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Adjuvanticity and Protective Immunity Elicited by *Leishmania donovani* Antigens Encapsulated in Positively Charged Liposomes

FARHAT AFRIN AND NAHID ALI*

Leishmania Group, Indian Institute of Chemical Biology, Calcutta 700032, India

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In the search for a leishmaniasis vaccine, extensive studies of cutaneous leishmaniasis have been carried out. Investigations in this regard with the visceral form are limited. As an initial step in the identification of the protective molecules, leishmanial antigens extracted from the membranes of *Leishmania donovani* promastigotes, alone or in association with liposomes, were evaluated for their immunogenicity and ability to elicit a protective immune response against challenge infection. Intraperitoneal immunization of hamsters and BALB/c mice with the leishmanial antigens conferred protection against infection with the virulent promastigotes. Encapsulation in positively charged liposomes significantly enhanced the protective efficacy of these antigens. The splenic parasite burden of hamsters was reduced by 97% after 6 months of infection. BALB/c mice exhibited 87 and 81.3% protection in the liver and spleen, respectively, after 4 months of infection. These protected animals elicited profound delayed-type hypersensitivity and increased levels of *Leishmania*-specific immunoglobulin G (IgG) antibodies. Protection in mice also coincided with elevated levels of IgM and IgA antibodies, which decreased with disease progression in the control-infected animals. Although both IgG1 and IgG2a antibodies were present in the sera of infected mice, IgG1 appeared to be the predominant isotype, suggesting a preferential induction of the Th2 type of immune response over that of Th1. Effective stimulation of all the IgG isotypes, particularly IgG2a, after immunization with liposome encapsulated antigens seems to be responsible for the significant levels of resistance against the disease. Taken together, these data indicate a potential for the liposomal antigens as a vaccine which could trigger both humoral and cell-mediated immune responses.

Leishmanias are obligate intracellular protozoan parasites of macrophages that cause a spectrum of human leishmaniasis, including localized and diffuse cutaneous, mucosal, and visceral leishmaniasis. Protozoa belonging to the *Leishmania donovani* complex, including *L. donovani*, *L. chagasi*, and *L. infantum*, cause the potentially fatal disease visceral leishmaniasis. Clinical and experimental studies demonstrate that generation of an effective cellular immune response is required for protection against this disease. Acute visceral leishmaniasis in humans and mice is associated with the production of specific and nonspecific circulating antibodies (14, 44). At the same time, there is repression of cell-mediated immunity, which is associated with an inability to generate gamma interferon (IFN-γ) and interleukin-2 (IL-2) (8), and decreased production of IL-1 and tumor necrosis factor (19). These immunological defects are restored after recovery from infection, and both humans and mice are resistant to reinfection (17, 41) by *L. donovani*. Epidemiologic studies of human visceral leishmaniasis suggest that up to 85% of infected individuals may spontaneously control infection (12, 20, 37). Different mouse strains are genetically resistant to visceral leishmaniasis, and susceptible strains develop spontaneous control of the disease (4, 5). These observations suggest that a protective immune response must be triggered by antigens of the parasite in the host.

Leishmania major, the causative agent of cutaneous leishmaniasis in humans, causes a spectrum of diseases in different inbred strains of mice, from self-resolving infection to uncontrolled parasite replication. Resistance or susceptibility to *L. major* infection depends on the expansion of one of two distinct subsets of $CD4^+$ lymphocytes (40, 47). Cells with a Th1 phenotype characterized by the secretion of IL-2 and IFN-γ are correlated with protection against *L. major*, whereas Th2 $CD4^+$ cells which produce IL-4, IL-5, and IL-10 promote disease progression (18). Such a distinction of the $CD4^+$ population based on differential cytokine profiles is not well defined for the human system (36). Hence, the nature of immune responsiveness during cutaneous leishmaniasis in humans is not understood as clearly as that in mice (30).

For *L. donovani* infection, even in the murine system, studies have failed to correlate the long-term outcome of the disease with differential expansion of the T-cell subsets (27). On the contrary, there are reports of Th1- and Th2-cell-associated cytokines in experimental visceral leishmaniasis (38). Similarly, studies of humans demonstrate that Th1- and Th2-like responses coexist in patients cured of visceral leishmaniasis (25, 28, 29). However, there is a correlation between decreased IFN-γ production during disease progression and its elevation with acquisition of resistance to *L. donovani* in both mice and humans (8, 41). IFN-γ may be produced by $CD4^+$, $CD8^+$, and natural killer cells, which have been shown to be important in the control of *L. donovani* infection (26, 52).

