Investigation into the Mechanism of Gamma Interferon-Mediated Inhibition of *Toxoplasma gondii* in Murine Astrocytes

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*Toxoplasma gondii* encephalitis is a common opportunistic infection of the central nervous system in AIDS patients. Gamma interferon (IFN-γ) alone or in combination with interleukin-1 (IL-1), IL-6, or tumor necrosis factor alpha significantly inhibits the growth of *T. gondii* in murine astrocytes, suggesting these are important nonimmune effector cells in the brain. Inhibition was found to be independent of a nitric oxide-mediated or tryptophan starvation mechanism. Both reactive oxygen intermediates and iron deprivation are IFN-γ-mediated mechanisms known to operate against intracellular parasites in other cell types. Astrocytes generated from mice genetically deficient in the production of reactive oxygen intermediates (*phox−/−* mice) were found to inhibit growth of *T. gondii* when stimulated with IFN-γ alone or in combination with other cytokines. The reactive oxygen inhibitor catalase and the reactive oxygen scavengers mananol and thiourea failed to reverse the IFN-γ-induced inhibition of *T. gondii* in astrocytes. These data indicate that IFN-γ-induced inhibition in astrocytes is independent of reactive oxygen intermediates. IFN-γ-induced inhibition could not be reversed by the addition of iron salts, ferric citrate, ferric nitrate, or ferric transferrin. Pretreatment of astrocytes with desferrioxamine also did not induce the inhibition of *T. gondii*. These data indicate that the mechanism of IFN-γ inhibition was not due to iron deprivation. IFN-γ had no effect on *T. gondii* invasion of astrocytes, but inhibition of growth and loss of tachyzoite vacuoles were evident in IFN-γ-treated astrocytes by 24 h after invasion. Overall, these data suggest that IFN-γ-activated astrocytes inhibit *T. gondii* by an as-yet-unknown mechanism.

Primary astrocyte culture. Murine astrocytes from C57BL/6 × SV129 mice or syngeneic mice deficient in phagocyte oxidase (*gp91phox−/−*) [33] were cultured from the brains of neonatal mice. Murine pups were sacrificed, the brains were removed from the cranium, and then the forebrain was dissected and the meninges were removed. The tissue was minced and incubated in 0.25% trypsin for 45 to 60 min at 37°C. The tissue was further disrupted by triturating in a 20-ml pipette. The dissociated cells were filtered through a 74-μm pore-size Nitex mesh, centrifuged at 200 × g, suspended in growth medium at a concentration of 105 cells/ml and then plated onto poly-L-lysine-coated dishes. Astrocytes were maintained in endotoxin-free minimum essential medium (MEM; GIBCO-BRL, Gaithersburg, Md.) supplemented with 20% fetal bovine serum (FBS; GIBCO-BRL), 5% glucose, and 100 U of penicillin and streptomycin (GIBCO-BRL) per ml. The growth medium was changed every 3 days. After 7 to 10 days in vitro a confluent layer of *T. gondii* was achieved. 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 (Sigma L-2895). Astrocytes were dissociated in trypsin-EDTA, replated onto poly-L-lysine-coated coverslips at 104 cells/cm2 in a 24-well plate, and cultured for 7 to 10 days after replating. These astrocytes were then infected with *T. gondii* ME49 as described below.
TABLE 1. Effect of cytokine treatments in phox−/−
murine astrocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Infection (mean ± SD)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.6 ± 4.1*</td>
<td>100</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>11.4 ± 1.0*</td>
<td>53.6</td>
</tr>
<tr>
<td>IFN-γ + TNF-α</td>
<td>8.9 ± 1.9†</td>
<td>43.2</td>
</tr>
<tr>
<td>IFN-γ + IL-6</td>
<td>7.0 ± 0.5†</td>
<td>34.0</td>
</tr>
<tr>
<td>IFN-γ + TNF-α + IL-6</td>
<td>8.8 ± 2.2*</td>
<td>42.7</td>
</tr>
</tbody>
</table>

* Cells were incubated with cytokines for 72 h prior to infection; all cytokines were added at 100 U/ml, and cells were fixed 48 h after infection. †, significance at the P < 0.05 level versus the control.

