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Oral Immunization with *Toxoplasma gondii* Antigens in Association with Cholera Toxin Induces Enhanced Protective and Cell-Mediated Immunity in C57BL/6 Mice

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Following oral immunization of C57BL/6 mice with a *Toxoplasma gondii* sonicate (TSo) in association with either cholera toxin (CT) or CT B subunit, the *T. gondii*-specific in vitro proliferation of splenic T lymphocytes was determined. Cytokines produced by these T cells were then characterized. After oral challenge with *T. gondii* 76K cysts, the percentage of cumulative survival was assessed, as was the number of brain cysts in the mice which survived. The TSo-specific proliferation of splenic T lymphocytes was greatly enhanced by the use of CT, whereas CT B subunit alone did not lead to amplification of splenic T-cell proliferation. The use of CT was associated with an increase of interleukin-2 (IL-2) and gamma interferon synthesis by TSo-stimulated splenic T cells, whereas no enhancement of IL-5 and IL-6 production was observed. IL-4 was not detected. A significant protection of mice immunized orally with TSo plus CT was observed in comparison with those immunized with TSo alone. This protection was associated with a large decrease in the number of brain cysts compared with the number found in naive mice infected orally with a sublethal dose of *T. gondii* 76K cysts. Further studies, using well-defined *T. gondii* proteins which are known to induce both mucosal and systemic immune responses, are needed to confirm the value of CT in the enhancement of protection against oral toxoplasmosis.

Toxoplasma gondii is an obligate intracellular protozoan parasite responsible for toxoplasmosis in humans and other warm-blooded animals. Although generally benign for healthy people, toxoplasmosis may cause abortion or neonatal malformations if contracted during pregnancy (18). Furthermore, this disease is often lethal for immunocompromised patients such as those with AIDS, those with neoplastic disease, or bone marrow or heart transplant recipients (18). In veterinary medicine, toxoplasmosis has great economic importance in many parts of the world due to cattle abortion and neonatal loss (27). Thus, an effective vaccine against *T. gondii* would be of great value to both human and veterinary medicine.

Toxoplasmosis is usually acquired perorally and induces specific immunoglobulin A (IgA) synthesis in the gut (5, 32) and milk (5). Secretory IgA antibodies are considered to be protective against intestinal coccidiosis (10), and McLeod et al. (31) have suggested that intestinal IgA antibodies participate in the protection against virulent challenge which is conferred by oral administration of a *T. gondii* temperature-sensitive mutant. A major mechanism of resistance against *T. gondii* is considered to be cell-mediated immunity (19, 21, 41). Thus, besides attempts to increase the mucosal IgA response, the ability to enhance the cellular immune response against *T. gondii* may be of great interest in the development of an efficient oral vaccine.

The adjuvant effect of cholera toxin (CT) on the mucosal immune response and particularly on intestinal IgA synthesis is now well documented (15, 28, 30, 46), but the ability of CT to stimulate a cellular response against a related antigen is poorly understood. Recent studies have investigated the mucosal and systemic cellular responses after priming by the oral administration of keyhole limpet hemocyanin (KLH) associated with CT or a combination of CT and its B subunit (CT-B) (7, 45).

In a previous study, we demonstrated that CT greatly enhances the intestinal anti-*T. gondii* antibody response following oral immunization of mice with a *T. gondii* sonicate (TSo) and CT (2). In this study, we have examined the effects of CT and CT-B on the systemic cellular response after oral immunization of C57BL/6 mice with a TSo in association with CT, CT-B, or both in combination. We have also investigated the involvement of CT in the induction of subsequent protection against *T. gondii* oral challenge. Thus, we assessed the *T. gondii*-specific proliferation and secretion of lymphokines (gamma interferon [IFN-γ], interleukin-2 [IL-2], IL-4, IL-5, and IL-6) by stimulated splenic T cells in vitro. Finally, we investigated the protection conferred after oral challenge with cysts of the 76K strain of *T. gondii* and examined the correlation of this protection with the number of brain cysts in those mice which survived.

