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Immune Responses and Resistance to *Toxoplasma gondii* in Mice Immunized with Antigens of the Parasite Incorporated into Immunostimulating Complexes

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Immunostimulating complexes were prepared with antigens extracted from tachyzoites of *Toxoplasma gondii* and were used to immunize mice. The major antigens incorporated into the immunostimulating complexes were the P30 and P22 antigens and an antigen with an approximate molecular weight of 6,000. Other antigens of molecular weights above 30,000 were also present. High antibody titers to *T. gondii* antigens and a delayed-type hypersensitivity reaction were noted for the immunized mice. Challenge of these mice with tachyzoites injected interperitoneally or with oocysts administered orally resulted in a statistically significant ($P < 0.001$) conditional probability of survival compared with that of controls. In contrast, the differences between immunized mice and controls challenged with tissue cysts did not attain statistical significance.

Infection with *Toxoplasma gondii* usually results from the ingestion of food contaminated with oocysts or cysts of the parasite. Oocysts are shed by cats, the definitive hosts, and cysts are present in tissues from chronically infected animals (8). Human infection is often subclinical, but when acquired during pregnancy, it may result in congenital infection (28). In immunocompromised individuals, including AIDS patients, infection with *T. gondii* may develop into a serious disease (2, 13). In animals, toxoplasmosis is also often subclinical, but it can, especially in sheep, result in abortion and stillbirth, with significant economic losses. Moreover, tissues from chronically infected animals are a source for human infection (8).

Vaccination attempts with live, attenuated, killed, or lysed parasites, as well as different antigenic fractions of the parasite, have been conducted with varying success (14). Vaccines with live organisms are currently in use (5, 6, 37), but they represent a potential hazard.

The immunostimulating complex (iscom) is a highly immunogenic formulation of amphipathic antigens within a matrix consisting of Quil A, cholesterol, and phospholipids (24). Iscoms with antigens of *T. gondii* have been previously shown to induce protective immunity in mice (26, 34).

The aims of the present study were to determine the antigens of tachyzoites incorporated into the iscoms and to determine their capacity to induce protective immune responses in mice against a natural mode of infection, namely, oral infection with oocysts and tissue cysts.

MATERIALS AND METHODS

***T. gondii*.** Strains RH, ME49 (1), and C56 (16) were used. Tachyzoites of the RH strain were used for the preparation of iscoms. Tissue cysts and tachyzoites of the C56 strain or oocysts of the ME49 strain (18) were used for challenge infections. Cysts were obtained from the brains of chronically infected mice, and tachyzoites were obtained from the peritoneal fluid of infected mice (16). Oocysts were kindly provided by J. P. Dubey, U.S. Department of Agriculture, Beltsville Agricultural Research Center, Beltsville, Md.

Preparation of iscoms. The method described by Lövgren et al. (21) was used. Briefly, tachyzoites were suspended in phosphate-buffered saline (PBS) with cholesterol and phosphatidylcholine (125 μg/ml of each) dissolved in the detergent MEGA-10 (0.25%, wt/vol) (Sigma Chemical Co., St. Louis, Mo.). The suspension was incubated for 30 min at room temperature and centrifuged at 2,000 × *g* for 15 min, and the supernatant was collected. Quil A (Spikoside; Iscotec, Luleå, Sweden) was added to the supernatant to a final concentration of 0.1%, and the solution was dialyzed against PBS. The iscom preparation was further purified by centrifugation for 18 h at 200,000 × *g* through a layer of 10% sucrose. The pellet was resuspended in PBS, and the morphology of the iscoms was examined by electron microscopy. Protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.) and bovine serum albumin as the standard. Iscom matrix, i.e., iscoms without antigen, was prepared as described previously (20) and used as the control. Iscoms and matrix were stored at −85°C until they were used.

Immunization. Swiss Webster, adult, female mice (Simonsen Laboratories, Gilroy, Calif.) were injected subcutaneously with iscoms containing 5 μg of protein. Each mouse received 3 immunizations administered at 6-week intervals. Control mice were injected with matrix containing 10 μg of Quil A per injection.

