In Vitro Effect of TNF-\(\alpha\) and IFN-\(\gamma\) in Retinal Cell Infection with *Toxoplasma gondii*

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**Purpuse.** *Toxoplasma gondii* is an intracellular protozoan parasite and the most common cause of infectious uveitis. This study was conducted to evaluate the in vitro effect of tumor necrosis factor (TNF-\(\alpha\)) and interferon (IFN)-\(\gamma\) in rat retinal cells infected with *T. gondii*.

**Methods.** Rat retinal cells, retinal pigment epithelial (RPE) cells, and retinal Müller glial (RMG) cells were in vitro infected with *T. gondii* RH strain tachyzoites. Cultured cells were stimulated with various concentrations of TNF-\(\alpha\) and IFN-\(\gamma\). The effect of TNF-\(\alpha\) and IFN-\(\gamma\) in *T. gondii* invasion and replication between retinal cells was determined through two different methods: measuring \([3\text{H}]-\text{uracil incorporation and counting infected cells by microscopic examination.}

**Results.** Infection by *T. gondii* was lesser within RPE cells than within RMG cells. IFN-\(\gamma\) significantly inhibits \([3\text{H}]-\text{uracil incorporation in RMG and RPE cells (respectively, 35%, 83%, and 87%}}\]

resulting in the replication of retinal cells infected with the parasite and NO. The toxoplasmic retinochoroiditis is partly due to the lysis of the retinal cells infected with the parasite and partly to the adjacent inflammatory response in the choroid and retina. The role of cytokines in toxoplasmic retinochoroiditis has been studied in vivo in animal models, where it has been posited that cytokine IFN-\(\gamma\) plays an important role in controlling the disease. IFN-\(\gamma\) is one of the major cytokines of the adaptive immune response produced by activated T cells on infection with various intracellular infectious agents (mycobacteria, virus, *T. gondii*). TNF-\(\alpha\) has considerable importance in the innate immune response to any infection and is well produced by activated T cells at levels that can also influence intraocular immunity. It is accepted that host resistance to toxoplasmic infection is due to IFN-\(\gamma\) generated by innate natural killer cells and adaptive CD4 and CD8 T lymphocytes.

**Materials and Methods**

**Cell Cultures.**

The cell cultures used in these experiments were primary cultures of retinal cells from Lewis rats. These rats were killed by cervical dislo-
cation between their 8th and 12th days of life. Their eyes were swiftly enucleated in sterile conditions; immediately put in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5% glucose, 1% glutamine, 1% penicillin/streptomycin, 1% amphotericin B (complete DMEM); and left overnight in the dark at room temperature.

The eyeballs were then incubated in 0.5 mL of complete DMEM containing 0.1% trypsin and 70 U/mL collagenase for 1 hour at 37°C. Next, they were put in a dish containing DMEM supplemented with 10% fetal calf serum (FCS) to inhibit the action of trypsin. Each eyeball was then dissected under a microscope to isolate the pigment epithelium and the neurosensory retina separately.

The neurosensory retinas were placed in 10-cm dishes (four to five per dish) containing 5 mL DMEM with 10% FCS and then broken down with forceps into small fragments that were incubated at 37°C with 5% CO₂ for 5 to 6 days in the same medium. The fragments were then carefully removed from the dish and the cells grew to confluence. RMG cell purity was checked by immunocytochemistry. Briefly, RMG cells (passages 2 to 3, approximately 4 days of culture subsequent to the last passage) appeared slightly positive for the marker GFAP (gliarial fibrillary acidic protein) and highly positive for the marker vimentin.

The pigment epithelium in suspension were centrifuged at 1300 rpm for 10 minutes. The tissue was dissociated by adding 0.1 mL trypsin per eye to the pellet assisted mechanically by passing the contents of the tube 10 times through a Pasteur pipette. The action of trypsin was then inhibited by 5 mL DMEM with 10% FCS. The pellet was rinsed and diluted in DMEM with 10% serum (2 mL for four to five retinas). This solution was placed in 60-cm dishes containing 2 mL DMEM + 10% FCS (four to five retinas per dish) and incubated 4 to 5 days at 37°C in 5% CO₂ until confluence.