TABLE 2. Effect of reactive oxygen intermediate on IFN-γ in murine astrocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Infected cells (mean ± SD)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.8 ± 2.0</td>
<td>100</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.9 ± 0.2*</td>
<td>16.1</td>
</tr>
<tr>
<td>Catalase</td>
<td>12.9 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>IFN-γ + catalase</td>
<td>1.5 ± 0.2*</td>
<td>11.6</td>
</tr>
<tr>
<td>Thiourea</td>
<td>10.2 ± 1.0</td>
<td>11.8</td>
</tr>
<tr>
<td>IFN-γ + thiourea</td>
<td>1.2 ± 0.4*</td>
<td></td>
</tr>
<tr>
<td>Mannitol</td>
<td>12.4 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>IFN-γ + mannitol</td>
<td>0.9 ± 0.1*</td>
<td>7.3</td>
</tr>
</tbody>
</table>

* Cells were pretreated with IFN-γ (100 U/ml) for 72 h prior to infection; catalase, thiourea, and mannitol were added 2 h after infection, and cells were fixed 48 h postinfection. †, significance at the P < 0.05 level versus the control.

TABLE 3. Effect of DFO on T. gondii in murine astrocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Infected cells (mean ± SD)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.8 ± 3.9</td>
<td>100</td>
</tr>
<tr>
<td>DFO</td>
<td>9.8 ± 3.7</td>
<td>91</td>
</tr>
<tr>
<td>DFO + ferric citrate</td>
<td>33.0 ± 4.2*</td>
<td>305</td>
</tr>
<tr>
<td>DFO + ferritin</td>
<td>29.6 ± 4.5*</td>
<td>274</td>
</tr>
</tbody>
</table>

* Cells were incubated with medium (control) or DFO (50 μM) for 72 h prior to infection; cells were infected and incubated in the presence of medium alone or with ferric citrate or ferritin transferrin and then fixed 48 h later. †, significance at the P < 0.05 level versus the control.

Culture of T. gondii. The ME49 strain of T. gondii was utilized. Tachyzoites were obtained by in vitro culture in human foreskin fibroblast cells. Parasites were harvested after 2 to 3 days in culture. Parasites were resuspended in MEM supplemented with 10% FBS and then incubated with murine astrocyte cultures infected with 5 × 10⁵ parasites per well, a target ratio of 5:1 (parasites/host cells), for 2 h to allow the parasites to invade. The astrocyte cultures were then washed to remove any extracellular parasites and incubated with medium alone or in the presence of reactive oxygen scavengers or iron salts as described below.

Chemicals and cytokines. Murine recombinant IFN-γ, interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and IL-6 were purchased from Genzyme (Cambridge, Mass.). All other reagents were purchased from Sigma (St. Louis, Mo.). Ferric citrate was prepared by mixing a 1:1 ratio of trisodium citrate with ferric chloride and then adjusting the pH to neutrality with 1 M NaOH.

Cytokine and chemical treatments. Murine astrocytes were stimulated with IFN-γ, TNF-α, or IL-6, alone or in various combinations. Cytokines were used at the following concentrations: IFN-γ at 100 U/ml, TNF-α at 100 U/ml, and IL-6 at 100 U/ml. Astrocytes were incubated with cytokines for 72 h prior to infection. Cultures were washed to remove the cytokines, infected with T. gondii as described above, and then incubated for the next 48 h without cytokines. In some experiments, reactive oxygen scavengers (catalase, thiourea, or mannitol) or iron salts (ferric citrate or ferric transferrin) were added following infection with T. gondii.

Microscopic analysis of T. gondii intracellular replication. The percentage of infected astrocytes was determined by counting the number of infected cells per 500 cells under both phase and immunofluorescent microscopy. In some experiments, growth of parasites was determined by counting the number of tachyzoites in 100 vacuoles. Each condition was tested in triplicate. Immunofluorescence was assessed using a 1:50 dilution of a commercial polyclonal rabbit anti- Toxoplasma antibody. DFO was also tested by counting the percent infected cells and the number of tachyzoites per vacuole at 2 and 24 h postinvasion, respectively. No significant difference was seen in the percent infected cells at 2 h between control and IFN-γ-treated cells (Table 5), indicating that IFN-γ pretreatment of astrocytes has no effect on the invasion of host cells. By 24 h, however, both the percent infected cells and the number of tachyzoites per vacuole were significantly less in IFN-γ-treated cells versus control cells (Table 5). The decrease

