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Impairment of the Cellular Immune Response in Acute Murine Toxoplasmosis: Regulation of Interleukin 2 Production and Macrophage-Mediated Inhibitory Effects

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Depression of the cellular immune response to *Toxoplasma gondii* has been reported in both mice and humans. The present study was undertaken to determine the kinetics and mechanism of the observed downregulation of interleukin 2 (IL-2) production during experimental murine toxoplasmosis. For these investigations, the cell-mediated immune response to the wild type (PTg) was compared with that to the less-virulent mutant parasite (PTgB), which is deficient in the major surface antigen, p30 (SAG-1). Spleen cells from infected A/J mice failed to proliferate in response to *Toxoplasma* antigens during the first week of infection. Both PTg- and PTgB-infected A/J mice exhibited a significant reduction in the concanavalin A (Con A)-induced lymphoproliferative response. Further, the response of splenocytes from mice infected with the wild-type parasite was significantly diminished compared with that of mice infected with PTgB. The lymphoproliferative response to Con A reached its nadir at day 7 and remained below control levels for at least 14 days postinfection. By day 21 postinfection, the response to Con A and to *Toxoplasma* antigens was restored to the level observed prior to day 7. Con A-stimulated culture supernatants of spleen cells from mice on day 7 postinfection contained significantly less IL-2 than normal mice. There was no significant difference in the numbers of binding sites or capacity of high-affinity IL-2 receptors between infected and normal mouse splenocytes as determined by Scatchard analysis. Exogenous IL-2 at different concentrations failed to restore the proliferative response of lymphocytes from infected mice to Con A. Adherent macrophages from 7-day-infected mice were able to suppress IL-2 production by normal splenocytes following stimulation with Con A. The inhibitory activity mediated by infected cells was reversed by the antibody to IL-10 but not transforming growth factor β. There were insignificant levels of nitric oxide production in both infected and normal splenocytes. These results indicate that during acute murine toxoplasmosis, there is a well-defined period (day 7) during which both the T-cell mitogen and parasite antigen-associated lymphoproliferative response are reduced. Further, there is a reduction in the production of IL-2 and an increase in IL-10, which appear to mediate, in part, the observed downregulation of immunity to *T. gondii*.

Toxoplasmic encephalitis caused by the obligate intracellular protozoan *Toxoplasma gondii* is the most common central nervous system infection in those afflicted with AIDS. It is primarily manifested when the $CD4^+$ T-cell population is depleted and in the setting of a falling $CD8^+$ T-cell count (27). Recent studies of mice with the murine LP-BM5 virus (MAIDS) have demonstrated increased mortality in those mice coinfected with *T. gondii*, suggesting that the parasite is able to induce a state of immunodepression in an already compromised host (15). Earlier studies of mice have demonstrated that cell-mediated immunity is depressed following parasite infection in the normal host (8, 23, 38). Similar observations have been made in humans (4, 26). The depression of lymphocyte proliferative responsiveness to the *Toxoplasma* antigen was observed in an infant with congenital toxoplasmosis (28) and in adults with acute toxoplasmosis (26).

Downregulation of T-cell-derived cytokines, in particular interleukin 2 (IL-2), during infection with other protozoan parasites, intracellular bacteria, or viruses has been demonstrated. For example, concanavalin A (Con A)-stimulated T-cell culture supernatant collected at 7 days postinfection with lymphocytic choriomeningitis virus contained a 30- to 40-fold-reduced amount of IL-2 (34). Similar observations have been reported during *Trypanosoma cruzi* infection in mice (7, 11).

The potential role of activated macrophages in downregulatory host immune response is well recognized. Activated macrophages are known to secrete a variety of factors with suppressive activities, including IL-10 (12, 14), transforming growth factor β (TGF-β) (42), nitric oxide (2, 30), and prostaglandin (32). Murine IL-10 has a variety of immune downregulatory properties, including decreased IL-2 production (14, 40) and the inhibition of T-cell proliferation by Con A in the presence of accessory cells (12).

Our laboratory has been interested in studying the host response to p30 (SAG-1), a major surface protein of *T. gondii*. This antigen has an apparent M_r of 30,000 and is important in controlling host immunity to the parasite (21). We have used monoclonal antibodies (MAb) directed at this protein to isolate a mutant parasite deficient in p30, PTgB (18, 22). In the current study, we use this antigen-deficient mutant to investigate the host immune response. Our results indicate that *T. gondii* is able to induce a transient state of reduced lymphocyte proliferation in mice infected with either wild-type or p30 antigen-deficient parasites. To better appreciate the mechanism underlying the impaired cellular immune response, splenocytes from *T. gondii*-infected mice were examined for

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the production of IL-2 and binding of the IL-2 receptor to its ligand (i.e., IL-2). We report that spleen cells from infected mice were defective in their capacity to both secrete IL-2 and respond to exogenous IL-2. In addition, we observed the suppressive effect of macrophages from mice acutely infected with *T. gondii* on the production of IL-2. The culture supernatant obtained from these macrophages contained increased levels of IL-10. Antibody to IL-10 can partially restore the suppressed Con A-proliferative response by this supernatant.

