Interleukin-4 and Transforming Growth Factor β Have Opposing Regulatory Effects on Gamma Interferon-Mediated Inhibition of *Cryptosporidium parvum* Reproduction

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It was shown previously that enterocytes activated by gamma interferon (IFN- γ) are efficient effector cells in the killing of *Cryptosporidium parvum*. How this function is regulated is not clearly understood, but transforming growth factor β (TGF- β) and the Th2 regulatory cytokines may play a role. Using an in vitro cell culture system, we investigated how the key regulatory cytokines interleukin-4 (IL-4), IL-10, IL-13, and TGF- β might modulate the effect of IFN- γ in inducing resistance to infection in enterocyte cell lines. The results showed that TGF- β can abolish the inhibitory effect on *C. parvum* development and that neither IL-13 nor IL-10 influenced the action of IFN- γ . In contrast, IL-4 cooperated with low concentrations of IFN- γ (1 and 10 U/ml) to enhance parasite killing. One mechanism that appeared to be involved in the combined activity of IFN- γ and IL-4 was intracellular Fe²⁺ deprivation, but induction of nitric oxide production was not involved. In one cell line, the extents and durations of phosphorylation of STAT1, a transcription factor involved in IFN- γ signaling, were similar when cells were stimulated with IFN- γ alone and with IFN- γ and IL-4, suggesting that the cooperative effect of the cytokines was not related to STAT1 activation. The effects of the presence of TGF- β and IL-4 on IFN- γ function did not appear to involve any alteration in the level of expression of IFN- γ receptors.

Intestinal epithelial cells (IEC) form a physical barrier against pathogenic microorganisms and also play an important role in the induction of innate and acquired host immune responses. A variety of immunomodulatory and antimicrobial chemicals can be produced by IEC following exposure to pathogens, suggesting that the cells are immunological sensors of infection (37). These cells can also respond to cytokines (10, 20, 29, 31, 33, 34), but the relevance of this in the regulation of mucosal immunity is poorly understood.

Host immunological control of the reproduction of intracellular microbial pathogens is mediated by CD4⁺ T-helper (Th) cells via cell-mediated or Th1 responses characterized by production of cytokines such as interleukin-12 (IL-12) and gamma interferon (IFN- γ) (1). In contrast, elimination of extracellular pathogens such as parasitic nematodes is usually mediated by Th2 cytokines involved in allergic or antibody-dependent responses (22). Proinflammatory cytokines generated by a Th1 response may induce phenotypic alterations in IEC that promote clearance of intracellular infection. IFN- γ , either alone

or in combination with other proinflammatory cytokines, has been shown to stimulate antimicrobial killing mechanisms in IEC in vitro (12, 29, 34). This could involve either increased production of microbicidal nitric oxide derivatives via the up-regulation of inducible nitric oxide synthase (iNOS) (29) or deprivation of available Fe²⁺ required for development of parasites within the IEC (12, 34).

The down-regulation of IFN- γ -driven Th1 inflammatory responses may involve the antiinflammatory cytokine transforming growth factor β (TGF- β) and Th2 cytokines such as IL-10 and IL-4 (3, 13, 17, 25). TGF- β and IL-10 are key regulators in the maintenance of immunological homeostasis in the gastrointestinal tract (18) and may also have an important role in ameliorating inflammation caused by the Th1 response (6, 35). IL-4, in contrast, has been found to be involved in induction of Th2-mediated pathology in intestinal helminth infection (22) and in some models of inflammatory bowel disease (5).

Cryptosporidium parvum, the agent of cryptosporidiosis, is a coccidian that parasitizes epithelial cells of the gastrointestinal tract of mammals. The principal clinical manifestation is a watery diarrhea that usually lasts several days, but malnourished or immunocompromised hosts may develop a chronic and eventually fatal illness (15). Infection is transmitted orally in feces by oocysts which release sporozoites in the intestine that undergo development leading to production of oocyst progeny (11).

Immunological control of cryptosporidial infection appears to be dependent on CD4⁺ T cells (9, 16, 24) and requires IFN- γ and IL-12 (i.e., Th1) activity (21, 38, 39). CD4⁺ intraepithelial lymphocytes from gut epithelia of *Cryptosporidium*

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muris-infected mice were shown to be potent effector cells of immunity, acting at least in part through IFN- γ activity (24). Pretreatment of human enterocyte cell lines with IFN- γ inhibits *C. parvum* reproduction (34), and the mechanisms of action of IFN- γ involved some decrease in cellular invasion by sporozoites and inhibition of intracellular development by depletion of available intracellular Fe²⁺.

