Gamma Interferon Suppresses Acute and Chronic Trypanosoma cruzi Infection in Cyclosporin-Treated Mice

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To determine if exogenous gamma interferon is effective in immunosuppressed mice infected with Trypanosoma cruzi, recombinant murine gamma interferon was administered to cyclosporin-treated mice with either acute or chronic T. cruzi infection. Gamma interferon significantly decreased parasitemia and prevented death in acutely infected mice. Parasitemias and mortalities of mice treated with both gamma interferon and cyclosporin were similar to those of immunocompetent controls. In chronically infected mice, cyclosporin treatment produced significantly more organ explant cultures positive for T. cruzi. Fewer positive cultures, particularly for spleen and heart, were obtained from cyclosporin-treated mice when they also received gamma interferon. Ketoconazole treatment of mice resulted in no positive cultures. Cyclosporin treatment did not prevent activation of peritoneal macrophages by parenteral gamma interferon, nor did it have a consistent effect on serum titers of alpha/beta or gamma interferon in response to a second challenge inoculum of T. cruzi. These data indicate that exogenous gamma interferon suppresses acute and chronic T. cruzi infection in cyclosporin-treated mice but that gamma interferon is not as effective as the relatively specific antimicrobial ketoconazole. Gamma interferon activates macrophages despite cyclosporin treatment, and its effects appear to be tissue specific.

Cyclosporin inhibits many T-cell-dependent functions including delayed hypersensitivity (33, 36), allograft rejection (4), and production of cytokines such as interleukins-1, -2, and -3 and gamma interferon (IFN-γ) (5, 6, 33). Cyclosporin is used widely for prevention of graft rejection in transplant recipients, with improved patient and transplant organ survival, but opportunistic infections still complicate therapy. Disease due to opportunistic infection may result from recent acquisition of a new pathogen or from reactivation of infection acquired before transplantation. Partial reconstitution of the immune system with cytokines such as IFN-γ has become feasible because of recombinant technology and potentially may prevent development of disease due to infection. IFN-γ is currently being used in therapeutic trials for patients with cancer or infected with human immunodeficiency virus or parasites (1, 8, 22, 25, 26). We investigated the effects of administration of recombinant murine IFN-γ (rmuIFN-γ) to cyclosporin-treated mice with either acute or chronic infection with Trypanosoma cruzi, an intracellular pathogen known to be inhibited by rmuIFN-γ in vitro and in vivo in immunocompetent mice (20, 30). The goals of the study were to determine if administration of IFN-γ could suppress parasitemia and prevent death in acutely infected, immunosuppressed mice and if IFN-γ could suppress chronic infection in immunosuppressed mice and/or regulate the distribution of T. cruzi to organs.

MATERIALS AND METHODS

Mice and T. cruzi. Swiss Webster and C3H/HEN mice were obtained from Charles River (Wilmington, Mass.) or Simonsen Laboratories (Fremont, Calif.) and were 6 to 8 weeks of age when used. C3H/HEN mice were used in experiments with the chronic infection since parasitemia that can be quantitated by light microscopy can be induced with relatively short courses of cyclosporin A treatment in this strain but not in Swiss Webster or C57BL/6 mice (20a).

The highly virulent Y strain (21) used in all experiments is maintained by weekly syringe passage in Swiss Webster mice and produces peak parasitemia on day 7 of infection, at which time blood is collected in Hanks balanced salt solution (HBSS; GibCO Laboratories, Grand Island, N.Y.) with 3.4% sodium citrate (Sigma Chemical Co., St. Louis, Mo.). The trypomastigotes in blood are counted in a hemacytometer, diluted with HBSS-citrate to the desired concentration, and then used to infect mice by intraperitoneal (i.p.) injection.

To produce chronically infected mice, 20,000 trypomastigotes from blood were injected i.p. into mice. Twenty-four hours later, primaquine (Sigma) was added to the drinking water (0.25 mg per day per mouse) for 6 days to prevent death due to the acute infection. Mice were considered to have passed into the chronic stage of infection when parasitemia was subpatent without mortality due to the infection, usually by 6 to 8 weeks of infection with the Y strain.

