

## Effect of Cytokines on Growth of *Toxoplasma gondii* in Murine Astrocytes

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Cytokines play a significant role in the regulation of *Toxoplasma gondii* in the central nervous system. Cytokine-activated microglia are important host defense cells in central nervous system infections. Recent evidence indicates that astrocytes can also be activated by cytokines to inhibit intracellular pathogens. In this study, we examined the effect of gamma interferon (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-6 (IL-6), and IL-1 on the growth of *T. gondii* in a primary murine astrocyte culture. Pretreatment of astrocytes with IFN- $\gamma$  resulted in 65% inhibition of *T. gondii* growth. Neither TNF- $\alpha$ , IL-1, nor IL-6 alone had any effect on *T. gondii* growth. IFN- $\gamma$  in combination with either TNF- $\alpha$ , IL-1, or IL-6 caused a 75 to 80% inhibition of growth. While nitric oxide was produced by astrocytes treated with these cytokines, inhibition of *T. gondii* growth was not reversed by the addition of the nitric oxide synthase inhibitor *N*<sup>G</sup>-monomethyl-L-arginine. Furthermore, IFN- $\gamma$  in combination with IL-1, IL-6, or TNF- $\alpha$  also induced inhibition in astrocytes derived from syngeneic mice deficient in the enzyme inducible nitric oxide synthase. This finding suggests that the mechanism of cytokine inhibition is not nitric oxide mediated. Similarly, the addition of tryptophan had no effect on inhibition, indicating that the mechanism was not mediated via induction of the enzyme indoleamine 2,3-dioxygenase. The mechanism of inhibition remains to be elucidated. Results from this study demonstrate that cytokine-activated astrocytes are capable of significantly inhibiting the growth of *T. gondii*. These data indicate that astrocytes may be important host defense cells in controlling toxoplasmosis in the brain.

*Toxoplasma gondii* is an important pathogen in the central nervous system and causes a severe encephalitis in patients with AIDS. Cytokines play an important role in the regulation of *T. gondii* replication in the central nervous system (17). Gamma interferon (IFN- $\gamma$ ) has been shown to prevent reactivation of *Toxoplasma* encephalitis in mice (30). Tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 (IL-1), and interleukin-6 (IL-6) are up-regulated in the brains of mice with chronic toxoplasmosis (9, 15, 16). Studies indicate that IFN- $\gamma$ , TNF- $\alpha$ , IL-1, and IL-6 may control the growth of *T. gondii* in the brain via activation of microglia (4, 5). Studies of mice indicate that cytokines induce anti-*Toxoplasma* activity in microglia via a nitric oxide (NO)-mediated mechanism (10).

Recent evidence indicates that cytokines can also activate astrocytes to inhibit growth of *T. gondii* (6, 8, 25, 27). For example, IFN- $\gamma$  has been shown to inhibit growth of *T. gondii* in the glioblastoma cell line 86HG39 (6). Inhibition was shown to be via induction of indoleamine 2,3-dioxygenase (IDO), resulting in the degradation of intracellular tryptophan (8). Pelloux et al. found that in the astrocytoma cell line GHE, TNF- $\alpha$  inhibited, IL-1 stimulated, and IFN- $\gamma$  and IL-6 had no effect on growth of *T. gondii* (25). Finally, in primary human astrocytes, IFN- $\gamma$  and IL-1 in combination have been shown to inhibit growth of *T. gondii* via the production of NO (27). Interpretation of these results is difficult due to variability found in astrocyte cell lines and differences between tumor cells and primary astrocytes.