MATERIALS AND METHODS

Parasite and antigen preparation. The P strain of *T. gondii* (PTg) and its derived mutant PTgB were used for all experiments. The P strain used in these experiments was cloned from the Me49 strain (20). The p30-deficient mutant strain, PTgB, was selected from a large population of chemically mutagenized wild-type P strain parasites (18) as previously reported. All parasites in these experiments were used between the 1st and 35th passage in vitro. Parasites were maintained in our laboratory by in vitro passage in human foreskin fibroblasts at 37°C in minimal essential medium (MEM) containing 10% newborn calf serum. To prepare *Toxoplasma* antigen (TGA), infected human foreskin fibroblast monolayers were scraped, and the tachyzoites were freed by forced extrusion through a 27-gauge needle. The lysate was centrifuged at 590 × *g* for 10 min, and the pellet was resuspended in serum-free medium. Tachyzoites were separated from host-cell debris by treatment with phytohemagglutinin-M, as previously reported, followed by passage through a glass-wool filter (19). The parasites were then washed twice in serum-free medium and centrifuged at 590 × *g* for 10 min, and the pellet was lysed by repeated freezing at −70°C and thawing at 37°C. This material was then centrifuged at 590 × *g* for 10 min, and the supernatant was collected and sterilized by passage through a polysulfone membrane filter with a 0.22-μm-pore diameter (Gelman, Ann Arbor, Mich.). The protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as a standard.

Mice and infection. Female A/J mice, 5 to 6 weeks old, were obtained from Jackson Laboratories, Bar Harbor, Maine). Mice were infected intraperitoneally with 2 × 10^4 parasites, and the proliferative response of splenocytes, in the presence of soluble TGA and mitogen, Con A (Sigma Chemical Co., St. Louis, Mo.), were analyzed at different times after infection. Experiments were extended up to 21 days postinfection with surviving PTgB- or PTg-infected mice. Each experimental group consisted of at least 12 mice.

Lymphocyte preparation and cell culture. Following parasite infection, mice from each group were sacrificed, and cell suspensions were prepared from spleens or lymph nodes following homogenization of these tissues in phosphate-buffered saline (PBS). Erythrocytes were lysed by hypotonic shock with 0.2% ammonium chloride. The cells were then washed with PBS, followed by Iscove's medium containing 2 mM L-glutamine, 10 mM HEPES (*N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, Utah). For proliferative assays, the cells were cultured in triplicate wells at a concentration of 2 × 10^5 cells per well in 96-well flat-bottom culture plates in a final volume of 200 μl of Iscove's medium–10% FBS containing TGA, Con A, or neither. In preliminary experiments, the lymphoproliferation in response to TGA (8.75 μg/ml) and Con A was determined (data not shown). Cells were cultured for either 4 days with TGA or 2 days with Con A (5 μg/ml) in a humidified chamber at 37°C containing 5% CO_2. Culture supernatants were obtained at 24 h after culture. For proliferation assays, cell cultures were pulsed with 0.5 μCi of [^{3}H]thymidine (ICN, Costa Mesa, Calif.) for 6 h at 37°C. Pulsed cells were harvested on glass filter strips with an automated multiple sample cell harvester and dried, and the radioactivity was determined by liquid scintillation.

Adherent and nonadherent cell preparations. Spleen cells were isolated from each group of mice, and cell suspensions were prepared as described above. Nonadherent cells were prepared by incubating either normal uninfected spleen cells (5 × 10^6/ml) or spleen cells (5 × 10^6/ml) from mice at day 7 postinfection in a 100-mm-diameter plastic petri dish (Corning Glass Works, Corning, N.Y.) at 37°C in a humidified atmosphere containing 5% CO_2. After 2 h of incubation, medium containing nonadherent cells was collected, and the cells were washed, separated by centrifugation (590 × *g* for 10 min), and resuspended at a concentration of 4 × 10^6/ml of Iscove's medium containing 10% FBS. The nonadherent cells were about 80% Thy 1.2 positive by fluorescence-activated cell sorter (Ortho Diagnostics) (FACS) analysis. For proliferation assays, the cells were cultured in triplicate wells at a concentration of 2 × 10^5 cells per well in a final volume of 200 μl of Iscove's medium–10% FBS with or without Con A.

The adherent cells were gently washed twice with culture medium containing 10% FBS. Cells were dissociated by the addition of 5 ml of 0.02% EDTA (Sigma Chemical Co.) for 5 min at 37°C. The cells were further dissociated by repeatedly pipetting EDTA solution over the entire surface of each petri dish. Once dissociated, the cells were washed twice with medium. The adherent cell population contained about 95% macrophages as determined by Wright-stained morphology. The number of macrophages was determined microscopically by counting five fields. For proliferation assays, different concentrations of dissociated adherent cells (10^5 or 3 × 10^5 cells per well) were cultured with spleen cells from age-matched normal uninfected mice (2 × 10^5 cells per well) in triplicate wells of 96-well culture plates in a final volume of 200 μl of Iscove's medium–10% FBS with or without Con A. Culture supernatants were obtained at 24 h after culture, and the IL-2 activity of these supernatants was tested by their ability to stimulate proliferation of an IL-2-dependent murine T-cell clone (16). All cultures were incubated at 37°C in a 5% CO_2 atmosphere for 48 h. [^{3}H]thymidine (0.5 μCi; ICN) was added to each well for the final 6 h of culture. Incorporated radioactivity was determined by liquid scintillation counting. Data were expressed as counts per minute.

Flow microfluorometry. Flow cytometric analysis of splenocytes was performed by single color, indirect fluorescence with the use of an FACS. Briefly, 10^6 spleen cells in suspension from uninfected or infected mice were incubated for 1 h at 4°C with either anti-CD4$^+$ GK 1.5 (TIB 207; American Type Culture Collection, Rockville, Md.), anti-CD8 (TIB 150; American Type Culture Collection) or anti-Thy 1.2 (CHO-13.4; American Type Culture Collection) MAb. Analysis of the γδ-heterodimer was accomplished with MAb (3A10) directed at this receptor (kindly supplied by Susumi Tonegawa, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge). Cells were washed with PBS and incubated with fluoroscein-conjugated anti-rat, anti-mouse, and anti-hamster immunoglobulin G. After 45 min, the cells were washed twice with PBS, fixed with 0.1% methanol-free formaldehyde, and then analyzed by flow cytometry.