Macrophages were challenged with T. cruzi obtained from L-cell tissue cultures (13). Tissue culture T. cruzi consisted of approximately 99% amastigotes and 1% trypomastigotes as assessed by light microscope inspection of morphology. Parasitemias were determined by inspection of blood smears with a light microscope (3).

Infection of macrophages with T. cruzi in vitro. Murine peritoneal macrophage monolayers were established as described previously (13) and challenged with tissue culture T. cruzi at a ratio of two to four organisms per macrophage. Monolayers were washed free of extracellular organisms after 1 h of incubation and then incubated for 48 to 60 h longer to allow either multiplication or destruction of T. cruzi within macrophages as assessed by light microscopy. Monolayers were fixed with methanol and stained with 5% Giemsa (Harleco, Gibbstown N.J.). Macrophages were counted to

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organisms were distributed if rmuIFN-γ and cyclosporin treatments modified organ distribution of T. cruzi in the chronic infection. Mice were anesthetized by inhalation of methoxyflurane (Pitman-Moore, Inc., Washington Crossing, N.J.) and bled to death by severing of the axillary artery. Organs were excised and put into 15-ml centrifuge tubes (Corning Glass Works, Corning, N.Y.) on ice. Each organ was washed three times with HBSS by vacuum aspiration of HBSS from the centrifuge tube, placed in a Petri dish (60 by 15 mm) (Costar, Cambridge, Mass.), and then diced by cross-cutting with scalpels until the tissue fragments were smaller than 1 mm in diameter. The tissue fragments were transferred to a 15-ml centrifuge tube and washed three times with HBSS. Tissues were suspended in 4 to 10 ml of tissue culture medium (RPMI 1640 [GIBCO] supplemented with 10% fetal calf serum [Hyclone, Logan Utah], penicillin [Sigma] at 100 U/ml, and streptomycin [Sigma] at 100 µg/ml) and placed into two wells each of a 24-well plate (16-mm well diameter; Costar) that contained confluent monolayers of mouse embryo fibroblasts. Cultures were incubated at 37°C in 5% CO₂ in air. Spleens were bisected and perfused with 10 ml of medium. Two milliliters of the perfusate was inoculated into each of two tissue culture wells that contained fibroblast monolayers.

The cultures were examined weekly for up to 8 weeks with a binocular tissue culture microscope (CK model; Olympus, Tokyo, Japan) for evidence of T. cruzi infection, i.e., either free amastigotes or trypanosomastigotes. A tissue was scored positive for T. cruzi when one or more wells contained T. cruzi.

IFN assay. A microtiter photometric assay (7, 37) in which a two- or threefold dilution of the test sample was added to confluent L929 cells in 96-well microculture plates (Costar) was used. After 4 to 6 h of incubation, monolayers were washed and encephalomyocarditis virus was added. After approximately 16 h, when control monolayers were 90% destroyed by virus replication, monolayers were stained with crystal violet and air dried, and retained dye was quantitated in a Titrtek Multiskan (Flow Laboratories, McLean, Va.) with a 600-nm filter. Optical density readings were used to calculate the IFN titer (6, 29). Antibody to rmuIFN-γ (R4-6A2, HB 170; American Type Culture Collection, Rockville, Md.) (35) and to IFN-α/β antiserum (Lee Biologics, San Diego Calif.) were used to identify the type of IFN.

Hydrogen peroxide assay. Hydrogen peroxide was assayed by reduction of phenol red in microtiter trays (28) as previously done by us (16). Peritoneal macrophage cultures in quadruplicate were incubated at 3 × 10⁵ cells per well (previously determined to be optimal) in 96-well Microtest II tissue culture plates (Becton-Dickinson, Oxnard, Calif.) in HBSS without phenol red (GIBCO). Phorbol myristate acetate (200 nM; Sigma) was incubated with the macrophages for 90 min to stimulate the respiratory burst, after which absorbances were determined in a Titrtek Multiskan with a 600-nm filter.

