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Intranasal Immunization with SAG1 Protein of *Toxoplasma gondii* in Association with Cholera Toxin Dramatically Reduces Development of Cerebral Cysts after Oral Infection

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SAG1 protein of *Toxoplasma gondii* was evaluated as a protective antigen in mucosal immunization with cholera toxin as an adjuvant. CBA/J mice intranasally immunized with a combination of SAG1 and cholera toxin exhibited significantly fewer cysts in the brain after oral infection with the 76K strain of *T. gondii* than control mice. This acquired protection lasted at least 5 months. Protected mice developed high levels of serum anti-SAG1 immunoglobulin G antibodies as well as an enhanced systemic cellular response, as assessed by the proliferation of splenocytes in response to SAG1 restimulation in vitro. This cellular proliferation was associated with an increase of interleukin-2 and interleukin-5 synthesis and with barely detectable gamma interferon production. Splenic immune T cells were shown to convey modest protection to recipients against development of brain cysts following oral infection with *T. gondii*. Significant production of anti-SAG1 immunoglobulin A was induced in intestinal secretions of protected mice. These results indicate that intranasal immunization with SAG1 and cholera toxin can induce mucosal and systemic immune responses and affords partial and long-lasting resistance against the establishment of chronic toxoplasmosis.

Toxoplasma gondii is a protozoan parasite responsible for toxoplasmosis in warm-blooded animals, including man. Infection commonly starts at the intestinal mucosal surface, spreads into the body, and is rapidly confined to some host tissues as chronic infection. Usually asymptomatic in hosts with intact immunity, toxoplasmosis may lead to severe or lethal damage when associated with immunosuppressive states such as AIDS, because of the reactivation of encysted parasites, or when transmitted to the fetus during pregnancy (15, 43). There is, therefore, obviously a need to develop effective immunoprophylaxis.

To date, the most promising vaccination procedures have been achieved by exposure to vaccinal strains of parasites lacking the ability to induce chronic infection (6). Such strains hold considerable interest for veterinary medicine, but the drawbacks linked to the use of live vaccine, such as the possible reversion to virulent forms, make this vaccine strategy undesirable for human application. Hence, efforts to develop a subunit vaccine composed of well-defined antigens are intensively encouraged.

Most of the candidate vaccines have been given parenterally in order to stimulate systemic immunity, which is known to be very efficient in the control of toxoplasmosis (1, 3, 10, 12, 32). In view of the natural penetration by *T. gondii* of the intestinal tract, induction of a first line of local defense would also be of great interest. Indeed, a great deal of evidence indicating that specific secretory immunoglobulins A (sIgA) display a protective role against many pathogens which colonize mucosal tissues or invade the host organism by crossing mucous membranes has been accumulated (36, 44, 52). Cellular immune mechanisms, such as cytotoxicity elicited by intraepithelial lymphocytes (IEL), may also contribute to intestinal protection (8). As parenteral procedures rarely induce a mucosal response, direct immunization of mucosal surfaces appears to be the most suitable way both to provide local immunity and, in most cases, to evoke a concomitant systemic response. Oral administration is the most commonly reported procedure. However, the gastrointestinal environment is hostile to the antigen, and oral vaccination requires administration of a massive amount of antigen. In view of the existence of a common mucosal immune system, the nasal route appears to be an interesting alternative vaccination route. Intranasal (i.n.) immunization has already been proven to generate specific antibody responses, extending to numerous external secretions and to serum, and also to confer protection (18, 19, 47, 54). It is convenient and has the advantage over oral administration that degradation and dilution of the antigen are less. Yet the administration of soluble antigens alone frequently fails to stimulate immune responses and may result in establishment of oral tolerance. Cholera toxin (CT), known to improve immunogenicity of antigens given perorally (21) and i.n. (47), is able to overcome these problems (13, 48).

Several studies have shown the value of the 30-kDa protein (SAG1) of *T. gondii* as a protective antigen following parenteral immunization (5, 10, 28). This protein is the major surface antigen of the proliferative form of *T. gondii*. Recent data indicate that it is involved in the process of invasion (37, 38). Moreover, it is one of the main target proteins recognized by sIgA in animal and human models of toxoplasmosis (7, 34). In this study, we have evaluated the immunogenicity of SAG1 in association with CT when given i.n. to CBA/J mice. We have established the time course of the antibody response in serum and gut washes. We have also studied the proliferation and cytokine patterns of immune spleen cells after specific restimulation in vitro. CBA/J mice are markedly resistant to acute toxoplasmosis infection, as they have very low mortality rates, but they are susceptible to cyst formation and development of toxoplasmic encephalitis in chronic infection. As a protective criterion, we chose to evaluate the decrease in brain cyst load 1 month after an oral challenge, the number of brain cysts

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being one of the important factors that determine development of toxoplasmic encephalitis (4, 46).

MATERIALS AND METHODS

Animals. Female CBA/J mice aged 8 to 10 weeks (IFFA Credo) were used throughout these experiments.

Parasites. Tachyzoites of the RH strain of *T. gondii* were harvested from peritoneal fluids of Swiss OF1 mice intraperitoneally infected 3 to 4 days earlier. Cysts of the 76K strain of *T. gondii* were obtained from the brains of orally infected Swiss OF1 mice.

