.01 ± 0.45 and 1.3 ± 0.3 μm (n = 3) as calculated by particle size analyzer for Lip-rgp63, Lip-rgp63-CpG ODN and empty liposome, respectively.
The encapsulation efficiency of rgp63 in Lip-rgp63 was 43 ± 4%. The encapsulation efficiency of rgp63 and CpG ODN in Lip-rgp63-CpG ODN were estimated to be 65 ± 3% and 94 ± 2.5%, respectively. The concentration of rgp63 and CpG ODN in liposomes was adjusted to 2 µg and 10 µg/50 µl after purification and calculation of encapsulation efficiency, respectively.

3.2. Characterization of the purified rgp63, Lip-rgp63 and Lip-rgp63-CpG ODN by SDS-PAGE

SDS-PAGE analysis of the purified rgp63 revealed a single protein band with MWt of 55 kDa (Fig. 1, lane 2). Also, the SDS-PAGE analysis of Lip-rgp63 and Lip-rgp63-CpG ODN after removing free rgp63 in the supernatant revealed rgp63 incorporation into the liposomes (Fig. 1, lanes 3 and 5, respectively).

3.3. Challenge results

Lesion development was monitored by weekly measurement of footpad thickness (Fig. 2). The lesion size progressed at a more rapid rate in control groups than in mice immunized with free rgp63 (P < 0.05), rgp63 plus CpG ODN in PBS (P < 0.01), Lip-rgp63 (P < 0.01) and Lip-rgp63-CpG ODN (P < 0.001). At 14 weeks after challenge in mice immunized with Lip-rgp63-CpG ODN, the lesion size was significantly smaller compared to mice immunized with free rgp63, rgp63 plus CpG ODN in PBS, and Lip-rgp63.

Fig. 3. Spleen parasite burden in BALB/c mice immunized SC, three times in 3 weeks intervals, with Lip-rgp63, Lip-rgp63-CpG ODN, soluble rgp63 plus CpG ODN in PBS, rgp63 in PBS, empty liposomes and PBS after challenge with virulent L. major promastigotes. A limiting dilution analysis was performed 14 weeks after challenge on the cells isolated from the spleen of individual mice and cultured in triplicate in serial eight-fold dilutions. The number of viable parasite per spleen was determined by ELIDA software based on limiting dilution assay method. The bar represents the average score ± S.E.M. (n = 4). (***P < 0.001 when the mice immunized with Lip-rgp63-CpG ODN are compared with mice received rgp63 plus CpG ODN in PBS.

Fig. 4. Levels of anti-gp63-specific IgG, IgG2a, and IgG1 in sera of BALB/c mice immunized SC, three times in 3 weeks intervals, with Lip-rgp63, Lip-rgp63-CpG ODN, soluble rgp63 plus CpG ODN in PBS, rgp63 in PBS, empty liposomes and PBS. Blood samples were collected from the mice 3 weeks after the last booster (A) and 14 weeks after challenge (B). The rgp63-specific IgG, IgG2a and IgG1 levels were assessed using ELISA method. Panel C indicates the ratio of IgG2a/IgG1 based on absorbance. The assays were performed in triplicate at 200-fold dilution for each serum sample. Values are the mean ± S.D. (**)P < 0.01 and (***)P < 0.001 when the mice immunized with Lip-rgp63-CpG ODN are compared with mice received rgp63 plus CpG ODN in PBS.
Fig. 6. Splenic T-cell responses of BALB/c mice immunized SC, three times in 3 weeks intervals, with Lip-rgp63, Lip-rgp63-CpG ODN, soluble rgp63 plus CpG ODN in PBS, rgp63 in PBS, empty liposomes and PBS. Twenty days after the last booster, their spleens were removed and the splenocytes were stimulated in vitro with either rgp63 (5 μg/ml) or SLA (10 μg/ml), concanavalin A (2.5 μg/ml), or with no stimulation. Production of IFN-γ (A) and IL-4 (B) were assessed by sandwich ELISA with supernatants removed after 72 h of in vitro incubation. Cells from 3 mice per group were pooled. Each bar represents the mean and SEM of triplicate wells. (***): P < 0.001 when the mice immunized with Lip-rgp63-CpG ODN are compared with mice received rgp63 plus CpG ODN in PBS.

