

## Gamma Interferon-Induced Inhibition of *Toxoplasma gondii* in Astrocytes Is Mediated by IGTP

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***Toxoplasma gondii* is an important pathogen in the central nervous system, causing a severe and often fatal encephalitis in patients with AIDS. Gamma interferon (IFN- $\gamma$ ) is the main cytokine preventing reactivation of *Toxoplasma* encephalitis in the brain. Microglia are important IFN- $\gamma$ -activated effector cells controlling the growth of *T. gondii* in the brain via a nitric oxide (NO)-mediated mechanism. IFN- $\gamma$  can also activate astrocytes to inhibit the growth of *T. gondii*. Previous studies found that the mechanism in murine astrocytes is independent of NO and all other known anti-*Toxoplasma* mechanisms. In this study we investigated the role of IGTP, a recently identified IFN- $\gamma$ -regulated gene, in IFN- $\gamma$  inhibition of *T. gondii* in murine astrocytes. Primary astrocytes were cultivated from IGTP-deficient mice, treated with IFN- $\gamma$ , and then tested for anti-*Toxoplasma* activity. In wild-type astrocytes *T. gondii* growth was significantly inhibited by IFN- $\gamma$ , whereas in astrocytes from IGTP-deficient mice IFN- $\gamma$  did not cause a significant inhibition of growth. Immunoblot analysis confirmed that IFN- $\gamma$  induced significant levels of IGTP in wild-type murine astrocytes within 24 h. These results indicate that IGTP plays a central role in the IFN- $\gamma$ -induced inhibition of *T. gondii* in murine astrocytes.**

*Toxoplasma gondii* is an important pathogen in the central nervous system, where it causes a severe and often fatal encephalitis in patients with AIDS. Cytokines play an important role in the regulation of *T. gondii* replication in the central nervous system (5, 10, 11). Gamma interferon (IFN- $\gamma$ ) has been shown to be the main cytokine preventing reactivation of *Toxoplasma* encephalitis in the brain (17, 18). Several studies have demonstrated that IFN- $\gamma$  can control the growth of *T. gondii* in the brain via the activation of microglia (3, 4). The anti-*Toxoplasma* activity in microglia is via a nitric oxide (NO)-mediated mechanism (7). IFN- $\gamma$  can also activate astrocytes to inhibit the growth of *T. gondii* (8). The mechanism of IFN- $\gamma$ -mediated anti-*Toxoplasma* activity in murine astrocytes has been found to be independent of the mechanisms previously demonstrated in other cells, e.g., mechanisms involving NO, tryptophan starvation, reactive oxygen intermediates, and iron deprivation (9).

IFN- $\gamma$  is thought to exert its effects largely by activation of IFN- $\gamma$ -responsive genes, of which over 200 have been identified (2). For most of these genes, their contributions in mediating the effects of IFN- $\gamma$  are unknown. One recently identified IFN- $\gamma$ -regulated gene is *IGTP* (19). It is representative of a family of at least six genes encoding 47- to 48-kDa proteins that contain a GTP-binding sequence and which are expressed at high levels in immune and nonimmune cells after exposure to IFN- $\gamma$ . Several of these proteins, including IGTP, localize to the endoplasmic reticulum (ER) of cells, suggesting that they may be involved in the processing or trafficking of immunologically relevant proteins, such as antigens or cytokines (20).

Recently it has been found that IGTP-deficient ( $\Delta$ IGTP) mice display a loss of host resistance to acute infection with *T. gondii* (21). In this study, we investigated the potential involvement of IGTP in IFN- $\gamma$  inhibition of *T. gondii* in murine astrocytes using primary astrocytes cultivated from IGTP-deficient mice.

### MATERIALS AND METHODS

**Primary astrocyte culture.** Murine astrocytes from C57BL/6  $\times$  SV129 mice or syngeneic mice, deficient in IGTP (21), were cultivated from the brains of neonatal (less-than-24-h-old) mice. Murine pups were sacrificed, the brains were removed from the cranium, the forebrains 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, and suspended in growth medium (GIBCO-BRL, Gaithersburg, Md.) supplemented with 20% fetal bovine serum (FBS) (GIBCO-BRL), 5% glucose, and 1% penicillin and streptomycin (GIBCO-BRL) 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 was reached. By this method, cells were found to be >95% astrocytes, as judged by positive staining for glial fibrillary acidic protein (GFAP). Cultures contained <5% microglia, as identified by staining with the lectin BS1-B4 (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, and cultured for 7 to 10 days after replating. These astrocytes were then infected with *T. gondii* ME49 as described below.