For this reason, we have chosen to study the effects of cytokines on growth of *T. gondii* in a primary astrocyte culture. In this study, we evaluated the effects of IFN- $\gamma$ , TNF- $\alpha$ , IL-1, and

IL-6 on the replication of *T. gondii* ME49 in a primary murine astrocyte culture. The effects of these cytokines individually, and the effects of IFN- $\gamma$  in combination with IL-1, TNF- $\alpha$ , and IL-6, on the growth of *T. gondii* were examined. The ability of each of these cytokines and cytokine combinations to induce nitric oxide production was assessed by using the Griess reagent. A nitric oxide-mediated mechanism of cytokine inhibition of *T. gondii* growth was addressed by using *N*<sup>G</sup>-monomethyl-L-arginine (NMMA), a nitric oxide inhibitor, and by using astrocytes derived from syngeneic mice deficient in the enzyme inducible nitric oxide synthase (iNOS). The IDO mechanism of inhibition was investigated via the addition of exogenous tryptophan. The aim of this study was to clarify the effect of cytokines on *T. gondii* replication in astrocytes and increase our understanding of the role of astrocytes in the host defense against *T. gondii* in the central nervous system.

### MATERIALS AND METHODS

**Primary astrocyte culture.** Murine astrocytes from C57BL/6  $\times$  SV129 mice or syngeneic mice, deficient in iNOS (iNOS<sup>-/-</sup>; gift of C. Nathan) (22), were cultivated from the brains of neonatal (less than 24 h old) mice. Murine pups were sacrificed, the brain was removed from the cranium, the forebrain was dissected and the meninges were removed. The tissue was minced and incubated in 0.25% trypsin for 5 min at 37°C. After 5 min, the trypsin was inactivated with a solution containing DNase and soybean trypsinase inhibitors, and the tissue was further disrupted by trituration in a 20-ml pipette. The dissociated cells were filtered through a 74- $\mu$ m-pore-size Nitex mesh, centrifuged at 200  $\times$  g, suspended in growth medium at a concentration of 10<sup>6</sup> cells/ml, and plated onto poly-L-lysine-coated dishes. Astrocytes were maintained in endotoxin-free minimal essential medium (BRL-GIBCO, Gaithersburg, Md.) supplemented with 20% fetal bovine serum (BRL-GIBCO), 5% glucose, and 100 U of penicillin and streptomycin (BRL-GIBCO) per ml. The growth medium was changed every 3 days. After 7 days in vitro, a confluent layer of 1  $\times$  10<sup>4</sup> to 2  $\times$  10<sup>4</sup> cells/cm<sup>2</sup> of astrocytes is reached. By this method, cells were found to be >95% astrocytes, as judged by positive staining for glial fibrillary acidic protein. Cultures contained <5% microglia, as identified by staining with the lectin BS1-B4 (catalog no. L-2895; Sigma, St. Louis, Mo.). Astrocytes were dissociated in trypsin-EDTA, replated onto poly-L-lysine-coated coverslips or 24-well plates at 10<sup>4</sup> cells/cm,

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and cultured for 7 to 10 days after replating. These astrocytes were then infected with *T. gondii* ME49 as described below.

**Culture of *T. gondii*.** Tachyzoites from *T. gondii* ME49 were obtained by in vitro culture in Vero cells. Parasites were harvested after 4 to 5 days in culture, resuspended in minimal essential medium supplemented with 10% fetal bovine serum, and used for infection of murine astrocyte cultures.

**Chemicals and cytokines.** Murine recombinant IFN- $\gamma$ , IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 were purchased from Genzyme (Cambridge, Mass.). NMMA and L-tryptophan were obtained from Sigma. [ $^3$ H]uracil and [ $^3$ H]tryptophan were purchased from Amersham Pharmacia Biotech (Arlington Heights, Ill.). The Griess reagent kit (catalog no. G-7921) was obtained from Molecular Probes (Eugene, Oreg.).

**Cytokine treatments.** Murine astrocytes were stimulated with IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$  (each at 100 U/ml), or IL-6 (1 ng/ml), alone or in combination, for 72 h prior to infection, and supernatants were removed for determination of nitric oxide production. Cultures were then infected with *T. gondii* and incubated for 48 h without cytokines, and growth was determined by the [ $^3$ H]uracil method described below. The percentage of infected astrocytes for each condition was determined by counting the number of infected cells per 500 cells under both phase and immunofluorescence microscopy. Testing for each condition was performed in triplicate. Immunofluorescence microscopy was performed with a 1:50 dilution of a commercial polyclonal rabbit anti-*Toxoplasma* antibody (DAKO, Carpinteria, Calif.) followed by detection with anti-rabbit fluorescein immunoglobulin G (Boehringer Mannheim, Indianapolis, Ind.) as previously described (13). All cultures were incubated in endotoxin-free medium, and no endotoxin contamination was detected in any of the experimental cultures.