RESULTS

Effect of cytokines on T. gondii in phox−/− murine astrocytes. Astrocyte cultures were generated from mice genetically deficient in generating reactive oxygen intermediates, stimulated with IFN-γ, either alone or in combination with TNF-α, IL-1, and IL-6 for 72 h, and then infected with T. gondii; the parasite growth was assessed 48 h later. All cytokine combinations significantly (P < 0.05) inhibited the growth of T. gondii in phox−/− murine astrocytes (Table 1). IFN-γ alone resulted in a 53.6% inhibition of growth compared to control cells, while IFN-γ in combination with TNF-α or IL-6 caused a slightly greater inhibition (34 to 43% of control; Table 1). The IFN-γ-induced inhibition in phox−/− astrocytes was comparable to the cytokine inhibition seen in normal murine astrocytes, whereas IFN-γ alone induced inhibition of 35 to 40% and a slight synergism was seen with IFN-γ in combination with other cytokines (18).

Effect of oxygen scavengers on IFN-γ-induced inhibition of T. gondii. To further test the role of an oxygen-dependent mechanism in the anti-Toxoplasma activity of IFN-γ in astrocytes, various inhibitors or scavengers were added to murine astrocyte cultures. Neither catalase, which converts hydrogen peroxide to water and oxygen, nor mannitol or thiourea, which are scavengers of hydroxyl radicals, reversed the inhibitory effect induced by IFN-γ (Table 2).

Effect of iron(III) on the IFN-γ-induced inhibition of T. gondii. To test whether the IFN-γ-induced inhibition of T. gondii in astrocytes is iron dependent, cells were incubated with the siderophore desferrioxamine (DFO). DFO (50 μM) did not induce inhibition of T. gondii in astrocytes; the addition of the iron salts, ferric citrate, or ferric transferrin to DFO did, however, cause a significant increase (two- to threefold) in the growth of T. gondii in astrocytes (Table 3). The role of iron in the IFN-γ-induced anti-Toxoplasma effect was further tested by the addition of ferric citrate at 5, 50, and 100 μM to IFN-γ-treated cultures. Ferric citrate did not reverse the IFN-γ-induced inhibition of T. gondii in astrocytes at any of the concentrations used (Table 4). These results indicate that the IFN-γ-induced anti-Toxoplasma effect is iron independent in astrocytes.

Effect of IFN-γ on invasion and growth of T. gondii. The effect of IFN-γ pretreatment of astrocytes on invasion and growth of T. gondii was also tested by counting the percent infected cells and the number of tachyzoites per vacuole at 2 and 24 h postinvasion, respectively. No significant difference was seen in the percent infected cells at 2 h between control and IFN-γ-treated cells (Table 5), indicating that IFN-γ pretreatment of astrocytes has no effect on the invasion of host cells. By 24 h, however, both the percent infected cells and the number of tachyzoites per vacuole were significantly less in IFN-γ-treated cells versus control cells (Table 5). The decrease
in the percentage of infected cells indicates that IFN-γ induces a microbicidal effect, while the decrease in the number of parasites per vacuole suggests that a microbicidal effect occurs by 24 h postinvasion.

**DISCUSSION**

IFN-γ is the main cytokine controlling *T. gondii* in the brain (35). Previous studies demonstrated that IFN-γ significantly inhibits *T. gondii* in astrocytes via a nitric oxide- and tryptophan-independent mechanism (19). In this study, the mechanism of IFN-γ-induced inhibition of *T. gondii* in astrocytes was further investigated. IFN-γ-induced inhibition was found to be independent of reactive oxygen intermediates, as evidenced by the inability of oxygen radical scavengers to reverse the inhibition and the fact that IFN-γ could also induce inhibition in astrocytes incapable of producing the reactive oxygen intermediates. The role of iron deprivation in IFN-γ-induced inhibition was also addressed. The inability of DFO to induce the inhibition of the growth of *T. gondii* and the inability of ferric salts to reverse the IFN-γ-mediated growth inhibition indicate that the IFN-γ-induced inhibition of *T. gondii* in murine astrocytes is independent of iron deprivation. IFN-γ was found not to affect invasion by *T. gondii* of astrocytes but was found to have a microbicidal and microbicidal effect that was evident by 24 h after invasion.