**Cryopreservation of primary murine astrocytes.** Murine astrocyte cultures prepared from mouse brain were cultivated in growth medium as described above. At 7 to 10 days after plating, cultures were trypsinized and then resuspended in growth medium with 10% dimethyl sulfoxide. Cells were then frozen to -70°C at -1°C/min using a Nalgene Cryo Freezing Container (catalog no. 5100-001; Nalge Nunc International, Rochester, N.Y.) and were stored in liquid nitrogen. Astrocytes frozen by this method were stable for months and routinely could be replated to attain a monolayer within 7 days that was GFAP positive.

**Culture of *T. gondii*.** Parasites were maintained by serial passage in confluent monolayers of human foreskin fibroblasts (ATCC CCD-27SK) grown in Dulbecco's modified Eagle's medium (pH 7.1) (GIBCO-BRL) supplemented with 10% FBS and a 1% penicillin-streptomycin solution (GIBCO-BRL). Parasites were

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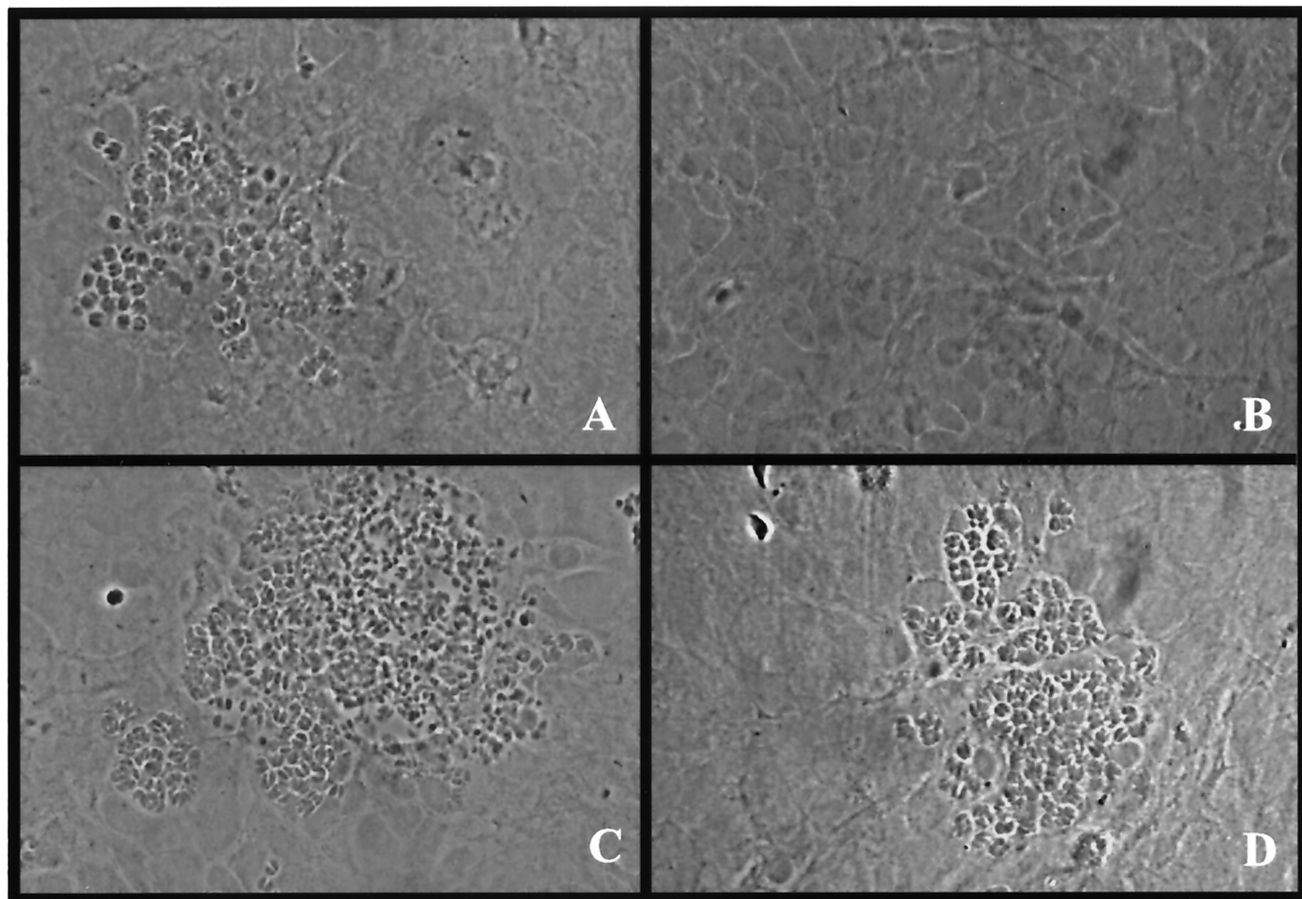