Drugs. Cyclosporin A (gift of Sandoz, East Hanover, N.J.) was dissolved in commercial virgin olive oil and administered to mice by subcutaneous injection in most experiments or by gavage. Controls received olive oil. rmuIFN-γ (gift of Genentech, Inc., South San Francisco, Calif.) was diluted to the desired concentration in HBSS and injected i.p. Ketoconazole powder (gift of Ianssen Pharaceutica, New Brunswick, N.J.) was dissolved in 0.2 N HCl, diluted to the desired concentration with distilled water, and administered by gavage (12).

Statistics. Differences in parasitemia and mortality were judged significant when P was <0.05 by Student’s t-test and the Mann-Whitney U test, respectively. The chi-square test was used to assess differences in organ explant cultures.

![FIG. 1. Representative experiment in which groups of seven Swiss Webster mice infected with 10⁴ trypomastigotes were treated with cyclosporin A at 150 mg/kg/day, rmuIFN-γ at 5,000 U i.p. every other day, cyclosporin A and rmuIFN-γ or vehicles. Treatment with rmuIFN-γ and cyclosporin A was started 48 h before infection and was continued until death. Mice treated with rmuIFN-γ alone had significant prolongation of time to death compared with untreated controls, and mice treated with cyclosporin and rmuIFN-γ had significant prolongation of time to death (P < 0.05) compared with cyclosporin-treated mice.](http://iai.asm.org/)

<p>| Table 1. Effect of rmuIFN-γ and cyclosporin on parasitemia in mice with acute T. cruzi infection* |
|-----------------------------------------------|-------------------|------------------|-------------------|</p>
<table>
<thead>
<tr>
<th>Day of infection</th>
<th>Parasitemia (10⁴ organisms/5 μl ± SEM)</th>
<th>Control</th>
<th>rmuIFN-γ</th>
<th>CSA</th>
<th>CSA + rmuIFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1.38 ± 0.51</td>
<td>0.28 ± 0.06</td>
<td>1.60 ± 0.24</td>
<td>1.56 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>11.63 ± 2.42</td>
<td>5.54 ± 0.33</td>
<td>21.64 ± 5.93</td>
<td>15.55 ± 3.31</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>50.56 ± 3.92</td>
<td>32.65 ± 5.23</td>
<td>112.46 ± 15.70</td>
<td>72.34 ± 8.76</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>88.79 ± 35.66</td>
<td>38.35 ± 10.06</td>
<td>352.0 ± 36.38</td>
<td>227.74 ± 35.1</td>
<td></td>
</tr>
</tbody>
</table>

* See text for details of experiment. Each value is derived from measurements from five mice. On days 7, 8, and 9, rmuIFN-γ treatment resulted in significantly lower parasitemias than control and cyclosporin treatment. On days 8 and 9, mice treated with both rmuIFN-γ and cyclosporin had significantly lower rates of parasitemia than cyclosporin-treated mice.

* CSA, Cyclosporin A.
to determine if death could be prevented and whether only two doses of rmuIFN-γ administered at the time of infection and 48 h later would be effective (Fig. 2). Significant \( P < 0.01 \) differences in survival resulted from rmuIFN-γ treatment of mice treated with cyclosporin, and survival was virtually identical to that of immunocompetent controls.

**Effect of rmuIFN-γ on chronic infection.** The foregoing experiments established that parenteral administration of rmuIFN-γ favorably affected cyclosporin-treated mice with acute *T. cruzi* infection. Experiments were then done to evaluate whether administration of rmuIFN-γ suppressed infection in cyclosporin-treated mice with chronic infection. C3H/HEN mice infected with *T. cruzi* for 3 months were treated with cyclosporin (150 mg/kg/day) for 7 to 14 days to induce sustained patent parasitemia, after which cyclosporin treatment was stopped and mice received either 50,000 U of rmuIFN-γ i.p. every other day for two to six injections or HBSS. Parasitemias were determined at least every other day. All rmuIFN-γ-treated and control mice died, most within 2 weeks of the start of rmuIFN-γ treatment. As judged by measurement of parasitemias and time to death, a favorable effect of rmuIFN-γ treatment was not evident (data not shown).

