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ORIGINAL PAPER

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Use of a recombinant antigen, SAG2, expressed as a glutathione-S-transferase fusion protein to immunize mice against *Toxoplasma gondii*

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Abstract The capacity of *Toxoplasma gondii* surface protein SAG2 to induce protective immunity against the parasite in mice was studied using recombinant SAG2 expressed as a glutathione-S-transferase (GST) fusion protein incorporated into immune stimulating complexes (iscoms). Immunization with the iscoms resulted in the production of antibodies recognizing SAG2 as well as GST. After oral challenge infection with *T. gondii* oocysts or tissue cysts, no protective effect was observed. On the contrary, mice immunized with fusion SAG2 or with GST iscoms died earlier than non-immunized control mice.

Toxoplasma gondii, an intracellular protozoan pathogen of humans and animals, has four major antigens in the surface membrane of the tachyzoite stage (Handman et al. 1980; Couvreur et al. 1988). These antigens are recognized by sera from naturally infected individuals (Santoro et al. 1985; Huskinson et al. 1989; Parmley et al. 1992), and at least one of them, SAG1 (P30), is involved in the process of host-cell invasion (Grimwood and Smith 1992; Mineo et al. 1993). They have been proposed as vaccine candidates, and it has been shown that immunization with SAG1 induces protective immunity in mice (Bülow and Boothroyd 1991; Kahn et al. 1991). We have previously shown that an experimental iscom vaccine, in which SAG1 and SAG2 are major components, induces protective immunity in mice against lethal challenge infection with *T. gondii* (Lundén et al. 1993). The gene encoding SAG2 has been cloned, sequenced, and expressed as recombinant proteins (Prince et al. 1990; Parmley et al. 1992). This enabled us to conduct the present investigation, in which a recombinant SAG2 glutathione-S-transferase (GST) fusion protein formulated into iscoms (immune stimulating complexes; Morein et al. 1984) was used to study the capacity of the recombinant antigen SAG2 to induce protective immunity.

Recombinant SAG2 fusion protein was produced as described by Parmley et al. (1992). In brief, a 438-bp gene fragment corresponding to the predicted amino acid sequence of the processed, native SAG2 antigen was produced by polymerase chain reaction. The fragment (designated v22) was spliced into the pGEX-2T vector and expressed in *Echerichia coli* as a GST fusion protein. After sonication of the culture a large proportion of the v22 fusion protein was in the soluble fraction and was purified by affinity to glutathione agarose. In addition, GST wild-type protein was prepared from bacterial cultures and purified by affinity to glutathione agarose.

Initial experiments using the dialysis method (Lundén et al. 1993) to incorporate the fusion protein into iscoms were unsuccessful, presumably due to the hydrophilic character of the protein. Therefore, we used the method of Morein et al. (1990) to incorporate essentially nonamphipathic proteins into iscoms by decreasing the pH to expose hydrophobic regions of the protein. The iscoms were prepared essentially as described by Sjölander et al. (1996). The protein was mixed with tritium-labeled cholesterol, phosphatidyl choline, and Quil A at a weight ratio of 1:1:1:5. The final concentrations of protein, Quil A, and MEGA-10 were 0.5 mg/ml, 0.25% (w/v), and 2% (w/v), respectively. After reduction of the pH to 2.5, the preparation was dialysed against phosphate-buffered saline (PBS) and the iscoms were isolated by centrifugation through a sucrose gradient. The protein content of the iscom preparation corresponded to 20–40% of the starting material, and precipitated unincorporated protein was demonstrated in lower fractions of the gradient.

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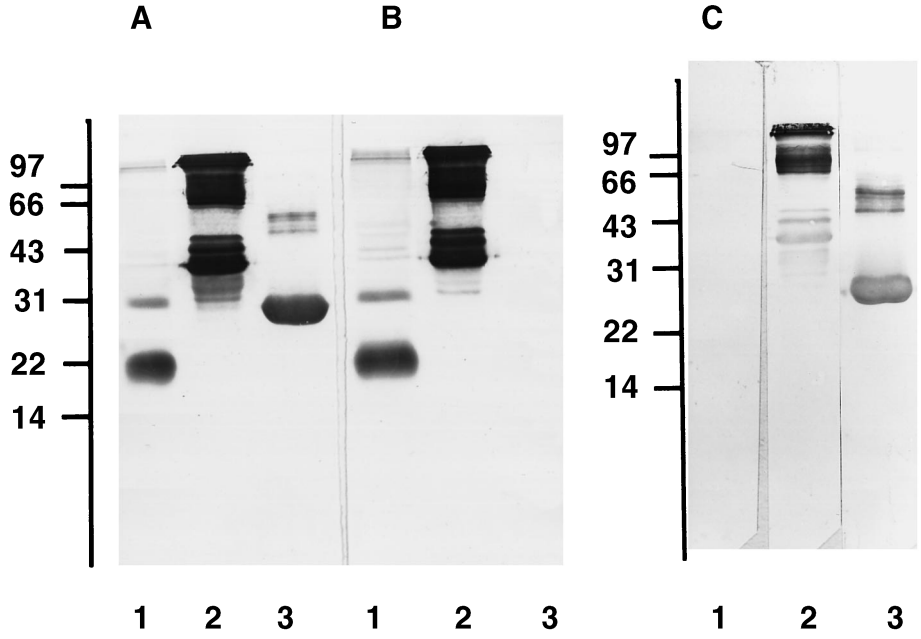

