INFECTION AND IMMUNITY, Sept. 1995, p. 3442–3447 Vol. 63, No. 9
0019-9567/95/$04.00+0

A *Toxoplasma gondii*-Derived Factor(s) Stimulates Immune Downregulation: an In Vitro Model

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Received 24 January 1995/Returned for modification 30 March 1995/Accepted 15 June 1995

Suppression of the T-cell lymphoproliferative response and downregulation of interleukin 2 (IL-2) production by *Toxoplasma gondii* has been observed following in vivo infection. In this study, an experimental in vitro murine system was developed to evaluate the kinetics of these responses. Normal splenocytes from uninfected mice were stimulated with either concanavalin A or an anti-CD3 monoclonal antibody and cocultured with *Toxoplasma* tachyzoites either directly or separated by a transwell. A progressive decline in the lymphoproliferative response was observed as the concentration of parasites in culture increased. Neither heat-killed nor formaldehyde-fixed parasites stimulated this downregulatory response by the splenocytes. A decline in IL-2 production was associated with the decrease in lymphocyte proliferation. The addition of an antibody to IL-10 or heat-inactivated anti-*Toxoplasma* sera to the culture supernatant partially neutralized the inhibitory effect on lymphocyte proliferation. Cytokine analysis of the responder splenocytes demonstrated a decrease in the message for IL-2 and IL-2 receptor and an increase in IL-10. Together, these observations suggest that during in vitro culture in a murine system, parasite antigens that stimulate the release of a soluble factor(s), such as IL-10, that inhibits proliferation of mitogen-stimulated T cells are expressed.

Suppression of the host immune system by different intracellular parasites is well recognized. Earlier studies from our laboratory and others have determined in mice that 7 days after *Toxoplasma gondii* infection, both the T-cell mitogen and parasite antigen-associated lymphoproliferative responses are reduced (3, 13, 18). Associated with this reduced lymphocyte response is a reduction in interleukin 2 (IL-2) and an increase in IL-10, which appear to mediate, in part, the observed downregulation by *T. gondii* (13). Both activated macrophages and lymphocytes secreting IL-10 (18) and the reactive intermediate, nitric oxide, inhibit T-cell proliferation in murine toxoplasmosis (3), and the expression of mRNA for IL-10 has been observed in the brains of mice infected with *T. gondii* (2). Murine IL-10 can downregulate the host immune response by decreasing the production of IL-2 (11, 24) and inhibiting mitogen driven T-cell proliferation (9). Reversal of the inhibitory response by exogenous IL-2 has been partially successful or ineffectual in evaluating the response of lymphocytes to either *Trypanosoma cruzi*-, *T. gondii*-, or malaria-infected mice (5, 6, 14). This discrepancy during acute *T. gondii* infection may be due in part to the use of different parasite strains.

Because immune cells from infected mice are subject to a wide range of regulatory processes in vivo, we developed an experimental model to evaluate the downregulatory response induced directly by the parasite following in vitro infection. In this report, splenocytes were isolated from uninfected mice and incubated with *T. gondii* in culture either in direct contact with the host cells or physically separated from the host cells by a semipermeable membrane. Our observations demonstrate that as the number of parasites in culture increases, there is a corresponding inhibition of both mitogen- and anti-CD3-driven lymphoproliferation. This hypoproliferative response could be partially neutralized by the addition of antibody to IL-10 or heat-inactivated anti-*Toxoplasma* rabbit serum to the culture, suggesting that *T. gondii* excretes or secretes a factor(s) that stimulates the production of the immune downregulatory cytokine, IL-10, in naive splenocytes.

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MATERIALS AND METHODS

Parasites. For these studies, either the wild-type P (PLK) strain of *T. gondii* (PTg) or the PTg-derived mutant, SAG1-deficient strain PTgB (15, 17) was used. Parasites were maintained in our laboratory by in vitro passage in human foreskin fibroblasts at 37°C in minimal essential medium containing 10% newborn calf serum. Parasites were purified from human fibroblast cell culture as previously described (13), and parasite viability was determined by trypan blue exclusion. For some experiments, parasites were heat killed or formalin fixed. For the heat-killed parasite preparation, purified parasites were suspended in serum-free medium at a concentration 2×10^8/ml and incubated at 56°C for 50 min, washed twice, and used fresh at various concentrations in 96-well plates. For the preparation of formalin-fixed parasites, purified parasites were suspended in phosphate-buffered saline (PBS) and incubated for 1 h with 0.1% formalin at room temperature, washed three times, and used at various concentrations (i.e., 10×10^5, 2×10^5, and 4×10^4 per well) and cell/parasite ratios (i.e., 1:5, 1:1, and 5:1). Controls were done in parallel, using live parasites at the same cell/parasite ratios (1:5, 1:1, and 5:1).