Assay of IL-2 and IL-10 production. Supernatants from cultures of Con A-stimulated splenocytes from normal uninfected or infected mice were collected at 24 h after the beginning of culture. IL-2 activity in the supernatants was

tested by their ability to stimulate proliferation of an IL-2-dependent murine T-cell clone (16). CTLL-2 cells (3.5×10^3 cells per well) were suspended in culture medium containing 10% FBS. CTLL proliferation, as indicated by incorporation of 0.5 μCi of [^{3}H]thymidine, was determined during the last 6 h of the 24-h culture period in the presence of serial twofold dilutions of standard recombinant (rIL-2) and experimental samples. Radioactive incorporation was determined by liquid scintillation spectroscopy. The response was analyzed by Probit analysis as described previously (16) compared with the 50% maximal response obtained with standard rIL-2 (kindly provided by Kendall A. Smith, Dartmouth Medical School, Hanover, N.H.). For IL-10 measurement, supernatants from 48-h splenocyte culture stimulated with Con A were assayed by enzyme-linked immunosorbent assay (ELISA) per the manufacturer's instruction (Endogen, Boston, Mass.), and the cytokine level was calculated by reference to standard units provided by the manufacturer.

Receptor-binding assay. A radioisotope assay was used to evaluate IL-2 receptor binding as previously described (43). Briefly, splenocytes from uninfected or infected (for 7 days) mice were cultured in the presence of 5 μg of Con A per ml for 48 h. The cells were washed in Iscove's medium containing 2 mM L-glutamine, 10 mM HEPES (pH 7.2), and 10% FBS. Serial dilutions of ^{125}I-IL-2 (provided by Kendall A. Smith) were incubated at 37°C with 4×10^5 to 6×10^5 spleen cells in a total volume of 200 μl of culture medium for 20 min. After incubation, the bound and free ^{125}I-IL-2 were separated by centrifugation at 8,500 *g* for 2 min through 20% paraffin oil (Fisher Scientific Co., Pittsburgh, Pa.) and 80% silicon oil (550 fluid; Contour Chemical Co., North Reading, Mass.). The tips of the tubes (400 μl, polyethylene; USA Scientific Plastics, Ocala, Fla.) containing the cell pellet and free radioactivity were cut off, and the radioactivity was determined by scintillation counting. The number of binding site and the dissociation constant (K_d) values were determined by Scatchard analysis as previously described (43). The number of binding sites per cell and the calculated dissociation constant values are based on the specific activity of 5×10^5 molecules per cpm.

Assays with anti-IL-10 and anti-TGF-β MAbs. MAb to IL-10 and TGF-β were used to investigate the effect of these cytokines on the proliferative cell response. Rat anti-mouse IL-10 antibody (Genzyme, Cambridge, Mass.) or anti-TGF-β antibody (mouse anti-TGF-β1, TGF-β2, or TGF-β3 MAb; Genzyme) was used at concentrations as recommended by the manufacturer. Splenocytes were prepared from either uninfected or infected (for 7 days) A/J mice as described above. Cells were cultured in triplicate wells at a concentration of 2×10^5 cells per well with either anti-IL-10 (4 μg/ml), anti-TGF-β (30 μg/ml) or normal rat immunoglobulin G (Sigma Chemical Co.). Cells were incubated either with anti-IL-10 or anti-TGF-β for 30 min at 37°C followed by the addition of either Con A or medium. After 48 h of incubation, the proliferation was determined by the amount of [^{3}H]thymidine incorporated. Data were expressed as counts per minute.

Nitrite determination. Nitrite in 24-h culture supernatants was measured by using Griess reagent (13). Briefly, 0.05 ml of supernatant was mixed with 0.05 ml of Griess reagent and then incubated for 10 min at room temperature, and A_{570} was determined with an automated plate reader. The nitrite concentration was calculated from a $NaNO_2$ (Sigma Chemical Co.) standard curve.

Statistical analysis. Probability significance was determined by two-tailed Student's *t* test, assuming equal variances. Statistical significance was set at *P* of <0.05 for all comparisons.

RESULTS

Splenocyte proliferative response to Con A. We first evaluated the in vitro lymphoproliferative response to Con A in A/J mice infected with either P wild-type or PTgB mutant parasites. Splenocytes were harvested at various times after infection and cultured for 48 h in the presence of Con A. Following incubation, lymphoproliferation was determined by the amount of [^{3}H]thymidine incorporated. As shown in Fig. 1, there was a gradual decline in the proliferative response to Con A during the first week of infection. This reduction in proliferation reached its nadir on day 7 postinfection in both PTg- and PTgB ($P < 0.05$)-infected mice compared with that in normal age-matched uninfected control mice. In addition, the Con A-induced response on day 7 was significantly lower in splenocytes obtained from PTg-infected mice than from PtgB-infected mice ($P < 0.05$). By day 14, this difference is no longer significant, although the Con A response in splenocytes from mice infected by either strain remained significantly lower than that in uninfected controls ($P < 0.05$). By day 21 postinfection, the difference in Con A-induced stimulation between infected and uninfected mice virtually disappeared.

Proliferative response to TGA. The ability of TGA to stimulate splenocytes from infected mice was then evaluated. As shown in Fig. 2A, at a concentration of 8.75 μg TGA per ml, splenocytes obtained from wild-type infected mice failed to proliferate in response to parasite antigen during the first week of infection. By days 14 and 21, however, the ability to proliferate in response to parasite antigen was significant ($P < 0.05$). Similar observation were made for splenocytes from the PTgB-infected mice (Fig. 2B).