There is also evidence of Th2 involvement in immunity to cryptosporidial infection, as IL-4^{-/-} mice or mice treated with anti-IL-5 or with anti-IL-4 plus anti-IL-5 antibodies had increased susceptibility to infection (2, 14). Also, the ability to eradicate infection has been associated with increased expression of IL-4-producing intraepithelial lymphocytes or IL-4 mRNA expression in the intestine (2, 28). Increased expression of IL-10 and TGF- β in the intestine during patent or resolving stages of *C. parvum* infection has also been reported (21, 35). The role that these cytokines have in regulating the antimicrobial activity of IEC induced by the Th1 response is unknown.

The aim of this study, therefore, was to employ an in vitro culture system for *C. parvum* to investigate the effect of cytokines known to modulate Th1 activity on IFN- γ -mediated killing of an intracellular pathogen by IEC. The results showed that TGF- β had an inhibitory effect on IFN- γ activity. The presence of IL-10, IL-13, and IL-4, in contrast, had no inhibitory effect, and, significantly, IL-4 showed a capacity to act synergistically with low concentrations of IFN- γ in parasite inactivation.

MATERIALS AND METHODS

Parasites. Oocysts of the Moredun isolate of *C. parvum* were maintained by passage with dexamethasone-treated mice, purified, and stored as described previously (30).

Cell lines and infection with *C. parvum*. Monolayers of adenocarcinoma enterocyte cell lines HT-29, Caco-2 (human), and CMT-93 (murine) were maintained in a humidified 37°C incubator with an atmosphere of 5% CO₂ and 95% air and passaged as described previously (34). The growth (complete) medium was Dulbecco's minimum essential medium with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin, 1 μ g of streptomycin/ml, 2 mM glutamine, and 1% nonessential amino acids (all reagents obtained from Sigma-Aldrich, Poole, Dorset, United Kingdom). Cells were grown on 13-mm-diameter glass coverslips in plastic 24-well plates (Costar, High Wycombe, Bucks, United Kingdom), and, when confluent, the medium was replaced with 300 μ l of complete medium containing 2 $\times$ 10⁵ oocysts. The cells were incubated at 37°C for 2 to 3 h to allow sporozoite excystation and invasion of cells. Wells were then washed to remove debris, replenished with 1 or 2 ml of complete medium, and returned to 37°C. At 24 h postinfection, the cells were washed with phosphate-buffered saline, fixed with methanol, stained with Giemsa, and mounted on slides (34). Parasites in 25 or 50 fields were counted microscopically at $\times$ 1,000 magnification, and mean values ($n = 4$) were obtained. In untreated coverslips, 150 to 200 parasites were observed. Percent inhibition of parasite development as a result of cytokine treatment was calculated from the mean counts from cytokine-treated samples and from control samples.

Cytokines. Lyophilized recombinant human or mouse IFN- γ , IL-4, IL-10, IL-13, and TGF- β were purchased from Peprotech, London, United Kingdom, reconstituted in buffers recommended by the manufacturer, and stored at -80°C. Cytokines were added to cells in the 24-well plates, starting at 24 h before infection unless otherwise stated.

Western blotting. Confluent Caco-2 cells in 6-well plates (Costar) were incubated overnight in complete medium with only 0.5% fetal calf serum, cytokine stimulated for either 30 min or 2 h for STAT1 expression and 24 h for IFN- γ receptor α (IFN- γ R α) expression, washed in phosphate-buffered saline (Sigma), and immediately lysed in boiling sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis loading buffer (50 mM TRIS [pH 6.8], 2% SDS, 0.1% bromophenol blue, 10% glycerol, 1% β -mercaptoethanol, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride). Lysates were passed several times through a 23-gauge needle and boiled for 5 min, and 50 μ l was subjected to electrophore-