**Determination of *Toxoplasma* growth.** Cultures were infected with  $10^5$  *T. gondii* tachyzoites per well (a 5:1 tachyzoite/host cell ratio) for 2 h. The monolayer was then extensively washed to remove extracellular tachyzoites and [ $^3$ H]uracil (2.5  $\mu$ Ci per well) was added. *T. gondii* growth was determined 48 h later by the [ $^3$ H]uracil incorporation method described below. Before cell harvesting, the appearance of the culture was checked to verify that *T. gondii*-induced cell lysis had not begun. The monolayer was washed three times with phosphate-buffered saline to remove any nonincorporated [ $^3$ H]uracil. The astrocyte monolayer was then lysed by incubation in 0.1% sodium dodecyl sulfate for 15 min at room temperature. Nucleic acids were precipitated by the addition of 3 M trichloroacetic acid. The contents of the wells were deposited on Whatman glass filters and washed extensively with 0.1 M trichloroacetic acid, and then radioactivity was determined with a liquid scintillation counter (21). For each experiment, controls included murine astrocytes cultured in the absence of *T. gondii*. The radioactivity of these samples was always near background levels, thus confirming that [ $^3$ H]uracil incorporation was specific to the parasite.

**Measurement of nitric oxide.** Supernatants from astrocyte cultures were collected after 72 h of incubation in cytokines, and nitrite was assayed with the Griess reagent kit (Molecular Probes). Briefly, a 150- $\mu$ l aliquot of culture supernatant was mixed with 50  $\mu$ l of the Griess reagent [0.05% *N*-(1-naphthyl) ethylenediamine dihydrochloride–0.5% sulfanilic acid in phosphoric acid] and diluted with 1.3 ml of water, and absorbance was measured at 548 nm in a spectrophotometer. The amount of nitrite in the sample was calculated from a sodium nitrite standard curve.

**Effect of NMMA or tryptophan on cytokine inhibition.** Murine astrocytes were cultured as described above except that in some experiments, either NMMA (final concentration of 400  $\mu$ M) or tryptophan (final concentration of 100  $\mu$ g/ml) was added to the culture at the time of cytokine addition. Tryptophan uptake by astrocytes was measured by monitoring [ $^3$ H]tryptophan uptake as described by Pfefferkorn (28). IDO activity was measured photometrically at 490 nm, by using the Ehrlich reagent to monitor the degradation of tryptophan to kynurenine as described by Däubener et al. (7).

**Statistics.** Within each experiment, all conditions were repeated in triplicate, and each experiment was replicated two to three times. Data were analyzed by nonparametric (Wilcoxon signed-rank test) and/or parametric (Student *t* test and analysis of variance) methods, using Sigma Stat version 1.0 (Jandel Scientific, San Rafael, Calif.).

## RESULTS

**Effect of IFN- $\gamma$ , TNF- $\alpha$ , IL-1, and IL-6 on growth of *T. gondii* in murine astrocytes.** Astrocytes were pretreated with cytokines and then infected with *T. gondii*, and growth was measured 48 h after infection. The effect of treating astrocytes with IFN- $\gamma$ , TNF- $\alpha$ , IL-1, and IL-6 alone and in combination is presented in Fig. 1. IFN- $\gamma$  inhibited the growth of *T. gondii* by approximately 65% ( $P < 0.05$ ), but there was no effect from IL-6, TNF- $\alpha$ , or IL-1 alone. IFN- $\gamma$  in combination with either IL-6, IL-1, or TNF- $\alpha$  inhibited the growth of *T. gondii* in murine astrocytes by approximately 75 to 80% ( $P < 0.05$ ), a level 10 to 15% greater than that observed after treatment with IFN- $\gamma$  alone ( $P < 0.05$ ). The addition of IL-6 did not reverse the inhibition of growth induced by either IFN- $\gamma$ -IL-1 or IFN-