Lymphocyte preparation and phenotyping. Splenocytes were obtained from 5- to 6-week-old female A/J mice. Erythrocytes were lysed by hypotonic shock with 0.2% ammonium chloride. The cells were washed in PBS and cultured in Iscove's medium containing 2 mM L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.2), and 10% fetal bovine serum (HyClone Laboratories, Logan, Utah). Viability of the lymphocytes was determined (>99.5%) by trypan blue exclusion. Unless otherwise noted, the cell concentrations were 2×10^5 cells per well in 96-well plates and 10^6 cells per well in 24-well plates.

For phenotypic analysis of the responder population, 10^6 splenocytes were incubated for 24 and 48 h with or without living *T. gondii* (10^6 parasite in a Transwell-COL chamber) in the absence or presence of concanavalin A (ConA; 5 μg/ml) or cross-linked anti-CD3 monoclonal antibody (MAb; 5 μg/ml). The washed cells were incubated with phycoerythrin (PE)-labeled anti-CD3ε_1, PE-labeled anti-CD4, or PE-labeled anti-CD8, fluorescein isothiocyanate labeled for 30 min at 4°C, washed with PBS containing 1% bovine serum albumin, fixed with 1% formaldehyde, and analyzed by flow cytometry (13). For controls, cells were stained with PE- or fluorescein isothiocyanate-labeled normal rat immunoglobulin G (IgG).

Lymphocyte proliferation assays were performed in triplicate, using cells cultured in Iscove's medium at a concentration of 2×10^5 spleen cells per well with different numbers of parasites to give a spleen cell/parasite ratio of 1:5, 1:1, or 5:1. In some experiments, a cell/parasite ratio of 4:1, 8:1, or 16:1 was used. Separation of responder from parasites was done by using a transwell system (Costar, Cambridge, Mass.). Mouse spleen cells (10^6) were cultured with various concentrations of live parasites while separated from the responder cells by a

TABLE 1. Primers used for PCR-assisted mRNA amplification

Primer	Sequence
β-actin (540 bp)	
5′	5′GTG CGC CGC TCT AGG CAC CAA 3′
3′	5′CTC TTT GAT GTC ACG CAC GAT TTC 3′
IL-4 (399 bp)	
5′	5′ATG GGT CTC AAC CCC CAG CTA GT 3′
3′	5′GCT CTT TAG GCT TTC CAG GAA GTC 3′
IL-2R p55 (700 bp)	
5′	5′ATG GAG CCA CGC TTG CTG ATG TTG 3′
3′	5′C CAT TGT GAG CAC AAA TGT CTC CG 3′
IL-2 (413 bp)	
5′	5′TTC AAG CTC CAC TTC AAG CTC TAC AGC GGA AG 3′
3′	5′GAC AGA AGG CTA TCC ATC TCC TCA GAA AGT CC 3′
IFN-γ (460 bp)	
5′	5′-TGA ACG CTA CAC ACT GCA TCT TGG-3′
3′	5′-CGA CTC CTT TTC CGC TTC CTG AG-3′
IL-10 (274 bp)	
Sense	226 GTGAAGACTTTCTTTCAAACAAAG 249
Antisense	476 CTGCTCCACTGCCTTGCTCTTATT 499

transwell at cell/parasite ratios of 1:1, 4:1, and 16:1. ConA was added at the start of culture and after 24 or 48 h of incubation at 37°C, the proliferation was measured by incorporation of [^{3}H]thymidine (0.5 μCi per well; specific activity, 40 to 60 mCi/mmol; Amersham), and the results were expressed as stimulation indices (SIs; SI = mean cpm for stimulated sample/mean cpm for unstimulated sample).

