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Preliminary evaluation of oligomannose-coated liposome vaccines against lethal protozoan infections in mice

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ABSTRACT

The oligomannose-coated liposome (OML) vaccine is known to induce cellular immunity specific for the encapsulated antigen in immunized mice. In the present study, we preliminarily evaluated the effect of the OML vaccine encapsulating the soluble protozoan lysate of *Toxoplasma gondii*, *Trypanosoma brucei gambiense*, or *Babesia rodhaini* on the corresponding protozoan infections in mice. After the challenge of *T. gondii*, the OML vaccine group avoided the high mortality resulting from acute infection that was dominantly observed in other control groups. During the infectious course, the development of the *T. gondii*-specific antibody, which is an indicator of humoral immunity, was constantly controlled at a lower level in the surviving mice of the OML vaccine group than in other lethally affected mice. On the other hand, other OML vaccines targeting for *T. b. gambiense* and *B. rodhaini* did not show any effect on these lethal infections in mice. The present preliminary study suggests that OML is a novel vaccine tool, at least for the control of acute toxoplasmosis.

Key words: oligomannose-coated liposome (OML), soluble protozoan antigen, antibody responses

INTRODUCTION

The oligomannose-coated liposome (OML) is effectively incorporated by a peritoneal macrophage probably via the macrophage mannose receptor (Ikehara and Kojima 2007; Shimizu *et al.* 2007), and its administration to mice can induce a strong cellular immune response, including a delayed type of hypersensitivity and the induction of cytotoxic T-lymphocytes, against the encapsulated antigen without any detectable toxicity (Fukasawa *et al.* 1998; Sugimoto *et al.* 1995). Recently, the effect of immunization with a soluble leishmanial antigen encapsulated within the OML was evaluated on *Leishmania major* infection in susceptible BALB/c mice (Shimizu *et al.* 2003). Intraperitoneal immunization of the OML vaccine more significantly suppressed the footpad swelling in infected mice and the parasite's growth in their local lymph nodes than in the control mice. Furthermore, protection against the *L. major* infection coincided with a dominant induction of the protozoa-specific helper-type 1 (Th1) immune response in the immunized mice (Shimizu *et al.* 2003; Shimizu *et al.* 2007).

Interferon (IFN)-γ is a crucial cytokine for the control of several protozoa, such as *Toxoplasma gondii* (Suzuki *et al.* 2000), *Trypanosoma brucei* (Hertz *et al.* 1998; Schleifer *et al.* 1993), or *Babesia microti*

(Igarashi *et al.* 1999), even though the outcomes of their infections were different, e.g. in nucleated cells, extracellularly, or in erythrocytes, respectively. The OML including IFN-γ with a skewed Th1 immune response might become an effective vaccine tool against these protozoan infections. In the present study, therefore, we preliminarily evaluated the vaccine effect of OML encapsulating the soluble protozoan lysate (antigen) of *T. gondii*, *T. b. gambiense*, or *Babesia rodhaini* on the corresponding infection in mice.

MATERIALS AND METHODS

Parasites and mice

The tachyzoite of *T. gondii* RH strain and the bloodstream form (BSF) of *T. b. gambiense* IL2343 strain were maintained by *in vitro* passages in Vero cells (Nishikawa *et al.* 2003) and a cell-free culture (Hirumi and Hirumi 1989; Hirumi and Hirumi 1991), respectively. The merozoite of *B. rodhaini* Australian strain was maintained by *in vivo* blood passages in mice (Igarashi *et al.* 1999). Specific pathogen-free female BALB/c mice were purchased from Nihon CLEA (Tokyo, Japan), and 8-wk-old mice were subjected to infectious experiments as described below.