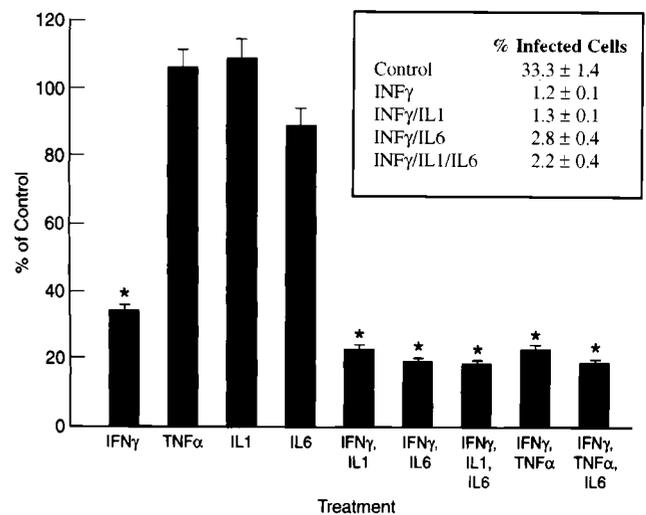


FIG. 1. Effect of cytokines on growth of *T. gondii* in mouse astrocytes. Cells were incubated with the cytokines IFN- $\gamma$  (100 U/ml), IL1 (1 ng/ml), IL-6 (100 U/ml), and TNF- $\alpha$  (100 U/ml) for 72 h before infection. Control cultures were incubated in medium alone. [ $^3$ H]uracil (2.5  $\mu$ Ci/ml) was added 2 h after infection, and cells were harvested 48 h later. Results are averages of three separate experiments. Bars indicate  $\pm$  standard error of the mean. \*, significance at the  $P < 0.05$  level versus control as calculated by Student's *t* test. (Insert) Percentages of cells (mean  $\pm$  standard deviation) infected with *T. gondii* ME49 as determined by microscopy (multiplicity of infection, 5:1). The difference between each cytokine treatment and the control was significant at  $P < 0.05$ ; there was no significant difference between any of the various cytokine treatments.

$\gamma$ -TNF- $\alpha$  in astrocytes. No inhibition of *T. gondii* growth was seen with either IFN- $\gamma$  alone or any of the IFN- $\gamma$  cytokine combinations when cytokines were added at the time of infection or 24 h prior to infection (data not shown).

Cultures pretreated with IFN- $\gamma$  alone and in combination with each of TNF- $\alpha$ , IL-1, and IL-6 were also assessed microscopically. The level of infection of cultures treated with cytokines was found to be  $<5\%$ , compared to 30 to 35% in the controls (Fig. 1, inset), which correlated with the results of the uracil uptake assay. The percent infected cells approximately doubled when IL-6 was added to other cytokines (i.e., 1.2% infected with IFN- $\gamma$  and 2.8% infected with IFN- $\gamma$ -IL-6). While not statistically significant, this finding raises the possibility that in astrocytes, as found for macrophages (2), IL-6 may reverse part of the activation due to other cytokines.

**NO production in cytokine-treated astrocytes.** NO production after treatment with each of the cytokine combinations is presented in Table 1. NO levels in the controls (i.e., no cytokines added) were between 2 and 3  $\mu$ M, and no increase in NO was seen with IFN- $\gamma$ , TNF- $\alpha$ , IL-1, or IL-6 treatment alone. All cytokine combinations resulted in a statistically significant elevation in NO above the control level ( $P < 0.05$ ): IFN- $\gamma$ -IL-1, 9.9  $\mu$ M; IFN- $\gamma$ -TNF- $\alpha$ , 15.2  $\mu$ M; and IFN- $\gamma$ -IL-6, 5  $\mu$ M. The addition of IL-6 to IFN- $\gamma$ -IL-1 decreased the NO levels induced by IFN- $\gamma$ -IL-1 slightly, while the addition of IL-6 to IFN- $\gamma$ -TNF- $\alpha$  had no effect on NO production.