Studies of animal models (10, 53, 57) and humans (16, 35, 39) have indicated that the development of a vaccine against the cutaneous form of leishmaniasis is feasible. Strategies for immunoprophylactic preparations include induction of protection with whole parasites, either live (53), attenuated (45), killed (16), or disrupted (35), and subcellular fractions (24, 48), with the aim of identifying and isolating protective antigens.

* Corresponding author. Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Rd., Calcutta 700032, India. Phone: 473-3491/0492/6793. Fax: 91-33-4730284/4735197.

Appreciable levels of protection against the disease have been achieved with soluble extracts as well as defined antigens and epitopes (1, 9, 46, 58). In contrast to the immunoprophylactic studies of cutaneous leishmaniasis, relatively few investigations of the visceral form have been carried out. Early studies have demonstrated reduction in the level of parasitemia with killed promastigotes in animal models (21, 22). However, although a few later studies have shown protection with purified antigens (42, 55, 56), investigators have reported difficulty in successful immunization against murine visceral leishmaniasis with fractionated and purified antigens in comparison to cutaneous models (23, 27). In the search for protective immunogens, we have extracted leishmanial antigens (LAg) from the membranes of *L. donovani* promastigotes and used them for immunizing hamsters and BALB/c mice against visceral leishmaniasis. The immunogenicity of leishmanial extracts can be effectively enhanced with adjuvants such as *Corynebacterium parvum* (49), liposomes (2, 24), and IL-12 (1). In this study we evaluated the efficacy of immunization with positively charged liposomes as adjuvants. These liposomes are strong immunopotentiators (32, 33), and at the same time they do not have the toxic effects of complete Freund's adjuvant, *C. parvum*, or IL-12. The data presented here indicate the vaccine potential of these liposome-associated antigens.

MATERIALS AND METHODS

***Leishmania* parasites.** *L. donovani* AG83 was originally isolated from an Indian kala-azar patient (15) and maintained in hamsters and BALB/c mice. The amastigotes were isolated from the spleens of infected animals in medium 199 (pH 7.4) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 25 mM HEPES, 100 U of penicillin G-sodium per ml, and 100 μg of streptomycin sulfate per ml and subcultured in the same medium at 22°C, at an average density of 2×10^6 cells/ml.

For infection, freshly transformed promastigotes were harvested in the stationary phase, washed in 0.02 M phosphate-buffered saline (pH 7.2) (PBS), and injected (2×10^7/0.2 ml of PBS) by the intravenous and intracardiac routes into mice and hamsters, respectively.

Antigen preparation. Stationary-phase promastigotes, harvested after the third or fourth passage in liquid culture, were washed four times in cold PBS and resuspended at a concentration of 1.0 g of cell pellet in 50 ml of cold 5 mM Tris-HCl buffer (pH 7.6). The suspension was vortexed six times for 2 min each, with a 10-min interval of cooling on ice between each vortexing. The parasite suspension was then centrifuged at $2{,}310 \times g$ for 10 min. The crude ghost membrane pellet obtained was suspended in 5 ml of 5 mM Tris buffer (pH 7.6) and sonicated three times for 1 min each at 4°C in an ultrasonicator. The suspension was finally centrifuged for 30 min at $4{,}390 \times g$. The supernatant containing LAg was harvested and stored at −70°C until used. The protein content in the supernatant was measured by the procedure described by Lowry et al. (34). The amount of LAg obtained from 1.0 g of cell pellet was 14 mg.

Preparation of liposomes. Liposomes were prepared with egg lecithin, cholesterol, and stearylamine at a molar ratio of 7:2:2. The thin, dry lipid film was dispersed in PBS for the preparation of empty liposomes. Vesicles with entrapped LAg were prepared by the dispersion of the lipid film in PBS containing 2 mg of LAg per ml. The mixture was vortexed, and the suspension was sonicated for 30 s in an ultrasonicator. Liposomes with entrapped antigen were separated from the free antigen by three successive washing in PBS with centrifugation ($105{,}000 \times g$, 60 min, 4°C). The protein content entrapped in the liposome was estimated by the method described by Lowry et al. (34), with bovine serum albumin as the standard, in the presence of 0.8% sodium dodecyl sulfate and appropriate blanks. The amount of associated LAg per milligram of egg lecithin was 36 μg.