FIG. 1. Effect of TGF- β on IFN- γ -mediated inhibition of *C. parvum* infection of Caco-2 cells. Cells were incubated with various concentrations of TGF- β (0.1 to 5.0 ng/ml) and 500 U of IFN- γ /ml starting at 24 h before infection with *C. parvum*. Results are expressed as percent inhibition of development (calculated from four values of cytokine-treated and control sample counts). Significant reversal of the effect of IFN- γ is noted at concentrations of 1 ng (*, $P < 0.03$) and 5 ng (**, $P < 0.009$) of TGF- β /ml.

sis in an SDS-10% polyacrylamide gel and transferred onto an enhanced chemiluminescence (ECL)-compatible nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, United Kingdom). Membranes were probed with primary antibodies (0.2 μ g/ml) overnight at 4°C, washed, and then probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (0.1 μ g/ml) for 1 h at room temperature. Blots were then developed using ECL reagent (Amersham Biosciences). Goat anti-human phospho-(Y701)-STAT1, goat anti-human phospho-(Y701)-STAT1p84/91, rabbit anti-human STAT1 p84/91, rabbit anti-human IFN- γ R α , and donkey anti-goat or anti-rabbit immunoglobulin G-horseradish peroxidase polyclonal antibodies were all obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Analyses of data. Results were analyzed by Student's *t* test or analysis of variance and are expressed as means $\pm$ standard deviations or 95% confidence intervals where stated.

RESULTS

TGF- β , but not IL-10, inhibits IEC activation by IFN- γ . Pollok et al. had previously shown that IFN- γ inhibited *C. parvum* development in two human cell lines, Caco-2 and HT-29 (34). The effect of TGF- β on this IFN- γ -mediated activation of IEC was investigated by comparing parasite reproduction in cells treated with IFN- γ or TGF- β alone or in combination starting at 24 h before infection. Figure 1 shows the result of an experiment with Caco-2 cells in which the presence of 500 U of IFN- γ /ml decreased parasite development by 36.8% $\pm$ 11.1% ($P < 0.004$). TGF- β alone did not significantly affect parasite development in cell lines but produced a dose-dependent curtailment of IFN- γ activity; when 1.0 ng of TGF- β /ml was used, the level of inhibition induced by IFN- γ decreased by a factor of 2.7 ($P < 0.03$), and 5.0 ng/ml reduced inhibition by a factor of 7.5 ($P < 0.009$). Similar results were obtained with HT-29 and CMT-93 cells (data not shown).

An advantage of using CMT-93 for studying in vitro parasite development is that the parasite stages, particularly with minute young trophozoites, are more readily observed with Giemsa staining when CMT-93 cells are used than when Caco-2 or HT-29 cells are used. This is because CMT-93 cells on a substratum are substantially more flattened and therefore

FIG. 2. Effect of TGF- β on the ability of IFN- γ to inhibit *C. parvum* invasion of CMT-93 cells. Cells were incubated with 500 U of IFN- γ /ml alone or in combination with different concentrations of TGF- β (in nanograms per milliliter) starting at 24 h before the addition of *C. parvum* oocysts. At 2 h after initiation of infection, cells were fixed and stained and intracellular trophozoites were counted. At 5 ng/ml, TGF- β effectively eliminated the effect of IFN- γ on invasion (*, $P < 0.0001$).

stain less densely. This feature of CMT-93 was used to measure the effect of IFN- γ on parasite invasion and the modulatory effect of TGF- β on IFN- γ activity. Oocysts were added to cells pretreated with cytokine(s), and at 2 h later, the intracellular parasites (mostly small trophozoites) present were counted. IFN- γ at 500 U/ml reduced the numbers of intracellular parasites in 25 fields by $27.2 \pm 6.0\%$ ($P < 0.001$). This IFN- γ activity was again inhibited by TGF- β in a dose-dependent manner (Fig. 2). At concentrations of 0.1 and 1.0 ng/ml, a diminution in the inhibitory activity of IFN- γ (from $27.2 \pm 6.0\%$ to $20.8 \pm 6.6\%$ [$P < 0.32$] and $19.4 \pm 6.7\%$ [$P < 0.13$], respectively) was noted. A concentration of 5.0 ng of TGF- β /ml significantly reduced the effect of IFN- γ on parasite invasion by a factor of 5.2% to $5.2 \pm 12.6\%$ ($P < 0.0001$). These results indicate that 500 U of IFN- γ /ml confers on CMT-93 cells a degree of resistance to invasion by sporozoites of *C. parvum* that is inhibited by the presence of TGF- β .