Preparation of T. gondii

The RH strain tachyzoites of T. gondii were propagated by intraperitoneal injection of IOPS/OFl female mice. After 3 or 4 days of infection, the mice were killed and the tachyzoites recovered under sterile conditions by injecting 10 mL of 9% NaCl solution in the peritoneal cavity and removing the contents with the same syringe. The tachyzoites were washed in PBS and centrifuged for 10 minutes at 2500 rpm. The pellet was then resuspended in culture medium and the tachyzoites counted with a hemocytometer. Viability was assessed via trypan blue exclusion. Parasites were 95% viable and samples contained less than one host cell per 300 parasites.

Cell Culture Infection with T. gondii

Cell cultures were grown in DMEM containing 10% SVF. One hour before infection, the medium was replaced by DMEM containing 5% SVF. When they grew to confluence, the cells were infected with the tachyzoites on 24-well dishes at a 5:1 ratio (250,000 tachyzoites per 50,000 cells). After 2 hours of infection, the supernatant was removed, the medium changed, and the cultures rinsed and reincubated for a variable period. In the last series of experiments, the cells were massively infected with a 15:1 ratio (750,000 tachyzoites for 50,000 cells) to assess tachyzoite penetration 6 hours after infection. The control experiments involved infection of NIH-3T3 human fibroblasts with T. gondii.

Immunocytofluorescence

The cells were fixed in 4% paraformaldehyde, saturated with 10% FCS, permeated with a 0.5% Triton solution, and stained with rabbit polyclonal anti-GFAP antibodies (gliaspecific, dilution 1/100; Dako, Trappes, France) for Müller cells, with mouse monoclonal anti-pan-cytokeratin antibodies for the pigment epithelial cells (dilution 1/50), and with mouse monoclonal anti-Toxoplasma or rabbit polyclonal anti-Toxoplasma antibodies. The second matching antibodies used (anti-rabbit or anti-mouse; dilution 1/100) were stained with FITC or TRITC. After they were rinsed, the preparations were mounted (Vectashield; Vector Laboratories, Compiegne, France) and examined under epifluorescence and confocal microscopes. The invasion of the different cellular types was quantified by counting the number of infected cells and the number of intracellular T. gondii in 200 cells randomly selected in each preparation. The purity of the cultures was checked by simultaneously using the anti-GFAP and anti-pan-cytokeratin antibodies.

Radioactive Uracil Incorporation

The radioactivity emitted by [³H]-uracil incorporated in the T. gondii DNA synthesis was measured to quantify tachyzoite proliferation within the infected cells, as described by Creuzet et al.¹¹ The cultures were infected with 250,000 tachyzoites per well (tachyzoite-to-host cell ratio, 5:1) for 2 hours, washed with PBS to eliminate extracellular tachyzoites, and incubated for various periods in DMEM containing 5% dialyzed calf serum and 4 μCi/mL (5.6·10⁶) uracil. At the end of the incubation period, the supernatant was eliminated, the cells were lysed for 15 minutes in 1 mL PBS containing 1% sodium dodecyl sulfate (SDS) and 1 mM uracil, and the nucleic acids were precipitated by adding 10% trichloroacetic acid (TCA). The contents of the wells were placed on fiberglass filters (GE/C: Whatman, Sarl, France) and washed three times with 5% TCA. The radioactivity was then determined with a scintillation counter. For each experiment, wells without T. gondii were included to check that uracil incorporation was specific to the parasite.

