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Protection against *Leishmania major* Infection by Oligomannose-Coated Liposomes

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Abstract—Liposomes coated with neoglycolipids constructed with mannopentaose and dipalmitoylphosphatidylethanolamine (Man5-DPPE) have been shown to induce cellular immunity against antigens encapsulated in the liposomes. To assess whether these neoglycolipid-coated liposomes can elicit protective immune response against challenge infection, effects of immunization with soluble leishmanial antigens encapsulated in the liposomes were evaluated using *Leishmania major* infection in susceptible BALB/c mice. Intraperitoneal immunization of mice with leishmanial antigens in the Man5-DPPE-coated liposomes significantly suppressed footpad swelling in comparison to the control, non-immunized mice, while progression of the disease was observed in mice administered antigens in uncoated liposomes and those administered soluble antigens alone, as seen with control mice. Similarly, the number of parasites decreased substantially in local lymph nodes of mice immunized with the antigen in the Man5-DPPE-coated liposomes. Protection against *L. major* infection in the immunized mice also coincided with an elevated ratio of antigen-specific IgG2a/IgG1 antibodies, which is a profile of T helper-type 1-like immune response. Taken together, these results indicate the possibility that Man5-DPPE-coated liposome-encapsulated antigens could serve as a vaccine that triggers protection against infectious disease.

Introduction

Cell-mediated immunity including delayed-type hypersensitivity and cytotoxic T-lymphocyte response has been found to be deeply involved in protection against infections with microbials and parasites such as *Treponema pallidum*, *Mycobacterium leprae*, and *Leishmania major*.[1–5] Thus, the induction of cell-mediated immunity to these pathogens is thought to be important in protection against infectious diseases.[6]

L. major, the causative agent of cutaneous leishmaniasis in humans, causes a spectrum of diseases in different inbred strains of mice. Resistance or susceptibility to the *L. major* infection depends on the expansion of one of two distinct subsets of $CD4^+$ lymphocytes, T helper-type 1 (Th1) cells or Th2 cells, during infection.[7] Th1 $CD4^+$ cells are involved in prevention of *L. major*, whereas Th2 cells promote disease progression. Interleukin-4 (IL-4) is a key cytokine for Th2 response, while gamma interferon (IFN-γ) promotes Th1 cell expansion.[8,9] In susceptible BALB/c mice, *L. major* causes a progressive disease that ultimately results in death preceded by the dominant production of IL-4 and a relatively low level of IFN-γ production.[9]

We have recently synthesized a neoglycolipid consisting of mannopentaose and dipalmitoylphosphatidylethanolamine (Man5-DPPE) and demonstrated that liposomes coated with Man5-DPPE (Man5-DPPE-coated liposomes) can induce a strong cellular immune response such as delayed type of hypersensitivity and induction of cytotoxic T-lymphocyte against antigens encapsulated in the liposomes without causing any detectable toxicity.[10,11] Although this data suggests that the Man5-DPPE-coated liposomes can be used as adjuvant for immunization against infectious diseases that require cellular immunity to prevent infection, whether or not

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immunization with the Man5-DPPE-coated liposomes is effective in actual infection has yet to be established. This study has investigated whether Man5-DPPE-coated liposomes can elicit a protective immune response against *L. major* infection in susceptible BALB/c mice.

Results

In order to investigate the effects of immunization with soluble leishmanial antigen (SLA)-encased liposomes coated with Man5-DPPE in *L. major* infection, we prepared SLA-encased liposomes coated with Man5-DPPE (Man5-coated SLA-liposomes), SLA-encased liposomes without Man5-DPPE (uncoated SLA-liposomes), and empty liposomes coated with Man5-DPPE (Man5-coated PBS-liposomes), respectively. Then, the molar ratios of lipid components in these liposomes were analyzed using high performance liquid chromatography (HPLC).[12] The molar ratios of dipalmitoylphosphatidylcholine (DPPC), cholesterol, and Man5-DPPE in Man5-coated SLA-liposome samples, uncoated SLA-liposome samples, and Man5-coated PBS-liposome samples are shown in Table 1. The ratio of Man5-DPPE in the Man5-coated SLA-liposome samples did not differ significantly from that in the Man5-coated PBS-liposome samples. In addition, the antigen concentration encapsulated in the Man5-coated SLA-liposome samples did not differ from that in the uncoated SLA-liposome samples. Liposomes containing Man5-DPPE aggregated with concanavalin A, a lectin that recognizes mannose residues, while uncoated liposomes did not (data not shown). The result indicates that mannose residues are expressed on the surface of the liposomes.

