Transforming Growth Factor-β, Reverse IFN-γ Activation of Intestinal Epithelial Cells during Cryptosporidium parvum Infection

Tarek K Zaalouk

Medical Parasitology Department, Faculty of Medicine, Al-Azhar University, Cairo, Egypt.

Corresponding Author: Tarek K Zaalouk, Mobile: (+20) 01123900070, E-mail: tkzaalouk@gmail.com

ABSTRACT

Background: The intracellular parasite Cryptosporidium parvum (C. parvum) is a causative agent of diarrhea in humans and potentially fatal in AIDS patients. The parasites develop in enterocytes and are transmitted mainly by the fecal-oral route. In developing countries, infections are more common in children and may be associated with malabsorption and malnutrition. Enterocytes are an active component of intestinal mucosal immunity and their resistance to infection can be mediated by more than one mechanism including responding to cytokine signals. IFN-γ activates various mechanisms in infected enterocytes to kill invading pathogens. In contrast, transforming growth factor β (TGF-β) is crucial in downregulating inflammation caused by the Th1 response.

Aim of Study: This study aims to investigate the role of IFN-γ and TGF-β in host immunity against C. parvum infection.

Methods: To determine the role of IFN-γ on the development of C. parvum in vitro, HT-29 monolayers were incubated for 24h with varying concentrations of IFN-γ. Following infection with C. parvum oocyst, the cells were re-incubated with cytokines for a further 24h before being fixed and stained with Giemsa. The parasites were then counted using a Zeiss Axioplan microscope at x1000 magnification with oil-immersion in 20 random fields across the diameter of the coverslip.

Results: IFN-γ was found to have a marked inhibitory effect on C. parvum infection. The effect of IFN-γ was partially reversed by TGF-β, which produces a significant dose-dependent antagonist of IFN-γ activity. IFN-γ mediated its action by modification of intracellular Fe2+ concentration.

Keywords: Cryptosporidium, HT-29 cells, IFN-γ, TGF-β.

INTRODUCTION

C. parvum is an intracellular, extracytoplasmatic protozoan parasite that colonizes the mucosal epithelium of the gastrointestinal (GI) tract causing a mild to severe, cholera-like diarrhea (1). Cryptosporidiosis may be fatal in AIDS patients (2) and may also be a serious complication among malnourished children in developing countries (3). Six percent of all diarrheal disease in immunocompetent individuals and up to 24 percent of diarrheas in immunosuppressed patients worldwide were attributed to cryptosporidiosis (4).

IEC plays a key role in the protection of the underlying biological compartments from both microflora and invading organisms. In response to infection, the epithelium activates mechanisms that help to maintain structural integrity, establish an inflammatory response, and contribute to parasite killing. Human or murine intestinal epithelial cell lines infected with C. parvum demonstrate an inflammatory response characterized in particular by the production of numerous chemokines (5, 6). Also, epithelial cells exhibit antimicrobial killing mechanisms that could affect the viability of Cryptosporidium. The antimicrobial peptides expressed by human epithelial cells, β-defensin-1 and -2, have been shown to induce lysis of C. parvum sporozoites and inhibit infection in vitro. Infection with C. parvum induced expression of a β-defensin-2 in the intestinal epithelium (7).

IFN-γ, the crucial cytokine produced during the early phase of infection with intracellular pathogens, activate various mechanisms in infected IEC to kill invading pathogens. The major source of IFN-γ in innate immunity is NK cells, that activated by cytokines, including IL-12, IFN-α/β, IL-15, TNF-α and IL-18 produced by ancillary cells such as dendritic cells and macrophages (8). The essential requirement for IFN-γ in controlling intracellular infection has been demonstrated by antibody-mediated neutralization of IFN-γ in vivo. In addition, gene knockout (KO) mice with deletion of either IFN-γ α receptor chain or the IFN-γ gene itself showed enhanced susceptibility to C. parvum infection (9). IFN-γ activity is mediated by two heterologous receptor subunits. A ligand-binding subunit IFNGR-1 and an accessory subunit IFNGR-2 (10). Heterodimerization of IFN-γ receptors leads to activation of tyrosine kinases, jak-1 and jak-2 (11), and subsequent phosphorylation of Stat-1 (signal transducer and activator of transcription). Stat-1 phosphorylation form gamma activation factor (GAF) homodimers which translocated to the nucleus and bind to gamma activation site (GAS) present in IFN-γ responsive gene (12).
This sequence of events leads to the activation of cell proteins transcription that mediates the activity of cytokine in the host immune response.