FIG. 1. Phase contrast of untreated and IFN- $\gamma$ -treated wild-type astrocytes versus untreated and IFN- $\gamma$ -treated  $\Delta$ IGTP murine astrocytes. (A) Untreated wild-type astrocytes; (B) IFN- $\gamma$ -treated wild-type astrocytes; (C) untreated  $\Delta$ IGTP astrocytes; (D) IFN- $\gamma$ -treated  $\Delta$ IGTP astrocytes, 48 h after infection. Almost no organisms were present in IFN- $\gamma$ -treated control astrocytes, but extensive replication was present in IFN- $\gamma$ -treated  $\Delta$ IGTP astrocytes.

harvested at 5 to 6 days postinfection, resuspended in minimal essential medium supplemented with 10% FBS, and used for infection of murine astrocyte cultures.

**Cytokine treatments.** Murine astrocytes were stimulated with IFN- $\gamma$  (Calbiochem) at 64 to 500 U/ml for 72 h prior to infection. Cultures were washed and then infected with  $5 \times 10^4$  *T. gondii* tachyzoites per well (a 5:1 tachyzoite/host cell ratio) for 2 h. Cultures were then washed to remove extracellular parasites and incubated for 48 h without IFN- $\gamma$ . Growth of *T. gondii* was assessed microscopically as detailed below. All cultures were incubated in endotoxin-free media, and no endotoxin contamination was detected in any experimental cultures.

**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 microscopies. Each condition was determined in triplicate. Immunofluorescence was performed using a 1:50 dilution of a commercial polyclonal rabbit anti-*Toxoplasma* antibody (DAKO, Carpinteria, Calif.) followed by detection with anti-rabbit fluorescein immunoglobulin G (IgG; Boehringer-Mannheim, Indianapolis, Ind.) as previously described (8, 9).

**Western blotting.** Total cellular protein lysates were prepared by washing the cells three times with ice-cold phosphate-buffered saline and then scraping them into ice-cold lysis buffer (1% [vol/vol] Nonidet P-40, 50 mM Tris [pH 7.5], 0.15 M NaCl, 5 mM EDTA) with plastic cell scrapers. Cellular protein lysates were separated on sodium dodecyl sulfate–10% polyacrylamide gels; the gels were transferred to an Immobilon-P membrane (Millipore, Bedford, Mass.) with a TEX50 wet transfer apparatus (Hoefer, San Francisco, Calif.) using 25 mM Tris, 480 mM glycine, and 20% (vol/vol) methanol buffer. Western blotting and detection were carried out using the ECL Detection System (Amersham International, Buckinghamshire, United Kingdom) according to the manufacturer's

protocols. The rabbit polyclonal anti-IGTP antiserum (19–21) was used at a 1:1,000 dilution; the peroxidase-conjugated anti-rabbit IgG secondary antibody (Boehringer Mannheim) was used at a 1:5,000 dilution.

**Statistics.** Within each experiment all conditions were repeated in triplicate wells, and each experiment was replicated two to three times (as indicated in the tables). Data were analyzed by the Student *t* test method using Sigma Stat Version 1.0 (Jandel Scientific, San Rafael, Calif.).

## RESULTS

**Growth of *Toxoplasma* in IFN- $\gamma$ -stimulated wild-type astrocytes versus growth in  $\Delta$ IGTP astrocytes.** Wild-type and  $\Delta$ IGTP murine astrocytes were stimulated with IFN- $\gamma$  for 72 h and infected with *T. gondii*, and growth of the parasite was assessed 24 h after infection. There was no difference in the rate of infection or growth of *T. gondii* in the untreated  $\Delta$ IGTP astrocytes compared to that of the wild-type astrocytes. At 48 h after infection, both wild-type and  $\Delta$ IGTP untreated astrocytes contained vacuoles with numerous tachyzoites (Fig. 1A versus C). In the IFN- $\gamma$ -treated astrocytes, however, a significant difference was observed between wild-type astrocytes and  $\Delta$ IGTP astrocytes. At 48 h after infection, the IFN- $\gamma$ -treated wild-type astrocytes had few visible parasites, while the IFN- $\gamma$ -treated  $\Delta$ IGTP astrocytes had numerous parasites and cells contained