Cytokine Treatment

The cell cultures were stimulated with LPS (10 and 100 ng/mL), rat recombinant IFN-γ (0.1, 1, and 10 ng/mL), rat recombinant TNF-α (0.1, 1, and 10 ng/mL), alone or in combination, 72 hours before the infection. After infection with T. gondii, the cultures were incubated in DMEM containing 5% and 5% SVF (for uracil incorporation and immunocytofluorescence, respectively) for 48 hours, without adding cytokines. The effect of cytokines on the infection was assessed according to two parameters: the penetration of tachyzoites within the host cells, and the proliferation of tachyzoites within the infected cells. Tachyzoite proliferation within the infected cells was quantified by using two methods: uracil incorporation and analysis of the preparations by immunocytofluorescence. The preparations were analyzed 24 hours after infection for Müller cells and 48 hours after infection for pigment epithelial cells. The penetration into the host cells was studied only in the Müller cells, by analyzing the preparations 6 hours after massive infection by immunocytofluorescence.

Quantification of NO Production

After 72 hours of cell culture stimulation with different cytokines, the supernatants were collected to quantify nitrite production by a colorimetric method based on the Griess reaction.¹² Put briefly, 100 μL of supernatant was added to 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylene diamine), and 10 minutes later the absorbance was measured at 540 nm. The nitrite level was quantified from a standard curve constructed with different concentrations of sodium nitrite. NO production was also measured 48 hours after infection.

Statistics

Within each experiment, all conditions were repeated in triplicate wells, and each experiment was performed three times. Data were analyzed by nonparametric (Wilcoxon signed-rank test) and/or parametric methods (Student’s t-test, analysis of variance; StatView; SAS Institute, Cary, NC).

RESULTS

Infection According to Cellular Type

The proliferation of tachyzoites within the host cells 24 hours after infection was assessed by uracil incorporation. The par-
Asites grew within both cell types studied, retinal pigment epithelium and retinal Müller cells (Fig. 1). Uracil incorporation was not significantly different between Müller cells and NIH-3T3. However, this incorporation was significantly less in the retinal pigment epithelial cells than in NIH-3T3 (25.4% ± 2.5%).

Cytokine Modification of the Infection

Proliferation of Tachyzoites within the Host Cell. Analysis by [3H]-Uracil Incorporation. The pigment epithelial cells were significantly less sensitive to infection with T. gondii when stimulated beforehand with IFN-γ or TNF-α (Fig. 2A). These cytokines triggered a dose-dependent inhibition reaction on tachyzoite proliferation. Inhibition was observed to increase with cytokine concentration in the culture medium. At 0.1 ng/mL, neither IFN-γ nor TNF-α had an effect. The effect appeared at concentrations of 1 ng/mL (30% inhibition with IFN-γ and 23% inhibition with TNF-α), and was highest at 10 ng/mL (75% inhibition with IFN-γ and 38% inhibition with TNF-α). IFN-γ at 10 ng/mL concentration had a higher inhibiting effect than did TNF-α at the same concentration. We observed a synergic effect between these two cytokines, wherein adding TNF-α significantly increased the inhibiting effect of IFN-γ. LPS had no effect on tachyzoite proliferation; however, it significantly increased the action of IFN-γ.

With regard to Müller cells, IFN-γ, but not TNF-α, had an inhibiting effect on tachyzoite proliferation (Fig. 2B). IFN-γ significantly inhibited [3H]-uracil incorporation by RMG cells (respectively, 35%, 83%, and 87% inhibition at 0.1, 1, and 10 ng/mL). Neither TNF-α nor LPS showed any synergic effect with IFN-γ in Müller cells. Thus, Müller cells appeared to be more sensitive to the inhibiting action of IFN-γ than did pigment epithelial cells.

Analysis by Immunocytofluorescence. The inhibiting effect of IFN-γ on T. gondii infection was also confirmed by microscopic confocal analysis (Fig. 3). The percentage of infected cells was 20% in the absence of stimulation and 7% after IFN-γ stimulation (Fig. 4A). The number of intracellular tachyzoites for 200 cells was significantly reduced when the cells were stimulated beforehand with IFN-γ (Fig. 4B).