BALB/c mice were immunized intraperitoneally with Man5-coated SLA-liposomes, uncoated SLA-liposomes, or SLA alone, boosted once, and then challenged subcutaneously with a lethal dose of *L. major* promastigotes. Immunization of susceptible BALB/c mice with Man5-coated SLA-liposomes significantly suppressed footpad swelling in comparison to the group of mice that received PBS (the control group) (Fig. 1). In contrast, treatment of BALB/c mice with SLA alone produced progressive footpad swelling, as in the control group. Immunization of mice with uncoated SLA-liposomes partially suppressed the swelling up to 3 weeks post-infection and then the size increased rapidly (Fig. 1A). Therefore, SLA itself did not protect against *L. major* infection even when SLA was encased within uncoated liposomes. Immunization of mice with Man5-DPPE-coated liposomes without SLA (Man5-coated PBS-liposomes) or co-immunization with Man5-coated

Table 1. Quantitative analyses of liposomes

Liposomes	Molar ratio (mol/mol)			SLA (μg/mg DPPC)
	DPPC	Cholesterol	Man5-DPPE	
Man5-coated SLA-liposomes	1.00	0.480	0.0052	3.8
Uncoated SLA-liposomes	1.00	0.497	0	4.1
Man5-coated PBS-liposomes	1.00	0.489	0.0042	0

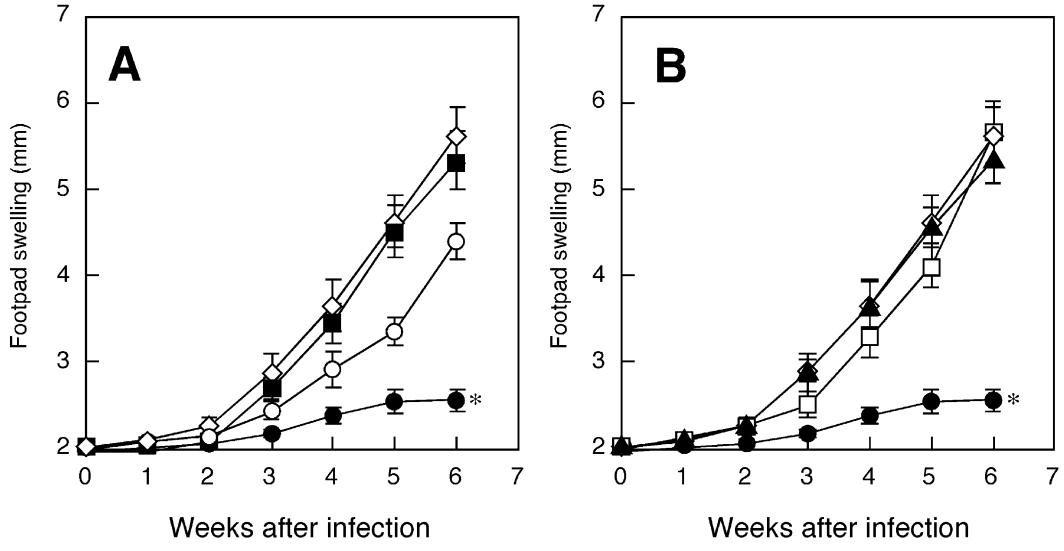

Figure 1. Immunization with Man5-coated SLA-liposomes to protect against infection with *L. major*. All groups of mice ($n=6$) were immunized intraperitoneally with Man5-coated SLA-liposomes (●), uncoated SLA-liposomes (E), SLA (A), Man5-coated PBS-liposomes (□), Man5-coated PBS-liposomes plus SLA (H), or PBS (B) 7 days prior to infection. After immunization, the mice were challenged in the left footpad with 5×10^5 *L. major* promastigotes. Weekly measurements of footpad thickness represent the average score ± SEM. *$p<0.001$ in a Student's *t*-test for the group of mice that received Man5-coated SLA-liposomes compared to the other group 6 weeks after infection. The results are representative of two individual experiments.