IEC express IFN-γ receptors that could involve in the immunologic control of infections. Nitric oxide (NO) production stimulated by IFN-γ has been described as an important immune mechanism against microbial and viral pathogens. Inducible nitric oxide synthase, iNOS; one of three isoforms that catalyzes the oxidation of arginine to produce nitric oxide, is upregulated by IFN-γ. iNOS production was proposed as a pathway in IFN-γ-mediated killing of the microsporidian Encephalitozoon intestinalis in IEC. Induction of indoleamine-2,3-dioxygenase (IDO) and consequent depletion of cellular tryptophan and/or limiting intracellular Fe⁺⁺ availability have also been implicated in mediating IFN-γ inhibitory effect against intracellular pathogens. Both tryptophan and iron are required for the intracellular development of organisms. However, the dominant mechanism of cytokine activity likely depends on the type of host cells.

The immunomodulatory effector TGF-β can be produced by IEC following exposure to pathogens and by infiltrating cells such as lymphocytes, macrophages, and platelets. TGF-β is crucial in the maintenance of immunological homeostasis in the GI tract and in ameliorating inflammation caused by the Th1 response. TGF-β may involve in the down-regulation of IFN-γ-driven Th1 inflammatory responses. This study aimed to employ an in vitro culture system for C. parvum to explore mechanisms involved in IFN-γ-mediated inhibitory effect against C. parvum and to investigate the effect of TGF-β on modulating this IFN-γ activity.

MATERIALS AND METHODS

- **Materials:** Plastic ware: All plastic consumables (e.g. flasks, pipettes, and tissue culture plates) were purchased from Vacsera Vaccination Centers, Cairo, Egypt.

**Ethical approval**

The study was approved by the Ethics Board of Al-Azhar University.

- **Cells and Reagents:** HT-29 (Human Colorectal Adenocarcinoma cell line) was obtained from Cell Culture Lap, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt. Dulbecco’s modified Eagle medium (DMEM), heat-inactivated fetal bovine serum, penicillin, streptomycin, glutamine, nonessential amino acids, phosphate-buffered saline (PBS), trypsin and all other chemicals were supplied by Vacsera Vaccination Centers, Cairo, Egypt. Lyophilized recombinant human IFN-γ and TGF-β were obtained from Sigma-Aldrich Ltd and reconstituted as recommended by the manufacturers. Cytokines were added to cells in the 24-well plates, starting at 24 h before infection unless otherwise stated.

- **Parasites preparation:** C. parvum oocysts were obtained from Theodor Bilharz Research Institute (TBD) Cairo, Egypt. Before use, oocysts were surface sterilized by suspension in 10% commercial bleach solution (0.55% sodium hypochlorite) for 10 minutes and subsequently washed 3 times in DMEM before enumeration in a Neubauer hemocytometer.

- **Cell culture and parasite infection:** HT-29 cell lines were grown at 37°C in 5% CO₂ in DMEM medium supplemented with 10% heat-inactivated fetal calf serum, 4 mmol/L glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% non-essential amino acids. Trypsinized cells were seeded into 24- well plastic tissue culture plates with 13mm diameter glass coverslips and grown to confluence over 5 days. Confluent cell monolayers were infected with 4X10⁵ viable C. parvum oocysts suspended in 200 µl culture medium. Cells were then incubated for 2 hours at 37°C to allow excystation and host cell invasion to occur. Following this period, unattached parasites and oocyst debris were removed by washing twice with the medium. Wells were subsequently filled with fresh medium and incubated at 37°C for the stated time. In experiments involving cytokines alone or in combination, unless otherwise stated, cells were cultured in the presence of cytokine at the stated concentrations for 24 hours before inoculation of C. parvum, and for a further 24 hours after inoculation. All experiments were done in triplicate.