Experiments were carried out to examine the effect of IL-10 on IFN- γ -mediated inhibition of *C. parvum* development. Unlike TGF- β , however, IL-10 was not found to have any inhibitory effect on the activity of IFN- γ (data not shown).

IL-4 and IFN- γ cooperate in inhibition of intracellular parasite development. A study was made of the effect IL-4 has on the inhibitory activity of IFN- γ in parasite reproduction. When IL-4 (10 ng/ml) was added to HT-29 cells 48 h before infection (i.e., 24 h before the addition of IFN- γ) or at the same time as IFN- γ (500 U/ml), there was no significant modulation of IFN- γ -mediated inhibition of *C. parvum* development (data not shown). To examine the effect of IL-4 further, the cytokine was added together with different concentrations of IFN- γ to cells 24 h before infection (Fig. 3). In cells cultured with IFN- γ alone, the level of inhibition increased (from $16.8 \pm 11.1\%$ [cytokine concentration, 1 U/ml] to $50.4 \pm 4.9\%$ [1,000 U/ml]) with increases in cytokine concentration. As before, IL-4 (10 ng/ml) in the absence of IFN- γ had little effect on parasite development (reduction of $13.3 \pm 11.1\%$) and did not down-regulate the inhibitory effect of IFN- γ on parasite development. On the contrary, IL-4 was shown to enhance

FIG. 3. Effect of IL-4 in combination with various concentrations of IFN- γ on the development of *C. parvum* in HT-29 cells. Cells were incubated with 10 ng of IL-4/ml in the presence or absence of IFN- γ starting at 24 h before infection. At low concentrations of IFN- γ (i.e., 1 and 10 ng/ml), the presence of IL-4 enhanced the inhibitory effect of IFN- γ more than threefold ($P < 0.001$).

inhibition by IFN- γ , particularly when low concentrations of IFN- γ (i.e., 1 or 10 U/ml) were employed. For example, 10 U of IFN- γ /ml provided a modest $20.3 \pm 10.4\%$ inhibition of development, but in the presence of IL-4, the level of inhibition was increased more than threefold to $62.6 \pm 5.4\%$ ($P < 0.001$). The inhibitory effect of 1,000 U of IFN- γ /ml was not significantly enhanced by the addition of IL-4. A similar effect was observed when Caco-2 cells were employed as host cells (data not shown).

In further experiments, parasite development was measured in HT-29 cells following treatment with a low concentration of IFN- γ (1 U/ml) and various concentrations of IL-4 (Fig. 4). Alone, neither IFN- γ nor the highest concentration of IL-4 used (20 ng/ml) was able to inhibit *C. parvum* development. However, in combination, IFN- γ and IL-4 produced significant

FIG. 4. Effect of a low concentration of IFN- γ combined with various concentrations of IL-4 on *C. parvum* development in HT-29 cells. Starting at 24 h before infection, the cells were treated with 1 U of IFN- γ /ml alone or combined with different concentrations of IL-4. Alone, both IFN- γ and IL-4 failed to cause significant inhibition of parasite replication, but when combined, significant inhibition was noted even at the lowest concentration of IL-4 (*, $P < 0.001$).

FIG. 5. Western blot studies of lysates from Caco-2 cells to show expression of the IFN- γ R α chain (A and B) or phosphorylation of STAT1 (C) after cytokine stimulation. Cells were serum starved overnight prior to stimulation. (A) IFN- γ R α expression after stimulation for 24 h with 10 U of IFN- γ /ml (γ) or 10 ng of IL-4/ml (IL-4) or a combination of these cytokines (γ /IL-4). Control (CON) cells were unstimulated. (B) IFN- γ R α expression after stimulation with 10 U of IFN- γ /ml (γ) or 5 ng of TGF- β /ml (TGF β) or a combination of the cytokines (γ /TGF β). Control cells were unstimulated. None of the cytokine combinations appeared to affect expression of the receptor. (C) Cells were incubated with either 10 U of IFN- γ /ml or 10 ng of IL-4/ml or a combination of the cytokines (γ /IL-4) for either 30 min or 2 h and blotted and probed with either anti-phospho-(Y701)-STAT1 (top) or anti-STAT1 p84/91(bottom). Control (Con) cells were unstimulated. IL-4 did not appear to influence STAT1 phosphorylation.

reductions in parasite reproduction ($P < 0.001$), and this effect was observed even at the lowest concentration of IL-4 employed (0.2 ng/ml).