PBS-liposomes plus antigen did not suppress the footpad swelling of the mice (Fig. 1B). These results indicate that the Man5-DPPE-coated liposomes act as adjuvant against *L. major* infection. In addition, both factors of Man5-DPPE on the liposomes and the encapsulation of leshmanial antigen in the liposomes are essential for induction of protection against *L. major* infection.

To determine whether the course of disease as assessed by footpad swelling correlated with the parasite burden, the number of viable *L. major* in draining lymph node cells was quantified 6 weeks after infection (Fig. 2). Mice injected with Man5-coated SLA-liposomes had at least a 10^2-fold reduction in the parasite burden in comparison to the control groups of mice. In contrast, the parasite burden in the lymph nodes was very high in the control mice. Treatment of the mice with SLA alone and Man5-coated PBS-liposomes plus SLA increased the parasite load in comparison to that for the control mice. Uncoated SLA-liposomes did not decrease the parasite load. Thus, these profiles paralleled the decrease in footpad swelling. These results strongly suggest that Man5-coated SLA-liposomes significantly prevented the growth of *L. major* in BALB/c mice.

It has been shown that susceptible BALB/c mice infected with *L. major* mount a Th2 response and produce IgG1 antibodies, whereas resistant mice suppress this activity and enhance Th1 response with the production of IgG2a antibodies.[13] To assess whether mice immunized with Man5-coated SLA-liposomes were protected against *L. major* infection due to the induction of Th1 response, the levels of the leishmanial antigen specific immunoglobulins in the serum of each mouse were measured 6 weeks after infection (Fig. 3). In the control mice that mounted a Th2 response, the IgG1 level appeared predominantly higher than the IgG2a level. Immunization of SLA alone or Man5-coated PBS-liposomes and co-immunization of Man5-coated PBS-liposomes plus SLA produced IgG1 dominant profiles, indicating that

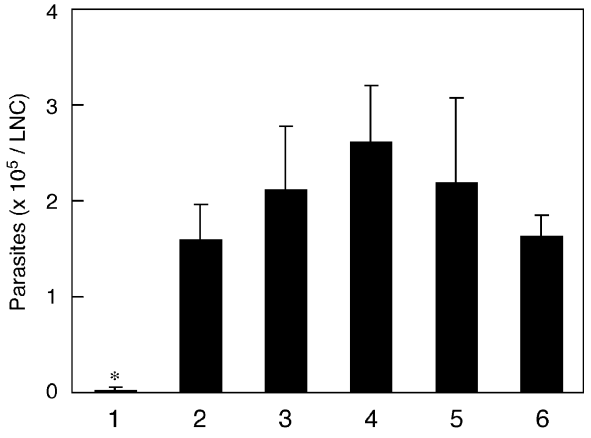

Figure 2. Suppression of *L. major* invasion of popliteal lymph nodes in BALB/c mice administered Man5-coated SLA-liposomes. Groups of mice were intraperitoneally immunized with Man5-coated SLA-liposomes (column 1), uncoated SLA-liposomes (column 2), SLA (column 3), Man5-coated PBS-liposomes (column 4), Man5-coated PBS-liposomes plus SLA (column 5), or PBS (column 6). A limiting dilution analysis was performed 6 weeks after infection on the cells isolated from popliteal lymph nodes of individual mice and cultured in triplicate in Schneider medium for 7 days at 27 °C in serial 5-fold dilution. The wells were assessed microscopically for *L. major* growth, and the number of viable parasites was determined from the well with the highest dilution. The bar represents the average score ±SEM of six mice per group. $*p < 0.01$ for the group with Man5-coated SLA-liposomes values compared to PBS controls 6 weeks post-infection in a Student's *t*-test.