Immune responses. Antibody responses were determined by enzyme-linked immunosorbent assay (ELISA) (1) with serum samples collected 21 days after the first and 7 days after the second immunizations. Toxoplasma iscoms (1 μg/ml) were used as the coating antigen, and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Caltag, Burlingame, Calif.) was used as secondary antibody. Sera from normal mice and from mice chronically infected with *T. gondii* were used as controls.

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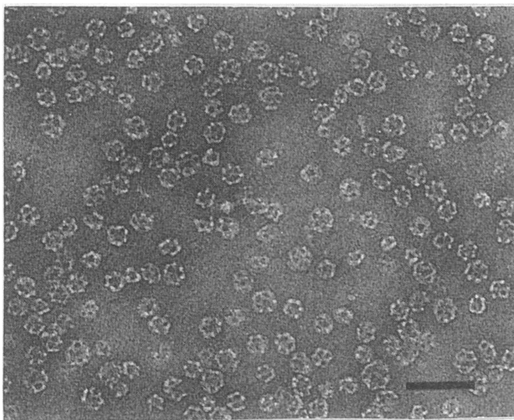


FIG. 1. Electron micrograph of iscoms containing *T. gondii* antigens showing the cage-like structures. Bar, 100 nm.

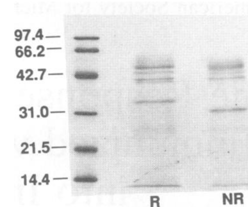


FIG. 2. SDS-PAGE of toxoplasma iscom stained with Coomassie brilliant blue G. Molecular weight markers (in thousands) are indicated to the left. Iscom was analyzed under reducing (R) and nonreducing (NR) conditions.

Cell-mediated immune responses were determined by the footpad assay as previously described (1).

Polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Iscoms or a lysate of RH tachyzoites were analyzed under both reducing and nonreducing conditions (12) by using the discontinuous buffer system of Laemmli (19). For immunoblotting, the proteins were transferred to nitrocellulose paper (Schleicher and Schuell, Keene, N.H.) as described by Towbin et al. (33). Identification of antigens in immunoblots was as described previously (12) with polyclonal sera from mice chronically infected with *T. gondii* or monoclonal antibodies (MAbs) 5D12, 6D10, and 16B1. MAbs 5D12 and 6D10 are directed against the P30 and P22 antigens, respectively, of the cell surface membrane of *T. gondii* (11, 12, 27). MAb 16B1 recognizes an antigen with an approximate molecular weight of 6,000 associated also with the membrane of the parasite (12, 29). Sera from normal mice were used as the control, and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Caltag) was used as the secondary antibody.

Challenge infection. Seventeen days after the third immunization, mice were challenged with tachyzoites, cysts, or oocysts. Eight immunized and eight control mice received 2.5×10^4 tachyzoites of the C56 strain (18) administered intraperitoneally. A second group, also with eight mice, was challenged orally by gavage with 10 cysts of the C56 strain. A third group of nine immunized and nine control mice was challenged orally by gavage with 250 sporulated (8) oocysts of the ME49 strain (1, 22). These challenge inocula were determined to be optimal by previous experiments. Survival was recorded up to 30 days after challenge. Thereafter, surviving mice were euthanized, and individual brains were examined microscopically for *T. gondii* cysts.

Statistical analysis. The conditional probability for survival for each group was calculated by the method of Kaplan and Meier and the method of Gehan for the determination of the significance of the differences between groups (3).

RESULTS

Characterization of antigens in the iscom. Electron microscopy of iscoms revealed the characteristic cage-like structure of antigens, each with an approximate diameter of 40 nm (Fig. 1). Sodium dodecyl sulfate (SDS)-PAGE of iscoms revealed several protein bands under reducing and nonreducing conditions (Fig. 2). One band in the area of the 30,000-molecular-weight (30K) marker noted in the nonreducing blot and one with a higher molecular weight noted in the reducing blot may correspond to the P30 antigen (Fig. 2). Diffusely stained bands noted above the 21.5K marker in both reducing and nonreducing blots may indicate the P22 antigen. One band at the front of the gel also was observed (Fig. 2).