Phenotypic analysis of mouse T-cell subpopulations. Splenocytes were isolated from infected mice at days 3, 7, and 14 postinfection, and the expression of T-cell phenotypes was analyzed by FACS. As shown in Table 1, there was a significant increase in the number of CD8$^+$ T cells in both wild-type- and PTgB-infected mice on days 7 and 14 postinfection. CD8$^+$ T-cell augmentation was most pronounced in wild-type-infected mice at day 14 following infection. Infection with either PTg or PTgB parasites induced a low level of γδ T-cell response (5%) in the A/J mice. By day 14 postinfection, 8.6% of the splenocyte population analyzed was positive for γδ-heterodimers.

Adherent cells suppress the Con A response of lymphocytes from uninfected or infected mice. Con A-stimulated proliferative responses of spleen cells from uninfected mice were studied in the presence of different concentrations of adherent cells (10^5 and 3×10^5 cells per well) obtained at day 7 postinfection from spleens of either wild-type- or PTgB-infected mice. Day 7 was chosen since the results indicated this was the time at which the proliferative response to Con A was most affected. As shown in Fig. 3, there was a significant decrease when normal spleen cells were incubated in the presence of adherent cells from infected mice ($P < 0.05$). This response was dependent on the number of adherent cells added to the culture. Similar results were obtained when spleen cells from mice infected with either the wild type or PTgB were cocultured with adherent cells from mice infected with either strain of *T. gondii* (data not shown).

IL-2 response during the course of primary infection. Splenocytes were obtained from mice at days 3, 7, 14, and 21 postinfection and stimulated with either parasite antigen or Con A. At 24 h, the culture supernatants were collected and IL-2 production was measured in an IL-2-dependent CTLL assay. In this bioassay, there was no detectable IL-2 produced

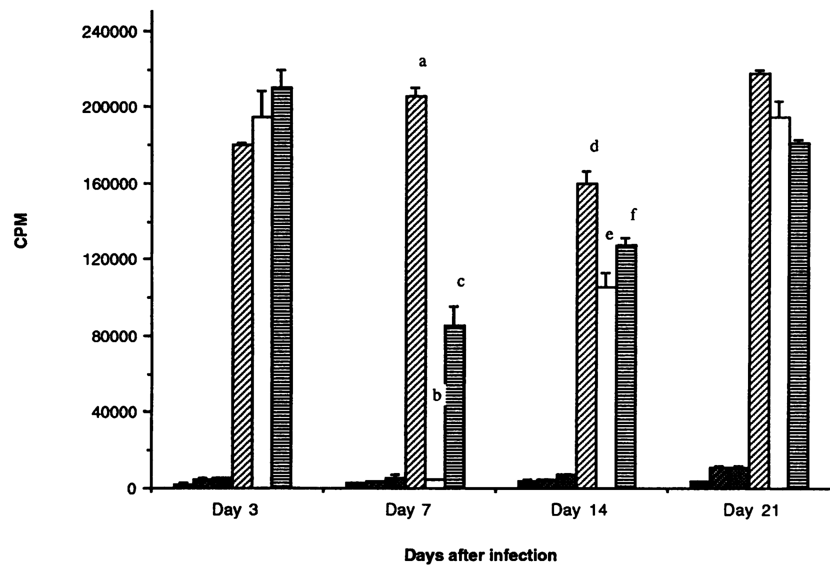

FIG. 1. Proliferative response to Con A of splenocytes from mice infected with either *T. gondii* PTg or PTgB. The spleen cells were obtained from uninfected mice or mice infected for 3, 7, 14, and 21 days and were cultured for 48 h in triplicate wells at a concentration of 2×10^5 cells per well in presence of Con A (5 μg/ml). Lymphoproliferation was determined on days 14 and 21 postinfection in only those PTg-infected mice which had survived the infection. The proliferation was determined by the amount of [^{3}H]thymidine incorporation, and the results are expressed as means ± standard deviations (error bars) for triplicate wells. ■ and ▨, normal uninfected mouse spleen cells without and with Con A, respectively; ▩ and □, PTg-infected spleen cells without and with Con A, respectively; ▦ and ▤, PTgB-infected spleen cells without and with Con A, respectively. This experiment was repeated five times. For comparisons b versus a, c versus a, b versus c, e versus d, and f versus d, (P was <0.05). For e versus f, P was >0.05.

by spleen cells from infected mice cultured for 24 h in the presence or absence of parasite antigen (data not shown).

As shown in Table 2, the quantity of IL-2 produced by infected mouse splenocytes after Con A stimulation was significantly lower than that produced by uninfected splenocytes. On all days evaluated, splenocytes from mice infected with the wild-type strain produced less IL-2 in response to Con A than splenocytes from mice infected with the mutant strain. The type of splenocyte cells involved in the reduced ($P > 0.05$) IL-2 production was evaluated. As shown in Table 3, coculture in the presence of adherent cells from infected mice significantly reduced the quantity of IL-2 detected in the supernatants of either uninfected or infected splenocytes.

Effect of exogenous IL-2 on splenocytes from infected mice. The ability of exogenous IL-2 to overcome the depressed proliferative response was evaluated for splenocytes from mice on day 7 postinfection. The biological activity of rIL-2 was confirmed by CTLL assay prior to use in this experiment. As shown in Fig. 4, exogenous IL-2 at different concentrations failed to reconstitute the depressed proliferative response to Con A of the spleen cells from acutely infected mice.

Determination of binding sites and capacity of high-affinity IL-2 receptors. To determine IL-2 receptor expression on lymphocytes from infected mice, splenocytes on 7 day postinfection were isolated and cultured in the presence of Con A for 48 h. IL-2 receptor expression was measured in a binding assay using radiolabeled rIL-2. A Scatchard analysis to determine the number of binding sites per cell and the dissociation constant (K_d) showed no significant differences between the wild-type- and PTgB-infected splenocytes. There was, however, a threefold decrease in the number of binding sites per cell in the infected mice (Table 4). Although splenocytes from both infected and uninfected mice express high-affinity receptors for IL-2, the difference in the K_d values between these groups was insignificant ($P > 0.05$).