Cross-linking of the anti-CD3 MAb (PharMingen, San Diego, Calif.) was performed by coating 24-well plates with 14 μg of goat anti-hamster IgG (Jackson Immunology Research Laboratories, West Grove, Pa.) overnight at 4°C. After washing, the wells were incubated with 5 μg of the anti-CD3 MAb at 37°C. Naive splenocytes were added to the wells and separated from live parasites by the semipermeable transwell membrane. DNA synthesis of proliferating lymphocytes was determined after 42 h by 6 h of [*methyl*-[^{3}H]thymidine (ICN, Costa Mesa, Calif.) incorporation.

Assays with an anti-PTg rabbit serum. Serum was obtained from rabbits immunized over a 3-week period with a total dose of 100 μg of PTg extract. The inoculum was prepared by repeat (four times) freezing-thawing of culture-derived tachyzoites purified over a nylon wool column. The antibody response to the PTg antigen was verified by enzyme-linked immunosorbent assay. The serum was heat inactivated at 56°C for 50 min and filtered before use. Splenocytes were prepared from uninfected A/J mice as described above. Parasites were incubated with heat-inactivated anti-PTg rabbit serum (dilution of 1:20) at 37°C across a semipermeable membrane from naive splenocytes that were incubated with either ConA or medium alone. Heat-inactivated normal rabbit serum (dilution of 1:20) was used as a control. After 48 h of incubation, lymphocyte proliferation was determined by [^{3}H]thymidine incorporation. Data were expressed as SIs.

Cytokine analysis and production of NO. The presence of IL-2 in 18-h culture supernatants was measured by proliferation of an IL-2-dependent murine T-cell clone as previously described (12). The response was analyzed by probit analysis and compared with the 50% maximal response obtained with standard recombinant IL-2. A MAb to IL-10 was used to investigate the effects of these cytokines on parasite-induced splenocyte proliferation in the presence of ConA. IL-10 activity was determined by an inhibition assay using a rat anti-mouse IL-10 antibody (12 μg/ml; Genzyme, Cambridge, Mass.) and normal rat IgG (Sigma Chemical Co., St. Louis, Mo.) as a control. Cells with parasites were incubated with anti IL-10 for 30 min at 37°C, after which either ConA or medium was added. After 48 h of incubation, proliferation was determined by [^{3}H]thymidine incorporation. Data were expressed as SIs.

Nitrite in 48-h culture supernatants was measured by using Griess reagent (10). Briefly, 0.05 ml of supernatant was mixed with 0.05 ml of Griess reagent (0.5% sulfanilamide and 0.05% *N*-1-naphthylenedimine hydrochloride in 2.5% H_3PO_4) and then incubated for 10 min at room temperature and read by a spectrometer (A_{570}). The nitrite concentration was calculated from a $NaNO_2$ (Sigma) standard curve. The presence of IL-4 in 24-h culture supernatants was measured by proliferation of a subline of the cell CTLL-2 line (CTLL-H) as described previously (25).

RNA extraction and reverse transcription. To establish the optimum time period for the expression of cytokine genes, PCR was performed with cells cultured for either 6 or 18 h. To do the assay, 8×10^6 splenocytes, an amount previously shown to be sufficient to allow for the detection of cytokine transcripts (19), were activated with ConA in the presence or absence of equal number of live parasites across a transwell at a cell/parasite ratio of 1:1. Extraction of RNA, preparation of cDNAs from splenocytes, and PCR-assisted mRNA amplification were done by a standard method as reported previously (19). Cytokine-specific primer pairs (Clontech, Palo Alto, Calif.) and the primer for mouse IL-10 (Stratagene, La Jolla, Calif.) were obtained from a commercial source and used according to the manufacturer's suggestions. The oligonucleotide primer sequences used for PCR-assisted mRNA amplification are shown in Table 1.

RESULTS

***Toxoplasma*-infected splenocytes inhibit lymphocyte proliferation.** Uninfected mouse splenocytes were cocultured with various concentrations of *T. gondii* in the presence of ConA, and the level of proliferation was determined. As the number of parasites in culture increased, there was a corresponding decrease in the lymphoproliferative response as well as in the production of IL-2 (Table 2). There was no significant difference in the degree of suppression induced by the wild-type or mutant, SAG1-deficient parasites. Similar observations were made when we used the transwell system in which the toxoplasmas were cocultured across the membrane from mitogen-driven naive splenocytes, as shown in Table 3. The greatest inhibition was observed at either a 4:1 (85%) or 1:1 (99%) cell/parasite ratio ($P < 0.005$). To define whether this suppression was dependent on the presence of live parasites, different concentrations of either heat-killed parasites or formaldehyde-fixed parasites were used in a similar assay. As shown in Fig. 1, none of these killed parasite preparations were able to inhibit the lymphoproliferative response. Further, the major surface protein SAG1 does not appear to be an important mediator of this response, since there was no discernible difference in the patterns of suppression induced by wild-type and mutant parasites. These findings indicate that the suppressive phenome-