MATERIALS AND METHODS

Parasites. Tachyzoites of the highly virulent RH strain of *T. gondii* (38) were obtained from the peritoneal exudate of Swiss OF1 mice infected 3 to 4 days earlier with a mixture of tachyzoites and TG 180 sarcoma cells (8). Brain cysts of the 76K strain of *T. gondii* (26) were obtained from orally infected Swiss OF1 mice. The virulence of the 76K strain was maintained by repeated passages every month in Swiss OF1 mice. Tachyzoites of the *T. gondii* temperature-sensitive mutant Ts4 (37) were maintained in vitro in MRC5 cell culture.

Mice. Inbred, 8- to 10-week-old C57BL/6 female mice

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(IFFA Credo) were used throughout. They were raised in an air-conditioned building (21°C and 60% relative humidity) in a room with controlled daily lighting and had free access to sterile food and water.

Preparation of TSo. Tachyzoites of the RH strain were washed, sonicated, and centrifuged as previously described (39). The supernatant from the last centrifugation, which was used as the source of antigen, was lyophilized in aliquots containing 10 mg of protein as determined by a protein assay reagent kit (Pierce) with bovine serum albumin (BSA) used as a standard. The lyophilized aliquots were stored at −20°C until use.

Peroral immunization. Lyophilized TSo (10 mg), previously described as optimum (2), was dissolved in 0.5 ml of bicarbonate buffer (0.15 M, pH 7.4, phosphate-buffered saline [PBS] containing 0.36 M sodium bicarbonate). CT (Sigma) and CT-B (Pasteur-Mérieux) were dissolved in bicarbonate buffer to give solution of 100 μg of CT or CT-B per ml. Four groups of mice were immunized orally with 10 mg of TSo alone or in association with 10 μg of CT, 10 μg of CT-B or 10 μg of CT-B plus 0.5 μg of CT. Three groups of control mice were administered either 10 μg of CT alone, 10 μg of CT-B plus 0.5 μg of CT, or the buffer alone. The dose of CT or CT-B was employed as already described by using KLH antigen (46). The antigen solutions were administered intragastrically to mice with a specially designed intubation needle. The immunization was repeated on days 10 and 20.

Vaccination with Ts4. One group of six mice was infected by intraperitoneal injection of 2×10^5 tachyzoites of the *T. gondii* Ts4 strain on three occasions 7 days apart.

Preparation of splenic T lymphocytes and antigen-presenting cells. Spleens (SPL) from orally immunized mice were harvested 8 days after the last immunization and pressed through a stainless steel mesh. SPL cells were washed with Hanks' balanced salt solution, and erythrocytes were lysed by hypotonic shock with a 0.83 M ammonium chloride-potassium carbonate solution. The SPL T cells were isolated by passage through a nylon-wool column (23), washed, and then resuspended in RPMI 1640 HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered culture medium (GIBCO) containing 5% fetal calf serum (Flow Laboratories), gentamicin (50 μg/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), and β-mercaptoethanol (5×10^{-5} M). For the antigen-presenting cell preparation, SPL cells from naive mice were obtained as described above but without passage through a nylon-wool column. Naive splenocytes were then irradiated (30 Gy) and suspended in culture medium.

In vitro T-cell proliferation and supernatant collection. SPL T cells (2×10^5) and antigen-presenting cells (5×10^5) were seeded in flat-bottomed 96-well culture plates (Falcon) in triplicate with 200 μl of twofold serial dilutions of 40 μg of TSo and BSA per ml in culture medium. Plates were incubated for 3 days at 37°C in a humidified 5% CO_2 atmosphere. Each well then received 18.5 kBq of [^{3}H]thymidine (specific activity, 37 GBq/mmol; TMM-79A; CEA), and incorporation into cellular DNA was measured 18 h later by liquid scintillation counting. Results (counts per minute) are the means ± standard errors (SE) from triplicate wells and are representative of at least three separate experiments. Culture supernatants were collected at 24 h (for IL-2 and IL-4 assays) and at 72 h (for IL-5, IL-6, and IFN-γ assays) as described elsewhere (19).