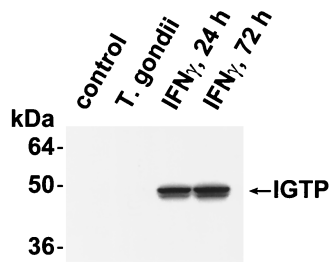


FIG. 2. Accumulation of IGTP in astrocytes exposed to IFN- $\gamma$ . Primary astrocytes were infected with *T. gondii* for 24 h at a multiplicity of infection of 5:1, were exposed to 100 U of IFN- $\gamma$ /ml for 24 or 72 h, or were exposed to control conditions. Protein lysates were then prepared and used for sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and blotting with IGTP antisera.

vacuoles with numerous tachyzoites (Fig. 1B versus D). The growth of *T. gondii* in IFN- $\gamma$ -treated  $\Delta$ IGTP astrocytes was comparable to that of untreated wild-type astrocytes, indicating that the IFN- $\gamma$ -mediated inhibition was reversed in  $\Delta$ IGTP astrocytes.

**Immunoblotting of IFN- $\gamma$  induction of IGTP in wild-type astrocytes.** Wild-type astrocytes were stimulated with IFN- $\gamma$  (100 U/ml) for 24 and 72 h and tested for induction of IGTP via Western blotting. IGTP was not detected either under control conditions or following infection with *T. gondii* (Fig. 2). However, wild-type astrocytes treated with 100 U of IFN- $\gamma$  for 24 or 72 h demonstrated strong IGTP induction (Fig. 2, lanes 3 and 4).

**Dose response of treated wild-type versus IFN- $\gamma$ -treated  $\Delta$ IGTP astrocytes.** A dose response of IFN- $\gamma$  stimulation in wild-type versus  $\Delta$ IGTP astrocytes was done to further investigate if IFN- $\gamma$ -mediated inhibition occurred in  $\Delta$ IGTP astrocytes at higher doses of IFN- $\gamma$ . Wild-type and  $\Delta$ IGTP astrocytes were pretreated with IFN- $\gamma$  at 64, 125, 250, and 500 U/ml for 72 h prior to infection. In wild-type astrocytes, *T. gondii* growth was significantly inhibited (10.6% of control) at 64 U/ml, whereas in  $\Delta$ IGTP astrocytes, 64 to 500 U of IFN- $\gamma$ /ml caused a minimal inhibition of growth (75 to 88% of control) (Table 1).

TABLE 1. Dose response of IFN- $\gamma$ -mediated inhibition in wild-type and  $\Delta$ IGTP astrocytes<sup>a</sup>

IFN- $\gamma$ treatment (U/ml)	<i>T. gondii</i> growth (% of control) in:	
	Wild-type astrocytes	$\Delta$ IGTP astrocytes
64	10.6 $\pm$ 2.2*	88.8 $\pm$ 14.5
125	3.6 $\pm$ 0.2*	88.2 $\pm$ 8.1
250	0.9 $\pm$ 0.05*	77.7 $\pm$ 12.9
500	0.9 $\pm$ 0.05*	75.6 $\pm$ 6.2

<sup>a</sup> Means  $\pm$  standard errors of the means of three separate experiments for wild-type and  $\Delta$ IGTP astrocyte cultures treated with IFN- $\gamma$  are shown. Control cultures were incubated in medium alone. Cells were incubated with cytokines for 72 h prior to infection. \*,  $P < 0.05$  for wild-type astrocytes versus IFN- $\gamma$ -treated astrocytes. There was no statistical difference between IFN- $\gamma$ -treated  $\Delta$ IGTP cells and untreated wild-type cells.