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

Cell/parasite ratio	production of IL-2 (pM)	
	Medium	ConA
No parasite	1.6 ± 0.6	56 ± 7
16:1	1.0 ± 0.01	27 ± 6
4:1	0.5 ± 0.01	20 ± 7
1:1	0.6 ± 0.01	8 ± 4

[a] Supernatants from 24-h cultures with various splenocyte/parasite ratios were tested for the presence of IL-2 by the CTLL-2 proliferation assay. The data shown are the means ± standard deviations of one typical experiment of four. IL-2 production in mice infected at a cell/parasite ratio of 1:1 was significantly lower than in uninfected mice ($P < 0.05$). For controls (medium only), the levels of IL-2 were below the detectable threshold (<2 pM).

TABLE 3. Spleen cell responses to different concentrations of live parasites in the presence of ConA[a]

Cell/parasite ratio	SI			% Inhibition[b] after stimulation with ConA	SI, ConA + IL-2	% Inhibition after stimulation with ConA + IL-2
	Medium	IL-2	ConA			
No parasite		1.4 ± 0.3	85 ± 7.0		85 ± 4.0	
16:1	1.5 ± 0.2	1.2 ± 0.2	24 ± 1.0	71	28 ± 0.7	67
4:1	0.6 ± 0.1	0.4 ± 0.2	12 ± 0.5	85	19 ± 0.8	77
1:1	0.3 ± 0.1	0.3 ± 0.1	0.8 ± 0.7	99	2 ± 0.7	97

[a] Normal mouse splenocytes were cultured in the presence of ConA (5 μg/ml) and exogenous IL-2 (2 nM) across a transwell membrane from parasites at various cell/parasite ratios. After 48 h of incubation at 37°C, proliferation was measured by the amount of [^{3}H]thymidine incorporated, and the results are expressed as SIs. Control cultures are transwell inserts containing medium with or without mitogen in the absence of parasites. Results are expressed as the means ± standard deviations of three experiments.

[b] Calculated as [(control − background) − (experimental − background)] control − background × 100.

non is dependent on the presence of live parasites and independent of the major tachyzoite surface protein, P30 (SAG1).

Lymphocyte proliferation in response to anti-CD3 was then used to distinguish if the inhibitory response was due to the effect of mitogen stimulation on the responding lymphocytes. CD3 molecules are important in the T-cell receptor-CD3 complex and are necessary for signal transduction following antigen binding. A transwell assay was performed to evaluate whether the inhibitory response was observed in the presence of cross-linked anti-CD3 (Table 4). Of note is that at the higher cell/parasite ratio (1:1), 92% inhibition was observed when the cells were stimulated with anti-CD3. These results indicated that the factor(s) responsible for the observed inhibitory effect was not dependent on the ConA-mediated response.

Kinetics of the lymphocyte inhibitory response by *T. gondii*. Splenocytes were evaluated at different time points following in vitro exposure to toxoplasmas for the ability to inhibit lymphocyte proliferation. Significant inhibition was observed with the 24-h culture at a cell/parasite ratio of 4:1 (77%; data not shown). However, consistent with the observations in Table 3, maximal suppression of DNA synthesis was noted when the responder cells were exposed to parasites for 48 h at a cell/parasite ratio either of 8:1 (97%) or 4:1 (99%). Exogenous IL-2 (2 nM) failed to reverse significantly the inhibitory effect mediated by the live parasites in both the 24- and 48-h cultures.