Cytokine analysis in culture supernatants. IFN-γ was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA) with a rat anti-mouse IFN-γ monoclonal antibody (Genzyme) and a polyclonal rabbit anti-mouse IFN-γ antibody (Immunogenex) (35). IL-2 was assayed by using the CTLL.2 cell line (20) by inhibiting the IL-4 activity with a monoclonal antibody, 11B11. Supernatants were measured for IL-4 with a commercial ELISA kit (Endogen). IL-5 was quantified by a sandwich ELISA, as described by Mosmann and Fong (35), using a pair of rat anti-mouse IL-5 monoclonal antibodies (TRFK4 and TRFK5; Pharmingen). The detection of IL-6 was performed with the 7TD1 cell line (44), kindly provided by the Ludwig Institute for Cancer Research (Brussels, Belgium). For all cytokine analyses, supernatants were pooled from three culture wells of antigen-stimulated lymphocytes and triplicate twofold serial dilutions of each pooled supernatant were prepared. Cytokine concentrations were determined by reference to standard curves constructed with fixed amounts of mouse recombinant IFN-γ (Genzyme), IL-2 (Roussel Uclaf), IL-4 (Endogen), IL-5 standard (lyophilized Th2 supernatant; gift from DNAX Research), or IL-6 (Immunogenex). The limits of sensitivity for the different assays were as follows: IFN-γ, 1 ng/ml; IL-2, 0.1 U/ml; IL-4, 0.1 U/ml; IL-5, 20 pg/ml; and IL-6, 1 pg/ml.

Peroral challenge. Brain cysts from mice infected chronically 1 month earlier with the *T. gondii* 76K strain were collected and diluted to contain a lethal dose of 90 cysts per 0.5 ml in PBS (corresponding to six 50% lethal doses [LD_{50}s]). Mice from each immunized or Ts4-vaccinated group were infected orally with this cyst suspension 18 days after the last immunization, and the mortality level was then assessed. At the same time, naive mice were infected orally with five cysts of the 76K strain of *T. gondii*, this being a nonlethal dose.

Quantitation of *T. gondii* cysts in mouse brains. Two months after oral challenge, brains from those mice which survived were removed and homogenized in 3 ml of PBS by using a mortar and pestle. For each brain suspension, the mean number of cysts from 10 samples (10 μl each) was then determined by light microscopy under ×10 magnification.

Statistical analysis. SPL T-cell proliferation was expressed as the mean counts per minute ± SE from triplicate wells. SE could not be calculated for the cytokine assays because the supernatants from triplicate wells were pooled. The number of brain cysts corresponds to the mean ± standard deviation of brain cyst counts of surviving mice for each group. The significance of the differences between experimental groups was determined by analysis of variance (Fisher's test) for proliferation assays and brain cyst counting and by use of the chi-square test for protection experiments. P values of ≤ 0.05 were considered significant.

RESULTS

***T. gondii*-specific SPL T-cell response.** *T. gondii*-induced proliferation was assessed with SPL T cells from mice immunized orally with TSo alone or with TSo in association with CT, CT-B, or both (Fig. 1). Significant *T. gondii*-specific blastogenesis was observed with stimulated T cells from mice fed TSo with or without adjuvants, while no T-lymphocyte stimulation was observed in cells from control mice given CT alone or CT in combination with CT-B or the buffer only ($P < 0.05$). No significant difference was observed between these three control groups. The TSo-specific SPL T-cell response of the mice which were immunized orally with TSo and CT was greatly enhanced in comparison with the response of those given TSo alone or TSo in combination with CT-B or CT-B plus CT ($P < 0.05$). In