## DISCUSSION

IFN- $\gamma$  has been shown to be the main cytokine preventing reactivation of *Toxoplasma* encephalitis in the brain (17, 18). Macrophages and microglia are phagocytic cells of hemopoietic origin and are important IFN- $\gamma$ -activated effector cells against *T. gondii* that exert potent anti-*Toxoplasma* activity via the induction of inducible NO synthase and the production of NO (1, 3, 4). NO is believed to be directly toxoplasmaicidal, resulting in intracellular killing and/or stasis of parasites. IFN- $\gamma$  has been shown to induce anti-*Toxoplasma* activity in a variety of nonhemopoietic cells via NO-independent mechanisms. For example, in human fibroblasts and retinal epithelial cells and rat retinal epithelial cells, IFN- $\gamma$  inhibition is due to the induction of indoleamine 2,3-dioxygenase, an enzyme that degrades intracellular tryptophan (14, 15, 16). In rat enterocytes, IFN- $\gamma$  inhibition was found to be due to iron starvation (6), while in human endothelial cells, IFN- $\gamma$  inhibition was found to be independent of reactive nitrogen intermediates, reactive oxygen species, or tryptophan starvation (22). In murine astrocytes we have found IFN- $\gamma$  inhibition was independent of all of the above mechanisms (8, 9). The anti-*Toxoplasma* mechanism operating in murine astrocytes, and possibly in other nonhemopoietic effector cells, remains to be elucidated.

In this paper we investigated the role of *IGTP*, a recently identified IFN- $\gamma$ -regulated gene, in the IFN- $\gamma$ -induced inhibition of *T. gondii* in murine astrocytes. We found that the inhibitory effect of IFN- $\gamma$  is ablated in astrocytes lacking the protein IGTP. In wild-type astrocytes, *T. gondii* growth was significantly inhibited at 64 U/ml, whereas in  $\Delta$ IGTP astrocytes, concentrations of up to 500 U of IFN- $\gamma$ /ml caused minimal growth inhibition. IGTP was highly expressed in wild-type astrocytes within 24 h of exposure to IFN- $\gamma$ , and expression remained high to at least 72 h. These data suggest that IGTP plays a central role in the IFN- $\gamma$ -induced inhibition of *T. gondii* in murine astrocytes. It is possible that small amounts of IFN- $\gamma$  in the central nervous system are important in maintaining astrocytes in an activated condition.

The function of IGTP is not known. IGTP is expressed at high levels in many IFN- $\gamma$ -stimulated cells, including immune cells such as macrophages, T cells, and B cells as well as nonimmune effector cells such as fibroblasts, hepatocytes (18), and as we have shown in this paper, astrocytes. IGTP is a GTP-binding protein that is membrane bound, and it localizes predominantly to the ER (20). There are many families of GTPases that associate with the ER and that are involved in protein processing or trafficking; therefore, it has been suggested that IGTP may regulate vesicular trafficking within the cell (20, 21).

*T. gondii* has a unique relationship with its host cell. Unlike other intracellular pathogens that reside in the cytoplasm, endosome, or lysosomal compartments, *T. gondii* resides within an intracellular compartment called the parasitophorous vacuole. The parasitophorous vacuole is a nonfusogenic compartment that does not intersect with the host cell endocytic or exocytic pathways (13). The nonfusogenic nature of the parasitophorous vacuole is crucial to survival of the intracellular stage, as fusion with lysosomes results in degradation of the parasite. Alterations in the host cell that induce trafficking of

the host cell endocytic or exocytic pathways to the parasitophorous vacuole could conceivably be detrimental to intracellular survival. IGTP, for example, may control parasite clearance through regulation of vesicular movement of antigen, cytokines, or other molecules to the parasitophorous vacuole.

The ability of nonhematopoietic cells to control *T. gondii* in vivo has, until recently, been unclear. A study employing chimeric mice demonstrated that resistance to the acute and chronic phases of *T. gondii* requires nonhematopoietic cells as well as those of hematopoietic origin (23). Host control of *T. gondii* and other pathogens is dependent on the induction of diverse effector molecules by IFN- $\gamma$  in a variety of nonhematopoietic cells. Consequently, murine knockouts of different IFN- $\gamma$ -induced effectors display various pathogen-specific susceptibilities, depending on the cells targeted by the pathogen and the cells in which the effectors are expressed. For example, IGTP-deficient mice are susceptible to *T. gondii* but not to *Listeria*. Our data demonstrate that IGTP is particularly important for astrocyte-mediated control of *T. gondii*, and they further underscore the critical importance of nonhematopoietic cells in resistance to toxoplasmosis. *T. gondii* infects several types of nonhematopoietic cells, including epithelial, endothelial, mesodermal, and neuronal cells, and the ability of the host to elicit effector mechanisms in these cells may relate to the pathogenesis of toxoplasmosis. For example, epithelial cells may be important effector cells in the acute phase of infection, endothelial cells in congenital toxoplasmosis, and astrocytes in cerebral toxoplasmosis. A better understanding of IFN- $\gamma$ -induced mechanisms in these cell types may be important in devising better treatment strategies for toxoplasmosis.

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