Immunization and challenge infection. Experimental groups consisted of 4-week-old hamsters and BALB/c mice. The animals were immunized by three intraperitoneal injections of 20 μg of LAg either in PBS or incorporated into liposomes at 2-week intervals in a total volume of 0.2 ml. Animals receiving only PBS or empty liposomes served as controls. At 10 days after the last immunization, the animals were challenged with freshly transformed promastigotes as described above.

Evaluation of infection. At the times designated in Results, the course of infection was monitored in the animals by estimating the organ parasite burden, expressed as Leishman-Donovan units and calculated by using the following formula: number of amastigotes per 1,000 cell nuclei × organ weight (in milligrams) (51).

DTH. Delayed-type hypersensitivity (DTH) was determined as an index of cell-mediated immunity. The response was evaluated by measuring the difference in the footpad swelling at 24 h following intradermal inoculation of the test footpad with 50 μl of LAg (800 μg/ml) from that of the control (PBS-injected) footpad.

ELISA. For the enzyme-linked immunosorbent assay (ELISA), microtiter plates were coated overnight at 4°C with 100 μl of LAg (25 μg/ml of 0.02 M phosphate buffer [pH 7.5]). Nonspecific binding sites were blocked with 1% bovine serum albumin in PBS, and the plates were washed with PBS containing 0.05% Tween 20. The wells were incubated overnight at 4°C with hamster or mouse sera. The plates were then incubated with a peroxidase-conjugated goat antibody directed against hamster (Southern Biotechnology Associates, Birmingham, Ala.) or mouse (Sigma Immunochemicals, St. Louis, Mo.) IgG. For the color reaction, the washed wells were incubated for 30 min with *o*-phenylenediamine dihydrochloride (0.8 mg/ml in 0.05 M phosphate citrate buffer [pH 5.0] containing 0.04% H_2O_2). Absorbance was determined at 450 nm in an ELISA reader.

Mice sera were further assayed for LAg-specific IgA, IgM, and IgG subclass antibodies by using a mouse antibody isotyping kit (Sigma Immunochemicals). LAg-coated wells incubated with mouse sera were treated overnight at 4°C with goat anti-mouse isotype-specific antibodies followed by peroxidase-conjugated rabbit anti-goat IgG (Sigma Immunochemicals). The plates were developed for color reaction and analyzed by measuring the optical density at 450 nm.

Statistical analysis. All data comparisons were tested for significance by using Student's *t* test; *P* values of <0.05 were considered significant.

RESULTS

Protection of hamsters against *L. donovani* infection. Hamsters were immunized intraperitoneally with either free LAg or LAg entrapped in liposomes, boosted twice, and then challenged intracardially with virulent *L. donovani* promastigotes. Control hamsters received PBS or empty liposomes. After 2, 4, and 6 months, groups of four hamsters were sacrificed, and the parasite loads in their spleens were quantified (51). As shown in Fig. 1A, hamsters given PBS or liposomes alone developed a progressive visceral infection. At 2 months, the splenic parasite burdens in all the groups were quite low. Immunization with free antigen resulted in partial protection at 4 (56.6%) and 6 (45%) months of infection. Higher levels of protection were obtained in the group immunized with LAg in liposomes at 4 months (86.7%), which attained significance at 6 months (97%; *P* of <0.05) in comparison to controls. The protection induced by LAg in liposomes at 6 months is also statistically higher than that with the free LAg (*P* of <0.05). The increase in parasite burden in the control hamsters paralleled an increase in the weight of the spleen (Fig. 1B). Similarly, the LAg-liposome-immunized group with significantly reduced parasite load had spleens comparable to those of normal, uninfected hamsters.

Acquisition of cell-mediated immunity. A positive DTH response towards LAg is an indication of cell-mediated immunity or resistance against the disease (8, 55). As shown in Fig. 2, immunization with LAg entrapped in liposomes induced the highest level of DTH. Two months after infection, the DTH responses in all the groups reached almost comparable levels. After 4 and 6 months, the DTH response in the group immunized with liposome-encapsulated LAg increased substantially followed by levels of DTH induced by free LAg. Control hamsters, however, did not show any significant increase in the DTH response at these time points. The strong DTH responses elicited by liposome-immunized hamsters correlated with the observed protection in this group.