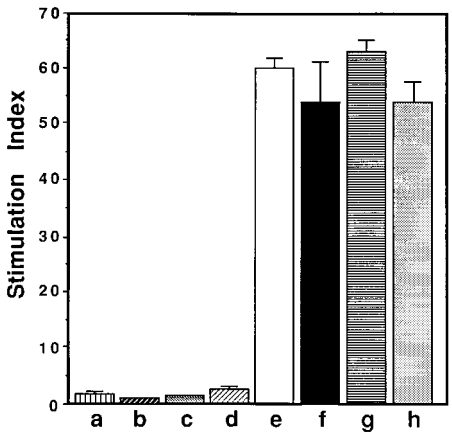

FIG. 1. Proliferative response of mouse splenocytes to *T. gondii* in vitro. Splenocytes were incubated in triplicate wells with either wild-type (bars a and e) or mutant (bars b and f) heat-killed parasites or wild-type (bars c and g) or mutant (bars d and h) formaldehyde-fixed parasites at a cell/parasite ratio of 1:5 in the absence (bars a to d) or presence (bars e to h) of ConA (5 μg/ml). After 48 h, lymphocyte proliferation was measured by [^{3}H]thymidine incorporation, and the results were expressed as SIs. Control splenocytes incubated with either wild-type or mutant live parasites in the presence or absence of ConA stimulation showed an SI of <5. Mean values for spleen cells from uninfected mice in the presence and absence of ConA were 121,656 ± 234 [SI = 60]) and 2,020 ± 30, respectively. This experiment was repeated three times.

Antibody neutralization of the inhibitory response. Previously, we reported that the inhibitory activity mediated by splenocytes obtained from infected mice could be partially neutralized by an antibody to IL-10 (13, 18). We next determined whether this cytokine was important in this in vitro model of *T. gondii*-mediated immune suppression. For this experiment, ConA-stimulated splenocytes (lower chamber) were cultured in the presence of an antibody to IL-10 across the transwell with different concentrations of live parasites (upper chamber). As shown in Table 5, an anti-IL-10 MAb was able to partially neutralize the inhibitory activity mediated by live parasites at cell/parasite ratios of both 16:1 (25%) and 4:1 (30%).

Alternatively, some other factor(s) such as nitric oxide may be partially responsible for the observed suppression (3, 18). However, under the conditions of this experiment, there was no detectable increase over the control in the degradation product of nitric oxide metabolism when cells were cultured with parasites at a ratio of 1:1 (99% inhibition) and stimulated with either ConA (0.7 and 1.2 μM NO at 24 and 48 h, respectively, compared with 0.5 and 1.0 μM NO at 24 and 48 h, respectively, for mitogen-stimulated splenocytes without parasites) or an anti-CD3 MAb.

To evaluate if the parasites were expressing a soluble antigen(s) responsible for the reduced proliferative response, a blocking assay was performed. Various numbers of parasites were incubated across a transwell with normal splenocytes in the presence of a 1:20 dilution of heat-inactivated rabbit anti-*Toxoplasma* serum, and the proliferative response to ConA

TABLE 4. Spleen cell responses to different concentrations of live parasites in the presence of a cross-linked anti-CD3 MAb[a]

Cell/parasite ratio	SI		% Inhibition after stimulation with anti-CD3
	Medium	Anti-CD3	
No parasite		40.6 ± 4.5	
16:1	2.6 ± 1.3	32.0 ± 5.7	21.0 ± 7.0
4:1	3.8 ± 2.4	16.0 ± 6.0	62.0 ± 10.0
1:1	2.4 ± 1.6	3.2 ± 1.8	92.0 ± 4.2

[a] Normal mouse splenocytes were cultured in the presence of anti-CD3 across a transwell membrane from parasites at various cell/parasite ratios. After 48 h of incubation at 37°C, proliferation was measured by the amount of [^{3}H]thymidine incorporated, and the results are expressed as SIs. Control cultures are transwell inserts containing medium with or without mitogen in the absence of parasites. Percent inhibition was calculated as for Table 3. Results are expressed as the means ± standard deviations of three experiments.

TABLE 5. Effects of an anti-IL-10 MAb on live parasite-stimulated splenocytes[a]

Cell/parasite ratio	SI			% Inhibition after stimulation with ConA	SI, ConA + anti-IL-10	% Inhibition after stimulation with ConA + anti-IL-10
	Medium	Anti-IL-10	ConA			
No parasite		1.4 ± 0.1	39.0 ± 2		39.3 ± 2	
16:1	0.5 ± 0.1	1.5 ± 0	10.0 ± 2	74	19.6 ± 3	50
4:1	0.7 ± 0	1.4 ± 0	1.0 ± 0	97	12.9 ± 1	67
1:1	0.5 ± 0.1	2.0 ± 0.1	0.1 ± 0	99	1.0 ± 0.05	97