Immunochemical analysis of iscoms by immunoblotting with polyclonal and monoclonal antibodies revealed that at least six antigens separated under nonreducing conditions were bound by antibodies in sera of chronically infected mice (Fig. 3A, lane 2). Each one of these antigens, except for one approximately 10K antigen, was recognized by antibodies in sera of mice immunized with the iscoms. Under reducing conditions, three approximately 34K, 24K, and 10K antigens and a group of antigens with molecular weights between 40,000 and 55,000 were recognized by antibodies in sera of chronically infected mice or mice immunized with iscoms (Fig. 3B). MAb 5D12 recognized the 34K antigen band as the P30 antigen and MAb 6D10 recognized the 24K antigen band as the P22 antigen under both reducing and nonreducing conditions (lanes 4 and 5 of Fig. 3). MAb 16B1 recognized the 10K antigen band as the 6K antigen under both reducing and nonreducing conditions (lanes 6 of Fig. 3).

Antibody and cell-mediated immune responses. One week after the second immunization, the antibody titers in mice immunized with iscoms ranged from 1:12,800 to 1:25,600 (Table 1). In immunoblots with iscom as antigen, antibodies in sera of mice immunized with iscoms recognized the same

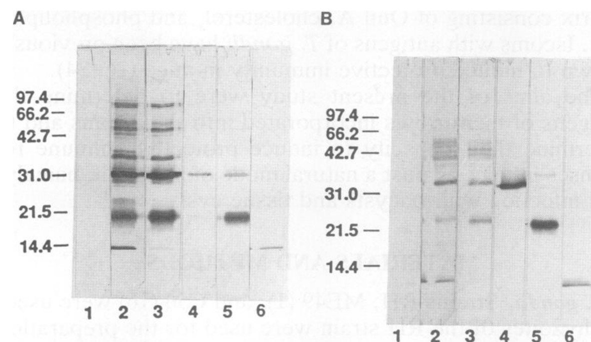


FIG. 3. Immunoblot analysis of toxoplasma iscom under nonreducing (A) and reducing (B) conditions. Lanes: 1, sera from nonimmunized control mice; 2, sera from mice chronically infected with *T. gondii*; 3, sera from mice immunized with toxoplasma iscom; 4, MAb 5D12 against the P30 antigen; 5, MAb 6D10 against the P22 antigen; 6, MAb 16B1 against a 6K antigen. Molecular weights (in thousands) are indicated to the left of each panel.

TABLE 1. Antibody and cell-mediated immune responses in mice immunized with iscom containing antigens of *T. gondii*[a]

Mouse group and no.	Result by:	
	DTH[b]	ELISA[c]
Immunized		
1	0.1	12,800
2	1.2	12,800
3	1.0	25,600
Control		
1	0.5	Neg
2	−0.1	Neg
3	0.1	Neg
Pos control[d]	ND	3,200
Neg control[e]	ND	Neg

[a] Abbreviations: DTH, delayed-type hypersensitivity; Neg, negative; Pos, positive; ND, not determined.
[b] Footpad assay. Each figure indicates the difference in thickness (in millimeters) between the footpad injected with antigen and the footpad injected with diluent.
[c] Highest dilutions with absorbances higher than those of negative controls.
[d] Pooled sera from chronically infected mice.
[e] Pooled sera from normal mice.

antigens which were detected by antibodies in sera of chronically infected mice, except for the 6K antigen (lanes 3 of Fig. 3). When a lysate of tachyzoites was used as antigen in a nonreducing gel, antibodies in sera of chronically infected mice recognized a large number of antigens, whereas antibodies in sera of mice immunized with iscoms recognized fewer antigens (Fig. 4). For two of three mice immunized with iscoms, the readings of the footpad swelling were higher than the readings for mice injected with matrix (Table 1).