Penetration of Host Cells by Tachyzoites. In the absence of cytokine stimulation, 39% of RMG cells were infected and several tachyzoites entered the cell cytoplasm. Tachyzoites’ capacity to penetrate Müller cells is reduced by

![FIGURE 1.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932957/)  

**FIGURE 1.** [3H]-uracil incorporation in retinal cells 24 hours after infection. Different types of cells (human fibroblasts NIH-3T3 [3T3] and RPE cells and RMG cells from Lewis rats) were incubated in DMEM supplemented with 3% FCS and infected for 2 hours with tachyzoites (ratio 1:5). [3H]-uracil incorporation (expressed in DPM) was assayed 24 hours after infection.

![FIGURE 2.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932957/)  

**FIGURE 2.** Influence of cytokines on tachyzoite multiplication among infected retinal cells: RPE (A) and RMG (B). Culture cells were stimulated 72 hours before infection with various cytokines (IFN-γ and/or TNF-α 0.1, 1, or 10 ng/mL) and/or with LPS at 100 ng/mL. IFN-γ and TNF-α were used at a concentration of 10 ng/mL when mixed with other cytokines. Cell culture without cytokine stimulation served as the control. [3H]-uracil incorporation was assayed 48 hours after infection (ratio 1:5). *P < 0.05; **P < 0.001.
The percentage of infected cells and the number of intracellular tachyzoites for 200 cells dropped from 39% to 19.5% and from 93 to 46, respectively, after the addition of IFN-γ (Fig. 5).

NO Production

The NO production of the studied cells was very small in the basal state when the cells were not stimulated (some 2 μM). NO production by both pigment epithelial cells and Müller cells increased significantly after stimulation with IFN-γ+TNF-α or IFN-γ+TNF-α+LPS combinations (Table 1). Neither IFN-γ alone nor TNF-α significantly increased NO production. The presence of T. gondii did not stimulate production (no significant difference between control wells without T. gondii and infected wells). In the second series of measure-

IFN-γ. The percentage of infected cells and the number of intracellular tachyzoites for 200 cells dropped from 39% to 19.5% and from 93 to 46, respectively, after the addition of IFN-γ (Fig. 5).

FIGURE 3. Confocal images of RPE and RMG cells infected with T. gondii. The proliferation of tachyzoites among RPE cells was assayed by immunocytofluorescence 48 hours after infection (ratio 5:1), without cytokine stimulation (A), and after stimulation with IFN-γ at 10 ng/mL 72 hours before infection (B). After stimulation with IFN-γ, the number of infected cells and the proliferation of tachyzoites within the cells were lower. The ability of tachyzoites to penetrate RMG cells was evaluated 6 hours after massive infection (ratio 15:1), without cytokine stimulation (C, D) and after stimulation with IFN-γ at 10 ng/mL 72 hours before infection (E). Tachyzoite proliferation among RMG cells was assayed 24 hours after infection (ratio 5:1) without cytokine stimulation (F). In the absence of prior stimulation with cytokines, the tachyzoites grew within the parasitophorous vacuoles and formed rosettes containing 10 to 20 tachyzoites each. After stimulation with IFN-γ, tachyzoite proliferation was lower, in most cases with only one parasitophorous vacuole per cell, and the vacuoles contained only 5 to 10 tachyzoites (data not shown).