Antigens. The SAG1 protein was purified by immunoaffinity according to a method described by Kasper et al. (24), with some modifications. The IgG anti-SAG1 monoclonal antibody (MAb) 1E5, described elsewhere (9), was coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions (Pharmacia). RH strain tachyzoites were lysed for 1 h at 4°C at a concentration of 2×10^8 parasites per ml in buffer A (50 mM Tris-HCl [pH 8.3], 0.5% Nonidet P-40, 150 mM NaCl, 2 mM EDTA) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 0.02 mg of aprotinin per ml). After centrifugation at 40,000 × *g* for 1 h, the supernatant was allowed to circulate at least four times on the 1E5 column previously rinsed with 10 volumes of buffer A. The column was subsequently washed with 10 volumes of 50 mM Tris-HCl (pH 8.3)–0.5% Nonidet P-40, 5 volumes of this wash buffer supplemented with 1 M NaCl and, finally, 5 volumes of 50 mM Tris-HCl (pH 8.3)–1% octyl-β-D-glucopyranoside. The SAG1 protein was eluted at room temperature with 2.5 volumes of 100 mM diethylamine-HCl (pH 11.5)–1% octyl-β-D-glucopyranoside and rapidly neutralized with 1 M Tris-HCl, pH 7.4. After concentration and exhaustive dialysis against 0.1% octyl-β-D-glucopyranoside in water, the purity of the preparation was determined by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis followed by either a silver staining technique or a transfer to a nitrocellulose paper subsequently probed with the MAb 1E5. The quantification of SAG1 was performed with a bicinchoninic acid protein assay reagent kit (Pierce). The protein was then lyophilized.

Immunization and harvesting of biologic fluids. For all experiments, mice received two equivalent doses i.n. at 28-day intervals of 10 μg of SAG1 alone, 10 μg of SAG1 plus 0.5 μg of CT (Sigma), 0.5 μg of CT alone, or buffer. When indicated, mice were given a third dose, identical to the first two, 4 months after the second immunization. Each immunogenic dose was diluted to a final volume of 10 μl in 0.1 M sodium bicarbonate and was instilled into the nostrils with a micropipette.

Blood was obtained by retroorbital puncture just before vaccination and at various times after each immunization, and intestinal secretions were collected by using the procedure previously described by Elson et al. (14). All samples were kept frozen (−20°C) until assayed for antibody activity.

Measurement of antibody responses. Serum IgG and intestinal IgA antibody responses to SAG1 and CT were measured by enzyme-linked immunosorbent assay (ELISA). Flat-bottomed wells of microdilution plates (Nunc) were coated overnight at 4°C with SAG1 or CT at 5 μg/ml in 50 mM sodium carbonate buffer, pH 9.6. After the plates were washed in 10 mM phosphate-buffered saline, pH 7.4, (PBS), nonspecific binding sites were blocked with PBS containing 4% bovine serum albumin (PBS–BSA 4%). Twofold serial dilutions in PBS of either test or preimmune samples were added to the wells and incubated for 1 h at 37°C. The plates were washed with 0.05% Tween 20 in PBS (PBS–T 0.05%) to remove unbound antibodies, and a goat anti-mouse Ig alkaline phosphatase conjugate was applied to each well for 2 h at 37°C. The conjugate was an anti-alpha chain of mouse IgA or an anti-gamma chain of mouse IgG (Sigma) as appropriate and was used in both cases at 1/1,000 in PBS–BSA 4%. After being washed in PBS–T 0.05%, the plates were developed with *p*-nitrophenylphosphate at 1 mg/ml in 1 M diethanolamine buffer, pH 9.8. The reaction was stopped 20 and 60 min later for IgG and IgA determination, respectively, by the addition of 3 N NaOH, and the optical density of each sample was read at 405 nm in a Titertek Multiskan ELISA reader (Flow Laboratories, Inc.). Titers were calculated by endpoint titration as the reciprocal of the highest test sample dilution giving an absorbance of 0.150 U over that of the preimmune sample at the same dilution.

Total IgA concentrations in secretions were evaluated by ELISA as described above, except that the plates were coated with a 1/500 dilution of goat anti-mouse alpha chain IgA (Sigma) and a mouse myeloma IgA (Sigma) was used to prepare a standard curve.

Results for each test group are expressed as the means of $\log_2$ titers ± standard deviations (SD) in sera and as the means ± standard errors of the means of specific IgA titers in relation to total IgA quantity to correct for variations in the IgA concentrations of gut washes.

IgG subclass determination. Analysis of serum anti-SAG1 and anti-CT IgG subclass was performed by ELISA as described above, except that sera were added to the plates at a single 1/2,000 dilution and alkaline phosphatase-conjugated anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (Cappel, West Chester, Pa.) were used at 1/500 in PBS–BSA 4%. The results are expressed as A_{405} values.

Measurement of antigen-specific proliferative response. Spleens were harvested and pressed through a stainless steel mesh. Single-cell suspensions were obtained by filtration through nylon mesh to remove any tissue debris. After the elimination of erythrocytes by hypotonic shock, the cells were resuspended in RPMI 1640 medium (GIBCO) supplemented with 5% fetal calf serum, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (25 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), β-mercaptoethanol (5×10^{-5} M), and gentamicin (50 μg/ml) and seeded in quadruplicate in flat-bottomed 96-well microtiter plates (Costar) at 5×10^5 cells per well in 200 μl of culture medium alone or with various concentrations of SAG1 or CT or 5 μg of concanavalin A (ConA) per ml. The plates were incubated for 3 days in 5% CO_2 at 37°C and pulsed with 1 μCi of [^{3}H]thymidine per well for an additional 18 h. Finally, the cells were collected onto glass fiber filters, and the radioactivity (counts per minute) was measured by liquid scintillation counting. The results are expressed as Δcpm, corresponding to the difference in mean counts per minute of stimulated cells and that of unstimulated cells.

Cytokine quantification. Spleen cells were cultured in 24-well plates at a density of 5×10^6 cells per well in 1 ml of culture medium alone or with either 1 μg of SAG1 per ml, 0.5 μg of CT per ml, or 5 μg of ConA per ml. Free-cell culture supernatants were harvested at intervals between 24 h and 4 days and assessed for interleukin-2 (IL-2), IL-4, IL-5, and gamma interferon (IFN-γ) activities.