[a] Normal mouse splenocytes were cultured across a transwell membrane with parasites at various cell/parasite ratios for 48 h in the presence of ConA and an anti-IL-10 MAb (12 μg/ml). Lymphocyte proliferation was measured by the amount of [^{3}H]thymidine incorporated, and the results are expressed as means ± standard deviations of three experiments. Rat IgG was used as an isotype-matched control and failed to block the either the lymphocyte response to ConA (SI = 39.2) or the inhibitory response by the parasites on the splenocytes. The experiment was repeated three times.

was determined. This dilution was sufficient to restore the proliferative response but did not alter the T-cell response of normal mice. As shown in Table 6, heat-inactivated anti-PTg rabbit serum was able to partially reverse the inhibitory effect on the responder cells. This effect was most apparent at the highest (16:1) cell/parasite ratio (36%) and not observed when the ratio reached 1:1. Neither an antibody to SAG1 (P30), the major surface protein of *T. gondii*, nor control rabbit serum was able to block the inhibitory response.

Phenotypic analysis and cytokine expression by *T. gondii*-infected splenocytes. A fluorescence-activated cell sorting analysis to determine the phenotype of the responder T cells was performed to better understand which T cells are most affected by the suppressive factors produced by the splenocytes in response to toxoplasmas. Parasites were cultured in a transwell and exposed to spleen cells obtained from uninfected mice at a cell/parasite ratio of 1:1. There was no difference in the expression of $CD3^+$, $CD4^+$, or $CD8^+$ T cells between uninfected control cells and cells cultured with live parasites at a 1:1 ratio for either 24 or 48 h (data not shown).

To ascertain the induction of cytokines, RNA was extracted from responder cells after 18 h of incubation across a transwell membrane with parasites and subjected to PCR. As shown in Fig. 2, ConA stimulation of uninfected mouse splenocytes resulted in the anticipated expression for IL-2, IL-2 receptor (IL-2R) p55, IL-4, and gamma interferon (IFN-γ). Of note is the absence of transcript for IL-10 in these preparations. When splenocytes were cultured in the presence of parasites (1:1 ratio, in the transwell system) and were stimulated with ConA for 18 h, the transcripts for IL-2, and IL-2R p55 were no longer detectable, but the message for IL-10 was evident. A transcript for IL-10 cannot be distinguished in the parasite-only lane (lane C). However, in the presence of ConA stimulation, enhanced expression of message for IL-10 can be observed (lane D).

DISCUSSION

Our in vitro observations suggest that live *T. gondii* can induce suppression of mitogen-driven lymphoproliferation of mouse splenocytes. The hypoproliferative response is mediated in part by IL-10 and cannot be significantly reversed by the addition of exogenous IL-2 to the responder cells during the first 24 or 48 h of in vitro infection. Our observations further suggest that under the conditions of this experimental murine model, the parasite is able to express parasite antigens that elaborate this response.

The observed hypoproliferative response is independent of both T-cell stimulus and parasite strain. In vitro exposure of splenocytes to live *T. gondii* can reduce both ConA and cross-linked anti-CD3-driven T-cell proliferative responses, similar to the observations reported for *T. cruzi* (1, 22, 23). However, the number of parasites able to induce this response differs considerably from the number of *T. cruzi* needed to do so. In our studies, this hypoproliferative response can be generated with 5 to 16 times fewer parasites than splenocytes. Remarkably, even fewer parasites (cell/parasite ratio of 64:1) can stimulate an 8 to 12% reduction in proliferation of normal splenocytes to mitogen stimulation. This finding suggests that the inhibitory molecules generated by the splenocytes in response to parasite antigen are very potent inhibitors of lymphocyte proliferation and furthermore require the simultaneous presence of both parasites and cells to be expressed.

Alteration in the production of IL-2 may be one mechanism whereby the parasite is able to affect the normal host response to infection. Previous reports from our laboratory and others have documented this response in vivo within 7 days following parasite infection in mice (3, 13, 18). The mechanism by which IL-2 production is decreased is probably multifactorial. However, IL-10 may affect the production of IL-2 in response to ConA. In our in vitro murine model, an antibody to IL-10 could partially neutralize the inhibitory effect. Murine IL-10 has been shown to inhibit cytokine production by impairing antigen-presenting cell function (11, 21), perhaps by down-regulating B7 expression on macrophages (9). Our data suggest that parasite antigens are able to stimulate the production of IL-10 in the presence of mitogen stimulation (Fig. 2). This possibility is consistent with previous reports from our laboratory describing studies in which the experimental ex vivo mouse model was used (13, 18). Those studies demonstrate that splenocytes obtained from infected mice at day 7 postinfection exert an inhibitory effect on the lymphoproliferative response in the presence of mitogen exposure. Similar observations have now been made in an in vitro human experimental model (5a). However, in both the mouse and human studies, the amplitude of this response is potentiated by the presence of mitogen.