Role of IL-10, TGF-β, and nitric oxide. To determine whether IL-10 was responsible for the observed depressed proliferative response to Con A, splenocytes obtained from either 7-day-infected or uninfected mice were cultured, and the supernatants were evaluated for the presence of IL-10. As shown in Table 5, the splenocytes obtained from mice infected with wild-type parasites produced considerable amounts of IL-10 in comparison to uninfected control splenocytes. Moreover, when the splenocytes from these infected mice were cocultured with the adherent cells obtained from 7-day-infected mice, the amount of IL-10 expressed in the supernatant was even greater. Antibody to IL-10 (at a concentration of 4 μg/ml) partially reversed (approximately 60%) the inhibitory response to Con A (Table 6). However, antibody to TGF-β failed to reverse the reduced proliferative response.

The culture supernatants from 7-day-infected mice were evaluated for the presence of nitric oxide. Splenocytes from either 7-day-infected mice or uninfected controls were stimulated with Con A, and the amount of NO produced was determined by the Greiss reaction (13). The same number of effector cells (2×10^5) that exhibited downregulatory activity in previous experiments were used. In this assay, there was no significant difference in the amount of NO produced by infected splenocytes at 24 h (0.5 μM) and 48 h (2.7 μM) postculture. An equivalent amount of NO was produced when the same number of normal spleen cells were stimulated with Con A. Either increasing the number of cultured cells or activating the macrophages with gamma interferon plus lipopolysaccharides enhanced the amount of detectable NO in the culture supernatant. Other times postinfection were not evaluated since there was no detectable NO production during the time of greatest reduction in the lymphoproliferative response to Con A.

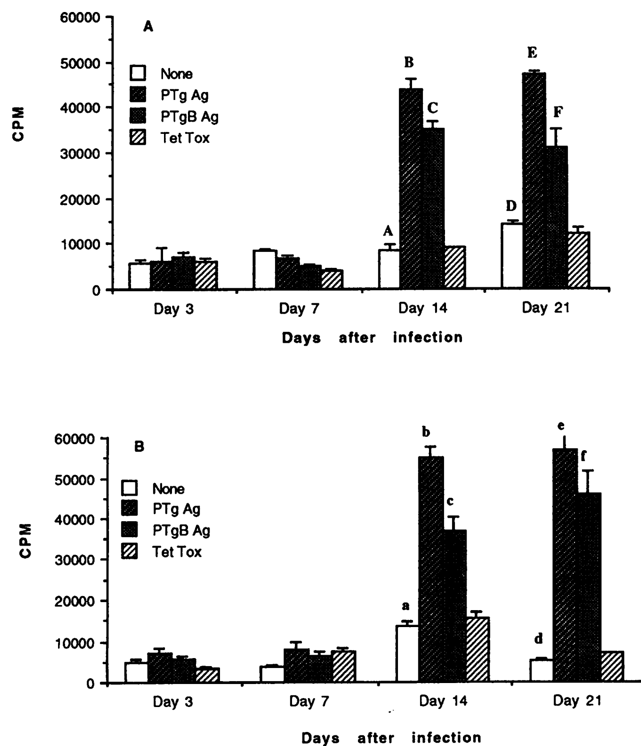

FIG. 2. Proliferative response to TGA of splenocytes from mice infected with PTg (A) or PTgB (B) parasites or age-matched normal uninfected mice. The splenocytes were cultured at a concentration of 2×10^5 per well in the presence of 8.75 μg of TGA per ml or of unrelated antigen, tetanus toxoid (Tet Tox). The results are expressed as means ± standard deviations (error bars) for triplicates. This figure shows the results of a set of four representative experiments. None, cells with medium only; PTg Ag or PTgB Ag, cells were cultured in the presence of PTg or PTgB antigen. Comparisons were made by using the two-tailed Student *t* test. In panel A, B versus A, C versus A, E versus D, and F versus D ($P < 0.05$); in panel B, b versus a, c versus a, e versus d, and f versus d ($P < 0.05$).

DISCUSSION

Our data suggest that a well-delineated period of lymphocyte hyporesponsiveness occurs following primary *T. gondii* infection in mice. During this period, there is an associated reduction of IL-2 in response to Con A stimulation. Although this reduced proliferative response and IL-2 production were observed during the first 14 days postinfection, it was most notable on day 7. These observations confirm previous reports indicating that a state of immunodepression occurs during the first 8 days of infection with *T. gondii* in both humans and experimental models (8, 23, 38, 39). Our studies extend these observations to at least 21 days, at which time the infected mice regain their ability to respond to both parasite antigen or Con A. Although both strains of parasite, the wild type and the mutant, have the ability to induce a state of hypoproliferation, the wild-type parasite had a greater influence on mediating this response. Whether these differences are due to a lower parasite burden or slower growth of the mutant remains unclear. Current data from our laboratory indicate that mice infected with the wild-type parasite die earlier and with greater frequency than those infected with the mutant parasite.

A decrease in the quantity of IL-2 was observed in the supernatant of cultured splenocytes from mice infected with either parasite strain following Con A stimulation. This is consistent with the finding of McLeod and coworkers, who also reported a similar depression of IL-2 during the acute phase of murine toxoplasmosis (29). We have observed an augmentation in $CD8^+$ T cells that accompanies the period of diminished lymphocyte reactivity to parasite antigen or Con A. It is possible that the depressed IL-2 response might be related to the rise in $CD8^+$ T cells, since $CD8^+$ lymphocytes usually produce high quantities of gamma interferon and little or no IL-2 (31, 44). Analysis of the absolute T-cell count failed to distinguish a significant difference in the number of T cells in infected and uninfected mice. However, this does not exclude a dissimilarity in the number of IL-2-producing cells between these mice.