- **Parasite enumeration by Giemsa staining:** Cell monolayers that grown on 13 mm glass coverslips were washed twice with PBS to remove any traces of medium and fixed in methanol for 1 min, stained with 10% Giemsa (Vacsera Vaccination Centers, Cairo, Egypt) in PBS (pH 7.2) for 2h, washed in de-ionized water and then mounted on glass slides. Using a Zeiss Axioplan microscope at X1000 magnification with oil-immersion the parasites were counted in 20 fields across the diameter of the coverslip starting from the periphery.

**Mechanism of action of IFN-γ:** To examine the role of iNOS activity in the IFN-γ-mediated inhibition of C. parvum development, HT-29 cells were cultured in medium containing IFN-γ in the presence or absence of the NOS inhibitor, N⁶-Monomethyl-L-arginine (N⁶-MMA, 250 µm), or with N⁶-MMA alone starting 24h before infection. Exposure of HT-29 cells to 250 µM of the NOS inhibitor N⁶-MMA was reported to inhibit iNOS enzyme activity in enterocytes. Similarly, the role of tryptophan depletion in IFN-γ mediated inhibition of C. parvum was examined by culturing of HT-29 cells in medium containing IFN-γ in the presence or absence of exogenous tryptophan or with exogenous tryptophan alone (250µg/ml) starting 24h before infection. Tryptophan was used in concentrations that were previously found to reduce the inhibitory effect of IFN-γ on T. Gondii. The role of...
cellular Fe$^{2+}$ in the IFN-$\gamma$-mediated control of infection was investigated by the treatment of HT-29 cells with IFN-$\gamma$, starting 24h before infection, in the presence or absence of FeSO$_4$, or with FeSO$_4$ alone (250$\mu$M). Exogenous iron was used in concentrations that were previously found to reduce the inhibitory effect of IFN-$\gamma$ on T. gondii and C. parvum.$^{19,21}$

The effect of TGF-$\beta$ on IFN-$\gamma$-mediated activation of IEC: Parasite development in cells treated with IFN-$\gamma$ (100 U/ml) or TGF-$\beta$ (1 and 5ng) alone or in combination starting at 24 h before infection were examined.

Statistical analysis: The obtained data were expressed as means ±SD. Significance analysis was carried out using a two-tailed Student’s t-test for unpaired means.

RESULTS

IFN-$\gamma$ inhibits C. parvum development in HT-29 cells: C. parvum development in HT-29 monolayers was confirmed by Giemsa staining of parasite asexual stages (Fig.1a). The maximal number of parasites were obtained at 6- 24 hours post-infection (135±7 and 155±11 parasites/20 fields respectively), but the parasite numbers declined at 48 h (83± 7 parasites/20 fields) and 72 h (35± 5 parasites/20 fields) (Fig.1b).

To determine the effect of IFN-$\gamma$ on the development of C. parvum in vitro, HT-29 monolayers were incubated for 24h with varying concentrations of IFN-$\gamma$. Following infection with C. parvum oocyst, the cells were re-incubated with cytokines for a further 24h before being fixed and stained with Giemsa. Intracellular parasites were quantified and the percentage inhibition induced by IFN-$\gamma$ was determined by calculating the reduction in the number of parasites compared with controls. IFN-$\gamma$ was found to have a marked inhibitory effect on C. parvum infection, since intracellular parasite development was reduced by 29±6, 40±9 and 54±11.7% following incubation with 10, 100 and 1000 cytokine U/ml respectively (Fig. 2).

IFN-$\gamma$ effect is time-dependent: Experiments were performed to determine the effect of IFN-$\gamma$ (100U/ml) when added at different time points before and after parasite inoculation. The level of inhibition was dependent on the exposure time of cell line to IFN-$\gamma$, and the greatest inhibitory effect of cytokine was obtained with cells exposed to IFN-$\gamma$ for 24 and 6h before parasite inoculation (41 ± 9 and 22 ± 3% respectively). Infection of the cells treated with IFN-$\gamma$ starting 2h after parasite inoculation was significantly reduced compared to cells exposed to the cytokine for 5 hours before the end of the experiment (11 ± 2 and 3 ± 1% respectively) (Fig. 3).