Reversal of suppression by exogenous IL-2 during acute *Toxoplasma* infection has been observed. In a study by Chan et al. (5), exogenous IL-2 was able to restore the reduced proliferative response of splenocytes obtained from RH strain-infected mice on day 3 but not day 6, whereas the proliferative response of mouse splenocytes infected with a less virulent strain of the parasite could be partially restored on both day 3 and day 6. In that study, splenocytes were used ex vivo postinfection. In our system, we used an in vitro model of infection with naive splenocytes. This approach may result in the expression of different cytokines or perhaps T-cell costimulatory regulatory factors that can affect the fate of the unresponsive lymphocytes.

TABLE 6. Effects of inactivated anti-PTg rabbit serum on live parasite-stimulated splenocytes[a]

Cell/parasite ratio	SI		% Inhibition after stimulation with ConA	SI		% Inhibition after stimulation with ConA + anti-PTg rabbit serum	SI		% Inhibition after stimulation with anti-P30 rabbit serum	SI		% Inhibition after stimulation with normal rabbit serum
	Medium	ConA		Anti-PTg rabbit serum	Anti-PTg rabbit serum +		Anti-P30 rabbit serum	Anti-P30 rabbit serum + ConA		Normal rabbit serum	Normal rabbit serum + ConA	
No parasite		95 ± 4			95 ± 7			91 ± 5			93 ± 4	
16:1	0.2 ± 0.0	26 ± 2	73	2 ± 0.0	63 ± 5	37	1 ± 0.01	23 ± 2	75	0.6 ± 0.1	26.0 ± 6	72
4:1	0.5 ± 0.0	8 ± 3	92	1 ± 0.0	22 ± 4	79	0.2 ± 0.01	9 ± 0.9	91	0.2 ± 0.1	4.0 ± 4	97
1:1	0.9 ± 0.0	2 ± 0.5	99	0.4 ± 0.0	6 ± 0.8	97	0.2 ± 0.01	7 ± 0.4	93		ND	

[a] Normal mouse splenocytes were cultured across a transwell membrane with parasites at various cell/parasite ratios for 48 h in the presence of ConA and rabbit anti-*Toxoplasma* serum (1:20). Lymphocyte proliferation was measured by the amount of [^{3}H]thymidine incorporated, and the results are expressed as means ± standard deviations of three experiments. The experiment was repeated three times. ND, not determined.

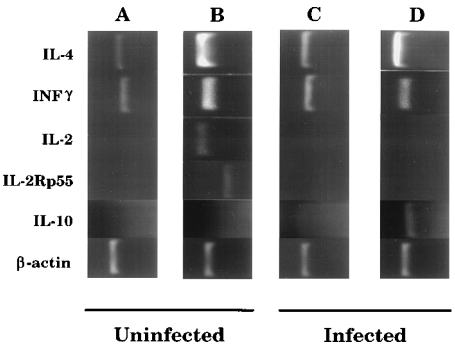

FIG. 2. Cytokine gene expression of splenocytes cocultured with *T. gondii*. Normal mouse splenocytes were incubated in the absence (lane C) or presence (lane D) of ConA (5 μg/ml) across a transwell membrane with parasites at a ratio of 1:1 for 18 h. Control splenocytes were incubated in the absence (lane A) or presence (lane B) of ConA (5 μg/ml) without parasites. After the period of incubation, the splenocytes were harvested, and mRNA was extracted and analyzed for cytokine gene expression by PCR.