Generation of antibody responses to immunization. Hamster sera were collected 10 days after the last immunization and at 2, 4, and 6 months of infection and assayed for antileishmanial IgG response by ELISA. Administration of liposome-associated LAg induced the highest antibody response before infection (Fig. 3). The antibody responses in the other groups increased gradually with infection, with the liposome-associated antigen eliciting the maximum stimulation. This result

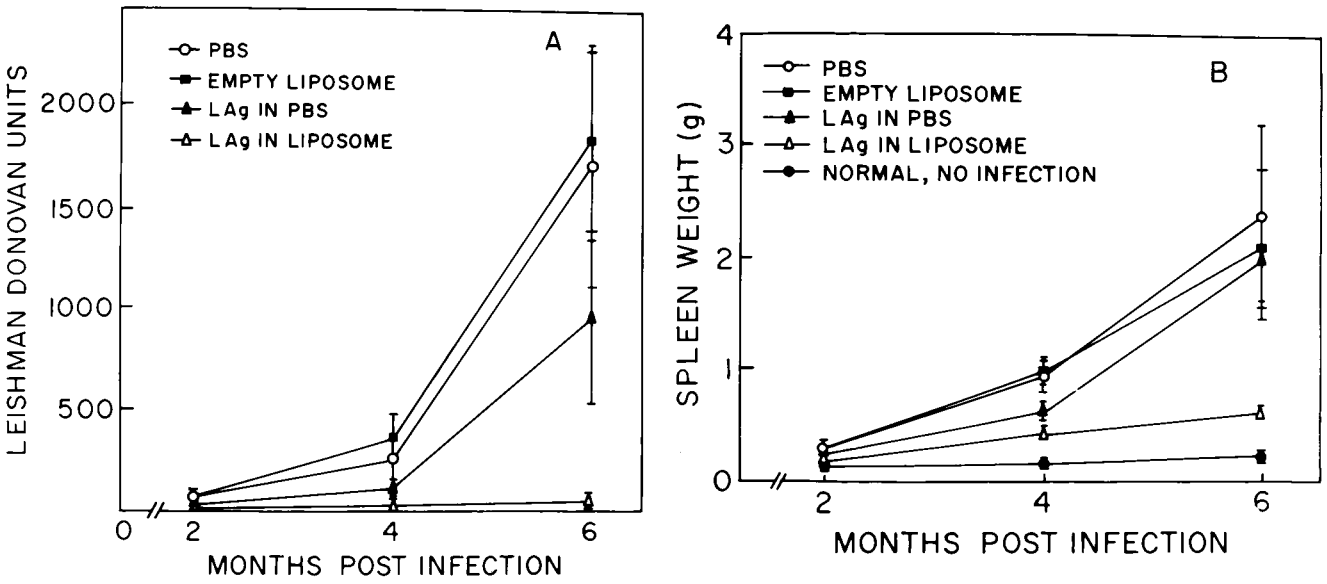

FIG. 1. Evaluation of protection against infection with *L. donovani* in immunized hamsters. Kinetics of spleen parasite burden (A) and changes in splenic weight (B) of hamsters immunized intraperitoneally three times at 2-week intervals with 20 μg of LAg in PBS or in association with liposomes as described in Materials and Methods. Control groups received PBS or liposome alone. At 10 days after the last immunization, the hamsters were challenged intracardially with 2×10^7 promastigotes of *L. donovani*. At the designated times, four hamsters from each group were sacrificed, and their parasite loads were determined by the weight and microscopic examination of impression smears of spleen tissues. The mean value ± standard error (error bar) is indicated for each group. The results are those from one experiment representative of two performed.

suggests a role for humoral immune response as well in liposome-mediated protection.