Mechanism of IFN-$\gamma$-mediated control of infection is dependent on deprivation of available cellular Fe$^{2+}$ but not on iNOS or tryptophan: Increasing the level of Fe$^{2+}$ in cell culture medium abrogated the inhibitory effect of IFN-$\gamma$ against C. parvum.

As the significant decrease in the percent of the inhibitory effect of the cytokine was noted in cells treated with both IFN-$\gamma$ (100U/ml) and exogenous Fe$^{2+}$ (250 $\mu$M) compared to cells treated with IFN-$\gamma$ alone (9% ± 5 and 40% ± 10% respectively). Fe$^{2+}$ alone did not affect C. parvum development (Fig. 4).

Exposure of HT-29 cells to 250 $\mu$M of the NOS inhibitor N6-MMA, did not affect the action of IFN-$\gamma$ on C. parvum, suggesting iNOS was not involved in killing C. parvum. No significant decrease in the percent of infection was noted in cells treated with both IFN-$\gamma$ and exogenous tryptophan compared to cells treated with IFN-$\gamma$ alone (37% ± 6% and 40% ± 10% respectively, Fig.4).

Similarly, HT-29 cells treated with exogenous tryptophan 250 $\mu$M did not affect C. parvum development and no significant decrease in the percent of infection was observed in cells treated with both IFN-$\gamma$ and exogenous tryptophan compared to cells treated with IFN-$\gamma$ alone (38% ±6% and 40% ± 10% respectively) (Fig. 4). This result indicated that In vitro IFN-$\gamma$ effect against C. parvum is independent of tryptophan.

TGF-$\beta$ abrogated IEC activation by IFN-$\gamma$: The effect of TGF-$\beta$ on IFN-$\gamma$-mediated activation of IEC during C. parvum infection was investigated by comparing parasite reproduction in cells treated with IFN-$\gamma$ or TGF-$\beta$ alone or in combination starting at 24 h before infection. IFN-$\gamma$ (100 U/ml) decreased parasite development by 40% ± 11% (P < 0.05). TGF-$\beta$ alone did not significantly affect parasite development in cell lines but produced a significant dose-dependent antagonist of IFN-$\gamma$ activity; when 1 and 5 ng of TGF-$\beta$/ml was used, the levels of parasite inhibition induced by IFN-$\gamma$ were 29%± 8% and 7% ±2% respectively (Fig. 5).

EXPLANATION OF FIGURES
Fig. 1a. *C. parvum* development in HT-29 cells. Monolayers were grown on glass coverslips in 24-wells plate, infected with 4x10^5 oocysts, and stained with Giemsa. After 24h of infection, both immature and mature asexual stages can be identified clearly by microscopy. Fig.1b. Time course of *C. parvum* infection in HT-29 cells. The maximal number of parasites was obtained 6-24h post-infection with no significant difference in infection observed between 6 and 24h incubation periods (P>0.05). Fewer parasites were obtained at 48h and 72h compared with 24h.

Fig. 2. Effect of concentration of IFN-γ on *C. parvum* infection of HT-29 cells. Cells were incubated with medium containing 100 U/ml IFN-γ starting from times between 24h before parasite inoculation to 19h after parasite inoculation. Parasite development was measured 24h post-infection. The maximum inhibitory effect of IFN-γ was observed in cells that were incubated with cytokine 24h before infection, and the cytokine did not affect when added 5h before the end of the experiment.

Fig. 3. Time-dependent inhibitory effect of IFN-γ on *C. parvum* infection in HT-29 cells. Cells were incubated with medium containing 100U/ml IFN-γ for 24 hours before parasite inoculation. Marked inhibition of infection was observed 24h post-infection with all IFN-γ concentrations tested.

Fig. 4. Effect of FeSO₄, iNOS, and tryptophan on IFN-γ-mediated inhibition of *C. parvum* infection. HT-29 cells were incubated with IFN-γ in the presence or absence of each reagent and parasite development was measured 24h postinfection. Only FeSO₄ had a
significant effect on the action of IFN-γ on inhibition of C. parvum infection.