Challenge experiments. Only one of nine immunized mice challenged with oocysts died during the observation period of 30 days, whereas eight of nine controls died between days 8 and 11 of infection. Also, when tachyzoites were used as the challenge, immunized mice survived longer than controls; seven of eight immunized mice died between days 12 and 25 of infection, while all eight control mice died between days 9 and 12. In contrast, challenge with cysts resulted in the deaths of six of eight immunized mice between days 10 and 21 and those of all controls between days 10 and 18. The conditional probability of survival for each different group is shown in Fig. 5. The differences between immunized and control mice were highly significant statistically ($P < 0.001$) for mice challenged with tachyzoites and oocysts but not for mice challenged with cysts ($P = 0.184$). However, immunization did not prevent infection, since *T. gondii* cysts were demonstrated in the brain of each surviving mouse.

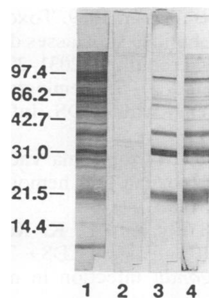


FIG. 4. Immunoblot with lysate of tachyzoites as antigen analyzed under nonreducing conditions. Lanes: 1, sera from mice chronically infected with *T. gondii*; 2, sera of normal mice; 3, sera from mice immunized with toxoplasma iscom once 3 weeks earlier; 4, sera from mice immunized with toxoplasma iscom twice, 7 and 1 week before collection. Molecular weights (in thousands) are indicated to the left.

DISCUSSION

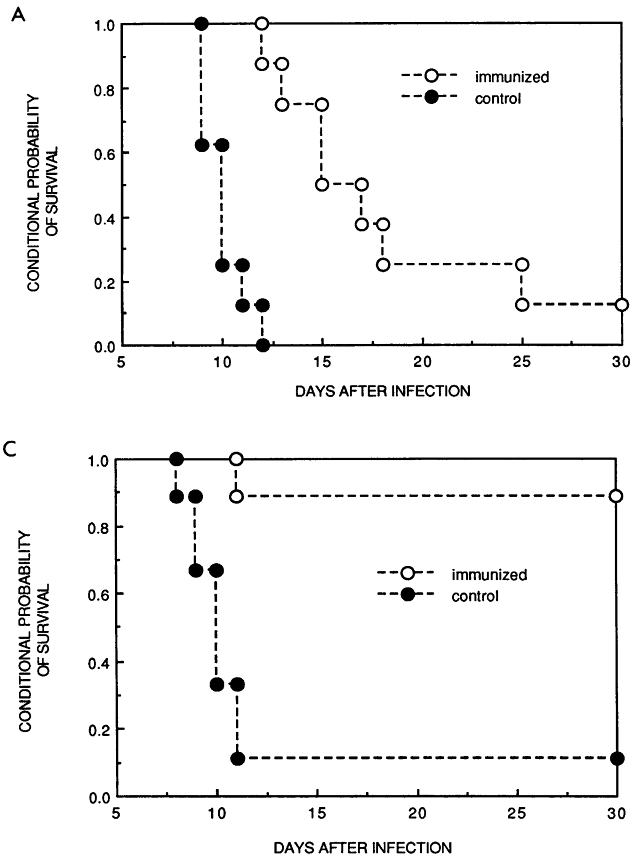
The iscom preparation used in this study contained a relatively large number of antigens of *T. gondii*. Because iscoms are formed by hydrophobic interaction and the procedure for their preparation selects for amphipathic molecules (24), it is likely that the four major iodinatable antigens of the cell surface membrane of *T. gondii* tachyzoites were present in the iscoms. These antigens are usually referred to as P43, P35, P30, and P22 (7, 10). Three of the antigens in the iscoms were identified as the P30, P22, and 6K antigens. The capacity of the P30 antigen to induce protective immunity in mice after incorporation into liposomes or the use of Quil A as adjuvant has been demonstrated previously (4, 15). The 6K antigen is mainly carbohydrate and is associated with the cell surface membrane of tachyzoites (29).