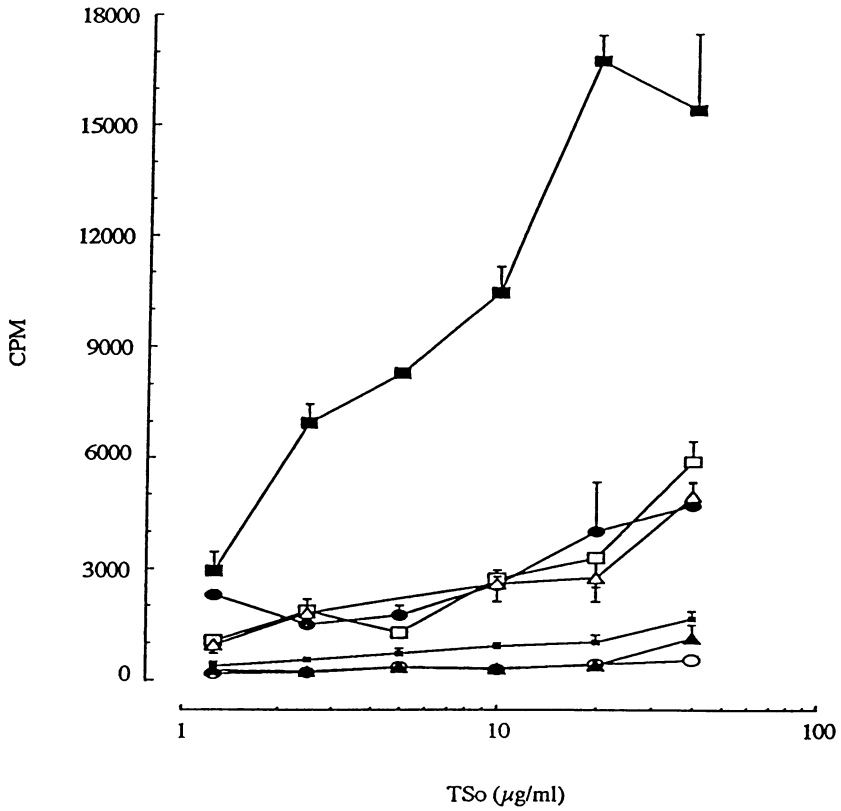

FIG. 1. In vitro TSo-specific proliferation of SPL T lymphocytes from mice immunized orally with 10 mg of TSo (●), 10 mg of TSo plus 10 μg of CT (■), 10 mg of TSo plus 10 μg of CT-B (□), 10 mg of TSo plus 10 μg of CT-B plus 0.5 μg of CT (△), 10 μg of CT (○), 10 μg of CT-B plus 0.5 μg of CT (▲), or buffer only (■) on three occasions 10 days apart. Results are the means ± SE from triplicate wells and are representative of at least three experiments.

contrast, oral administration of TSo plus CT-B or TSo plus CT-B plus CT did not increase the TSo-induced T-cell response in the SPL cells compared with that in the group given TSo alone. No significant difference was observed between the SPL T-cell proliferation of mice fed with TSo plus CT-B and those fed with TSo plus CT-B plus CT. Finally, no T-cell proliferation was observed with cells from any group of mice following in vitro incubation with BSA (between 102 ± 15 and 594 ± 165, cpm ± SE).

Cytokine release by *Toxoplasma*-stimulated SPL T lymphocytes. The presence of lymphokines was assayed in the supernatants of SPL TSo-specific T cells from mice immunized orally with TSo alone, TSo plus CT, or CT alone (Table 1). IFN-γ production occurred with stimulated T cells from mice immunized orally with TSo and CT, whereas no detectable IFN-γ secretion was observed in T-cell supernatants of mice fed TSo alone. Increased IL-2 secretion was observed with SPL T cells from mice given TSo plus CT compared to that for T cells from mice given TSo alone. No IL-4 production was detected in the supernatants of SPL T cells from any of the three groups of mice. Although IL-5 and IL-6 were detected in the supernatants of SPL T lymphocytes from mice fed TSo alone, no increased secretion of these cytokines was observed in the T-cell supernatants from mice immunized orally with TSo and CT. Finally, no cytokine detection, except nonspecific IL-6 production, was observed with TSo-stimulated SPL T cells from mice given CT alone.