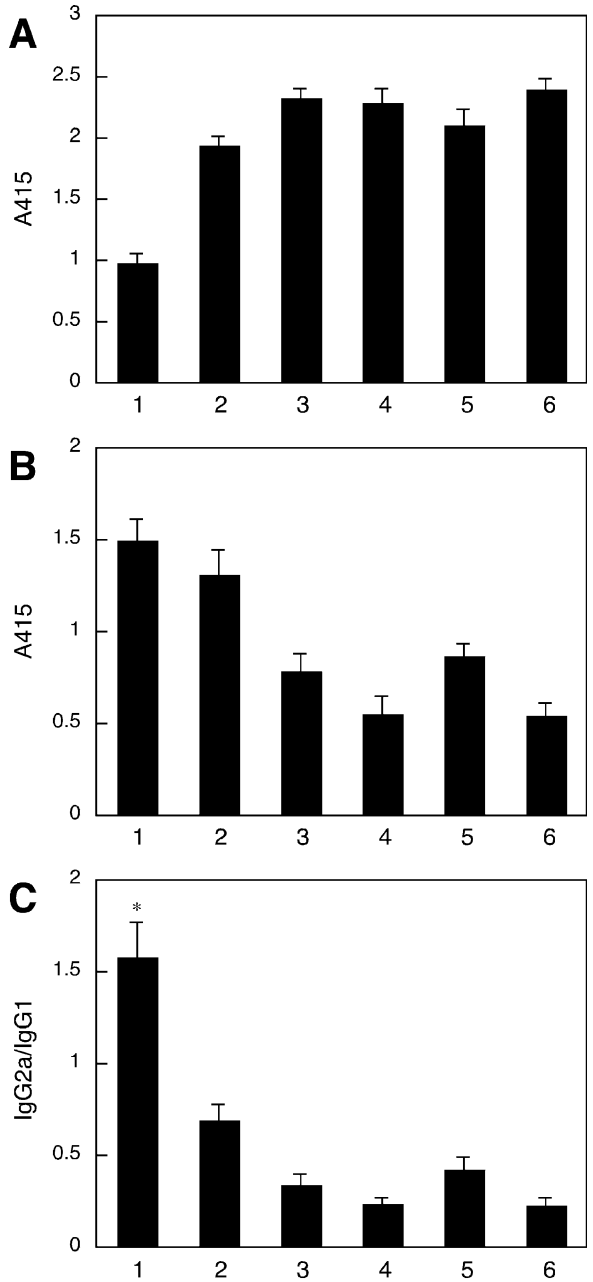

Figure 3. Levels of SLA specific IgG2a and IgG1 antibodies in sera of mice. SLA specific serum IgG2a and IgG1 levels were assessed via ELISA 6 weeks after infection. The assays were performed in triplicate at 50-fold dilution for each serum sample. Groups of mice were intraperitoneally immunized with Man5-coated SLA-liposomes (column 1), uncoated SLA-liposomes (column 2), SLA (column 3), Man5-coated PBS-liposomes (column 4), Man5-coated PBS-liposomes plus SLA (column 5), or PBS (column 6). Panels A and B displayed absorbance at 415 nm of SLA-specific IgG1 and IgG2a antibodies in the sera, respectively. Panel C indicates the ratio of IgG2a/IgG1 based on the absorbance. Values are the mean±SEM of six mice per group. $*p < 0.01$ when comparing the IgG2a/IgG1 ratio for the group of mice that were administered Man5-coated SLA-liposomes versus those from the control mice that were administered PBS.

the Th2-like response was induced in these groups of mice. Immunization with uncoated SLA-liposomes displayed a slight increase in IgG2a. However, the IgG2a/IgG1 ratio did not change significantly in comparison to that of the control mice, suggesting that these mice also mounted a Th2-like response. In contrast, the levels of IgG1 significantly decreased and the levels of IgG2a increased in the groups of mice that elicited an effective immune response after administration of Man5-coated SLA-liposomes. The ratio of IgG2a/IgG1 of this group significantly increased in comparison of that for the control groups, indicating that SLA-specific IgG2a antibodies relatively outnumber IgG1 antibodies. The enhancement of IgG2a production and suppression of IgG1 production suggested that Th1-like response overshadowed Th2-like response in the mice immunized with Man5-coated SLA-liposomes.