Induction of protective immunity in BALB/c mice. In view of the above results, we investigated the protective efficacy of LAg-liposomes in a susceptible, inbred model of visceral leishmaniasis, BALB/c mice. The animals were immunized and challenged as described in Materials and Methods for detailed evaluation of the protective responses. After 2 and 4 months, groups of five control or immunized mice were sacrificed, and the levels of parasite burden in their liver and spleen smears were studied (Fig. 4). The parasites appeared first in the liver and increased progressively, while the splenic parasite load began to rise much later in the control (PBS- and empty liposome-immunized) mice. Intraperitoneal injections with free LAg induced partial resistance to challenge with *L. donovani* promastigotes, as observed in the liver (7.4%) at 2 months of infection, which became statistically significant at 4 months, in both the liver (55.5%, P of <0.01) and the spleen (54.2%, P of <0.05), in comparison to controls. Mice immunized with LAg entrapped in liposomes stimulated higher levels of protection in the liver (61.7%) at 2 months, which was further enhanced at 4 months in both the liver (87%, P of <0.001) and the spleen

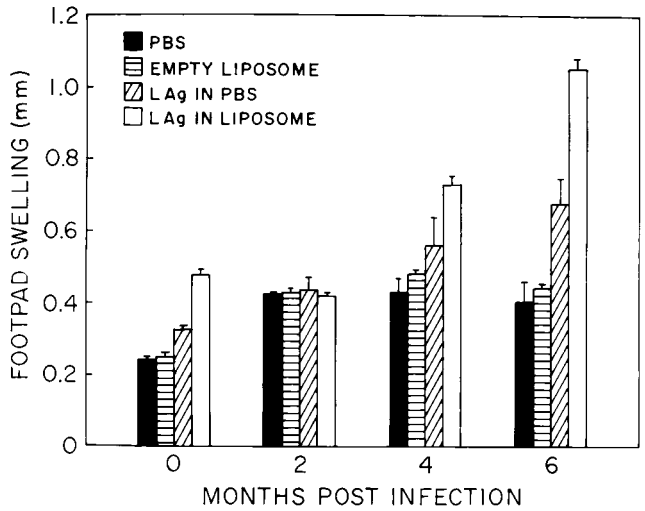

FIG. 2. Kinetics of parasite-specific DTH expressed by immunized hamsters with subsequent challenge infection. The test footpad was injected with 50 μl of LAg (800 μg/ml). The DTH response is expressed as the difference (in millimeter) between the thickness of the test and control (PBS-injected) footpads at 24 h. Each bar represents the mean ± standard error (error bar) for four hamsters.

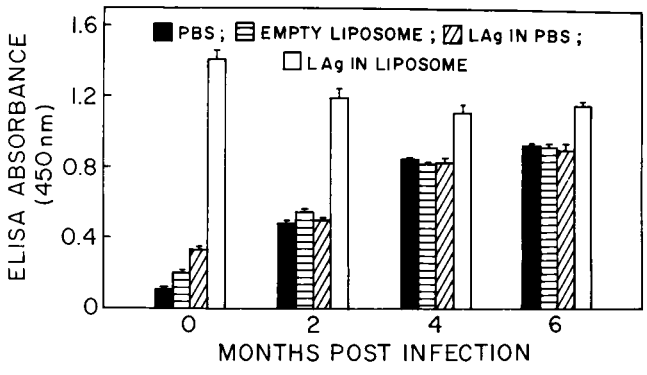

FIG. 3. Parasite-specific serum antibody response following immunization and infection of hamsters. Microtiter plates coated with LAg were incubated overnight with a 1:3,000 dilution of sera from hamsters immunized with PBS, empty liposomes, LAg in PBS, and LAg entrapped in liposomes. The ELISA was carried out as described in Materials and Methods. The results are expressed as the means ± standard errors (error bars) for four samples per group at each data point.