IFN-γ, IL-4, and IL-5 concentrations were evaluated by sandwich ELISA. IFN-γ was assayed by using a hamster anti-mouse IFN-γ MAb at 4 μg/ml (Genzyme, Boston, Mass.) as the capture antibody, a polyclonal rabbit anti-mouse IFN-γ antibody at 1/2,000 (Immunogenex, Los Angeles, Calif.) as the second antibody, and an alkaline phosphatase-conjugated polyclonal goat anti-rabbit Ig antibody at 1/1,000 (ICN, Costa Mesa, Calif.) as the developing antibody. IL-4 was quantified with a rat anti-mouse IL-4 MAb at 1 μg/ml (Pharmingen, San Diego, Calif.), a biotinylated rat anti-mouse IL-4 at 2 μg/ml (Pharmingen), and alkaline phosphatase-conjugated streptavidin at 1/1,000 (Amersham, Little Chalfont, United Kingdom). IL-5 production was evaluated with a rat anti-mouse IL-5 MAb at 1 μg/ml (Pharmingen), a biotinylated rat anti-mouse IL-5 at 2 μg/ml (Pharmingen), and alkaline phosphatase-conjugated streptavidin at 1/1,000 (Amersham). Cytokine concentrations were determined by reference to standard curves constructed with fixed amounts of mouse recombinant IFN-γ (Genzyme), IL-4 (Pharmingen), and IL-5 (Pharmingen). Briefly, 50 μl (each) of serially diluted recombinant cytokines and samples to be tested was added to precoated and preblocked plates for 1 or 2 h. Similar volumes of each second and developing antibody were added for 1 h in PBS–BSA 4%, and multiple washings were performed between each incubation.

Supernatants were monitored for IL-2 with a commercial ELISA kit (Genzyme), except that the substrate solution used was 0.1% H_2O_2–0.1% orthophenylenediamine in citrate (0.1 M)–phosphate (0.1 M) buffer, pH 4.5. The reaction was read at 492 nm.

The sensitivity limits for the different assays were as follows: IL-2, 15 pg/ml; IL-4, 10 pg/ml; IL-5, 15 pg/ml; IFN-γ, 0.8 ng/ml.

Adoptive transfer of splenic T lymphocytes. Single-cell suspensions from spleens were prepared as described above and were enriched for T cells by passage through nylon wool columns. The proportion of T lymphocytes in the nonadherent cell population was estimated by FACScan analysis to be 88%. Isolated cells (5×10^7) were injected into a tail vein of each recipient mouse 1 day before toxoplasma infection.

Measurement of resistance to challenge infection. CBA/J mice were infected orally with 100 cysts of the 76K strain obtained from the brains of chronically infected mice. One month after challenge, mice were sacrificed and brains were recovered. Each brain was homogenized in 5 ml of PBS with a pestle and mortar. The mean number of cysts per brain was determined microscopically by counting 6 samples (10 μl each) of each homogenate. The results are expressed as means ± SD for each group.

Statistical analysis. Levels of significance of the differences between groups were determined by the Student *t* test.

RESULTS

Protection against oral challenge. Mice received two i.n. doses of the SAG1 antigen alone or together with CT. To compare the efficiencies of the regimens to confer protection against toxoplasmosis infection, brains of immunized mice were checked for the number of cysts following peroral challenge (Fig. 1). Control groups of mice received CT or buffer only. Mice given SAG1 plus CT exhibited strong resistance to cyst formation in comparison with buffer-treated and CT-immunized groups, showing 85% fewer cysts in the brain. This protection was highly significant ($P < 0.01$). The delivery of SAG1 alone slightly reduced cerebral cyst numbers but failed to provide significant protection compared with the results obtained with mice inoculated with buffer only ($P > 0.05$).

We next examined the ability of i.n. vaccination to achieve long-term protection. Groups of mice primed with two i.n. immunizations were given a booster dose 4 months later by the same route. Five months after the boost, the mice were orally

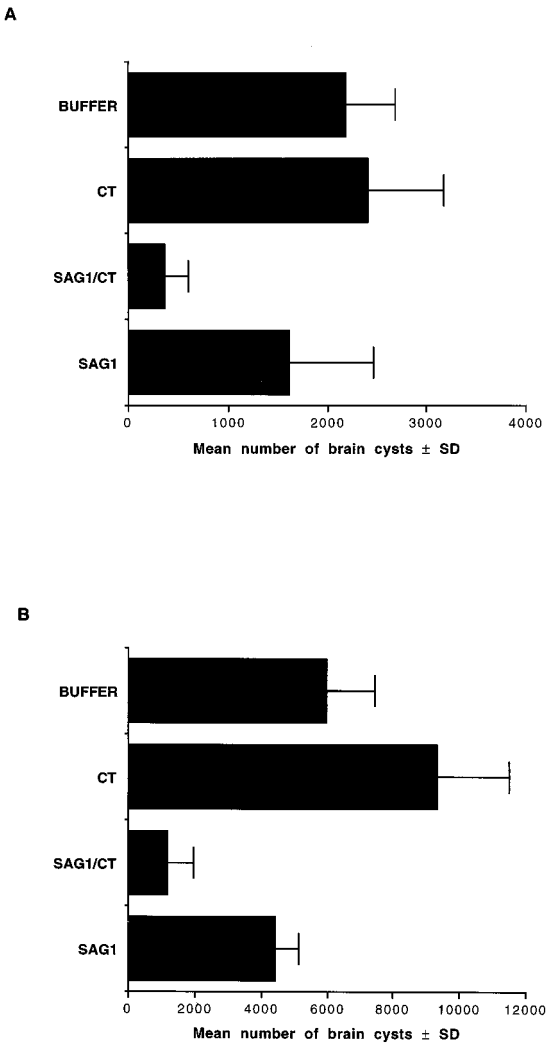


FIG. 1. Assay for protection against oral challenge. (A) Mice given two i.n. doses of SAG1 alone or SAG1 plus CT were orally infected with 100 cysts of the *T. gondii* 76K strain 13 days later. Brain parasite load was evaluated 1 month after infection. Animals receiving only CT or buffer served as controls. Values are means ± SD of two similar experiments performed with groups of 12 mice. (B) In a separate experiment with four mice per group, mice received a single booster dose 4 months after the first two immunizations. Five months later, they were orally challenged, and brain cycts were enumerated 1 month after challenge. The results are representative of two experiments.