Our results demonstrated that the addition of exogenous IL-2 had little effect on restoring the reduced proliferative response of these splenocytes. Failure to revert this response has previously been reported in viral (33), bacterial infection (3), and other parasitic infection, in particular with *T. cruzi* (17). In those studies, infection with either lymphocytic choriomeningitis virus or salmonellae resulted in reduced IL-2 production that could not be reversed by the addition of exogenous IL-2. In contrast to our results, Chan et al. reported restoration of suppressed proliferative response of spleen cells from mice infected for 3 or 6 days with C56, an avirulent strain of *T. gondii* (8). It is possible that differences in parasite strains and the times tested may be responsible for this discrepency. Also, in malaria infection, suppressed lymphoproliferative responses are restored by exogenous IL-2 (10). The failure of lymphocytes to proliferate in response to exogenous IL-2 led us to examine the IL-2 binding property of the receptors on lymphocytes from infected animals. The results from these

TABLE 1. Phenotypic analysis of mouse T-cell populations

Day postinfection	Spleen cell type	% T-cell phenotypes (mean ± SD) with MAb:				
		Thy 1.2	GK 1.5 (CD4)	TIB 150 (CD8)	3A10 (γδ T cells)	Negative
0	Uninfected	32 ± 1	24 ± 5	13 ± 3	0 ± 0	3 ± 1
3	PTg infected	31 ± 3	18 ± 3	14 ± 5	0.3 ± 0.2	5 ± 2
	PTgB infected	32 ± 6	22 ± 4	15 ± 4	1 ± 0.6	7 ± 2
7	PTg infected	38 ± 4	23 ± 5	19 ± 4	5 ± 0	1 ± 0.5
	PTgB infected	34 ± 2	24 ± 4	19 ± 2	6 ± 0	3 ± 2
14	PTg infected	37 ± 3	30 ± 2	25 ± 5	9 ± 0.9	2 ± 0
	PTgB infected	24 ± 0	10 ± 0	19 ± 0	0 ± 0	2 ± 0.5

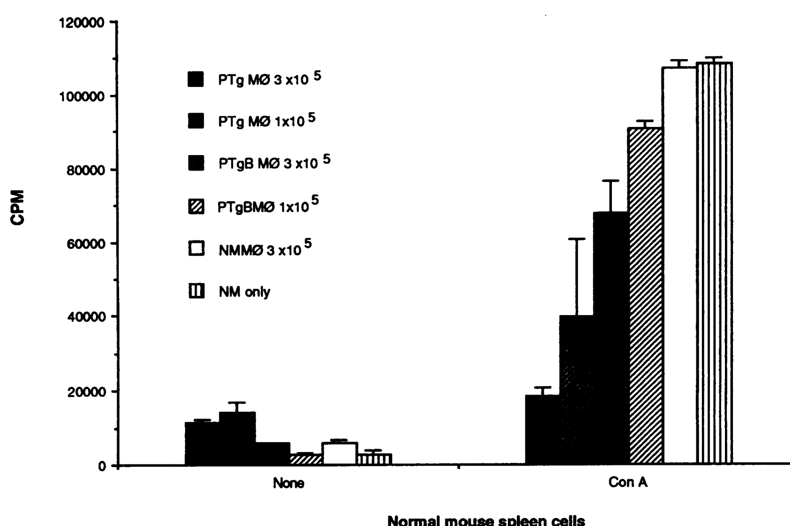

FIG. 3. Proliferative response to Con A of uninfected normal spleen cells cultured with adherent cell populations obtained from mice on day 7 postinfection or uninfected control mice. Adherent macrophages were isolated and cultured as described in Materials and Methods. Proliferation was determined by the amount of [^{3}H]thymidine incorporated, and the results are expressed as means ± standard deviations (error bars) of triplicates. Data show a representative experiment with five mice per group. PTg MØ, adherent cells from PTg-infected spleens; PTgB MØ, adherent cell from PTgB-infected spleens; NM MØ, adherent cells from normal uninfected mouse spleens, NM only, uninfected spleen cells with or without Con A. This experiment was repeated three times.

experiments showed a threefold decrease in the number of IL-2 binding sites per cell in both PTg- and PTgB-infected mice compared with cells from uninfected mice. However, the binding affinity, as determined by the K_d values, remained the same for cells from uninfected mice as well as from PTg- and PTgB-infected mice. It is possible that among the nonresponding cell population, a defect in the IL-2 signaling pathway may exist. Studies are currently underway to evaluate this possibility.

The results of our study demonstrated that macrophages from infected mice reduce the lymphoproliferative response when added to the culture by affecting IL-2 production of normal uninfected splenocytes in response to Con A. This macrophage-mediated downregulation of IL-2 production has also been reported for bacterial infection (3). The inhibitory activity was not limited to macrophages since the nonadherent spleen cells from mice on day 7 postinfection also inhibited T-cell proliferation and Con A-induced IL-2 production, although to a lesser degree (data not shown).