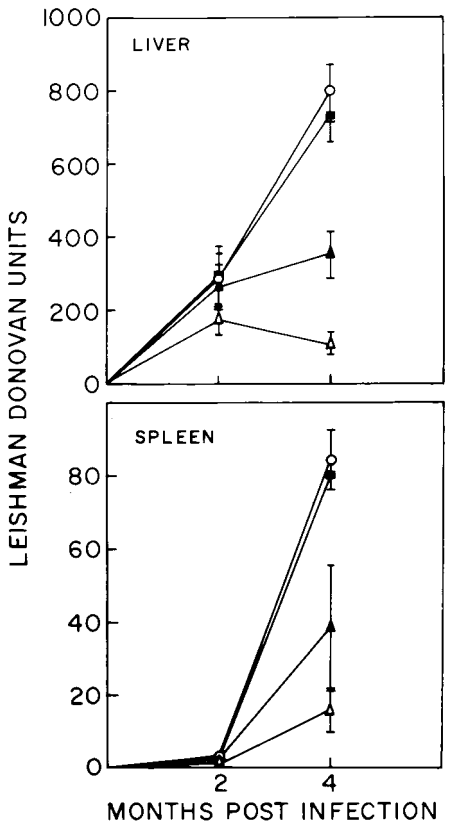

FIG. 4. Kinetics of protection against *L. donovani* in livers and spleens of BALB/c mice immunized with PBS (○), empty liposomes (■), LAg in PBS (▲), and LAg in liposomes (△). After 3 intraperitoneal immunizations, mice were challenged intravenously with *L. donovani* promastigotes, and the parasite burdens at 2 and 4 months of infection were calculated as described in Materials and Methods. The mean values ± standard errors (error bars) for five mice per group are given. The results are those from one experiment representative of two performed.

(81.3%, P of <0.001) compared with the control mice. Further, for the liver, the level of protection conferred by LAg in liposomes was significantly higher (P of <0.001) than that induced by the free antigen after 4 months of infection. There was no significant difference in the weights of the livers in control and protected mice. However, the liposome-associated LAg-immunized mice exhibited a significant decrease in the splenic weight compared with that for the controls (data not shown). Taken together, these observations indicated that immunization with LAg could result in significant protection against *L. donovani* infection, particularly, when LAg is entrapped in liposomes.

DTH responses in immunized mice. Immunization of BALB/c mice with LAg in liposomes induced strong *Leishmania*-specific DTH responses followed by levels induced by free LAg (Fig. 5). At 2 months after infection, DTH responses in protected animals did not differ significantly from those in the controls. However, at 4 months, the DTH levels of liposome-immunized animals increased significantly, whereas those of the controls did not exhibit any marked elevation.

Specific antibody responses. To understand whether the protection in immunized mice correlated with a specific pattern of immunoglobulin production, mice sera were assayed for IgG, IgM, IgA, and IgG isotype levels with specific ELISAs. While liposome-antigen-immunized mice produced significant

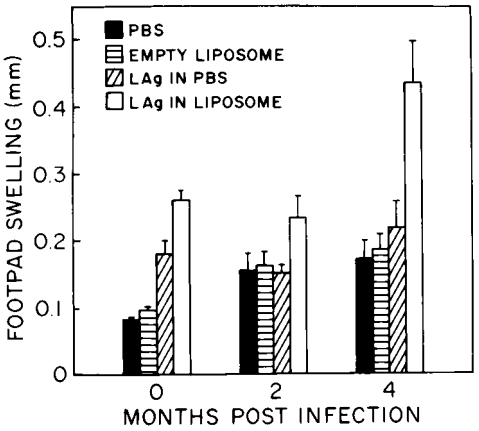

FIG. 5. DTH response to LAg in immunized and challenged mice expressed as footpad swelling at 24 h. Each bar represents the mean ± standard error (error bar) for five mice.

levels of IgG immediately after immunization, there were no measurable levels of IgG in sera from LAg immunized as well as control mice (Fig. 6). With progressive infection, the empty liposome- and PBS-immunized animals elicited strong IgG responses, as expected. However, the levels remained significantly lower than those induced by the LAg-liposome immunogen. Free antigen could also stimulate a substantial IgG response but only after exposure to *L. donovani* infection. The initial stimulation of IgM and IgA antibodies in the control mice was followed by a drop as the disease progressed. However, enhanced levels of IgM and IgA antibodies were observed in the protectively immunized animals.

Leishmania-specific IgG isotype analysis showed strong levels of IgG1 antibody in the control mice at 2 months of infections (Fig. 7). Though there was low levels of production of IgG2a, IgG2b, and IgG3 antibodies in the initial phase, the responses in these subclasses were considerably increased at 4 months. Nevertheless, the IgG1 subclass response remained the highest. Immunization with free antigen induced significant stimulation of all the isotypes only after challenge infection. However, the liposomal-LAg formulation could effectively boost the production of all the isotypes after immunization and further promote the maximum levels of these antibodies with subsequent challenge infection. The effective stimulation of the IgG subclass antibodies after immunization, particularly the IgG2a isotypes which have been implicated to have a protective role against *L. major* (6), might be responsible for the induction of the protective immunity observed in this group.