FIGURE 4. Influence of cytokines on RMG cell infection determined by immunocytofluorescence 24 hours after infection. Cultures of RMG cells were stimulated 72 hours before infection with IFN-γ at 10 ng/mL, more or less associated with LPS at 100 ng/mL, or with TNF-α at 10 ng/mL. Cell culture without cytokine stimulation served as the control. The cells were fixed 24 hours after infection (ratio 5:1), stained with antibodies, and examined with epifluorescence and confocal microscopes. The percentage of infected cells (A) and the number of tachyzoites (B) were counted among 200 cells randomly selected from each preparation.
Ocular toxoplasmosis, whether acquired or congenital, typically manifests itself in the form of a chorioretinitis reflecting the inflammation and necrosis of an area including the neuroretina, the pigment epithelium, and the choroid in response to the release of tachyzoites. Cysts and free forms of tachyzoites were found in the neuroretina and the pigment epithelium. The two types of retinal cells we have chosen to study, Müller cells (RMG) and pigment epithelial cells (RPE), play an important role in the local immune response by producing certain cytokines (TNF, IL-1) and acting as antigen-presenting cells.\(^{13,14}\) We therefore chose to study the penetration and the proliferation of \textit{T. gondii} in these cells and to assess variations in response to the action of IFN-\(\gamma\) and TNF-\(\alpha\). In our experiments \textit{T. gondii} infected the Müller cells and the pigment epithelial cells, and the infection was lesser within the RPE cells than within the RMG cells. \textit{T. gondii} proliferation was also inhibited by IFN-\(\gamma\), TNF-\(\alpha\), and the IFN-\(\gamma\)+TNF-\(\alpha\) combination in the RPE cells, but only by IFN-\(\gamma\) or the IFN-\(\gamma\)+TNF-\(\alpha\) combination in the RMG cells. Pretreatment of RMG cells with IFN-\(\gamma\) prevented infection (decreasing the percentage of infected cells) and also induced a postinfection defense mechanism (decreasing the number of parasites per infected cell 6 hours after massive infection, as well as decreasing parasite multiplication among cells 48 hours after infection). It is possible that the infection’s reduction could be partly due to a consequence of TNF- and IFN-\(\gamma\)-mediated apoptosis of RPE or RMG cells. Nevertheless, we showed that the number of RMG cells infected and the number of tachyzoites per cell decreased significantly when the cells were pretreated with IFN-\(\gamma\) (Fig. 4), and this effect was not influenced by the mediated apoptosis. The literature does not mention any study on the penetration and proliferation of \textit{T. gondii} in RMG cells, and only one team has studied the interactions between \textit{T. gondii} and RPE cells. Nagineni et al.\(^{15}\) studied the capacity of \textit{T. gondii} to infect human RPE cells, but the percentage of infected cells was not specified, the authors having used the size of the lysis plaques to assess the infection intensity. They observed that IFN-\(\gamma\) and TNF-\(\alpha\) reduced the intensity of infection in RPE cells in a dose-dependent way. When they studied the infection of the astrocytes of human cerebral tissue, which are glial cells just like RMG cells, Pelloux et al.\(^{16}\) found 16% of infected cells 24 hours after infection, which is close to the 20% we obtained with RMG cells. With regard to the action of IFN-\(\gamma\), their results were different from ours, as they noted no inhibiting effect on tachyzoite proliferation. Two hypotheses can explain these differences. First, the incubation time of the cells with the tachyzoites was shorter, the cells having been treated with IFN-\(\gamma\) for 24 hours before infection (compared with 72 hours in our experiments). Second, the astrocytes used by the authors were of human origin, whereas we worked on rat cells. Another team, using murine astrocytes preincubated 72 hours before infection with different cytokines, found an inhibiting effect for IFN-\(\gamma\) and the IFN-\(\gamma\)+TNF-\(\alpha\) combination on \textit{T. gondii} proliferation, as we did with RMG cells.\(^{17}\) However, no inhibiting effect was observed when TNF was used alone, a result similar to ours with RMG cells. In our study, we used dissociated RPE cells, retaining no cells of the original single layer. The barrier function played by RPE cells is mostly dependent on the integrity of tight junctions. It is known that IFN-\(\gamma\) induces alterations of RPE tight junctions.\(^{18}\) Studies have shown that IFN-\(\gamma\) stimulates expression of intercellular adhesion molecule (ICAM)-1 in human RPE cells.\(^{19}\) ICAM-1 secretion by RPE cells might actively participate in immune reactions in the retina by recruiting and activating lymphocytes, contributing to the immunopathologic process in inflammatory diseases.