Protection induced by TSo with or without CT against an oral challenge with *T. gondii* cysts of the 76K strain. Eighteen days after the last immunization, mice were challenged orally with 76K strain *Toxoplasma* cysts corresponding in quantity to six times the LD_{50} and the percentage of cumulative survival was then calculated (Fig. 2). Mortality first occurred on day 8 after challenge, except for mice vaccinated with the Ts4 strain, which all survived until the end of the experiment (day 60; $P < 0.001$). On day 8, the percentage of survival was greater in the groups of mice fed TSo plus CT (96.4%), TSo alone (81.4%), and CT alone (90.9%) compared

TABLE 1. Cytokine response profile of TSo-specific T lymphocytes from mice immunized orally with TSo or CT or both[a]

Oral immunization regimen	IFN-γ (ng/ml)	IL-2 (U/ml)	IL-4 (U/ml)	IL-5 (pg/ml)	IL-6 (pg/ml)
TSo	<1.0	0.49	0.12	68	100.8
TSo + CT	93.6	4.30	0.16	55	89.9
CT	<1.0	0.24	0.12	<20	108.3

[a] Mice were immunized orally with 10 mg of TSo with or without 10 μg of CT or with 10 μg of CT alone on three occasions 10 days apart. IL-2 and IL-6 detections were performed with the CTLL.2 and the 7TD1 cell lines, respectively. IL-4, IL-5, and IFN-γ release was assessed by ELISAs. For each test, standard curves were produced by using recombinant cytokines. The detection limits of the assays are 1 ng/ml (IFN-γ), 0.10 U/ml (IL-2 and IL-4), 20 pg/ml (IL-5), and 1 pg/ml (IL-6). The results are representative of at least three experiments.

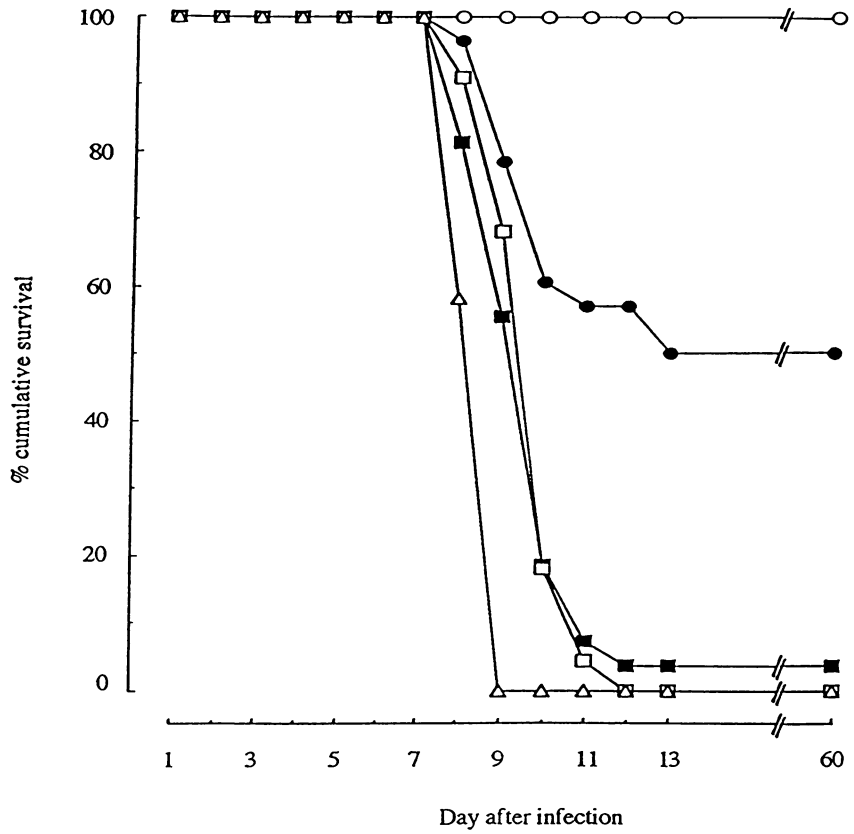

FIG. 2. Protection of mice immunized orally with 10 mg of TSo (■, $n = 27$), 10 mg of TSo plus 10 μg of CT (●, $n = 28$), 10 μg of CT (□, $n = 22$), or buffer only (△, $n = 12$) on three occasions 10 days apart or infected intraperitoneally with 2×10^5 tachyzoites of *T. gondii* Ts4 (○, $n = 6$) on three occasions 7 days apart. Oral challenge was performed 18 days after the last immunization with 90 cysts of the *T. gondii* 76K strain (six LD_{50}s).