DISCUSSION

In the present study, we investigated the potential of *L. donovani* promastigote antigens alone or in association with positively charged liposomes to elicit a protective immune response against challenge with infective promastigotes in hamsters and BALB/c mice. Our previous studies have demonstrated that positively charged liposomes enhance the immunogenicities of the associated antigens (32). We have shown that similar liposome entrapment of the LAg increases their immunogenicity, resulting in significant levels of immunoprotection. This work indicates the vaccine potential of LAg entrapped in positively charged liposomes in experimental visceral leishmaniasis.

We demonstrated that immunization with the LAg with li-

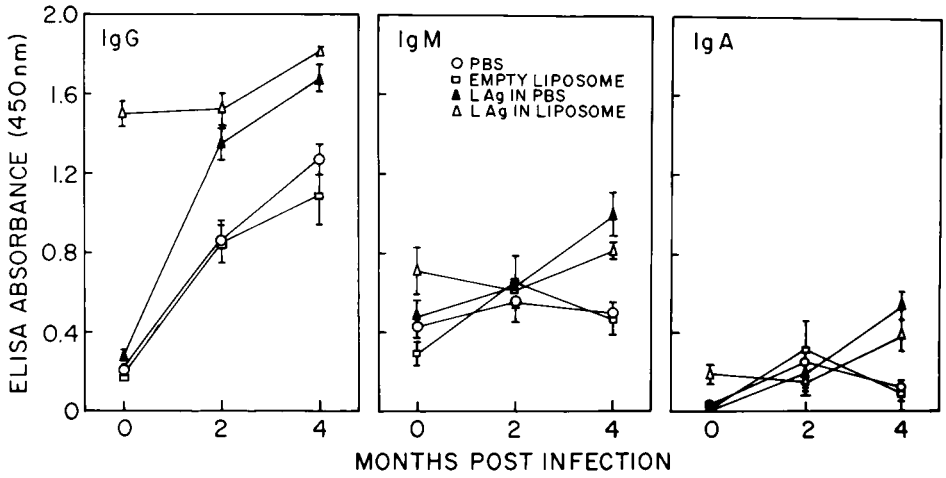

FIG. 6. Parasite-specific IgG, IgM, and IgA serum antibodies in immunized mice infected with *L. donovani*. LAg-coated microtiter plates were incubated overnight with a 1:1,000 dilution of sera, and the ELISA was carried out as described in Materials and Methods. Data are mean absorbance values ± standard errors (error bars) for five samples.

posomes as an adjuvant system induced significant protection in hamsters against infection with *L. donovani* promastigotes (Fig. 1). These vaccinated hamsters showed no increase in the weights of the spleens. Parasite burden analysis further confirmed these results, in that a very low number of parasites were observed in the spleens of the immunized hamsters challenged with promastigotes, suggesting almost complete protection. Reproducible levels of protection in BALB/c mice (Fig. 4) by the antigens in positively charged liposomes indicate the efficacy of this vaccine candidate.

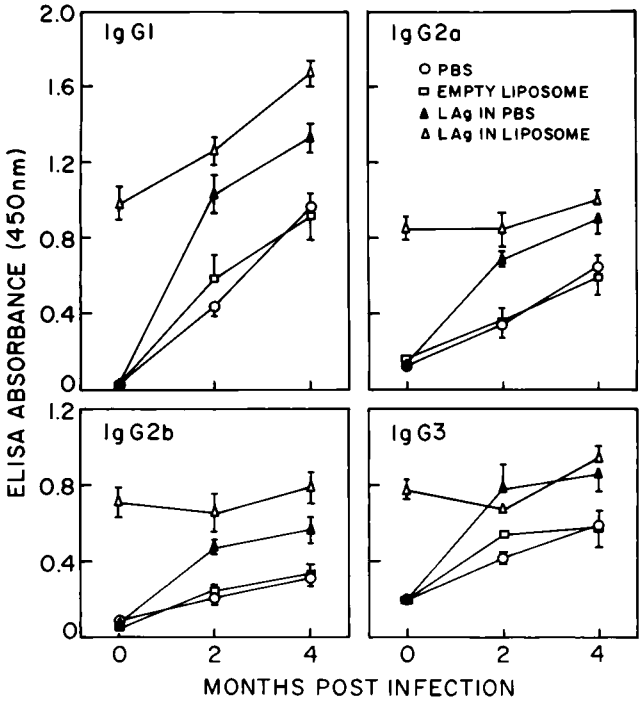

FIG. 7. Kinetics of IgG isotype levels in immunized mice before and after infection. The isotypes were assayed by ELISA, as described in Materials and Methods, with a serum dilution of 1:1,000. Data are presented as the mean absorbance values ± standard error (error bars) of five mice.