Immunity to *T. gondii* is by both humoral and cell-mediated immune responses. The latter response is regarded as the major component (9), and $CD8^+$ cytolytic T lymphocytes are major mediators (15, 31). Iscoms are potent inducers of humoral as well as cell-mediated immune responses (24, 26), including major histocompatibility complex class I restricted cytotoxic T cells (25, 32). In this study, immunization with iscoms resulted in high antibody titers and a delayed-type hypersensitivity reaction.

Immunization with iscoms resulted in marked protection against death due to an infection with oocysts. The protection against tachyzoites, expressed as a prolonged survival time, was significant and was in accordance with that in previous reports (26, 34).

The different results noted with mice challenged with oocysts and those challenged with cysts may have been due to differences in the challenge inoculum. For the oocyst challenge, a previously standardized inoculum which killed two out of three normal mice was used. This type of titration, however, could not be done accurately for the cyst challenge, possibly because of the wide variation in the numbers of bradyzoites within each cyst. Another factor that may have influenced the results was antigenic differences among tachyzoites, sporozoites, and bradyzoites. Since iscoms were prepared with tachyzoites, the immune response may have been directed against these forms. Both the P22 and P30 antigens, two of the major antigens of the iscom preparation, have been previously reported to be specifically expressed in tachyzoites but not in bradyzoites or sporozoites (16, 17). Furthermore, antigenic differences between *T. gondii* strains have been previously reported (35, 36). The fact that in this study one strain was used to prepare iscoms, whereas two others were used for challenge, also might have influenced the results.

Immunization resulted in increased survival, but it did not prevent disease and the colonization of *T. gondii* throughout the body. All mice became ill after being challenged, and cysts were found in the brains of survivors. Similar results were observed with pregnant sheep immunized with toxoplasma iscoms; after challenge infection, there was a reduc-



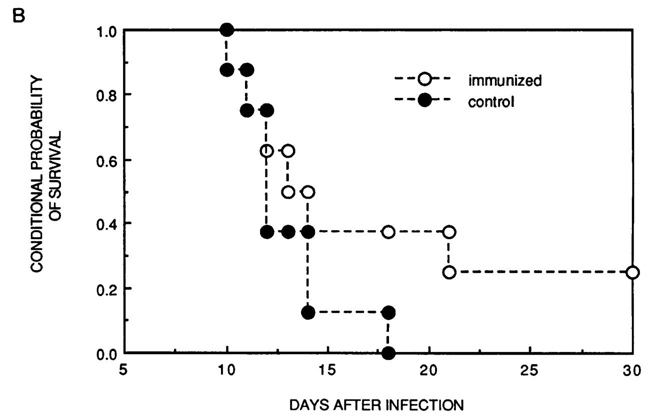


FIG. 5. Conditional probability of survival (Kaplan-Meier curves) for mice immunized with toxoplasma iscom and controls injected with iscom matrix and challenged with toxoplasma tachyzoites administered intraperitoneally (A) or cysts (B) or oocysts (C) administered orally.

tion in lamb mortality, but the lambs were born with precolostral antibodies, indicating intrauterine infection (6). If a vaccine against *T. gondii* is to be developed for the prevention of animal disease or to decrease the transmission of *T. gondii* from animal products, it will have to prevent parasitemia and the colonization of tissues. It is possible to achieve this by incorporation of other antigens into iscoms. Another strategy could be to try to stop the infection at the first barrier, the gastrointestinal mucosa, by inducing local immunity through oral administration of a vaccine with specific bradyzoite and sporozoite antigens. Various methods to incorporate proteins and peptides into iscoms have been previously developed (23, 30), and iscoms may generate protective immunity when given orally (25). These new technologies will be useful to further examine new strategies to prevent *T. gondii* infection.

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