with Lip-rgp63-CpG ODN, lesion size was significantly (P < 0.01) smaller than all the other groups. The lesion size in mice immunized with free rgp63 was progressed and only partial protection was observed in this group. At 11 weeks after challenge in mice immunized with rgp63 plus CpG ODN in PBS, only a limited progress in lesion size was observed and protection level was higher than mice immunized with free rgp63 (P < 0.05). The results also showed that there was no significant difference between mice immunized with rgp63 plus CpG ODN and mice immunized with Lip-rgp63. In control groups the lesion thickness reached a plateau after 12 weeks (Fig. 2) but the disease progressed by metastasis to other organs.

3.4. Splenic parasite burden after challenge

The number of viable L. major was quantified in the spleen of different groups of mice at 14 weeks after challenge (Fig. 3). The lowest number of live parasites (P < 0.001) was seen in mice immunized with Lip-rgp63-CpG ODN.
compared with the other groups. Mice immunized with Lip-rgp63 and rgp63 plus CpG ODN in PBS showed significantly \((P<0.001)\) lower parasite burden than control group (PBS or control empty liposomes); however, the parasite burden in these groups were higher than \((P<0.05)\) the group of mice immunized with Lip-rgp63-CpG ODN. There was no significant difference between mice received PBS and mice immunized with empty liposomes.

3.5. Antibody response

In order to determine the type of immune response generated in immunized mice, the anti-rgp63- and anti-SLA-specific IgG, IgG1 and IgG2a antibodies were titrated before (Figs. 4A and 5A) and after (Figs. 4B and 5B) challenge with \(L.\ major\) promastigotes.

As shown in Figs. 4A and 5A, the sera from mice immunized with Lip-rgp63-CpG ODN and Lip-rgp63 before challenge showed significantly higher levels of IgG2a antibody compared with the other groups \((P<0.001)\). However, the level of IgG1 in mice immunized with Lip-rgp63 was significantly higher than mice immunized with Lip-rgp63-CpG ODN \((P<0.001)\). The ratio of IgG2a/IgG1 in sera of mice immunized with Lip-rgp63-CpG ODN was significantly higher than the other groups \((P<0.001;\) Figs. 4C and 5C). The level of IgG1 and IgG2a in mice immunized with free rgp63 or rgp63 plus CpG ODN was also more than controls \((P<0.05)\).

The level of IgG1 in mice immunized with free rgp63 was significantly \((P<0.05)\) higher than the mice immunized with rgp63 plus CpG ODN in PBS. The ratio of IgG2a/IgG1 in mice immunized with rgp63 plus CpG ODN in PBS was significantly \((P<0.05)\) higher than mice immunized with rgp63 \((P<0.05)\).

Challenge with \(L.\ major\) promastigotes induced elevation of IgG, IgG1 and IgG2a antibodies in all the groups of mice compared with the antibody titers before challenge (Figs. 4B and 5B). The sera from mice immunized with Lip-rgp63-CpG ODN and Lip-rgp63 contained significantly elevated levels of specific IgG2a antibody compared with the other groups \((P<0.05)\). Meanwhile, even though the level of IgG2a antibody in mice immunized with Lip-rgp63 was the same as mice immunized with Lip-rgp63-CpG ODN but the level of IgG1 in mice immunized with Lip-rgp63 was significantly \((P<0.001)\) higher than the mice immunized with Lip-rgp63-CpG ODN. The significantly \((P<0.001)\) highest IgG2a/IgG1 ratio was seen in mice immunized with Lip-rgp63-CpG ODN compared to the other groups. The IgG2a/IgG1 ratio in mice immunized with rgp63 plus CpG-ODN in PBS was significantly \((P<0.05)\) higher than mice immunized with rgp63 \((P<0.05)\).