Immunization against experimental visceral leishmaniasis has been reported to be less easily achieved than for cutaneous models (27). Hence, although there has been considerable interest in antigens stimulating immune responses to other *Leishmania* species, for example *L. major* (1, 24, 48) and *L. amazonensis* (9), few investigators have explored the potentials of *L. donovani* antigens. Those studied include 80-, 72-, and 70-kDa antigens, the former two of which confer partial protection upon immunization of mice (23, 55). Crude promastigote extracts have been successfully used for vaccination against cutaneous leishmaniasis in murine models with various adjuvants (1, 24, 49). The LAg used in our studies are strong immunogens by themselves inducing partial protection. Their immunogenicity is enhanced by their incorporation in neutral liposomes (2). Liposomes are effective adjuvants for stimulation of both humoral and cell-mediated immunity (13, 31, 33, 43). Apart from the mode of antigen association, the surface charge of the liposome influences the activation of the immune system. In an earlier report we showed that positively charged liposomes are as strong an immunopotentiator as Freund's adjuvant (32). This liposome-antigen preparation has been successfully used herein against challenge with *L. donovani* infection.

Cellular immune responses play a paramount role in the pathogenesis and healing of leishmaniasis. Both clinically (17) and in experimental systems (3), active visceral leishmaniasis is associated with parasite-specific T-cell unresponsiveness. Resistance against the disease by successful chemotherapy as well as asymptomatic infections can be assessed by acquisition of skin test positivity. Low level of parasite-specific DTH responses in control infected animals correlated with disease progression in both hamsters and mice. These animals made resistant by immunization with antigen-liposomes expressed strong DTH even after pathogenic challenge.

Apart from diminished cellular responses, visceral leishmaniasis is associated by the production of high levels of antibody, which is observed prior to the detection of parasite-specific T-cell response (14). This antibody response has been exploited for the diagnosis of *L. donovani* and *L. chagasi* infec-

tions (7). However, their protective role is still controversial. The presence of parasite-specific antibodies in self-limiting subclinical cases as well as in patients after cure suggest a possible role for the antibodies in resistance which remains to be determined. Our studies demonstrate an activation of the humoral as well as cell-mediated response with liposome-encapsulated LAg which correlated with resistance against the disease. Similar elicitation of DTH as well as antibody response with protection has been reported for a recombinant *L. chagasi* antigen (56). These observations are at variance with the reports that protection against leishmaniasis is induced by a strong DTH and almost undetectable amounts of antibodies (6, 49). Again, under certain immunization conditions, parasite-specific DTH might not correspond with protection (46, 54).

The $CD4^+$ subsets, Th1 and Th2, also play a role in B-cell differentiation, proliferation, and isotype regulation (50). Susceptible mice infected with *L. major* mount a Th2 response and produce IgG1 antibodies, whereas resistant mice suppress this activity and enhance IgG2a responses (6). In contrast to the dichotomous immune response observed in murine cutaneous leishmaniasis, infection with *L. donovani* in BALB/c mice elicited both IgG1 and IgG2a responses suggesting an induction of Th1- and Th2-type responses. However, higher levels of IgG1 than of IgG2a since the initiation of the disease demonstrates the possible predominance of Th2 potentiation over that of Th1 in murine visceral leishmaniasis. Immunization of mice with liposome-associated antigens stimulated the production of all the IgG subclass antibodies. An elevation of IgG2a in parallel with IgG1 with antigen-entrapped liposomes prior to infection may play a functional role in resistance against the disease in this group. The occurrence of both IgG1 and IgG2a subclasses has been reported in *L. infantum*-infected dogs (11), and studies with mice and humans demonstrate the coexistence of Th1- and Th2-like responses in visceral leishmaniasis (28, 38). *L. donovani* antigens thus have the potential to trigger IgG1 and IgG2a responses. Identification of the antigens eliciting these responses may help with the design of future vaccines against visceral leishmaniasis.

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