**Effect of NMMA on cytokine inhibition of *T. gondii* growth in murine astrocytes.** Addition of NMMA decreased nitrite levels in all cytokine combinations to background levels ( $<2$   $\mu$ M) in all cultures (Table 1). The presence or absence of NMMA during the 72-h pretreatment with cytokines prior to infection of astrocytes with *T. gondii* did not affect the cytokine-mediated inhibition of *T. gondii* growth due to IFN- $\gamma$ -IL-1, IFN- $\gamma$ -IL-6, IFN- $\gamma$ -IL-1-IL-6, IFN- $\gamma$ -TNF- $\alpha$ , or IFN- $\gamma$ -TNF- $\alpha$ -IL-6 (Table 2) or to the individual cytokines (data not shown).

TABLE 1. Effect of cytokines on NO production in mouse astrocytes

Treatment	Nitrite concn (μM) <sup>a</sup>
Control.....	2.0 ± 1.2
IFN-γ.....	1.9 ± 0.8
TNF-α.....	1.3 ± 0.5
IL-1.....	1.4 ± 0.6
IL-6.....	0.7 ± 0.7
IFN-γ, IL-1.....	9.9 ± 2.3 <sup>b</sup>
IFN-γ, IL-6.....	5.0 ± 0.8 <sup>b</sup>
IFN-γ, IL-1, IL-6.....	6.3 ± 1.7 <sup>b</sup>
IFN-γ, TNF-α.....	15.2 ± 1.0 <sup>b</sup>
IFN-γ, TNF-α, IL-6.....	13.8 ± 0.7 <sup>b</sup>
IFN-γ, IL-1, NMMA.....	2.3 ± 1.7
IFN-γ, IL-6, NMMA.....	2.1 ± 1.7
IFN-γ, IL-1, IL-6, NMMA.....	1.8 ± 1.1
IFN-γ, TNF-α, NMMA.....	2.2 ± 1.6
IFN-γ, TNF-α, IL-6, NMMA.....	2.6 ± 1.6

<sup>a</sup> Mean ± standard deviation of three separate experiments for NO in the supernatants of astrocyte cultures treated with IFN-γ (100 U/ml), IL-1 (1 ng/ml), IL-6 (100 U/ml), and TNF-α (100 U/ml), in the presence or absence of NMMA (400 μM); control cultures were incubated in medium alone. Cytokines were added to cultures 72 h prior to infection, and NMMA was added at the time of cytokine addition. 0 = level of detection (<1.0 μM).

<sup>b</sup> P < 0.05 from the value of the control group as calculated by Student's *t* test.

**Effect of cytokines on growth of *T. gondii* in iNOS<sup>-/-</sup> murine astrocytes.** Treatment of iNOS<sup>-/-</sup> astrocytes with IFN-γ-IL-1, IFN-γ-IL-6, IFN-γ-IL-1 plus IL-6, IFN-γ-TNF-α, or IFN-γ-TNF-α plus IL-6 inhibited the growth of *T. gondii* significantly (75 to 80%) (Table 3), as was seen with syngeneic control murine astrocytes. In addition, as found for control astrocytes, the addition of IL-6 had little or no effect on the degree of inhibition caused by either IFN-γ-IL-1 or IFN-γ-TNF-α. As expected, no nitric oxide was detected in the supernatants from the iNOS<sup>-/-</sup> astrocytes after treatment with any of the cytokine combinations (Table 3).