Since the model described above may have been too harsh to determine if rmuIFN-γ had a favorable effect, experiments were then done to determine if cyclosporin and rmuIFN-γ treatment had an effect on explant cultures of chronically infected mice and to evaluate in comparison the activity of an antimicrobial agent, ketoconazole, known to cure acute murine *T. cruzi* infection (19) and to suppress the chronic infection (12). Four groups of C3H/HEN mice infected for 3 months were treated with cyclosporin (150 mg/kg/day), rmuIFN-γ (50,000 U i.p. twice a week) and cyclosporin, ketoconazole (60 mg/kg/day by gavage) and cyclosporin, or olive oil for 4 weeks. Treatment was followed by sacrifice and explant culturing during the next 2 weeks. The average time from end of treatment to explant culture was the same for all groups. During treatment, all the mice treated with cyclosporin developed only patent parasitemia, none of the mice treated with ketoconazole and cyclosporin developed patent parasitemia, and some mice treated with either olive oil or the combination of rmuIFN-γ and cyclosporin developed patent parasitemia.

Skeletal muscle, omental fat, bladder-ureter, spleen, diaphragm, and heart were sampled for explant culture (Table 2). In controls, cultures of skeletal muscle and spleen were frequently positive, whereas few other organs were positive. Cyclosporin treatment produced positive cultures in a large proportion of organs \( P < 0.01 \) compared with controls. rmuIFN-γ treatment of cyclosporin-treated mice produced

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**TABLE 2. Effect of cyclosporin, rmuIFN-γ and cyclosporin, and ketoconazole and cyclosporin on explant cultures of C3H/HEN mice infected with *T. cruzi***

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. positive/no. cultured</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td>None (control)</td>
<td>6/7</td>
</tr>
<tr>
<td>CSA</td>
<td>7/7</td>
</tr>
<tr>
<td>CSA + rmuIFN-γ</td>
<td>4/4</td>
</tr>
<tr>
<td>CSA + Keto</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* C3H/HEN mice infected with *T. cruzi* for 3 months were treated with cyclosporin (150 mg/kg/day), rmuIFN-γ (50,000 U i.p. twice a week) plus cyclosporin, ketoconazole (60 mg/kg/day by gavage) and cyclosporin, or olive oil for 4 weeks. The mice were then sacrificed, and explant cultures of the indicated organs were done. CSA, Cyclosporin A; Keto, ketoconazole.

\( \text{P} < 0.05 \) compared with CSA for spleen and heart combined.

\( \text{P} < 0.01 \) compared with control.
TABLE 3. Effect of administration of rmuIFN-γ on explant cultures of chronically infected Swiss Webster mice

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>No. positive/no. cultured (%)</th>
<th>Spleen</th>
<th>Skeletal muscle</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>rmuIFN-γ</td>
<td>2/8 (25)</td>
<td>6/8 (75)</td>
<td>1/8 (12)</td>
<td></td>
</tr>
<tr>
<td>None (control)</td>
<td>4/4 (100)</td>
<td>3/4 (75)</td>
<td>0/4 (0)</td>
<td></td>
</tr>
</tbody>
</table>

* Swiss Webster mice were infected with 10³ Y strain trypomastigotes from the blood and treated with primacine for 10 days. After 4 months of infection, mice were treated with 50,000 U of rmuIFN-γ twice a week for 1, 2, 3, or 4 weeks, after which two experimental mice and one control mouse were sacrificed and explant cultures were done. Controls received phosphate-buffered saline. The negative skeletal-muscle cultures were observed in mice treated with rmuIFN-γ for 2 and 4 weeks.

fewer positive cultures of spleen and heart (P < 0.05 for spleen and heart cultures combined) but had no apparent effect on other organs. The effect of ketoconazole was substantial, as none of the explanted organs were positive despite concurrent treatment with cyclosporin (P < 0.01 compared with controls). Thus, cyclosporin was a potent immunosuppressive agent in chronic infection, as assessed by explant cultures. rmuIFN-γ appears to have a limited effect, especially when compared with the antimicrobial agent ketoconazole.