Preparation of soluble protozoan antigens

For the purification of *T. gondii* tachyzoites, the infected Vero cells were washed twice with cold phosphate-buffered saline (PBS), and the final pellet resuspended in cold PBS was passed through a 27-gauge needle (TERUMO, Tokyo, Japan) and then through a 5.0-μm pore filter (Millipore, Bedford, MA, USA) (Nishikawa *et al.* 2003). The BSF of *T. b. gambiense* grown in the cell-free culture was washed twice with cold PBS. For the purification of *B. rodhaini* merozoites, the infected red blood cells (IRBCs) were collected from the infected mice and washed twice with PBS, discarding the buffy coat fraction. To clarify the merozoites, the pellet of IRBCs was suspended with a 0.83% ammonium chloride solution and then incubated at 37°C for 10 min (Nishikawa *et al.* 2001). After resolution of the red blood cells (RBCs) membrane, the merozoites were washed 3 times with cold PBS. Once the parasites had been prepared, the pellet of each parasite was disrupted with 3 repetitions of freeze-thawing and subsequent sonication. After centrifuging the extracts at 10,000 x g for 30 min at 4°C, the supernatants were filtered through a 0.2 μm pore cellulose acetate membrane (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and then kept at –80°C until use. These clarified lysates of *T. gondii*, *T. b. gambiense*, and *B. rodhaini* were designated as STgA, STbgA, and SBrA, respectively.

Preparation of liposomes

Dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylcholine (DPPC), and cholesterol were purchased from Sigma (St. Louis, MO, USA). Oligomannose [M3: Manα1-6(Manα1-3)Man] was purchased from Dextra Laboratories (Reading, UK). Neoglycolipids (M3-DPPE) were prepared by conjugating the M3 with DPPE as described previously (Mizuochi 1991; Mizuochi *et al.* 1989). OML was prepared as described previously (Shimizu *et al.* 2007; Shimizu *et al.* 2003). Briefly, a chloroform-methanol (2:1 v/v) solution containing 1.5 μmol of DPPC and cholesterol was placed in a conical flask and then dried by rotary evaporation. Subsequently, 2 ml of ethanol containing 0.15 μmol of M3-DPPE was added into the flask and evaporated to prepare a lipid film containing the neoglycolipid. Two hundred microliters of PBS containing 5 mg/ml STgA, STbgA, SBrA, or bovine serum albumin (BSA: control antigen) was added on the dried lipid film, and multilamellar vesicles were prepared by intense vortex dispersion. The multilamellar vesicles were extruded 10 times through a 1 μm pore polycarbonate membrane (Nucleopore, Pleasanton, CA, USA). Liposomes encapsulating the target antigen were separated from the free antigen by 3 successive rounds of washing in cold PBS with centrifugation at