**Effect of tryptophan on cytokine inhibition of *T. gondii* growth.** Tryptophan (100 μg/ml) added to cultures stimulated with cytokines did not reverse inhibition of *T. gondii* growth caused by any of the cytokine treatments in normal or in iNOS<sup>-/-</sup> astrocytes (Table 4). Tryptophan uptake by astrocytes was verified by [<sup>3</sup>H]tryptophan incorporation (28). No

TABLE 2. Effect of NMMA on cytokine inhibition of *T. gondii* growth in murine astrocytes

Treatment	Inhibition of <i>T. gondii</i> growth (% of control) <sup>a</sup>	
	Medium only	Cytokines + NMMA
IFN-γ, IL-1	22.4 ± 3.1	24.7 ± 1.1
IFN-γ, IL-6	20.4 ± 2.3	21.0 ± 0.8
IFN-γ, IL-1, IL-6	18.2 ± 1.5	15.2 ± 4.1
IFN-γ, TNF-α	23.3 ± 3.7	16.6 ± 2.5
IFN-γ, TNF-α, IL-6	14.3 ± 2.9	16.8 ± 2.5

<sup>a</sup> Mean ± standard deviation of three separate experiments for astrocyte cultures treated with IFN-γ (100 U/ml), IL-1 (1 ng/ml), IL-6 (100 U/ml), and TNF-α (100 U/ml), in the presence or absence of NMMA (400 μM), and for control cultures incubated in medium alone. Cells were incubated with cytokines for 72 h prior to infection, and NMMA was added at the time of cytokine addition; [<sup>3</sup>H]juracil (2.5 μCi/ml) was added 2 h after infection, and cells were harvested 48 h later. No endotoxin contamination was detected in any of the cultures.

TABLE 3. Effect of cytokines on growth of *T. gondii* in iNOS<sup>-/-</sup> murine astrocytes

Treatment	Inhibition of <i>T. gondii</i> growth (% of control) <sup>a</sup>
IFN-γ, IL-1.....	22.8 ± 2.3
IFN-γ, IL-6.....	21.1 ± 0.8
IFN-γ, IL-1, IL-6.....	23.3 ± 3.1
IFN-γ, TNF-α.....	22.2 ± 1.8
IFN-γ, TNF-α, IL-6.....	19.2 ± 5.9

<sup>a</sup> Mean ± standard deviation of two separate experiments for astrocyte cultures treated with IFN-γ (100 U/ml), IL-1 (1 ng/ml), IL-6 (100 U/ml), and TNF-α (100 U/ml). Cells were incubated with cytokines for 72 h prior to infection; [<sup>3</sup>H]juracil (2.5 μCi/ml) was added 2 h after infection, and cells were harvested 48 h later. Control cultures were incubated in medium alone. All values are significantly different (P < 0.05) from the control group as calculated by Student's *t* test. Nitrite was not detectable (i.e., <1.0 μM) in any sample.

IDO activity was detected in astrocytes with or without cytokine stimulation.

DISCUSSION

The cytokines IFN-γ, TNF-α, IL-1, and IL-6 are known to be important in controlling the replication of *T. gondii* in the brain. The importance of cytokine activation of microglia in regulating *T. gondii* infection in the brain is well established, but the role of astrocytes is less well understood. Variable effects on the growth of *T. gondii* have been demonstrated in different astrocyte tumor lines with IFN-γ, TNF-α, IL-1, and/or IL-6 treatment. In the glioblastoma cell line 86HG39, IFN-γ alone has been shown to inhibit the growth of *T. gondii*, while in the glioblastoma cell line 87HG31, the combination of TNF-α and IFN-γ was required to inhibit growth (6, 8); in GHE astrocytoma cells, TNF-α but not IFN-γ inhibited the growth of *T. gondii* (25). Additionally, in astrocytoma cells, IL-1 was found to stimulate growth and IL-6 had no effect on *T. gondii* growth (25).

In the present study using primary murine astrocytes, we have demonstrated that pretreatment with IFN-γ alone or in combination with IL-1, IL-6, or TNF-α but not IL-1, IL-6, or TNF-α alone significantly inhibited the growth of *T. gondii* in these cells. This result is consistent with findings for glioblastoma cell line 86HG39 (6) but in contrast to those for astro-