![Graph showing inhibition of C. parvum infection](https://ejhm.journals.ekb.eg/)

**Fig. 5.** Effect of TGF-β on IFN-γ-mediated inhibition of C. parvum infection. HT-29 cells were incubated with various concentrations of TGF-β (1 and 5 ng/ml) and 100 U of IFN-γ/ml starting at 24 h before infection with C. parvum. A significant reversal of the effect of IFN-γ is noted at concentrations of 5 ng of TGF-β/ml.

**DISCUSSION**

IEC plays a central role in innate immunity by activating mechanisms that help to maintain structural integrity, establish an inflammatory response, and contribute to parasite killing. Previous studies with transgenic mice, which lack IFN-γ activity, suggest that IFN-γ plays an important role in protective immunity against cryptosporidiosis. However, the mechanism(s) by which IFN-γ exerts its effect on intestinal cryptosporidiosis remains to be determined. In this work, the role of IFN-γ in mediating enterocyte resistance to C. parvum infection, and the possible mechanism of this effect was explored. Also, the role of TGF-β in the regulation of IFN-γ activity was investigated.

In the present study, the parasite has been shown to develop in the HT-29 cell line. After 24 h of infection, many developing and mature intracellular parasites were observed. The maximal number of parasites was obtained 6-24h post-infection.

IFN-γ is a key cytokine in immunity to Cryptosporidium⁷⁸, but the mechanism of cytokine in modulation enterocytes activation against C. parvum infection still unclear. Finding in the present study indicated that IFN-γ treated intestinal cells demonstrated marked resistance to infection by the parasite. The effectiveness of IFN-γ on intestinal cell activation was dose and time-dependent. IFN-γ inhibitory effect against C. parvum was highest when cells were treated with cytokine 24h before parasite inoculation. A smaller but significant inhibition of infection was observed when cells were treated with IFN-γ around the time of parasite inoculation and the cytokine had no effect when used 5h before the end of the experiment. These results may reflect the minimum duration period required for IFN-γ to induce phenotypic effects on cells (6-12 hours)⁷¹.

Studies of potential anti-cryptosporidial mechanisms of enterocytes mediated by INF-γ indicated that the effect of IFN-γ on C. parvum development was not dependent on mechanisms involving nitric oxide production or restriction of tryptophan. In contrast, the addition of exogenous Fe²⁺ to IEC cultures reduced the inhibitory effect of IFN-γ, suggesting that deprivation of available cellular Fe²⁺ was an important anti- cryptosporidial mechanism mediated by IFN-γ. This mechanism has previously been described in studies of T. gondii and C. parvum infections of IEC⁹⁹,²¹. Cellular iNOS activity was reported to mediate the action of cytokine against Listeria monocytogenes infection in Caco-2 cells⁳¹. Also, cellular tryptophan depletion was involved in mediating IFN-γ inhibitory action on T. gondii infection of fibroblasts⁴⁰. These differences may reflect the host species of enterocytes or sensitivity of the respective organisms to NO activity as well as the variable requirements for endogenous tryptophan and Fe²⁺ by the different parasites.

Upregulation of TGF-β expression in the human intestine during cryptosporidiosis has been reported previously⁴⁰ and a potential source of the cytokine is the IEC. TGF-β may have a healing role, as it has been shown to hinder the disruption of the epithelial barrier by C. parvum infection or by IFN-γ-⁴³,⁴⁴. Finding in the present study demonstrated that TGF-β produces a significant dose-dependent antagonist of IFN-γ activity. The present results suggest, therefore, that the presence of TGF-β at the infection site can have important antagonistic effects on the microbicidal activity of IEC. The mechanism(s) of action of TGF-β on IEC is unclear at present; whereas in macrophages a contributory factor in TGF-β-induced down-regulation of IFN-γ activation was reduced expression of the IFN-γ R, this was not observed with IEC²¹. Further studies are required to elucidate the full spectrum of the mechanism of TGF-β in reversing IFN-γ activation of IEC during C. parvum infection.

**CONCLUSION**

Taken together, the data from the present study suggest that enterocytes actively react to IFN-γ cytokine to increase their resistance against C. parvum infection, a reduction in the availability of cellular Fe²⁺ by IFN-γ may mediate this effect. TGF-β significantly reverses IFN-γ -mediated activation of enterocytes.

**REFERENCES**