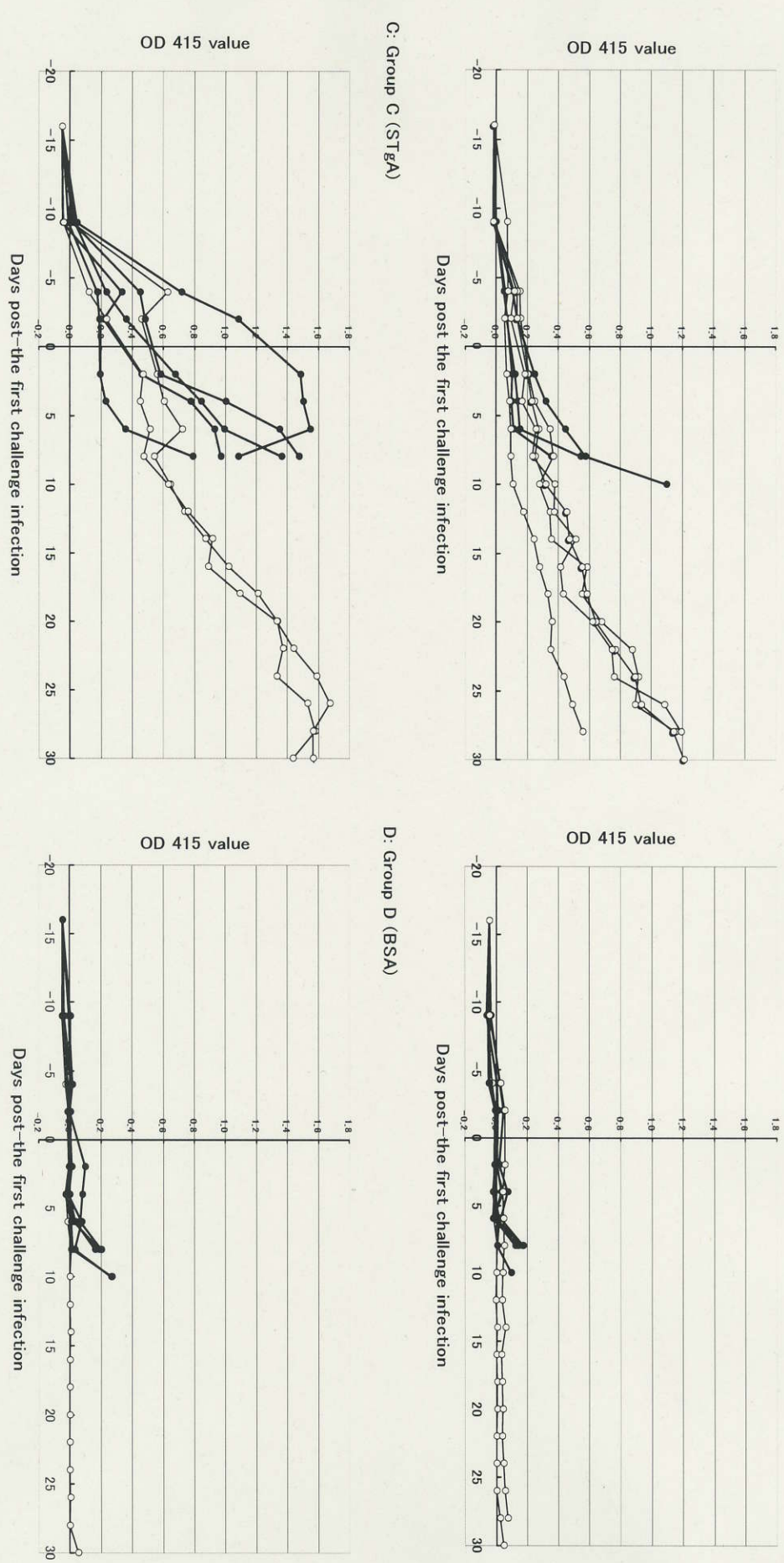

Fig. 1: Dynamics of *T. gondii*-specific antibody in each mouse of groups A (OML vaccine group), B (OML control group), C (STgA group), and D (BSA group) after the challenge infection of *T. gondii* RH strain. The dynamics were compared on the absorbance at 415 nm between the surviving (open circle) and lethally affected mice (filled circle) in each panel.

Table 1. Survival rate in OML vaccine trial.

Protozoa	Group	Survival rate (%)*
T. gondii		
	A: STgA in OML	4/7 (57)
	B: BSA in OML	2/7 (29)
	C: STgA	2/7 (29)
	D: BSA	1/7 (14)
T. b. gambiense		
	A: STbgA in OML	0/7 (0)
	B: BSA in OML	0/7 (0)
	C: STbgA	0/7 (0)
	D: BSA	0/7 (0)
B. rodhaini		
	A: SBrA in OML	0/7 (0)
	B: BSA in OML	0/7 (0)
	C: SBrA	0/7 (0)
	D: BSA	0/7 (0)

*Survived mouse number/total number (%)

20,000 x g for 30 min at 4°C. The amount of encapsulated antigen was measured using a modified Lowry protein assay reagent (Pierce, Rockford, IL, USA) in the presence of 0.3% sodium dodecyl sulfate (SDS).

OML vaccine trials in infectious experiments

For each OML vaccine trial against *T. gondii*, *T. b. gambiense*, or *B. rodhaini* infection, BALB/c mice (n = 7 per group) were immunized subcutaneously twice with 3 μg of each protozoan antigen (STgA, STbgA, or SBrA) encapsulated within OML (Group A: OML vaccine group), 3 μg BSA encapsulated within OML (Group B: OML control group), 3 μg of each protozoan antigen (Group C: STgA, STbgA, or SBrA group), or 3 μg BSA (Group D: BSA group) in 100 μl PBS on days 16 and 9 after each protozoan infection. On day 0, all mice of each vaccine trial were injected intraperitoneally with 500 tachyzoites of *T. gondii*, 1,000 BSF of *T. b. gambiense*, or 1 x 10^6 IRBC of *B. rodhaini*. The survival rate of each group was determined until day 30 after the challenge infection. For *T. b. gambiense* and *B. rodhaini* infections, the development of parasitemia was observed daily until the mice died, as described previously (Igarashi *et al.* 1999; Kuboki *et al.* 2006). All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine (Hokkaido, Japan).