TABLE 4. Effect of tryptophan on cytokine inhibition of *T. gondii* growth

Treatment	Inhibition of <i>T. gondii</i> growth (% of control) <sup>a</sup>			
	Control astrocytes		iNOS <sup>-/-</sup> astrocytes	
	Medium	Cytokines + tryptophan	Medium	Cytokines + tryptophan
IFN-γ	35.5 ± 2.3	38.7 ± 3.4	32.3 ± 2.3	31.0 ± 2.1
IFN-γ, IL-1	28.4 ± 3.9	33.3 ± 3.5	21.1 ± 1.1	15.6 ± 2.0
IFN-γ, IL-6	21.5 ± 2.9	29.6 ± 1.4	20.5 ± 1.7	17.0 ± 4.1
IFN-γ, IL-1, IL-6	20.6 ± 2.1	22.8 ± 5.4	25.5 ± 4.9	19.5 ± 1.7
IFN-γ, TNF-α	23.5 ± 3.5	22.5 ± 5.1	23.4 ± 0.7	16.8 ± 5.6
IFN-γ, TNF-α, IL-6	21.0 ± 2.2	23.7 ± 1.1	15.0 ± 3.8	12.5 ± 1.6

<sup>a</sup> Mean ± SD of two separate experiments for astrocyte cultures treated with IFN-γ (100 U/ml), IL-1 (1 ng/ml), IL-6 (100 U/ml), and TNF-α (100 U/ml) in the presence and absence of tryptophan (100 μg/ml) and for control cultures incubated with medium alone. Cells were incubated with cytokines for 72 h prior to infection, and tryptophan was added at the time of cytokine addition; [<sup>3</sup>H]juracil (2.5 μCi/ml) was added 2 h after infection, and cells were harvested 48 h later. No statistically significant difference was detected between any of the cultures as determined by Student's *t* test and analysis of variance.

cytoma cell line GHE (25). The absence of IFN- $\gamma$ -induced inhibition in GHE astrocytoma cells may be due to the fact that in this study, cells were pretreated with IFN- $\gamma$  for only 24 h. Peterson et al. (26) found that pretreatment with IFN- $\gamma$  for 24 h did not activate murine astrocytes to inhibit *T. gondii*, which is consistent with our observations. We found that murine astrocytes needed to be pretreated with IFN- $\gamma$  for 48 to 72 h to induce inhibition. Similarly, Däubener et al. (6, 8) found optimal inhibition with IFN- $\gamma$  when glioblastoma cells were pretreated for 72 h. Treatment of astrocytes with cytokines after infection did not induce inhibition. Thus, priming of astrocytes by IFN- $\gamma$  is required for anti-*Toxoplasma* activity. This phenomenon has also been reported for monocytes (3).

IFN- $\gamma$  in combination with either IL-1, TNF- $\alpha$ , or IL-6 inhibited *T. gondii* growth in murine astrocytes. The effect of adding either IL-1, IL-6, or TNF- $\alpha$  to IFN- $\gamma$  significantly (by 15 to 20%) increased inhibition of growth induced by IFN- $\gamma$  alone. Similarly, in microglia, IFN- $\gamma$  activation is enhanced by TNF- $\alpha$ , IL-1, or IL-6 (4, 5). In macrophages, IL-6 has been reported to reverse the inhibition caused by IFN- $\gamma$ -IL-1 (2). In our study, IL-6 did not reverse the inhibition caused by either IFN- $\gamma$ -IL-1 or IFN- $\gamma$ -TNF- $\alpha$  in murine astrocytes. The effect of IL-6 is of interest due to the implication that IL-6 plays an important role in the immunopathogenesis of *Toxoplasma* encephalitis (31).

IFN- $\gamma$  in combination with IL-1 and other cytokines has been shown to stimulate nitric oxide production via the enzyme iNOS in both human and murine astrocytes (14, 19). Treatment of primary murine astrocytes with IFN- $\gamma$  in combination with other cytokines also resulted in the production of nitric oxide. However, inhibition of *T. gondii* growth was found to be nitric oxide independent, as demonstrated by the inability of NMMA to reverse the inhibition and the ability of cytokines to inhibit *T. gondii* growth in iNOS<sup>-/-</sup> astrocytes.