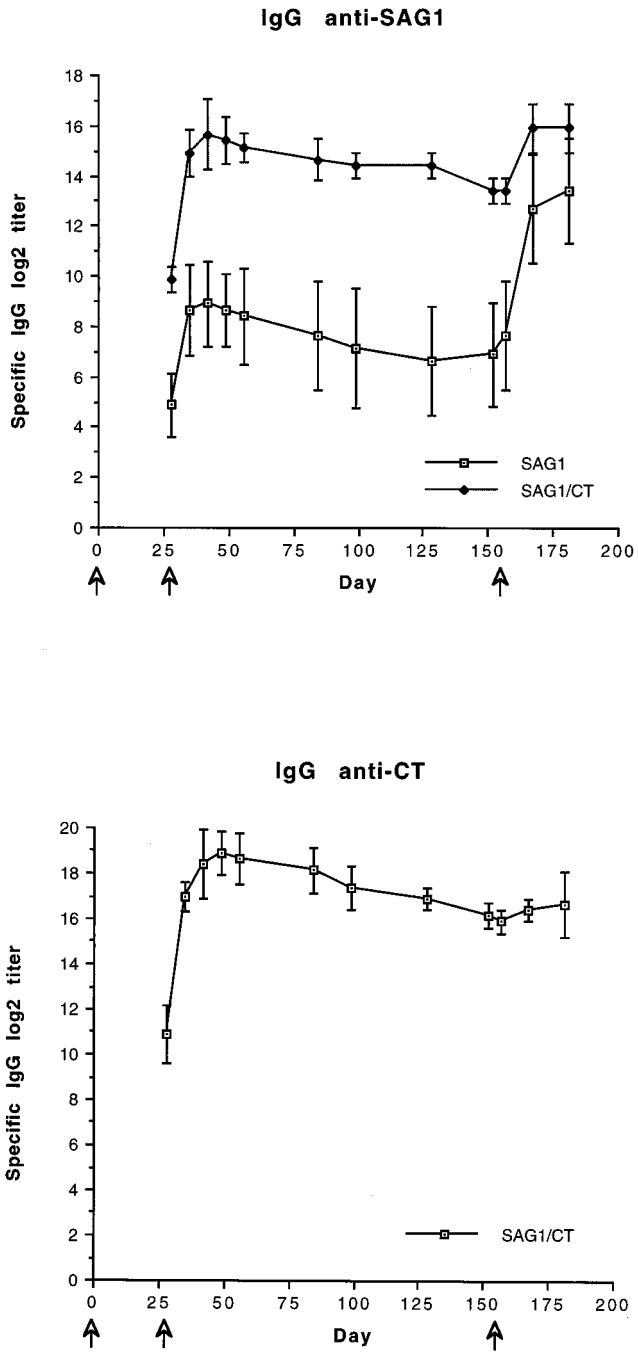


FIG. 2. Time course of serum IgG antibody responses to SAG1 and CT in groups of four mice immunized i.n. on three occasions (arrows) with either SAG1 plus CT or SAG1 alone. Titers are expressed as the reciprocal $\log_2$ of the endpoint dilution. Results are the means of titers ± SD. Only responding groups are depicted. Results from one of two similar experiments are shown.

challenged. Cerebral parasite burden from the SAG1-CT group was still dramatically low compared with that for mice treated with buffer or CT only, showing 80% fewer cerebral tissue cysts ($P < 0.01$). Again, the difference between nonimmunized and SAG1 groups was not significant. It must be emphasized that the general cyst burden was higher in mice from the long-lasting-protection experiments. We suspect that this was related to the ages of the mice rather than to modification of the virulence of cysts used for the challenge.

These results confirmed both the protective role of SAG1 antigen and the potent i.n. adjuvanticity of CT.

Antibody responses. To investigate the duration and immunological memory of systemic and mucosal antibody responses induced by i.n. vaccination, mice from the long-term-protection experiments were assayed for serum IgG and intestinal IgA antibodies to both SAG1 and CT prior to challenge. Sera and intestinal secretions were harvested at different times after each inoculation.

During the observation period preceding the third immunization, mice given SAG1 with CT displayed an average 100-times-greater anti-SAG1 activity in sera than those administered SAG1 alone (Fig. 2). In both groups, a specific IgG antibody response was generated 4 weeks after the first inoculation and secondary immunization resulted in typical pat-

terns of memory response with a fast and pronounced increase in antibody levels to the antigen which persisted over time. Upon the third vaccination, anti-SAG1 titers from SAG1-immunized mice tended to reach those of mice coadministered SAG1 and CT. The time course of serum anti-CT IgG antibody production followed the kinetic response to SAG1, exhibiting a considerable enhancement of IgG level after the second vaccination, which was maintained for the remainder of the experiment. No systemic response to SAG1 was manifested in the CT group of mice, indicating that no polyclonal antibody activity could be attributed to the adjuvant (data not shown).

While only one of four mice given SAG1 alone exhibited extremely low and short-lived gut levels of anti-SAG1 IgA (data not shown), all mice subjected to the SAG1-CT-immunizing regimen mounted an appreciable intestinal IgA response to SAG1, but with wide interindividual variation (Fig. 3). No response could be detected after the first immunization, and two immunizations were required to detect antibodies to SAG1 in intestinal secretions. The peak production occurred soon after the secondary inoculation and rapidly decreased over time, yet specific IgA production remained demonstrable for 4 months prior to the third immunization. After this, although an increase in the intestinal response to SAG1 could be registered, it did not reach the level of that observed after the second immunization. Previous investigators have suggested that repeated i.n. applications of CT could induce a nonspecific increase in sIgA levels and obscure the specific secretory response (54), but in our case, total IgA levels were comparable after the second and the third immunizations whereas IgA titers specific to SAG1 dropped. i.n. inoculation of SAG1 with CT also generated an intestinal response to CT. Specific activity was already detectable after the first immunization but was very low compared with anti-CT levels measured following the second vaccination. As for antibody production to SAG1, no greater response to CT was demonstrable after the third vaccination.