Fig. 1 Immunoblot analysis of antibody responses in mice immunized with either v22 iscoms (*A*) or GST iscoms (*C*) against nonreduced *Toxoplasma gondii* tachyzoite lysate (*lanes 1*), v22 fusion protein (*lanes 2*), or GST wild-type protein (*lanes 3*). Blots in *panel B* were probed with mAb 6D10 against the SAG2 (P22) antigen. Molecular weights (kDa) are indicated to the *left*

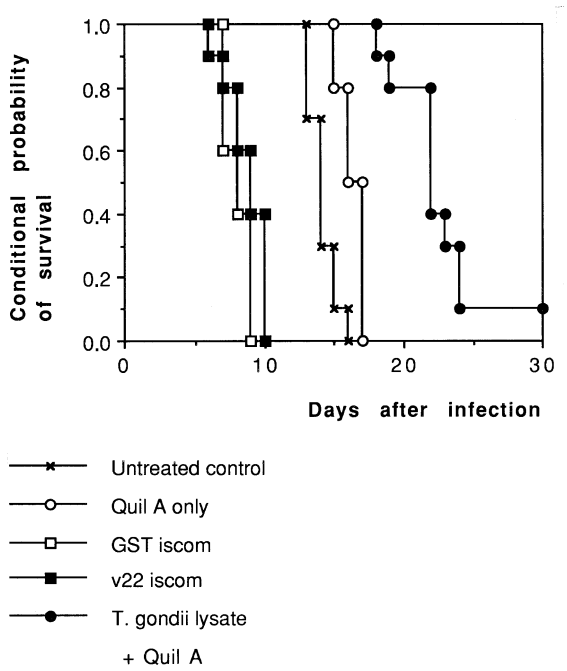

Fig. 2 Conditional probability of survival (Kaplan-Meier curves) for mice immunized with either v22 iscoms or GST iscoms and challenged orally with *T. gondii* oocysts. Control mice were immunized with *T. gondii* tachyzoite lysate mixed with Quil A, were injected with Quil A only, or were left untreated before the challenge infection

Because this protocol failed to incorporate GST into iscoms, this protein was coupled to preformed iscom matrix using *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) as the coupling reagent as described by Sjölander et al. (1996). In brief, matrix was prepared from tritium-labeled cholesterol and phosphatidyl ethanolamine (PE) and modifed for coupling by incubation with SPDP (5 × moles PE). SPDP was then reduced by incubation with dithiothreitol (DTT; 250 moles excess to SPDP), and excess DTT, SPDP, and low-molecular-weight reaction products were removed from the matrix preparation by gel filtration. GST was also modified with SPDP (25 mol SPDP/mol protein) before gel filtration. The eluted protein was then incubated with the activated and reduced matrix, and the iscoms were isolated by centrifugation through a sucrose gradient. Approximately 20% of the initial amount of protein was recovered in the iscoms. Some unincorporated protein was detected in the upper fractions of the gradient, but the major loss occurred during gel filtration of the SPDP-modified protein.

Swiss Webster adult female mice (Simonsen Laboratories, Gilroy, Calif.; kept in accordance with *Principles of laboratory animal care*, NIH publication 86–23, revised 1985) were divided into 2 groups of 20 mice, and the mice in each group were injected with either 0.65 μg v22 iscom or 0.8 μg GST iscom. These doses corresponded to approximately 10 μg Quil A. A group of 10 mice were given 1 μg *T. gondii* (RH strain) tachyzoite lysate mixed with 10 μg Quil A, another group of 10 mice received 10 μg Quil A only, and 10 additional mice served as untreated controls. There were five immunization episodes, with intervals comprising 4 weeks between the first and the second episode, 6 weeks between the second and the third episode, 2 weeks beween the third and the fourth episode, and 6 weeks between the fourth and the fifth episode. All immunizations were carried out subcutaneously in a volume of 0.2 ml.

After the fourth immunization, two mice immunized with v22 iscom and two immunized with GST iscom were bled and the antibody response was analyzed by immunoblotting and enzyme-linked immunosorbent as-

say (ELISA). For the ELISA (Araujo 1991), either the fusion protein, GST, or a lysate of RH-strain tachyzoites was used as the coating antigen at a concentration of 30 μg/ml. In the blotting analysis (Lundén et al. 1993), which was performed under nonreducing conditions, the monoclonal antibody 6D10 (Prince et al. 1990) was used as a marker for the SAG2 antigen. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Caltag, Burlingame, Calif.) was used as the secondary antibody in both assays.