Discussion

The present study investigated the immuno-potentiation for leishmanial antigens encased in the Man5-DPPE-coated liposomes to induce protective response in BALB/c mice against challenges with the lethal dose of *L. major* promastigotes. Both footpad swelling and parasite load at the local lymph nodes decreased substantially in the mice immunized with SLA encased within Man5-DPPE-coated liposomes. On the other hand, these parameters of parasite infection did not change in mice immunized with SLA encased in uncoated liposomes or SLA alone. Therefore, immunization with SLA encased within Man5-DPPE-coated liposomes triggered a strong protective response to *L. major* infection. The increased IgG2a/IgG1 ratio in sera of immunized mice with Man5-coated SLA-liposomes demonstrated that the protective response was probably due to the predominance of a Th1-like response.

Reports have stated that the liposomes produced from charged lipids affect *Leishmania* as a resistant immuno-adjuvant.[14,15] Although the mechanisms involved in immuno-potentiation by liposomes remain vastly speculative, their adjuvant activity is probably due to their efficient interaction with antigen-presenting cells (APCs)[16,17] as a result of the charge. As in the case of the charged liposomes, the adjuvant activity of the Man5-DPPE-coated liposomes is thought to be due to the efficient interaction with APC since the mannose receptor is expressed on the surface of APCs.[18,19] Carbohydrate-protein interaction such as mannose versus. lectin is much stronger and more specific than interaction by charge. Therefore, the Man5-DPPE-coated liposomes seem to be a more suitable adjuvant than the charged liposomes.

The adjuvant action of the Man5-DPPE-coated liposomes is thought to be due to their facilitation of antigen delivery to APCs through interaction between the mannose receptor on cell surfaces and oligomannose exposed on Man5-DPPE-coated liposomes. Therefore, the composition of liposomes, particularly the amount of Man5-DPPE in the liposomes, might be important in elucidating the adjuvant activity. This study used liposomes containing DPPC, cholesterol, and Man5-DPPE in a molar ratio of 1:0.48:0.0052 (Table 1). Even though the ratio of Man5-DPPE in total lipids is very low, the Man5-DPPE-coated liposomes induced protection against leishmanial infection. A substantially higher amount of Man5-DPPE in the liposomes may improve the effective adjuvant activity of the Man5-DPPE-coated liposomes. In addition, the liposomes coated with Man2-DPPE or Man3-DPPE might be able to elicit protective immune response, since we have shown that the liposomes coated with these neoglycolipids also induce a delayed type of hypersensitivity response.[10] These lines of inquiry are now being pursued.

Conclusion

This paper demonstrated that the administration of pathogen-specific antigens encapsulated in liposomes coated with Man5-DPPE was able to induce a protective immunity against murine leishmaniasis; liposomes coated with Man5-DPPE are thus considered to offer promise for use as an adjuvant for the treatment of related infectious diseases.

Experimental

Animals and parasites

Specific pathogen-free female BALB/c mice (6 weeks of age) were purchased from SLC (Shizuoka, Japan). Infections were induced using stationary-phase promastigotes of *L. major* (MHOM/SU73/5KSKH) grown at 27 °C in Schneider medium, pH 6.5 (Wako Pure Chemical Industries; Osaka, Japan) supplemented with 20% heat-inactivated fetal calf serum (HyClone; Logan, UT, USA). Mice were injected in the left hind footpad with 5×10^5 stationary-phase *L. major* promastigotes, and the course of the disease was monitored via weekly measurement of footpad thickness with a dial gauge caliper. SLA was prepared from promastigotes of *L. major* in four cycles of freezing and thawing in phosphate buffered saline (PBS), were then subjected to sonication at 4 °C with 20 cycles at 1-s blasts (Microson Ultrasonic Homogenizer), followed by centrifugation at 100,000*g*. The antigen was passed through a 0.2-μm filter and stored at −80 °C until use. The protein concentration was determined using Modified Lowry protein assay reagent (Pierce, USA).