Cellular proliferative response. Sixteen days after the second immunization, single-cell preparations from spleens were prepared to assess the systemic proliferative responses to either SAG1 or CT.

Although only weak proliferation of cells from SAG1-immunized mice was recorded in response to the highest in vitro SAG1 concentration, splenocytes from mice given a combination of SAG1 and CT showed a dose-dependent proliferative response to SAG1 restimulation (Fig. 4). This proliferation was specific, since no cellular expansion of CT-primed or nonimmune splenocytes was found when the cells were cultured with SAG1. Furthermore, in vitro challenge with CT evoked specific proliferation of cells from mice treated with CT alone or in the presence of SAG1. These results demonstrate that i.n. administration of a mixture of SAG1 and CT triggered a systemic cell-mediated immunity both to SAG1 and to the adjuvant.

Cytokine production. The supernatants of cultured immune splenocytes were evaluated for the production of IFN-γ, IL-2, IL-4, and IL-5 in response to both SAG1 and CT (Table 1). SAG1-CT-primed splenocytes responded to SAG1 stimulation by an increased production of IL-2 and IL-5 compared with that for unstimulated splenocytes or SAG1-triggered splenocytes from naive mice. Surprisingly, only a poor IFN-γ response to restimulation by SAG1 in vitro could be detected. Similarly, CT-immune splenocytes from mice sensitized either with CT alone or with CT plus SAG1 gave rise to a high release of IL-2 and a concomitant production of IL-5 to CT stimulation in vitro whereas only low IFN-γ activity was evidenced. No specific release of IL-4 from any culture supernatant was demonstrable. Also, SAG1-primed splenocytes were found to produce undetectable amounts of all cytokines tested. As a further assessment of the relative contribution of Th1- or Th2-type cytokines, we analyzed serum IgG subclass anti-SAG1 and anti-CT responses. Serum samples were taken on the day of spleen recovery. As shown in Fig. 5, similar patterns of IgG responses to the antigen and the adjuvant were observed with SAG1-CT- and CT-immunized groups, with a clear dominance of IgG2a over IgG1 antibodies, indicative of IFN-γ activity. No

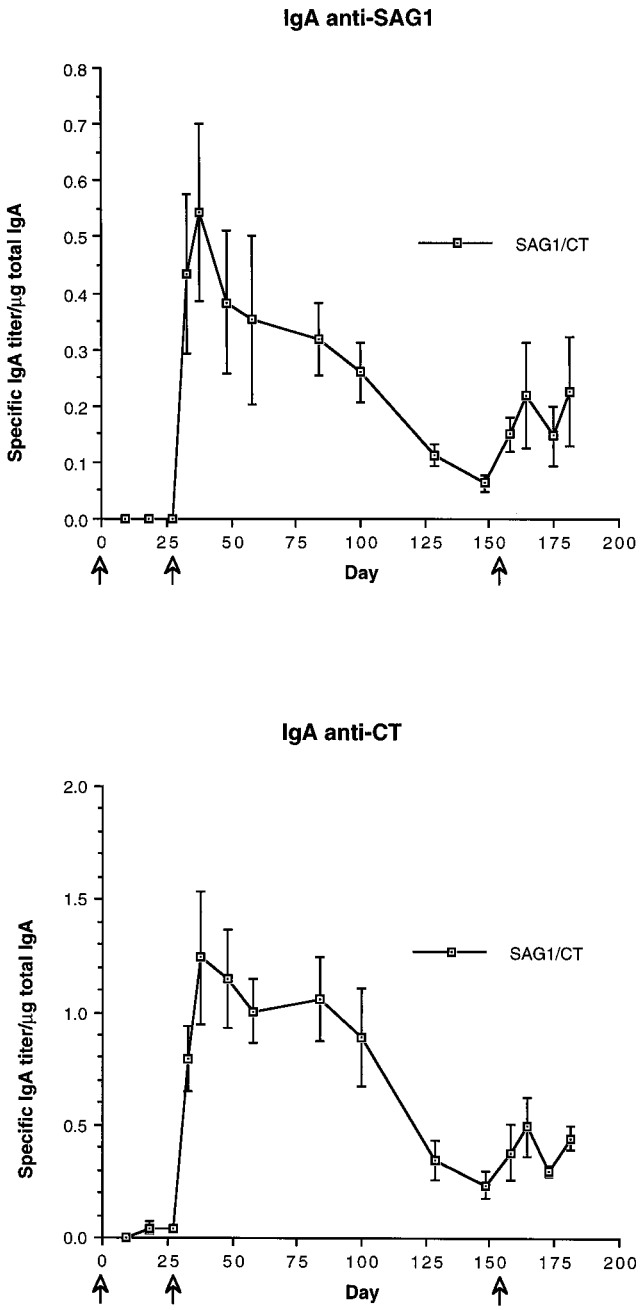


FIG. 3. Time course of intestinal IgA antibody responses to SAG1 and CT in groups of four mice immunized i.n. on three occasions (arrows) with either SAG1 plus CT or SAG1 alone. Titers are expressed as the reciprocal of the endpoint dilution. Results are the means ± standard errors of the mean of the titers in relation to the total IgA levels. Only responding groups are depicted. Results from one of two similar experiments are shown.