IL-13 and IL-4 have some similar biological functions, and experiments were performed to determine whether IL-13 could also cooperate with IFN- γ . Results indicated, however, that IL-13 had no effect on IFN- γ activity (results not shown).

Effect of cytokines on IFN- γ receptor signaling. As shown in the experiments described above, IL-4 had synergy with IFN- γ in clearing *C. parvum*-infected epithelial cells and TGF- β inhibited IFN- γ activity. It was considered possible that these cytokines can affect expression of the IFN- γ R. To determine whether this is the case, a study was made of the expression of IFN- γ R α in Caco-2 cells following growth in cultures with IL-4 or TGF- β alone or together with IFN- γ . Visual inspection showed that the level of expression of IFN- γ R α after treatment with TGF- β or IL-4, however, appeared to be unaltered, and this was confirmed by densitometry (Fig. 5A and B). These findings indicate that TGF- β and IL-4 do not affect expression of the IFN- γ R on Caco-2 cells.

The actions of IFN- γ are primarily mediated through the activation of the transcription factor STAT1 following phos-

FIG. 6. Effect of exogenous Fe²⁺ on the cooperative activity of IFN- γ and IL-4 in inhibition of *C. parvum* infection of HT-29 cells. FeSO₄ (500 μ M) was added to cell cultures incubated with both cytokines. The inhibitory effects of both a high concentration of IFN- γ (1,000 U/ml) (stripes) and a combination of low concentrations of IFN- γ (1 U/ml) and IL-4 (10 ng/ml) (spots) were reduced by the addition of 500 μ M FeSO₄ (*, $P < 0.001$).

phorylation, and IL-4 has been shown to inhibit STAT1-mediated transcription in immune cells (8). It was of interest, therefore, to determine whether the synergistic activity of the combination of IFN- γ and IL-4 involves an altered pattern of STAT1 phosphorylation. Figure 5C shows that in Caco-2 cells, STAT1 was phosphorylated 30 min after stimulation with either IFN- γ or IL-4 alone whereas no phosphorylation was detected after 2 h. The combination of IFN- γ and IL-4 did not appear either to increase the amount of STAT1 phosphorylation or to prolong the length of time that STAT1 remained phosphorylated. These observations indicated that IL-4 did not influence the phosphorylation of STAT1 in Caco cells in the presence of a low concentration of IFN- γ .

The combined action of IFN- γ and IL-4 on enterocytes is independent of iNOS. A synergistic action of IFN- γ and IL-4 on activation of macrophages to kill *Leishmania* has been reported, and the antimicrobial effect was due to increased iNOS activity (4). Pollok et al. previously demonstrated that known inhibitors of NOS (*N*-nitro-L-arginine [L-NNA]) or iNOS (1400 W) used at concentrations previously shown to block NOS activity in enterocytes (7) failed to impede IFN- γ activity against *C. parvum* (34). Similarly, in the present study, neither L-NNA nor 1400 W affected the inhibitory effect of IFN- γ or a combination of IFN- γ and IL-4 (data not shown). This result suggests that the antimicrobial activity conferred on enterocytes by cooperation between IFN- γ and IL-4 is not dependent on NOS activity.

IFN- γ plus IL-4 inhibits intracellular parasite development by Fe²⁺ deprivation. It has been shown previously that an important mechanism involved in IFN- γ -induced inhibition of the intracellular development of *C. parvum* or the related parasite *Toxoplasma gondii* in enterocytes was depletion of available cellular Fe²⁺ (12, 34). In those studies, the addition of exogenous Fe²⁺ to cultures reduced the inhibitory effect of IFN- γ on both parasites. Figure 6 shows results of an experiment in which the role of Fe²⁺ in the IFN- γ -plus IL-4-induced anti-cryptosporidial activity of HT-29 cells was investigated. The presence of IFN- γ alone at a high concentration (1,000 U/ml) inhibited parasite reproduction by 42.4% $\pm$ 11.5%, and

this effect was inhibited by 500 μ M FeSO₄. As in earlier experiments, a combination of IFN- γ (1 U/ml) and IL-4 (10 ng/ml) decreased parasite development substantially (48.8% $\pm$ 5.3%), and, as with IFN- γ treatment, the inhibitory effect of the combined IFN- γ and IL-4 was lost when FeSO₄ was present ($P < 0.001$). In the absence of cytokines, the same concentration of FeSO₄ had no effect on *C. parvum* infection, indicating that FeSO₄ has no damaging effect on host cells. This result suggested that a combination of IL-4 and IFN- γ can starve enterocytes of available Fe²⁺ required for *C. parvum* growth.