3.6. \textit{In vitro} cytokine production by splenocytes

The supernatant of cultured splenocytes were analyzed to titrate the level of IFN-\(\gamma\) and IL-4, lymphokines indicative of Th1 and Th2 response, respectively. The significantly \((P<0.001)\) highest level of IFN-\(\gamma\) and a significantly \((P<0.001)\) lowest level of IL-4 were detected in supernatant of splenocytes restimulated with either rgp63 or SLA in group of mice immunized with Lip-rgp63-CpG ODN (Fig. 6).

4. Discussion

Despite a decade of global efforts, few preparations of first generation \textit{Leishmania} vaccine have reached phase 3 clinical trials. The results of phase 3 clinical trials showed an acceptable level of safety, but only limited efficacy was seen and no further improvement in efficacy was shown with multiple injections \[27,28\]. The main reason for this failure may be the lack of an appropriate adjuvant.

The most effective adjuvant used with various \textit{Leishmania} antigens in murine model is IL-12 \[29-31\], but various issues such as safety and cost presently preclude this interleukin from the list of candidate adjuvants to be used in human \[31\]. Therefore, effective adjuvants or new antigen delivery systems are required to improve the efficacy of candidate vaccines against leishmaniasis. In this study, CpG ODN 1826 containing two CpG motifs with a phosphorothioate backbone was used as a Th1 immunostimulatory adjuvant based on the other studies \[20,21\].

CpG ODN has been used in soluble form with \textit{Leishmania} antigen in previous anti-\textit{Leishmania} vaccine studies \[32-34\]. Rhee et al. \[32\] used soluble CpG ODN 1826 as an adjuvant with either a recombinant antigen or heat-killed \textit{Leishmania} antigen and the results showed a more potent and durable protection with soluble CpG ODN 1826 than IL-12 in susceptible BALB/c mice infected with \(L.\ major\). In the present study, co-encapsulation of CpG ODN and rgp63 antigen in liposomes was investigated as an alternative approach to enhance the adjuvant effect of CpG ODN. When CpG ODN is used in liposomal form the proximity of adjuvant to antigen was maintained and the \textit{in vivo} degradation of CpG ODN is reduced \[14\].

As an indication of protection, the size of footpad swelling and the number of live \(L.\ major\) parasite in the spleen of infected mice were assessed and compared between different groups of mice. The smallest size of footpad swelling was seen in the mice immunized with Lip-rgp63-CpG ODN compared to all other groups (Fig. 2). Although there was a significant \((P<0.05)\) difference between mice immunized with rgp63 plus CpG ODN in PBS and mice that received rgp63 alone at week 11 post-infection, the difference was not significant thereafter. The mice immunized with Lip-rgp63-CpG ODN showed significantly \((P<0.001)\) smaller footpad swelling as compared to other groups up to 14 weeks after challenge. The number of viable \(L.\ major\) at week 14 after challenge in the group that received Lip-rgp63 or rgp63 plus CpG ODN was lower than control groups but the significantly \((P<0.001)\) lowest number of viable \(L.\ major\) was
observed in group of mice received Lip-rgp63-CpG ODN. A correlation was observed between footpad swelling and the number of live *L. major* in the spleen. The ability of soluble CpG ODN to induce protection against leishmaniasis was previously reported by other investigators [32,34,35]. BALB/c mice vaccinated with ALM (autoclaved *L. major*) plus CpG ODN in soluble form were protected against *L. major* challenge up to 6 weeks [32]. A significantly smaller lesion size was seen in *L. major*-infected BALB/c mice immunized with SLA plus CpG ODN [35] and in another study, 40% of BALB/c mice vaccinated with intact freeze-thawed *L. major* plus CpG ODN were protected against *L. major* challenge [34]. However, the results of this study showed that liposomal form of CpG ODN with Ag induces more potent protection compared to soluble form of CpG ODN and Ag (Fig. 2).