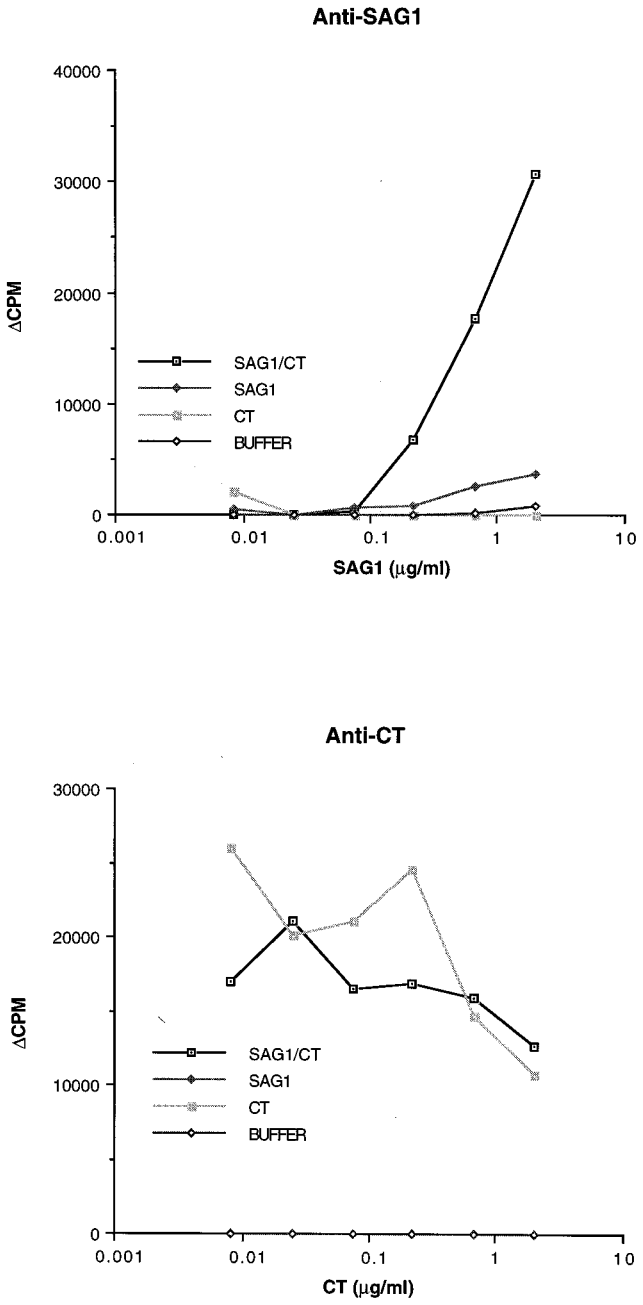


FIG. 4. In vitro proliferation of splenocytes from mice given two i.n. doses of SAG1 with or without CT, CT alone, or buffer in response to SAG1 or CT restimulation. Results are expressed as Δcpm, calculated by subtracting the mean counts per minute of unstimulated cells (<10,000 cpm) from the mean counts per minute of stimulated cells. The results are representative of at least four experiments.

IgG3 was detectable in any group; conversely, high levels of IgG2b were detected.

Adoptive transfer of immune T lymphocytes. To investigate the contribution of the systemic T-cell response to the reduction of brain cyst development, T lymphocytes from spleens of SAG1-CT-immunized mice were isolated 2 weeks after the second immunization and intravenously transferred to naive syngeneic mice (5×10^7 T lymphocytes per mouse). Control mice received either an equivalent number of T lymphocytes from nonimmunized donors or no cells. One day later, all recipients were subjected to an oral challenge with 100 cysts of strain 76K, and 1 month later they were assessed for the presence of cerebral tissue cysts.

T lymphocytes obtained from vaccinated mice conveyed a significant but low level of protection to the recipients compared with that for naive T lymphocytes ($P < 0.01$). No difference was seen between groups of mice given splenic T cells from a naive donor and those having received no lymphoid cells (Fig. 6). Cytokine assay of adoptively transferred T lymphocytes, cocultured with naive splenocytes treated with mitomycin to serve as antigen-presenting cells, ascertained the specific production of IL-2 and IL-5 to SAG1 restimulation, whereas the production of IFN-γ became barely detectable (not shown).

DISCUSSION

The development of prophylactic means capable of inducing both local and systemic immunities is a valuable approach for use against invasive injurious microorganisms such as *T. gondii*. Mucosal rather than conventional delivery of vaccine candidates has been found to be the most relevant vaccination strategy to achieve this dual goal under conditions of enhancement of the immunogenicity of nonreplicating antigens and avoidance of oral tolerance. The use of CT as an adjuvant completely fulfills these requirements.

Our group has therefore previously shown that oral administration of a *T. gondii* sonicate in association with CT to C57BL/6 mice provided 50% survival from a lethal challenge (2). The need for vaccines to have a controlled composition prompted us to repeat the mucosal vaccination trials with well-defined antigens. In the present study, we show that i.n. delivery of the purified major surface tachyzoite protein, SAG1, together with CT, to CBA/J mice effectively prevents brain cyst formation following oral challenge with the 76K strain of *T. gondii*. Systemic and mucosal antibody responses as well as systemic cellular immunity to both SAG1 and CT were shown to be mounted in these protected mice.

Recently, Brown et al. (4) clearly demonstrated that the presence of the L^d gene in mice is correlated with resistance to brain cyst formation. The putative mechanism of this resistance might be better control of the parasitemia stage through the early stimulation of $CD8^+$ T cells by protective peptides of *T. gondii* presented in the context of the *H-2* L^d molecule. In our study, SAG1-CT-immunized CBA/J mice (*H-2*^{k}), despite being a cyst-susceptible strain, effectively downregulated the development of brain cysts upon oral infection. Since brain tissue cyst bradyzoites were shown not to express SAG1, the most likely explanation for SAG1-induced protective immunization is also a limitation of tachyzoites reaching the brain. This protection was extended up to 5 months after the end of immunization. This finding provides further support for previous observations that SAG1 protein is a relevant vaccine candidate for the induction of protective immunity.