The precise mechanism(s) of reduced IL-2 production during primary *T. gondii* infection remains unclear. The results of our study demonstrate that macrophages from mice that are acutely infected with *T. gondii* can indeed inhibit Con A-stimulated T-cell proliferation and can also affect production of IL-2. The suppressive effect of macrophages on in vitro lymphocyte proliferation in response to mitogens or parasite antigens was previously suggested for malaria (24), trypanosomiasis (41), and toxoplasmal infections (8). Whole spleen cell populations from either *T. gondii*-infected mice (day 7) or age-matched normal uninfected animals irradiated and added to the culture of splenocytes from uninfected mice in the presence of Con A resulted in decreased Con A-induced T-cell proliferation, accompanied by reduced IL-2 production. The mechanisms of suppressor function of macrophages are not clearly understood. In addition to the production of soluble factors with negative regulatory activity, a recent study also suggested that macrophage-mediated downregulation of the

TABLE 2. Production of IL-2 by Con A-stimulated spleen cells[a]

Spleen cell type	IL-2 production (pM) on day postinfection indicated			
	3	7	14	21
Uninfected	60 ± 7	94 ± 7	50 ± 4	80 ± 8
PTg infected	55 ± 4	<5	54 ± 2	40 ± 5
PTgB infected	90 ± 7	18 ± 5	61 ± 3	53 ± 6

[a] Supernatants from 24 h cultures with Con A (5 μg/ml) of spleen cells obtained from PTg- or PTgB-infected mice and age-matched control mice. The supernatants were tested for the presence of IL-2 by the CTLL-2 cells proliferation assay as described in Materials and Methods. The data shown are the results (± standard deviations) of one typical experiment of four experiments. On day 7 postinfection, IL-2 production in splenocytes from PTg-infected mice was significantly lower than that from PTgB-infected animals ($P < 0.05$) or from uninfected mice ($P < 0.05$). For each time point, cultures in medium only had levels of IL-2 that were below the detectable threshold (<2 pM).

TABLE 3. Production of IL-2 by Con A-stimulated cocultured spleen cells[a]

Spleen cell type	IL-2 production (pM) with:			
	Con A	Macrophages		
		PTg-infected	PTgB-infected	Uninfected
Uninfected	245 ± 59	21.8 ± 2	69 ± 7	240 ± 30
PTg infected	47 ± 6	19.5 ± 1.4	25 ± 4	43 ± 6
PTgB infected	60 ± 11	24 ± 5	22 ± 1.3	52 ± 8

[a] Uninfected spleen cells or spleen cells (2 × 10^5) from infected mice were cocultured with adherent macrophages (3 × 10^5) from PTg- or PTgB-infected (for 7 days) or uninfected spleens in the presence of Con A. Supernatants from 24-h cultures with Con A of cocultured cells were obtained, and the quantity of IL-2 produced was determined by the CTLL-2 cell proliferation assay. The proliferation was measured by [^{3}H]thymidine incorporation. The response was analyzed by Probate analysis as described previously (16) compared with the 50% maximal response obtained with standard IL-2. The data shown are results (± standard deviations) of one typical experiment of four experiments.

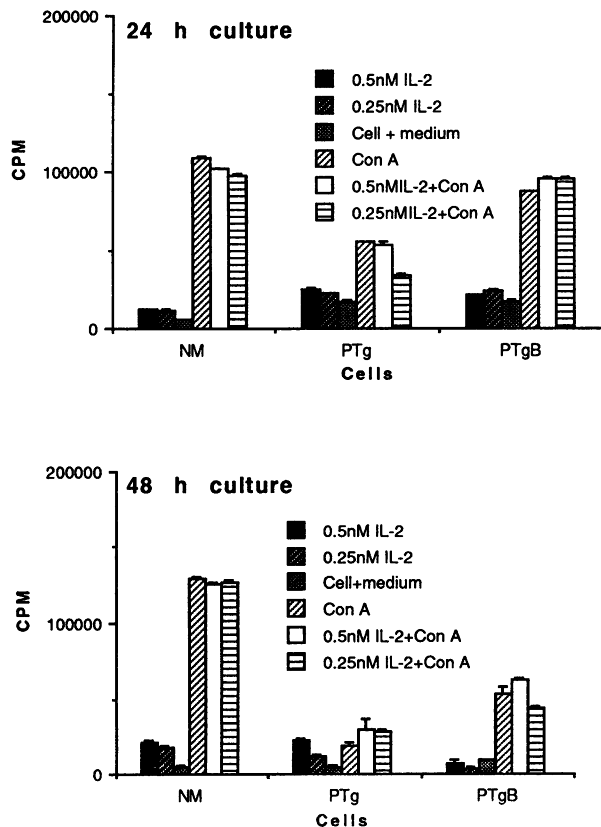

FIG. 4. Effect of exogenous IL-2 on Con A-stimulated proliferative response of splenocytes from PTg- or PTgB-infected mice. Different concentrations of rIL-2 were added to the splenocytes of mice on day 7 postinfection with either PTg or PTgB parasites and uninfected control mice. The cells were cultured for either 24 or 48 h with or without Con A in the presence of rIL-2. Proliferation was determined by the amount of [^{3}H]thymidine incorporated, and the results expressed as counts per minute. Data present a representative experiment with three mice per group. NM, normal mouse spleen cells; PTg, PTg-infected spleen cells; PTgB, PTgB-infected spleen cells. This experiment was repeated four times.

T-cell function can occur by a cell contact-dependent mechanism, through a process of macrophage activation involving the CD14 antigen (25).

The various soluble factors produced by macrophages that may be implicated in downregulation of the immune response include IL-10 (12, 14), TGF-β (42), nitric oxide (2, 30), and prostaglandin (32). Murine or human IL-10 has been shown to exert an antagonistic effect on IL-2 production (14, 40). Our results detected an increased level of IL-10 in the supernatant of Con A-stimulated splenocytes from infected mice (Table 5). The inhibitory activity of this supernatant can be partially reversed by antibody to IL-10 (Table 6), suggesting that this cytokine may be responsible in part for the reduced lymphoproliferation as well as the alteration in production of IL-2 in response to Con A.