with that obtained with the control group of mice given buffer only (58.3%). On day 9, no mice from the control group were still alive, whereas the percentages of survival in the other groups were as follows: 78.5% in mice immunized with TSo plus CT, 55.5% in mice fed TSo alone, and 68.1% for the group of mice treated with CT alone. From day 9 to day 12, the percentage of surviving mice decreased rapidly in the groups of mice given TSo or CT alone compared with that in the group of mice immunized with TSo and CT. By day 12, none of the mice fed CT alone was alive. From day 13 until the end of the experiment, the percentage of cumulative survival in the groups of mice fed TSo alone (3.7%) or in combination with CT (50%) remained constant. This result demonstrates a significant protection of mice immunized orally with TSo in association with CT ($P < 0.01$), in contrast to a lack of protection observed with mice fed CT or buffer only. Finally, no significant protection was observed with the groups of mice immunized orally with TSo in association with CT-B ($n = 5$) or CT-B plus CT ($n = 10$) (data not shown).

Brain cyst counts in surviving mice. Two months following oral challenge, the brain cysts from surviving mice were counted and compared with the number of brain cysts from naive C57BL/6 mice infected orally with a nonlethal dose of five cysts from the 76K strain (Fig. 3). The number of cysts in brains of mice fed TSo and CT was significantly lower than that observed in the brains of nonimmunized mice ($P < 0.05$). A significantly lower number of cysts was also observed in brains from mice vaccinated with the Ts4 strain compared with those observed in brains from nonimmunized mice or TSo-plus-CT-immunized mice ($P < 0.05$).

DISCUSSION

The adjuvant effect of CT on the mucosal immune response to bacteria and viruses is now well recognized (9, 28). We have recently reported that CT significantly enhances the anti-*T. gondii* secretory IgA response following oral immunization of mice with TSo and CT (2). This enhancement of the intestinal secretory IgA response is of great interest because IgA antibodies have been considered, in some cases, to be protective against oral infection with parasites, bacteria, or viruses (10, 24, 28). The protective immune response following a primary *T. gondii* infection is complex and probably involves humoral as well as cellular mechanisms. The T-cell response is of critical importance in toxoplasmosis not only in terms of cellular immune defense against the parasite (19, 21, 41) but also as help and regulation of the IgA antibody response (13). Thus, the potential for enhancing the cellular immune response against *T. gondii* would be highly valuable in the development of an effective vaccine.

In this study, we have determined the effect of CT and CT-B on the systemic cellular response to *T. gondii* after oral immunization of C57BL/6 mice with TSo in association with CT, CT-B, or a combination of both. The TSo-specific

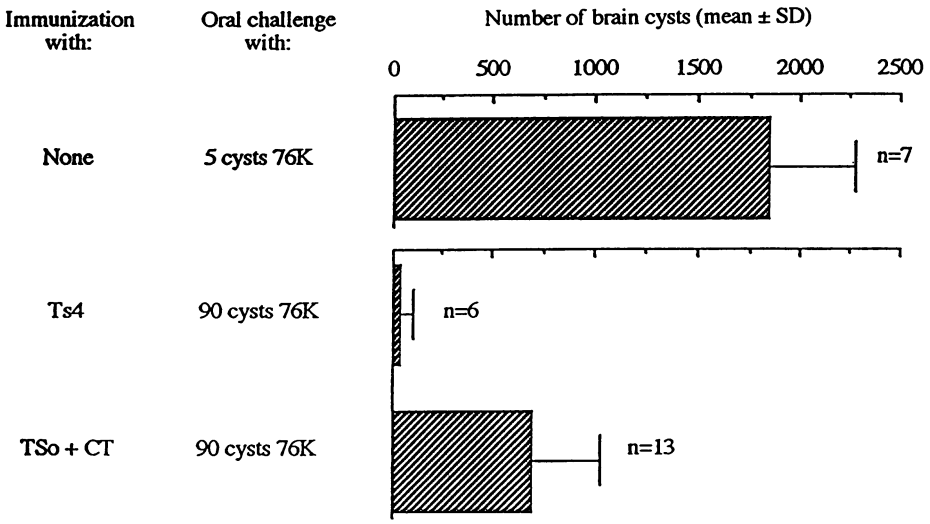

FIG. 3. Number of brain cysts in surviving mice immunized orally or Ts4 vaccinated and further challenged orally with 90 cysts of the *T. gondii* 76K strain and in naive mice infected orally with 5 cysts of the *T. gondii* 76K strain (nonlethal dose). Results are the number of brain cysts in each mouse determined 2 months after challenge or after infection.