**FIGURE 5.** Influence of cytokines on massive RMG cell infection determined by immunocytofluorescence 6 hours after massive infection. Cultures of RMG cells were stimulated 72 hours before infection with IFN-\(\gamma\) at 10 ng/mL, more or less associated with LPS at 100 ng/mL, or with TNF-\(\alpha\) at 10 ng/mL. Cell cultures without cytokine stimulation served as the control. The cells were fixed 6 hours after massive infection (ratio 15:1), stained with antibodies, and examined under epifluorescence and confocal microscope. The percentage of infected cells (A) and the number of tachyzoites (B) were counted among 200 cells randomly selected on each preparation.
We also confirmed the capability of RPE and RMG cells to produce NO under certain conditions (for instance, when simultaneously stimulated with IFN-γ and TNF-α more or less associated with LPS). Goureau et al. showed that NO synthetase, which transforms L-arginine into NO in RMG cells, corresponds to the inducible form of the enzyme (as in the macrophages). This NO production by the activated cells could be a defense mechanism against T. gondii but also has a toxic effect on the retina’s neuronal cells. In vivo, an increase in NO production is observed in the aqueous humor and the vitreous body of rats with experimentally induced uveitis. The responsibility of NO in the development of uveitis appears to be independent of NO production. Both cytokines were used alone at different concentrations (0.1, 1, or 10 ng/mL). IFN-γ and TNF-α were used at a concentration of 10 ng/mL in combination or with LPS at 100 ng/mL. Cell culture with no cytokine stimulation served as control.

| Table 1. Influence of Cytokines on NO Production by RPE and RMG Cells |
|-------------------------|-------------------------|-------------------------|-------------------------|
|                         | RPE                     |                         | RMG                     |
|                         | Before Infection | After Infection | Before Infection | After Infection |
| Control                 | <2                     | <2                     | <2                      | <2                      |
| LPS 100                 | <2                     | <2                     | <2                      | <2                      |
| IFN-γ 0.1               | <2                     | <2                     | <2                      | <2                      |
| IFN-γ 1                 | <2                     | <2                     | <2                      | <2                      |
| IFN-γ 10                | 5 ± 1                  | <2                     | <2                      | <2                      |
| TNF-α 0.1               | <2                     | <2                     | <2                      | <2                      |
| TNF-α 1                 | <2                     | <2                     | <2                      | <2                      |
| IFN + TNF               | 93.75 ± 2.2            | 7.8 ± 0.4              | 25 ± 1                  | <2                      |
| LPS + IFN               | 10.97 ± 0.7            | <2                     | <2                      | <2                      |
| LPS + TNF               | 3.66 ± 0.3             | <2                     | <2                      | <2                      |
| LPS + IFN + TNF         | 88.9 ± 1.2             | 11 ± 0.4               | 37.1 ± 0.7              | 5 ± 0.4                 |

NO production was measured in the culture supernatant 72 hours after cell culture stimulation with various cytokines (before infection) and 48 hours after infection with tachyzoites. IFN-γ and TNF-α were used alone at different concentrations (0.1, 1, or 10 ng/mL). IFN-γ and TNF-α were used at a concentration of 10 ng/mL in combination or with LPS at 100 ng/mL. Cell culture with no cytokine stimulation served as control.

NO-2 concentration (μM)

In conclusion, we have demonstrated the inhibiting role of IFN-γ in the penetration and proliferation of RH strain tachyzoites in retinal cells, pigment epithelial cells, and Müller cells, in a dose-dependent way and according to a mechanism that seems independent of NO production. Inhibition by TNF-α applies only in RPE cells (not RMG cells), also in a dose-dependent way and also according to a mechanism that appears to be independent of NO production. Both cytokines IFN-γ and TNF-α inhibited T. gondii replication in RPE cells, whereas only IFN-γ had an anti-Toxoplasma activity within RMG cells. The differences in cytokine response may be the reason that RPE cells are less efficiently infected by T. gondii than RMG cells.

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References