DISCUSSION

A potentially important manifestation of the Th1 mucosal immune response that controls the acute stage of *C. parvum* infection is the stimulation of an antimicrobial killing capacity in IEC by IFN- γ (12, 20, 29, 34). The present investigation demonstrated that TGF- β inhibited this function whereas IL-4 cooperated with IFN- γ in parasite killing. The presence of either IL-13 or IL-10 had no effect on the activation of IEC by IFN- γ .

In cases of *C. parvum* infection, upregulation of TGF- β expression in the human intestine has been reported previously (35) and a potential source of the cytokine is the IEC (23, 35). TGF- β may have a healing role, as it has been shown to hinder disruption of the epithelial barrier by *C. parvum* infection or by IFN- γ (33, 36). The inhibitory effect of TGF- β on IFN- γ activation of IEC is also characteristic of its anti-inflammatory role (6, 18, 27, 32). 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 (32), this was not observed with IEC.

Like TGF- β , IL-10 is an important anti-inflammatory cytokine that promotes oral tolerance and prevents inflammatory bowel disease in mice (18). IL-10 also down-regulates activation of macrophages by proinflammatory cytokines (25). The failure of IL-10 to down-regulate activation of IEC by IFN- γ may not be surprising, however, as it similarly failed to inhibit increased expression of iNOS induced by combinations of IFN- γ and other proinflammatory cytokines in these cells (20).

The finding that the presence of IL-4 did not inhibit but, instead, enhanced IFN- γ -mediated inhibition of parasite development in IEC contrasts with the role of IL-4 as a cytokine that antagonizes IFN- γ functions (13, 20, 25). Two possible mechanisms for the synergistic effect on IEC of low concentrations of IFN- γ and IL-4 were studied. However, neither the expression of IFN- γ R α nor the phosphorylation of STAT1 in IEC was affected by a combination of the cytokines. It was recently reported that IL-4 can also phosphorylate STAT1 although STAT6 is the transcription factor that promotes IL-4-mediated activation (26), and this observation was repeated in the present investigation.

In line with its role as a Th1 antagonist, IL-4 has been shown previously to inhibit activation in IEC of iNOS mediated by combinations of IFN- γ and other pro-inflammatory cytokines (20). In contrast, cooperation of IL-4 and IFN- γ was found

necessary for maintenance of iNOS activity in human airway epithelial cells (19). Furthermore, IFN- γ and IL-4 were reported to act synergistically to increase expression of the polymeric immunoglobulin receptor of enterocytes (31). Significantly, IL-4 and IFN- γ were shown to act synergistically in killing *Leishmania major* parasites within macrophages, and this effect was associated with increased iNOS activity (4). In the present study, the antimicrobial effect was not due to nitric oxide production by IEC. However, addition of exogenous Fe²⁺ to IEC cultures prevented the inhibitory effect of IFN- γ , suggesting that deprivation of available Fe²⁺ was an important antimicrobial mechanism. This mechanism has previously been described in studies of *T. gondii* and *C. parvum* infections of IEC (12, 34), although it is possible that the exogenous Fe²⁺ may be (at least in part) overriding another mechanism.

Results of a previous study of *C. parvum* development in IL-4-deficient mice indicated that IL-4 had a protective effect in the latter stages of infection (2). During this late stage of infection, the IFN- γ response waned and increasing numbers of IL-4-producing CD4⁺ T cells accumulated in the intestine. Hence, the synergistic activation of IEC by IL-4 and low concentrations of IFN- γ observed in the present study suggests that, physiologically, their cooperation in stimulating IEC might be most effective late in infection. As the Th1-mediated inflammatory response becomes down-regulated, therefore, pathology is reduced but efficient inactivation of parasites by IEC is maintained.

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