The anti-T. cruzi effect of rmuIFN-γ on spleen compared with other organs was also demonstrated in nonimmunosuppressed, chronically infected Swiss Webster mice (Table 3). When the results given in Tables 2 and 3 are combined, spleen cultures from rmuIFN-γ treated mice are seen to be significantly less frequently positive than spleens from cyclosporin-treated and control mice (P < 0.01).

Effect of cyclosporin on serum titers of IFN-γ. Since levels of IFN activity in serum of acutely infected mice are low and difficult to measure (11, 20a, 34), a protocol similar to that used by Nakane et al. (24) to induce higher levels of IFN-γ in serum of mice acutely infected with Listeria monocytogenes was used. Swiss Webster mice were infected i.p. with 10³ trypomastigotes from blood and were treated with either cyclosporin (150 mg/kg subcutaneously or by gavage) or olive oil on days −1, 0, and +1. Six days later, the mice were injected intravenously with 10³ tissue culture T. cruzi, and 6 hours later, serum was collected. Control mice produced both IFN-α/β and IFN-γ in response to the second injection of T. cruzi. In a total of 10 experiments, cyclosporin treatment had no consistent effect on the serum titer of total IFN activity or the titers of IFN-α/β or IFN-γ. Typical titers of IFN activity were 6 to 64 U/50 µl of serum sample. Treatment with anti-IFN-α/β typically decreased the titer fourfold, whereas treatment with anti-IFN-γ either did not change the titer or decreased it two- to fourfold. Acidifying the sample to pH 2 decreased the titer twofold. In addition, injection of 50,000 U of rmuIFN-γ i.p. on day 2 of infection did not affect IFN response in the serum, regardless of cyclosporin treatment.

Effect of cyclosporin on activation of macrophages by parenteral rmuIFN-γ. We reported previously that cyclosporin treatment of mice acutely infected with T. cruzi did not prevent activation of peritoneal macrophages caused by the infection (18). To evaluate the effect of cyclosporin treatment on the activation of peritoneal macrophages by parenteral rmuIFN-γ, mice were treated with cyclosporin (150 mg/kg/day subcutaneously) for 3 days and were injected i.p. with 50,000 U of rmuIFN-γ on the third day. Peritoneal macrophages were harvested on the fourth day, challenged in vitro with tissue culture T. cruzi, and incubated for 48 to 60 h. The results indicate that cyclosporin treatment did not prevent activation of peritoneal macrophages, as assessed by the number of infected macrophages and number of organisms per infected macrophage. In a typical experiment, 40.6% ± 0.6% (mean ± standard error of the mean) of resident macrophages were infected with an average of 9.8 ± 1.0 amastigotes per infected macrophage. For mice injected with rmuIFN-γ and treated with either cyclosporin or olive oil, the respective values were 9.4% ± 0.1% and 0.7% ± 0.3% infected macrophages and 1.1 ± 0.1 and 1.4 ± 0.4 amastigotes per infected macrophage. Cyclosporin treatment also did not appear to inhibit the ability of injected rmuIFN-γ to induce peritoneal macrophages to produce hydrogen peroxide in response to phorbol myristate acetate. In this protocol, in which mice were treated with olive oil or cyclosporin at 150 mg/kg subcutaneously on days −1 and 0 and injected with 50,000 U of rmuIFN-γ or diluent i.p. on day 0, macrophages were harvested 24 h later and challenged with phorbol myristate acetate. The mean change in absorbance (± standard error of the mean) was 0.036 ± 0.007 for rmuIFN-γ-treated mice, 0.043 ± 0.009 for rmuIFN-γ- and cyclosporin-treated mice, and 0.013 ± 0.012 for resident macrophages.