The mechanisms of IFN-γ-induced inhibition of *T. gondii* which have been demonstrated in other cell types include reactive oxygen intermediates, induction of nitric oxide production, tryptophan starvation, and iron deprivation. In human mononuclear phagocytes, IFN-γ induces toxoplasmoidal activity via reactive oxygen intermediates (28). In murine macrophages and microglia, IFN-γ activates inhibition of *T. gondii* via L-arginine-dependent production of nitric oxide (1, 5). In nonmyeloid cells, IFN-γ-induced inhibition of *T. gondii* was found to occur via tryptophan degradation in human fibroblasts and retinal pigment cells (29, 32), while in enterocytes inhibition occurred via iron deprivation (37).

In murine astrocytes, we have previously shown that IFN-γ-induced inhibition of *T. gondii* was independent of nitric oxide intermediates and tryptophan degradation (19). We found in the present study that IFN-γ-induced inhibition of *T. gondii* in astrocytes was also independent of reactive oxygen derivatives and iron deprivation. Astrocytes have been shown to produce superoxide via a neutrophil-type NADPH oxidase during recovery from hypoxia (21, 36). The respiratory burst as an antitoxoplasmic mechanism in astrocytes has not previously been investigated. The finding that reactive oxygen intermediates do not play a role in the antitoxoplasmic activity of astrocytes is consistent with studies that have found that p47phox⁻/⁻ mice, which lack an inducible oxidative burst, are able to control both the acute and chronic stages of *T. gondii* infection (2). Iron deprivation, a common antimicrobial mechanism, was also not found to be the mechanism of IFN-γ-induced inhibition of *T. gondii* in astrocytes. These data indicate that the IFN-γ-induced inhibition of *T. gondii* in astrocytes occurs via an unknown mechanism.

IFN-γ is known to induce a diverse array of effects on cells (5, 6). IFN-γ is a 34-kDa glycoprotein that binds to a membrane receptor. The IFN-γ receptor is ubiquitously expressed (5, 6). IFN-γ is a 34-kDa glycoprotein that binds to a membrane receptor. The IFN-γ receptor is ubiquitously expressed (5, 6). IFN-γ is a 34-kDa glycoprotein that binds to a membrane receptor. The IFN-γ receptor is ubiquitously expressed (5, 6). IFN-γ-regulated response genes are themselves transcription factors and are required for the induction of other secondary components of the cellular response to IFN-γ. More than 200 IFN-γ-regulated genes have been identified (6). The function of many of these genes is known, and they have been identified as being involved in a diverse range of distinct cellular programs which collectively orchestrate the immune response. For example, IFN-γ induces the expression of major histocompatibility complex (MHC) I and II molecules, which are involved in antigen presentation; the induction of enzymes, resulting in the respiratory burst; nitric oxide and tryptophan degradation, which have antimicrobial effects; and the induction of expression of ICAM molecules and chemokines, which are involved in leukocyte-endothelium interactions. The function of many of the other known IFN-γ response genes, however, is not understood.

While the mechanism of IFN-γ-induced inhibition of *T. gondii* in astrocytes is not understood, it was found that IFN-γ resulted in a microbicidal and microbicidal effect that was evident by 24 h after invasion. IFN-γ has a wide variety of effects on the physiology of cells, including cell shape changes, an antiproliferative effect, and the induction of mitogen-activated protein kinases, which may regulate some of these effects (4, 5, 26). One possible mode of action of IFN-γ in *T. gondii* may be through disruption of the intracellular organization of the cytoskeleton or other host cell organelles, which may in turn affect the parasitophorous vacuole, an organelle essential for the intracellular survival of *T. gondii*. The acquisition of host cell cytoskeleton, endoplasmic reticulum, and mitochondria around the parasitophorous vacuole of *T. gondii* is well documented, and inhibition of lysosomal fusion with the parasitophorous vacuole is also known to be essential for intracellular survival (24, 34). In support of this, IFN-γ was found to interfere with the intracellular development and survival of the parasite in astrocytes, and it is possible that this effect of IFN-γ is due to the disruption of interactions of the parasitophorous vacuole with the host cell organelles.

Whatever the mechanism of IFN-γ-induced inhibition of *T.
IFN-γ-MEDIATED INHIBITION OF T. GONDII

33. Pollock, J. D., D. A. Williams, M. A. C. Gifford, L. L. Li, D. Du, J. Fisherman,

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