Although TGF-β may mediate depression of immunity by inhibiting cytokine production (9) during *T. cruzi* infection (36), it does not appear to play a role in downregulation of immunity under the conditions in these experiments. However, the inability to reverse the inhibitory effect of the infected splenocytes by MAb to TGF-β does not exclude its potential importance under other experimental conditions during *T. gondii* infection. Nitrogen intermediates, in particular, nitric oxide, have also been shown to inhibit the proliferative response of T cells (30). Recent studies suggest that nitric oxide mediates, in part, the suppression observed in trypanosomiasis (35). Earlier reports (1) have demonstrated the potential role of this metabolite during infection with *T. gondii*. Using the same number of Con A-stimulated splenocytes (2×10^5) that exhibit downregulation of immunity, we were unable to detect a significant increase in NO production over that by control. It has been shown, however, that IL-10, whose production was augmented with the same number of infected spleen cells, can be a potent inhibitor of NO production (5, 6). With greater numbers of cells (5×10^6), a significant increase in the amount of NO produced by infected splenocytes (5.5 and 10 μM at 24 h and 48 h, respectively) compared with that by uninfected spleen cells (2 and 5.3 μM at 24 and 48 h, respectively) was demonstrated. Thus, we believe that NO does have a potential role in downregulation of immunity; but in our system, at the number of cells utilized, it is below the level of significance and may not contribute to the observed effect. Studies with the NO inhibitor, NMMA (N^G-monomethyl-L-arginine), using more effector cells ($>2 \times 10^6$) than were utilized throughout these experiments indicate that the inhibitory effect by the infected splenocyte supernatant can be maximally reversed by approximately 20 to 25% (22a).

Other than IL-10 induction, which appears to be partially responsible for some of the immunosuppressive features we have observed, other factors that may influence IL-2 production could include (i) suppression of the IL-2 gene at the transcriptional level (37), (ii) an increased rate of degradation

TABLE 4. ^{125}I-labeled IL-2 binding assay results for high-affinity receptors[a]

Spleen cell type	No. of binding sites/cell	K_d (pM)
Uninfected	3,968 ± 2,258 A	45 ± 36 D
PTg infected	1,305 ± 1,077 B	40 ± 28 E
PTgB infected	1,498 ± 1,081 C	40 ± 27 E

[a] The results for binding of ^{125}I-labeled IL-2 to its receptor on Con A-activated uninfected and PTg- or PTgB-infected (for 7 days) splenocytes from two mice are shown. The number of high-affinity binding sites and dissociation constant values (K_d) were determined by Scatchard analysis. Comparisons were made using the two-tailed student's t test: B versus A ($P < 0.05$), C versus A ($P < 0.05$), B versus C ($P > 0.05$), E versus D ($P > 0.05$), and F versus D ($P > 0.05$).

TABLE 5. Production of IL-10 by splenocytes from *T. gondii*-infected or uninfected mice after in vitro stimulation with Con A

Spleen cell type	IL-10 (U/ml)
PTg infected	5.2 ± 1.0
Uninfected	<1
PTg infected + macrophages[b]	7.3 ± 2.0
Uninfected +[c]: PMØ[d]	
Infected macrophages	3.5 ± 0.5
Uninfected macrophages	<1

[a] Spleen cells (2×10^5) from mice on day 7 postinfection with PTg parasites or from uninfected mice were cultured in the presence of Con A. Supernatants from 48-h cultures with Con A were assayed for IL-10 production by ELISA, and the results are expressed as the means ± standard deviations of duplicates.

[b] Spleen cells from mice 7 days after infection cultured with macrophages obtained from mice 7 days after infection.

[c] Uninfected +, uninfected mouse spleen cells cultured with macrophages obtained either from mice 7 days after infection or from uninfected mice.

TABLE 6. Effects of anti-IL-10 or anti-TGF-β antibody on Con A-stimulated splenocytes[a]

Spleen cell type	IL-2 production (pM) with:					
	Medium only	anti-IL-10 MAb	anti-TGF-β MAb	Con A	anti-IL-10 MAb + Con A	anti-TGF-β MAb + Con A
Uninfected	2,429 ± 145	2,746 ± 251	3,670 ± 1,349	87,566 ± 9,296	99,781 ± 9,062	86,909 ± 6,670
PTg infected	5,736 ± 390	5,009 ± 1,469	4,821 ± 2,329	22,934 ± 246	73,823 ± 4,030	22,261 ± 2,818

[a] The proliferative response of splenocytes to Con A in the presence of either rat anti-murine IL-10 or anti-TGF-β MAb is shown. Spleen cells were obtained from PTg-infected mice on day 7 postinfection and were cultured (2×10^5 cells per well) with Con A for 48 h in the presence of anti-IL-10 antibody (4 μg/ml) or anti-TGF-β antibody (30 μg/ml). Rat immunoglobulin G was used as an isotype-matched control and did not show any effects on the controls (2,533 ± 239 and 86,900 ± 5,670, respectively) or experimental group (5,810 ± 2,808 and 22,400 ± 245, respectively). The proliferation was measured by [^{3}H]thymidine incorporation, and the results are expressed as the means ± standard deviations of triplicate wells. The experiment was repeated three times.

of IL-2 mRNA, (iii) the lack of IL-2-producing cells, and (iv) production of other soluble, inhibitory factors. In summary, our observations suggest that a state of transient immunodepression occurs during acute murine toxoplasmosis. This condition appears to be mediated principally by macrophages from infected mice. These macrophages are able to inhibit the proliferative response of naive cells in response to Con A. Further, these macrophages appear to affect the production of IL-2, perhaps through the production of IL-10, which is at least partially responsible for the observed effect.

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