Different experimental methods demonstrated that specific sIgA plays an important role in vivo in controlling several mucosally acquired infections (44, 52). Direct evidence for a role of anti-*T. gondii* intestinal IgA in the prevention of oral acquisition of toxoplasmosis has not yet been established; however, the ability of milk IgA from women chronically infected with *T. gondii* to reduce infection of cultured enterocytes lends support to this view (34). The production of intestinal sIgA to SAG1, which has been found to be involved in the binding step of *T. gondii* to host cells (37), could thus be of great impor-

TABLE 1. Cytokine production of splenocytes from mice immunized i.n. with SAG1 alone or in association with CT[a]

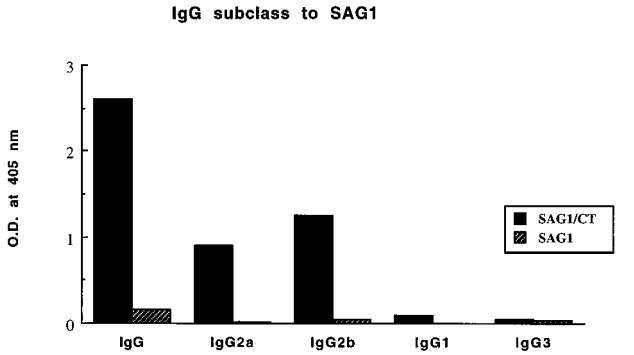
Immunization regimen	Amt of IL-2 (pg/ml)				Amt of IFN-γ (ng/ml)				Amt of IL-5 (pg/ml)			
	SAG1	CT	No Ag[b]	ConA	SAG1	CT	No Ag	ConA	SAG1	CT	No Ag	ConA
SAG1-CT	464	932	44	1,096	4.6	3.4	1.8	26.7	274	1,080	<31	291
SAG1	64	40	88	1,033	<0.8	<0.8	1.7	17.8	<31	<31	<31	274
CT	88	1,104	84	1,204	<0.8	1.4	1.5	28.9	<31	937	<31	343
Buffer	85	53	76	800	<0.8	<0.8	0.9	8.5	<31	<31	<31	189

[a] Mice were given two i.n. doses of SAG1 with or without CT, CT alone, or buffer. Splenocytes recovered 16 days after the last immunizations were cultured with SAG1, CT, or ConA. The culture supernatants were examined for cytokine production. Results for peak levels of cytokine content in the supernatant are given. Values for IL-2 are taken from day 2 of culturing, values for IFN-γ are taken from day 3, and values for IL-5 are taken from day 4. Results from one of four similar experiments are shown.

[b] Ag, antigen.

tance. Our results indicated a significant production of gut IgA to SAG1 in mice immunized with SAG1 and CT, whereas mice having received similar doses of antigen alone failed to develop a demonstrable IgA response in the gut and to acquire protection. The intestinal IgA response from protected mice gradually declined over time, but it persisted at a low level over several months. This finding is in agreement with numerous studies reporting rather short-lived local antibody response after either naturally acquired infection or mucosal vaccination (26, 27) and probably indicates that after lodging in effector sites, antigen-sensitive cells do not continue to secrete large amounts of antibody in the absence of further local stimulation (33). When mice received a single booster dose 4 months after the first two immunizations, only a modest intestinal immune response to SAG1 and CT could be observed. Though these booster responses were at low levels, it is tempting to suggest that they represent anamnestic responses, since just a single dose was sufficient to enhance specific IgA responses over the background at this time whereas initially the first immunization generated very little intestinal IgA to CT and no detectable response against SAG1. Several studies clearly demonstrated the existence of an anamnestic mucosal IgA response to protein antigens (40, 42, 47, 53). Yet Wu and Russell (54) recently reported the absence of a standard anamnestic salivary IgA response after either i.n. or intragastric vaccination. It seems that successful generation and maintenance of memory mucosal cells are highly dependent on appropriately timed doses of the vaccine, as judged from the series of several priming immunizations usually used by investigators to generate long-lasting mucosal memory (33, 49). Thus, in our study, repeated immunizations might have evoked stronger disseminated memory. Moreover, our results do not preclude the possibility that the intestinal lamina propria of i.n. primed mice could be immunologically competent to mount an early secondary response at the time of infection by sessile memory cells activated in situ (41).

On the other hand, i.n. immunization with a SAG1-and-CT mixture is clearly effective to prime mice for serum antibody memory responses. This finding contrasts with the above-described low-level intestinal memory responses and could sug-



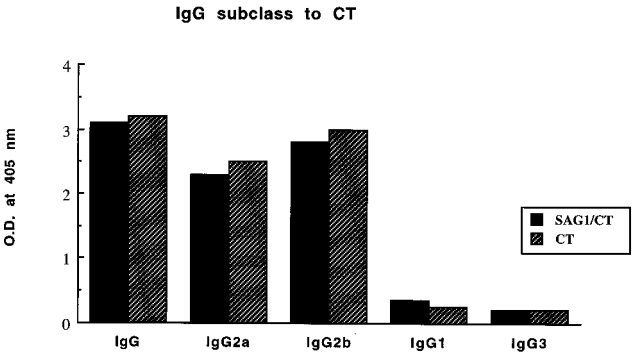


FIG. 5. Serum IgG subclass profile in mice twice immunized i.n. with SAG1 or CT or both. O.D., optical density.

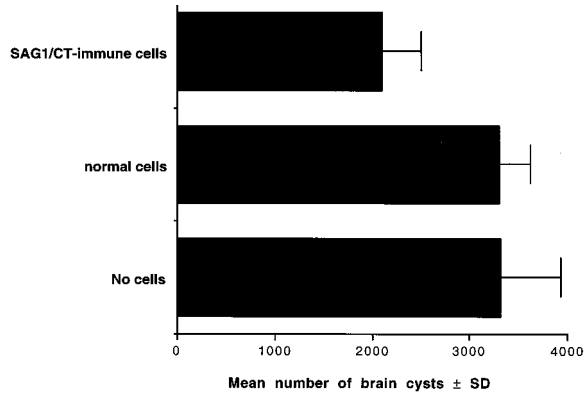


FIG. 6. Number of brain cysts in nontransferred mice and mice adoptively transferred with splenic T lymphocytes from SAG1-CT-immunized or nonimmunized mice and further challenged with 100 cysts of the *T. gondii* 76K strain. Results from one of two similar experiments are shown.