DISCUSSION

rmuIFN-γ treatment of immunocompetent mice with acute T. cruzi infection has been shown previously to significantly decrease parasitemias (20) and prevent death (30). Results in this report extend these findings for acute infection in that rmuIFN-γ suppressed parasitemias of cyclosporin-immunosuppressed mice to levels of parasitemia observed in immunocompetent mice. Furthermore, rmuIFN-γ prevented death due to T. cruzi in immunosuppressed mice when the inoculum of T. cruzi was sufficiently small. High inocula of T. cruzi were chosen to ensure measurement of parasitemia by light microscopy. Small inocula probably are more analogous to the subcutaneous inocula that cause clinical infection and likely result in gradual systemic release of organisms, a situation that contrasts with the effect of the i.p. injections used in this study. The salutary results of treatment may have been limited by the doses of rmuIFN-γ used. Lower mortality and parasitemia might have occurred if higher dosages of rmuIFN-γ had been used, possibly because of greater anti-T. cruzi activity in nonphagocytic cells. Alternatively, higher dosages may not be more effective than a threshold dose. In particular, higher dosages of rmuIFN-γ are not likely to be more effective by further enhancing the direct anti-T. cruzi activity of macrophages. The prime target of IFN-γ appears to be the macrophage (14, 20, 31), and the doses of rmuIFN-γ used in our experiments were sufficient to activate macrophages to kill T. cruzi and toxoplasma 24 h after a single injection in vivo (10, 14; see Results above). Since only two doses of rmuIFN-γ prevented death from murine T. cruzi (Fig. 2) and toxoplasma infection (14), the major effect of IFN-γ is likely activation of macrophages early, before endogenous IFN-γ and/or other cytokines generated in response to the infection activate macrophages. Higher dosages of rmuIFN-γ or more injections later in the acute infection may not improve macrophage function with respect to controlling T. cruzi infection.

Interestingly, Reed (38) found that rmuIFN-γ treatment of non-cyclosporin-treated mice acutely infected with T. cruzi restored plaque-forming-cell responses. Thus, treatment
early in infection may restore some cell-mediated and humoral immune responses. Reed’s studies did not determine whether the effect of rmIFN-γ was directly immunoregulatory or a result of the anti-T. cruzi activity of rmIFN-γ.

The route or method of administration of rmIFN-γ may be important. Murray recently reported that constant subcutaneous infusion of rmIFN-γ with an osmotic pump was superior to i.p. injection of rmIFN-γ in reducing liver parasite burden in a murine model of acute visceral leishmaniasis (23).

Assessment of the effects of rmIFN-γ administration in chronic T. cruzi infection is more difficult than such assessment in acute infection. Chronically infected mice were treated with cyclosporin to induce parasitemia before rmIFN-γ treatment soas to use parasitemia and mortality as measures of treatment effectiveness, but this harsh model did not show a beneficial effect of rmIFN-γ. Alternatively, rmIFN-γ treatment was started earlier in cyclosporin-treated mice, and explant cultures were used to assess outcome. rmIFN-γ treatment significantly reduced the number of spleen and heart cultures that were positive for T. cruzi but had little effect on other organs. These results suggest that the anti-T. cruzi effects of IFN-γ may be organ, tissue, or cell specific. Peritoneal macrophages are activated by endogenous mechanisms to kill T. cruzi in chronic T. cruzi infection (9, 20a). Thus, the benefit of rmIFN-γ treatment in chronic infection may need to be mediated through effects on cells other than macrophages.

Ketoconazole is a very potent anti-T. cruzi agent in vitro and in vivo. Ketoconazole can cure the majority of acutely infected mice (19), but it appears to only suppress chronic infection (12). As judged by explant cultures, ketoconazole was much more potent than rmIFN-γ. For serious T. cruzi infection, ketoconazole or standard anti-T. cruzi agents likely are preferable therapeutic agents, although possibly in combination with rmIFN-γ as it has been used in the treatment of humans with visceral leishmaniasis (1, 27). However, in transplant and other immunosuppressed patients, prevention of disease due to infection may be a reasonable goal. For that purpose, when diverse intracellular pathogens threaten the patient, rmIFN-γ or other immunomodulatory agents may be effective and preferable to a number of different pathogen-specific agents, with their potentials for adverse effects.

ACKNOWLEDGMENTS

We thank Jon Green for help in establishing the IFN assays. This work was supported in part by the Research Service of the Department of Veterans Affairs.

REFERENCES