The cytokine secretion profile of the proliferating splenocytes was assessed to determine the type of immune response generated. The splenocytes were stimulated in vitro with either rgp63 or SLA to see if this effect was antigen specific. The highest level of IFN-γ was seen in the splenocytes of mice immunized with Lip-rgp63-CpG ODN as compared to all other groups (*P* < 0.001). Although the level of IFN-γ was significantly higher in mice immunized with rgp63 plus non-encapsulated CpG ODN (*P* < 0.05) or in mice immunized with Lip-rgp63-CpG ODN (*P* < 0.001) compared to control groups, but there was a significant (*P* < 0.001) difference in IFN-γ production by cells from mice immunized with Lip-rgp63-CpG ODN compared to mice immunized with rgp63 plus non-encapsulated CpG ODN (Fig. 6A). A significantly (*P* < 0.001) lower IL-4 production was seen in group of mice immunized with Lip-rgp63-CpG ODN compared to the group of mice immunized with rgp63 plus non-encapsulated CpG ODN (Fig. 6B). The results of cytokine production analysis showed that liposomal CpG ODN induces preferentially a more potent Th1 type response compared to soluble CpG ODN. The effect of CpG ODN on antigen-specific cytokine-producing cell was also examined in other reports and established that CpG ODN boosted the response to other protein or peptide-based vaccines when used in liposomal form [16,36,37].

Analysis of IgG1 and IgG2a antibody isotypes is used as a marker of Th2 and Th1 immune response, respectively [38]. The level of IgG1 and IgG2a antibodies was assessed in different groups of mice before and 14 weeks after challenge. The results showed that the mice immunized with rgp63 alone primarily elicited an IgG1 antibody response especially when the level of anti-gp63 antibodies was determined (Fig. 4A). However, addition of CpG ODN to the rgp63 in liposomal form resulted in a significant increase (*P* < 0.001) in the production of IgG2a antibodies (IgG2a/IgG1 ratio by 2–4 folds) as compared to the other groups of mice (Figs. 4C and 5C). Immunization of mice with rgp63 plus non-encapsulated CpG ODN resulted in only a 1.5-fold increase in IgG2a/IgG1 ratio. The antibody profile was consistent with cytokine profile and showed that liposomal CpG ODN induced a more potent Th1 type of immune response than soluble CpG ODN. A similar shift in antibody isotype profile was observed when a variety of other antigens were co-administered with CpG ODN [21,39,40].

In the current study, positively charged liposomes consisting of DDAB were used. The positively charged vesicles provide an initial strong trigger mediating recognition and uptake by DCs which is even superior to the presence of opsonins adsorbed to the particle surface [41]. Moreover, DDAB has been reported as an effective adjuvant for eliciting cell-mediated response [42].

The improved adjuvant effect of CpG ODN was shown either by linking of CpG ODN directly to the antigen [43,44] or co-encapsulating CpG ODN in liposome vesicles [16], particularly cationic liposomes [45,46]. These findings suggest that optimal immune stimulation occurs when antigen and adjuvant are presented to the immune system in a close spatial and temporal proximity. Moreover, improved adjuvant effect of CpG ODN when encapsulated in liposome might be due to the fact that TLR9, the receptor for CpG motifs [47], localizes to endosomal/vacuolar/vesicular compartments but not to the cell surface [15]. Hence, internalization of CpG ODN is a prerequisite to activate TLR9 for initiation of signaling [48]. This mechanism, together with the finding that lipofection of CpG ODN into spleen cells enhances its immune stimulatory effects [49], suggest that encapsulation of CpG ODN in liposomes can enhance uptake by TLR9-expressing DCs to produce elevated levels of IL-12 and downstream responses [46]. Therefore, it is concluded that co-encapsulation of rgp63 and CpG ODN in cationic liposome might be an appropriate strategy to induce a more potent Th1 immune response and improve protection against leishmaniasis.

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References