gest that the SAG1 antigen gains access to different inductive sites to generate local and systemic responses. The fact that the first immunization was sufficient to evoke high antigen-specific IgG titers in sera along with almost undetectable amounts of intestinal IgA seems to support this hypothesis. It is generally accepted that upon i.n. inoculation, antigens reaching the superficial lymph nodes after crossing the nasal mucosa preferentially induce a systemic response, whereas antigens reaching the nose-associated lymphoid tissue generate a clearer sIgA response (31). As the B subunit of CT was shown to increase the transepithelial influx of coadministered antigen into the nasal mucosa (17), we can hypothesize that the passage of the SAG1 protein through the mucosa might be predominant, leading to a stronger antibody response in sera. In addition, while vaccination with the adjuvant was always superior to the inoculation of SAG1 alone, three i.n. immunizations with SAG1 were shown to elicit almost equivalent specific antibody titers. The ability of the antigen alone to stimulate systemic humoral immunity efficiently while not inducing a detectable intestinal response also suggests a loss of epithelial integrity. This may be explained partly by the presence of detergent in association with SAG1, which was necessary to keep it in suspension. Further investigations will be needed to determine whether under our conditions the detergent could act as a nasal absorption enhancer, as has previously been described for different surfactants (20). Studies performed with different animal models to determine the role of humoral immunity in the control of *T. gondii* infection have led to the general assessment that serum antibody responses play a partial role in protection against *T. gondii* infection. MAbs to *T. gondii* membrane surface antigens have been shown to convey partial protection against toxoplasmosis in recipient mice (23). It is thus likely that anti-SAG1 sera could contribute to the limitation of the systemic spread of *T. gondii*.

Protective immunity against *T. gondii* infection is generally considered to be cell mediated, with a special involvement of $CD8^+$ T cells and IFN-γ (16). Under our experimental conditions, splenocytes from mice vaccinated with SAG1 and CT proliferated in response to SAG1 restimulation and at the time selected released IL-2, IL-5, and only extremely low levels of IFN-γ. This seems to indicate that i.n. immunization with SAG1 and CT adjuvant could prime cells with the ability to produce Th1 and Th2 types of cytokines. Although in vitro studies have clearly shown that CT stimulates Th2 clones and inhibits Th1 clones (39), some debate exists as to whether CT as a mucosal adjuvant favors the development of Th2-type responses in both mucosal and systemic tissues or not. Some investigators reported consistently higher frequencies of antigen-specific Th2-type cells, whatever the antigen coadministered with CT, whereas others observed induction of both Th1 and Th2 lymphokines (22, 35, 50, 55). Our results might support the latter observation; however, the lack of IL-4 despite IL-5 production and the presence of IL-2 along with low or undetectable levels of IFN-γ production deserve further investigation. Previous reports indicated that parenteral immunization with SAG1 and the adjuvant Quil A resulted in the total prevention of latency in brains from one mouse strain and was strongly related to the induction of IFN-γ-secreting and cytotoxic $CD8^+$ splenocytes (25, 28, 29). In our study, SAG1-CT-immune splenocytes produced only barely detectable amounts of IFN-γ in response to SAG1 restimulation. This finding could explain why the passive transfer of SAG1-CT-sensitized T lymphocytes from spleens to recipient syngeneic mice conferred only partial protection against oral challenge. On the other hand, analysis of serum anti-SAG1 isotype distribution of the SAG1-CT group revealed a high predominance of IgG2a over IgG1, which is considered to be indicative of IFN-γ activity (45). One possible explanation is that cervical lymph nodes rather than the spleen may be predominantly involved in IFN-γ production along with serum IgG2a synthesis. Alternatively, as IFN-γ-independent pathways for the production of IgG2a were recently shown to exist (51), we can speculate that the protective immunity in SAG1-CT-immunized mice might be independent of IFN-γ synthesis. A recent study reports that IFN-γ-independent protective mechanisms can be operative during the acute stage of toxoplasmosis (30). However, further experiments are required to resolve this question. Finally, other protective immune mechanisms could account for the 80% reduction of brain parasite colonization observed in mice given SAG1 and CT. Because the specific intestinal response occurred only in protected mice, it may be speculated that this could be a relevant protective mechanism in vivo. However other effectors such as gut IEL, not studied here, could have been stimulated and be protective. Indeed, previous work has shown that intestinal IEL from mice infected orally with *T. gondii* displayed cytotoxic activity against infected targets in vitro and specifically produced IFN-γ (8). IFN-γ was subsequently found to activate enterocytes to render them capable of inhibiting replication of the parasite (11). Investigations are currently in progress in our laboratory to determine whether intestinal IEL are efficiently primed by i.n. administration of SAG1 and CT.

The rationale of this study was to take advantage of the common mucosal immune system to evoke immunity in the intestinal tract where *T. gondii* enters the body while achieving priming at a distant and less-hostile inductive site, the nose-associated lymphoid tissue. Our results support previous work showing that the i.n. route of antigen deposition is an effective way of providing a disseminated mucosal antibody response as well as systemic immunity. We also showed that SAG1 could be used as an efficient mucosal immunogen. However, we did not succeed in totally preventing the establishment of latency, which constitutes a permanent risk for the transmission of the parasite. With regards to the more obvious notions that the common mucosal immune system is not so common but rather compartmentalized and that greater priming is obtained at the site of the applied antigen, it may be pertinent to test the intragastric route of immunization with SAG1 for its ability to evoke protection against *T. gondii* infection despite the need for a means to ensure the integrity of the vaccine preparation. We are currently carrying out oral vaccination of CBA/J mice with polylactide coglycolide microspheres containing SAG1 to evaluate this hypothesis. It could also be useful to combine other vaccine candidates such as an antigen specific to the bradyzoite stage of the parasite in order to enhance protective immune responses.

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