proliferation of splenic T cells was greatly enhanced by the use of CT as an adjuvant, whereas CT-B alone did not lead to the amplification of T-cell blastogenesis. This adjuvant effect is in agreement with that observed by others (7, 45) as regards KLH-specific T-cell response in the spleen. The association of CT plus CT-B did not increase *T. gondii*-induced T-cell proliferation, in contrast to previous reports showing that CT plus CT-B is an efficient adjuvant for both humoral (46) and cellular (7, 45) KLH-specific immune responses. In general, CT-B alone has been shown to be a poor adjuvant (46), although CT-B has been shown to induce protection against influenza virus infection by vaccine inoculated intranasally (42). It seems that the addition of a small quantity of CT (0.5 μg) to CT-B (10 μg) was sufficient to obtain an enhancement of a KLH-specific immune response to a level similar to that obtained with a larger quantity of CT alone (10 μg) (46). In our study, although the dose of CT and CT-B was the same as that used by Wilson et al. (46), the dose and the nature of antigens were different (5 mg of KLH versus 10 mg of TSo). Thus, the adjuvant effect of CT or CT-B may be influenced by certain critical parameters, including dose, route, timing of delivery, and nature of the associated antigen (e.g., crude antigenic extract versus well-defined antigen or parasite versus virus). Recently, the adjuvant mechanism of CT has been partially attributed to an increase in gut permeability; in contrast, CT-B, which lacks the adenylate cyclase-cyclic AMP-activating property of CT, failed to increase gut permeability (29). Since CT-B has no effect on TSo-specific blastogenesis in this study, the mechanism of increased permeability could be a critical parameter in the in vivo adjuvant action of CT on a TSo response. Several factors have been suggested to be responsible for the effect of CT. Thus, besides increased gut permeability (29), other mechanisms, such as enhanced switching of IgC_H genes to favor both IgG and IgA (30), increase in antigen-presenting cell function (3), or inhibition of suppressive mechanisms (16), may all contribute to the complexity of CT's adjuvanticity. In our study, the detection of a TSo-specific cellular immune response in the SPL indicates that tissues outside the gut-associated lymphoid tissue are affected by mucosal priming by TSo in association with CT.

Furthermore, we have evaluated the SPL T-cell subtype, stimulated by TSo by means of the cytokine patterns produced by these T lymphocytes. Cytokine detection showed that the use of CT led to an enhancement of IL-2 and IFN-γ secretion by TSo-stimulated SPL T cells, whereas no increase of IL-4, IL-5, nor IL-6 was observed. Thus, according to the Th dichotomy (34), it seems that the in vivo use of CT with TSo enhances the Th1-type cytokine response of TSo-induced splenic T cells. The fact that, following *T. gondii* oral infection, the in vitro mesenteric cellular immune response to TSo is dominated by a Th2-type cytokine pattern whereas a predominant Th1 cytokine response is observed in the spleen argues in favor of this hypothesis (6). Furthermore, it has already been observed that a predominance of IL-2 at nonmucosal sites compared to a predominance of IL-4 at mucosal sites (11) suggests that a preferential stimulation of a particular Th subtype could be influenced by the cell microenvironment. Moreover, the adjuvant effect of CT on KLH is associated with a stimulation of a plasma IgG and secretory IgA response (15), a Th1 and a Th2 function, respectively. However, $CD8^+$ cytotoxic splenocytes have been shown to produce a pattern of cytokines similar to that of Th1 (17). Recently, Clarke et al. (7) have shown that both $CD4^+$ and $CD8^+$ cells can be generated in vivo by oral immunization with KLH and CT. The increase of IL-2 and IFN-γ production, probably by both $CD4^+$ and $CD8^+$ lymphocytes, following TSo plus CT oral immunization is of great interest in toxoplasmosis. Thus, for example, $CD4^+$ lymphocytes appear to play a synergistic role with $CD8^+$ cells in protective immunity against *T. gondii* (1, 19), since IL-2 release by *T. gondii*-specific $CD4^+$ cells from Ts4-vaccinated mice enhances the antigen-specific induction of IFN-γ in a $CD8^+$ cell population (19). $CD8^+$ cytotoxicity (21, 25) aided by the helper activities of $CD4^+$ cells (1) and the microbicidal or microbiostatic activity of IFN-γ-activated macrophages (40), fibroblasts (36), endothelial cells (47), and enterocytes (12) are two major mechanisms of resistance to *Toxoplasma* infection.