At 1 week after the last immunization episode, mice immunized with v22 or GST iscoms were divided into two groups. Each mouse in one subgroup was inoculated orally with 3,000 sporulated oocysts of the Me49 strain of *T. gondii*, and each mouse in the other subgroup was inoculated orally with 10 tissue cysts of the C56 strain. These challenge inocula had previously been determined to be optimal. Control mice that had been immunized with *T. gondii* lysate together with Quil A, injected with Quil A alone, or left nonimmunized were inoculated orally with 3,000 oocysts of the Me49 strain per mouse. Mortality was recorded for up to 30 days after the challenge. Differences in the duration of survival between groups were assessed by the two-tailed Mann-Whitney *U*-test.

Immunization with each iscom preparation elicited a substantial antibody response. After four immunizations with v22 iscoms the antibody titers against the v22 fusion protein and wild-type GST exceeded 1:10,240. In tests against *T. gondii* antigens, titers of 1:2,560 were measured. Immunization with GST iscoms also induced the production of antibodies recognizing the v22 fusion protein and GST (titers 1:10,240 and 1:2,560, respectively), whereas antibodies reacting with *T. gondii* lysate were not detected in sera from these mice.

Immunoblotting analysis confirmed that sera from mice immunized with either v22 or GST iscoms recognized both of these proteins, whereas only sera from mice immunized with v22 iscoms reacted with *T. gondii* antigen. The band stained by the anti-v22 serum was in the same position as the band stained by the monoclonal antibody against the SAG2 antigen of *T. gondii* (Fig. 1).

Challenge of mice immunized with either v22 iscom or GST iscom with oocysts resulted in 100% mortality before day 10 of infection. No significant difference in the time to death was observed between these two groups ($P = 0.1736$). The nonimmunized controls died between days 13 and 16 after inoculation, and the mice injected with Quil A died between days 15 and 17 after infection. This prolongation of the time to death as compared with that of mice immunized with v22 or GST iscom was statistically significant ($P < 0.001$). Immunization with *T. gondii* lysate mixed with Quil A resulted in a significantly prolonged survival of 18–30 days ($P < 0.001$; Fig. 2). Challenge with tissue cysts gave similar results. All mice immunized with either v22 or GST iscom died between days 13 and 22 after infection, and there was no statistically significant difference between the two groups ($P = 0.2123$).

Increased susceptibility to infection after immunization with parasite antigens has been described for a number of parasite infections, including toxoplasmosis. Thus, immunization of mice with the major surface antigen of *T. gondii* (SAG1) together with Freund's adjuvant has resulted in increased susceptibility to challenge infection (Kasper et al. 1985). However, when SAG1 was incorporated into liposomes (Bülow and Boothroyd 1991) or mixed with Quil A (Kahn et al. 1991), remarkable protection was achieved. These contradicting results have been attributed to the adjuvants used, to the immunization protocols, and to the mouse strains used. That such factors play important roles has been shown in murine leishmaniasis. In this system it has been shown that the variation in resistance is due to the induction of a Th2 cell response instead of a protective Th1 cell response (Scott 1989; Locksley and Scott 1991). The reverse effect observed in the present study is not clearly explained by the choice of adjuvant. Results similar to those presented herein have also been obtained using Freund's incomplete adjuvant (S.F. Parmley, unpublished data). Iscoms, in general, elicit immune responses that are characterized by production of high levels of Th1-associated cytokines such as interleukin (IL)-2 and interferon gamma (IFN-γ). They also have the capacity to activate major histocompatibility complex (MHC) class-I-restricted cytotoxic T-cells (Morein et al. 1995). Thus, the immune response induced by iscoms is similar to that associated with protective immunity to *T. gondii* (Denkers et al. 1993). In a previous study it was shown that iscoms containing *T. gondii* antigens could induce protection against lethal challenge infection (Lundén et al. 1993).

Theoretically, the negative effect of immunizing with v22 could be due to immunosuppressive properties of the SAG2 antigen. However, since immunization with GST iscoms also resulted in increased susceptibility to infection, it seems more likely that the observed effect was caused by this protein. Other recombinant antigens expressed as GST fusion proteins have been shown to afford protective immunity, for example, in vaccination experiments against *Babesia bovis* infection in cattle (Wright et al. 1992) and against *Taenia ovis* in sheep (Johnson et al. 1989). However, although no serologic cross reaction between GST and *T. gondii* antigen was detected, GST, or the immune response to it, might in some way interact specifically with *T. gondii* or with mechanisms involved in resistance against the parasite. Since the GST expression system is widely used and has several practical advantages, it would be of great interest to investigate further this possible interaction with *T. gondii*.

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