IFN- $\gamma$  has been shown to inhibit *Toxoplasma* growth via induction of the enzyme IDO, which results in the degradation of tryptophan in human fibroblasts, glioblastoma cells, retinal epithelial cells, and macrophages (8, 12, 23, 24, 29). Additionally, the IDO pathway has been shown to be activated by IFN- $\gamma$  and TNF- $\alpha$  in some glioblastoma cells and native human astrocytes (7). In our study, the addition of tryptophan did not reverse the inhibition caused by the IFN- $\gamma$  alone or IFN- $\gamma$  in combination with either TNF- $\alpha$ , IL-1, or IL-6, and no increase in IDO activity could be detected in astrocytes following cytokine treatment. These data suggest that in murine astrocytes, cytokines inhibit *T. gondii* via an IDO-independent pathway.

IFN- $\gamma$ -induced inhibition of *T. gondii* in endothelial cells has been shown to be mediated by an IDO-independent mechanism and was also demonstrated not to be mediated via nitric oxide or reactive oxygen intermediates (32). The presence of some other mechanism(s) induced by cytokines is not surprising given the many diverse effects that cytokines have on astrocyte functions (1, 20). For example, IL-1 induces a reactive astrocyte phenotype characterized by the expression of TNF- $\alpha$ , IL-6, and colony-stimulating factor in astrocytes (20). IFN- $\gamma$  also induces many changes in cell physiology, including metabolic and cytoskeletal changes. The cytokine-induced inhibition of *T. gondii* in astrocytes may be due to some of these generalized effects on host cell function. It is possible that iron starvation (11) or other reactive oxygen intermediates can be induced by IFN- $\gamma$  alone or in combination with other cytokines and that these mechanisms, which are the focus of our current investigations, are responsible for the cytokine-mediated inhibition of *T. gondii* growth in murine astrocytes. Whatever the mechanism(s) involved, this study clearly demonstrates that cytokine-activated astrocytes induce significant anti-*Toxoplasma*

activity and that IFN- $\gamma$  is the primary cytokine mediating this inhibition.

The ability of cytokines to activate astrocytes to inhibit replication of *T. gondii* may also be involved in the mechanism of reactivated *Toxoplasma* infections in AIDS. For instance, evidence suggests that in patients with AIDS, a shift from a Th1 to a Th2 cytokine profile occurs in the brain (18). The presence of the Th1 cytokines IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 in the brain would presumably activate astrocytes to exert anti-*Toxoplasma* activity. Concomitantly, a shift to a Th2 cytokine profile, which is accompanied by a decrease in IFN- $\gamma$  and subsequent decreases in TNF- $\alpha$  and IL-1, might then promote growth of *T. gondii* in astrocytes. The effect of Th2 cytokines on *T. gondii* in astrocytes is not known, but data from our previous study (13) showed that astrocytes, when unstimulated by IFN- $\gamma$  or other cytokines, serve as excellent host cells for *T. gondii*, supporting extensive replication resulting in the lysis and continual reinfection of astrocytes. This finding, in conjunction with the Th1/Th2 shift hypothesis, suggests that astrocytes may play a pivotal role in the pathophysiology of *Toxoplasma* encephalitis in the brains of patients with AIDS.

In conclusion, we found in murine astrocytes, IFN- $\gamma$  alone or in combination with IL-1, TNF- $\alpha$ , or IL-6 significantly inhibited growth of *T. gondii*. Although IFN- $\gamma$ -IL-1 and IFN- $\gamma$ -TNF- $\alpha$  induced NO production, inhibition was not found to be via an NO-mediated mechanism. Cytokine-mediated inhibition was also not due to induction of IDO. The NO/IDO-independent pathway responsible for inhibition of *T. gondii* growth is currently under investigation in our laboratory. Given that IFN- $\gamma$  has been shown to be the main cytokine controlling growth of *T. gondii* in the brain and that TNF- $\alpha$ , IL-1, and IL-6 are up-regulated in the brains of mice with chronic toxoplasmosis, results from the present study indicate astrocytes are most likely an important effector cell in limiting *T. gondii* replication in the brain.

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