Enzyme-linked immunosorbent assay (ELISA)

Sera were collected at approximate intervals during the vaccine trial only for *T. gondii* infection. For the detection of *T. gondii*-specific antibody in ELISA, 50 μl per well of 30 μg/ml STgA in a coating buffer

(50 mM carbonate-bicarbonate, pH 9.6) was coated on flat-bottomed 96-well microliter plates (Nunc, Postboks, Denmark) and then incubated overnight at 4°C. After washing once with a washing buffer (0.05% Tween 20 in PBS), the residual binding areas were blocked with 100 μl per well of a blocking solution (5% skim milk in PBS) at 37°C for 1 hr. After the blocked plates were washed once with the washing buffer, 50 μl of the collected serum diluted in the blocking solution (1:25) was added and then incubated at 37°C for 1 hr. After washing 6 times with the washing buffer, each well was incubated with 50 μl of horseradish peroxidase-conjugated goat anti-mouse immunoglobulins antibody (DakoCytomation, Glostrup, Denmark) diluted in the blocking solution (1:5,000) at 37°C for 1 hr. Subsequently, the plates were washed 6 times with the washing buffer and then incubated with 100 μl per well of a substrate solution [0.3 mg/ml of 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid), 0.1 M citric acid, 0.2 M sodium phosphate, and 0.03% H_2O_2] in a dark room at room temperature for 1 hr. Finally, a stopping solution (1% SDS) was added into each well, and the absorbance at 415 nm was measured using an MTP-500 microplate reader (Corona Electric, Ibaragi, Japan).

RESULTS AND DISCUSSION

The results of the OML vaccine trials, determined by the survival rates of all groups, are summarized in Table 1. On day 30 after the challenge of *T. gondii*, the OML vaccine group (Group A) showed a higher survival rate of 57%, as compared to that of the BSA group (Group D, 14%). The OML control and STgA groups (Groups B and C, respectively) showed a slightly higher survival rate of 29% than that of group D. In the cases of other vaccine trials against *T. b. gambiense* and *B. rodhaini* infections, the corresponding OML vaccines did not contribute to the increase of the survival rates (Table 1) and the decrease of parasitemia development (data not shown).

The development of the protozoa-specific antibody was monitored during the vaccine trial against *T. gondii* infection. Accordingly, the antibody responses on day 2 after the infection were detected at low levels in the OML vaccine group (Fig. 1A), while those in the STgA group showed relatively higher and variable levels on the same day (Fig. 1C). After the challenge infection, however, the antibody response showed the characteristic dynamics of the last surviving mice in groups A and C. Namely, the last survived mice showed slightly gradual increases of the antibody titers until 30 days after the infection (Figs. 1A and C, open circles). In contrast, the lethally affected mice exhibited radical increases of the titers until 8 days after the infection, at which time, they began to die (Figs. 1A and C, filled circles). On the other hand, the OML control and the BSA groups seldom showed detectable antibody responses during the infectious experiment (Figs. 1B and D), although only the lethally affected mice in these groups displayed instant increases in the titers immediately before death (filled circles). Even though all surviving mice were subsequently re-challenged with *T. gondii* on day 31 after the first infection, they did not die until day 60.

The present preliminary study indicates that the OML vaccine is effective against acute toxoplasmosis in mice. After the challenge of *T. gondii*, only the OML vaccine group avoided the high mortality of acute infection that was dominantly observed in other control groups. In addition, the development of the *T. gondii*-specific antibody, which is an indicator of humoral immunity, was constantly controlled at a lower level in the surviving mice of the OML vaccine group than in other lethally affected mice. These results suggest that the protozoa-specific Th1 immune responses were induced by immunization of OML encapsulating STgA and the responses would lead to the survival against toxoplasmosis.

The OMLs encapsulating STbgA and SBrA did not exhibit any vaccine effect on acute infections of *T. b. gambiense* and *B. rodhaini* in mice. However, the reason for this remains unclear. Possibly, the OML

vaccines constructed in the present study might not be effectively inducible for protozoa-specific Th1 immunity in the mice. Alternatively, even if the OML vaccines were inducible, the Th1 immunity might be originally incomplete for the control of these protozoan infections. Therefore, further studies are required to examine the ability of these OML vaccines to induce protozoa-specific Th1 immunity and to develop suitable vaccines against acute trypanosomiasis and babesiosis. In conclusion, the present preliminary study suggests that OML is a novel vaccine tool, at least for the control of acute toxoplasmosis.

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