In this in vitro model, IL-10 appears to be an important cytokine involved in the downregulatory response. Cytokine analysis of the responding splenocyte population demonstrates the downregulation of genes expressing IL-2 and IL-2R p55, whereas the message for IL-10 was evident when uninfected splenocytes were cocultured with live parasites at a cell/parasite ratio 1:1 in the presence of ConA stimulation (Fig. 2). This finding is consistent with recent observations from our laboratory that demonstrate similar downregulation of mRNA for IFN-γ expression and upregulation of IL-10 expression in 7-day infected splenocytes cultured in the presence of ConA. We have recently demonstrated (13, 18) that significant neutralization of the reduced proliferative response can be achieved with anti-IL-10 concentrations ranging from 10 μg/ml ($P < 0.05$) to 80 μg/ml ($P < 0.01$). Furthermore, this antibody is able to increase the amount of IL-2 in the culture supernatant of parasite-infected splenocytes stimulated with ConA (12a). The control antibody had no significant effect on reducing the lymphoproliferative effect of the 7-day infected culture supernatant ($P > 0.05$). Together, these findings suggest that the effect of an antibody to IL-10 is not parasite dose dependent once a threshold value of IL-10 in the culture supernatant is reached.

In this in vitro study, messages for IFN-γ and IL-4 were detectable whether uninfected splenocytes were stimulated with or without ConA in the presence or absence of live parasites. The inhibitory role of IL-4 on IL-2 production might also be considered (26). It has been reported that IL-10 can affect proliferation and IL-2 production of human peripheral blood T cells, while the production and mRNA levels of IL-4 and IFN-γ are not affected by IL-10 (7). Moreover, IL-10 does not appear to affect expression of the TCR T-cell receptor-CD3 complex (7).

Since this response can be only partially neutralized by an antibody to IL-10, other factors or cytokines may contribute to this effect. Nitrogen intermediates, in particular nitric oxide, have been shown to inhibit the proliferative response of T cells (20). Although ex vivo studies by us and others suggest that NO production may be in part responsible for the observed immunosuppression in mice 7 days postinfection, our in vitro studies indicate that this mechanism is at best only a minor constituent of the downregulatory response. Another molecule that may be involved in this response is transforming growth factor β (TGF-β). Earlier studies in our laboratory have failed to dem-

onstrate blocking of the inhibitory effect ex vivo by antibody to TGF-β, suggesting that TGF-β has only a limited role in the downregulatory response (13).

From our observations, it appears that live parasites are required for the production of inhibitory factor(s). In our study, neither heat-killed nor formalin-treated parasites were capable of inhibiting the host cell immune response. The reduced lymphoproliferative response could be partially neutralized (38%) at a cell/parasite ratio of 16:1 when an anti-*Toxoplasma* rabbit serum was added to the culture. Since live parasites excrete or secrete parasite antigens (4), we suggest that *T. gondii* expresses specific molecules that stimulate this immunosuppressive response in this in vitro mouse model. Further, this parasite antigen appears to be soluble since it can exert its immunological effect across a transwell membrane. Circulating *Toxoplasma* antigens have been detected in the sera of infected mice (reviewed in reference 16). It is perhaps one of these circulating antigens that is responsible for the observed immunosuppressive response. Active infection of the macrophages with toxoplasmas may accentuate the immunosuppressive response, since reversal was only partially achieved with anti-*Toxoplasma* sera.

The results of these studies demonstrate that *T. gondii* can stimulate the production of various immune factors, including IL-10, during an in vitro experimental model of infection. Recent studies by Gazzinelli et al. have demonstrated the importance of IL-12 both in vitro and in vivo during acute and chronic toxoplasmosis in mice. When peritoneal macrophages are exposed to either live parasites or soluble tachyzoite antigens in vitro, they are able to produce IL-12 as early as 2 days postinfection (11a). In our studies, exposure of macrophages to parasite antigens results in the production of IL-10, which appears to be responsible in part for the observed immunosuppression in mice in both an in vivo and an in vitro system. In contrast, immunosuppression by human monocytes may be partially mediated by IFN-γ (5a). By either mechanism, this parasite is able to induce a state of transient immune suppression. This downregulatory response may prevent the host from immune hyperreactivity; alternatively, it may be a mechanism that allows the parasite to survive in a hostile environment.

ACKNOWLEDGMENTS

We thank Kendall A. Smith for the IL-4 assay, Alice Givan and Gary Ward for expert help with flow cytrometric analysis, and Ellie Sarrica for help in preparing the manuscript. We also express our sincere appreciation to Jacqueline Channon, Tadashi Matsuura, and Imtiaz Khan for careful review and discussions regarding the manuscript.

This work was supported by National Institutes of Health grants AI19613 and AI35956.

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