Finally, we have investigated whether this enhancement of the systemic cellular response to *T. gondii* with CT can lead to a significant protection of C57BL/6 mice following a

lethal oral challenge. Our results from oral immunization with TSo plus CT demonstrate that this adjuvant greatly enhances the protection of C57BL/6 mice against a subsequent oral challenge with *T. gondii* cysts, in contrast to the absence of protection obtained with CT-B as an adjuvant. Moreover, the protection induced by TSo plus CT seems to be associated with a substantial reduction in the number of brain cysts in the surviving mice, since a higher number of brain cysts was found in mice infected orally with a lower nonlethal dose of 76K cysts. It is of note that the number of brain cysts recovered from mice orally infected with *T. gondii* cysts is the same whatever the number of cysts used for oral infection (data not shown). Thus, that protection and the decrease in the number of brain cysts seem to be associated with an enhancement of *T. gondii*-specific splenic T-cell stimulation. Araujo (1) has shown that $CD4^+$ as well as $CD8^+$ T cells actively participate in the development of resistance to *T. gondii* and in the mechanisms controlling brain cyst formation in mice. It has already been reported that the adjuvanticity of CT is restricted by *H-2* major histocompatibility complex genes and that a more pronounced immune response is obtained with the $H\text{-}2^b$ congenic strain of mice (14). The fact that increased immunity seems to be closely related to the mouse strain used must be borne in mind for further oral vaccination strategies using CT as an adjuvant. Nevertheless, the use of C57BL/6 mice ($H\text{-}2^b$ haplotype) in our experiment was not only for the high responsiveness of these animals to CT's adjuvanticity but also because the C57BL/6 strain is highly susceptible to *T. gondii* oral infection (22) and is therefore a pertinent model for protection studies. Recently, a high degree of protection has been observed after parenteral immunization of outbred mice with SAG1 incorporated in liposomes (4) or in the presence of Quil A (25) and was correlated with a lack of intracerebral cyst development (25). However, protection is clearly dependent on the mouse strain used since, in the same experiment (25), a lower protection was obtained with the C57BL/6 strain, with a smaller reduction in the number of brain cysts. These data together with our work raise the problem of (i) defining an adequate antigen delivery system in order to obtain efficient protection as well as (ii) choosing the right model of protection to test vaccine efficiency.

Only one previous study (31) has reported the possibility of using the oral route, this being the natural site of entry for *T. gondii*, to increase the survival of mice against an oral challenge. In our study, we have demonstrated the great potential of CT as an adjuvant in the protection of mice immunized orally with TSo against a subsequent lethal oral infection. This oral protection is associated with an enhancement of splenic T-cell stimulation, IFN-γ and IL-2 secretion, and a substantial decrease in the number of brain cysts, together with an increase in the mucosal IgA response as previously described (2). The long-term immunological memory induced by CT but not by CT-B after oral immunization with KLH, as recently described (43), confirms the value of using CT as an adjuvant for developing an efficient oral vaccine against *T. gondii*. It will be important to test the adjuvant effect of CT on well-defined *T. gondii* immunogens for which the genes have been cloned (33) and which are known to induce both mucosal and systemic immune responses (5, 6) as well as to determine the long-term immunological memory induced.

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