Preparation of liposomes

DPPC, cholesterol, and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Mannopentaose (Man5) with the structure Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man was from Dextra Laboratories (UK). Neoglycolipid (Man5-DPPE) was prepared by conjugation of Man5 with DPPE as described previously.[20,21] All other chemicals were of analytical reagent grade.

Liposomes were prepared as described previously.[10,11] Briefly, a chloroform–methanol (2:1, v/v) solution containing 5 μmol of DPPC and 2.5 μmol of cholesterol were placed in a conical flask and rotary evaporated to prepare a lipid film; 200 μL of PBS or PBS containing SLA (5 mg protein/mL) were added to the dried lipid film and multilamellar vesicles were prepared by intense vortex dispersion. The multilamellar vesicles were extruded 19 times through 1-μm pore polycarbonate membranes (Nuclepore, Pleasanton, CA, USA). Liposomes with encased SLA were separated from free SLA by three successive rounds of washing in PBS with centrifugation (20,000*g*, 30 min, 4 °C). The liposomes were then coated with oligomannose via incubation with Man5-DPPE at 4 °C for 24 h to yield Man5-DPPE-coated liposomes. After incubation, free Man5-DPPE was removed by centrifugation. The molar ratios of the lipid components of liposomes were determined using high-performance liquid chromatography (HPLC), as described previously.[12] The amount of encapsulated antigen was determined with Modified Lowry protein assay reagent (Pierce) in the presence of 0.3% sodium dodecyl sulfate using bovine serum albumin as the standard.

Immunization and infection

BALB/c mice ($n=6$ per group) were immunized intraperitoneally two times with (i) 1 μg SLA encased within Man5-coated liposomes in 100 μL of PBS (Man5-coated SLA-liposomes), (ii) 1 μg SLA encased within uncoated liposomes (uncoated SLA-liposomes), (iii) 1 μg SLA in PBS, (iv) empty Man5-coated liposomes (Man5-coated PBS-liposomes), (v) Man5-coated PBS-liposomes plus 1 μg SLA, or (vi) PBS. A primary dose was followed by a booster dose at 7 days. Mice were challenged 7 days after the second immunization with 5×10^5 stationary growth phase *L. major* promastigotes subcutaneously in the left hind footpad, and the course of the disease was monitored by weekly measurement of footpad thickness with a dial gauge caliper.

Quantitative parasite cultures

Viable *L. major* parasites in infected tissues were enumerated by a limiting dilution assay.[22] Cells from draining lymph nodes of individual mice were suspended at a ratio of 4×10^5 cells/0.2 mL in Schneider medium with supplements, and serial 5-fold dilutions of the cell suspensions were placed in flat-bottom 96-well microtiter plates in triplicate and kept at 27 °C for a week. Wells containing motile parasites were identified under a light microscope. Data reported is the calculated mean and standard deviation of dilution factors of the last positive well multiplied by the number of 4×10^5 lymph node cells.

Antibody isotype assay

The mice were bled 6 weeks after infection, and the serum was pooled from individual mice. For the assay, flat-bottom 96-well plates were pre-coated with 50 μL of 2.5 μg/mL of SLA overnight at 4 °C, and the levels of IgG1 and IgG2a in the serum which diluted to 50-fold with PBS were evaluated in the plates using a mouse IgG isotyping kit (Zymed Laboratories Inc., San Francisco, CA, USA) in accordance with the manufacturer's instructions.

Statistic analysis

The statistical significance of differences between groups was assessed via a Student's *t*-test; p values <0.05 were considered significant.

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