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Inhibition of Growth of *Toxoplasma gondii* in Cultured Fibroblasts by Human Recombinant Gamma Interferon

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The growth of *Toxoplasma gondii* in cultured human fibroblasts was inhibited by recombinant human gamma interferon at concentrations of 8 to 16 U/ml. The interferon was titrated by observing a total inhibition of parasite plaque formation 7 days after infection. Inhibition of the growth of *T. gondii* in the early days after infection was measured by marked reductions in the incorporation of radioactive uracil, a precursor that can only be used by the parasites. This assay showed that when cells were pretreated with gamma interferon for 1 day and then infected, inhibition of *T. gondii* growth could be readily detected 1 or 2 days after infection. When the pretreatment was omitted and parasites and gamma interferon were added at the same time, no inhibition of parasite growth could be detected 1 day later, although it was apparent after 2 days. Cultures from which the gamma interferon had been removed by washing after a 1-day treatment showed inhibition of *T. gondii* growth. Gamma interferon had no effect on the viability of extracellular parasites, but it did inhibit the synthesis of host cell RNA and protein by ca. 50% 3 days after treatment. This degree of inhibition is unlikely, of itself, to compromise the growth of *T. gondii*. Recombinant alpha and beta interferons had no effect on the growth of *T. gondii*.

Interferons are currently assigned to three different classes (reviewed in reference 4). Alpha interferons are produced by leukocytes in response to viral infection or to treatment with double-stranded RNA. These same stimuli elicit beta interferons from fibroblasts. In contrast, gamma interferons are produced by T-lymphocytes in a general response to lectins or in a specific response to antigens to which they have previously been sensitized. All of these interferons are potent inhibitors of viral growth. The role of the various interferons in blocking the growth of *Toxoplasma gondii* is unclear. Both chicken and mouse beta interferons were observed to suppress the growth of this parasite in homologous host cells (17). However, no effect on the growth of *T. gondii* was seen when mouse interferon was used to treat L-cells (18). Some inhibition was found when rabbit interferon was used to protect cultured rabbit cells, but it was uncertain whether the effect could be ascribed to the interferon (18). These experiments made use of interferon that was induced in vivo and was probably a mixture of alpha and beta interferons. More recently, no effect of human alpha or beta interferons on the course of infection of human fibroblasts with *T. gondii* was detected (1).

Studies of the action of unpurified gamma interferon against *T. gondii* are complicated by the variety of lymphokines produced by lectin or specific-antigen stimulation of T-cells. One of these lymphokines is macrophage-activating factor (which could be identical to gamma interferon), and activated macrophages are known to kill *T. gondii* (2). If gamma interferon has an antiviral-like effect on *T. gondii*, this effect must be demonstrated in cells other than macrophages. Several reports have suggested that lymphokines produced by mouse spleen lymphocytes can suppress the growth of *T. gondii* in mouse fibroblasts and kidney cells or in permanent cell lines (12, 13, 20). These lymphokines were induced by stimulating lymphocytes from toxoplasma-immune animals with *T. gondii* antigens. The active factor termed toxoplasma growth-inhibiting factor was observed to

have the approximate molecular weight and lability at pH 2 and the isoelectric point expected of gamma interferon (20, 21). These studies encouraged us to examine the effect of highly purified human recombinant gamma interferon (8).

MATERIALS AND METHODS

Our cloned line of the RH strain of *T. gondii* was used in all experiments. The parasites were maintained by serial passage in human fibroblast monolayer cultures and titrated by plaque formation in these cultures (15). The results of these titrations are reported in PFU. The PFU/parasite ratio was ca. 0.5 when parasites just released from host cells by forced passage through a 27-gauge needle were used. That is, approximately half of these parasites were viable in the plaque assay. The human fibroblast cells were grown in Eagle (7) minimal essential medium that contained antibiotics and 10% fetal bovine serum as previously described (15). The serum concentration was reduced to 3% for interferon treatment and for infection of human fibroblast cells. All experiments with interferon were carried out with confluent monolayer cultures in trays that contained wells of 1.6 or 2.3 cm in diameter. To prepare cultures with *T. gondii* plaques for photography, the monolayers were fixed with methanol-acetic acid-water (50:7:43, by volume) for 10 min and then stained with 200 mg of Coomassie blue per ml in the fixation solution.

The extent of the growth of *T. gondii* in monolayer cultures was determined by measuring the incorporation of [³H]uracil into acid-precipitable material. Wells that were to be labeled 24 h after infection received 5×10^4 PFU, whereas those to be labeled 48 h after infection received 8×10^3 PFU. Infected and uninfected control cultures in 24-well trays were labeled by adding to the existing medium 50 μ l of medium that contained a total of 5 μ Ci. After a 2-h incubation, the radioactive medium was dumped from the well tray, and the monolayers were washed by immersing the entire tray in a large beaker of Hanks (9) balanced salt solution. The monolayer was then fixed to the plastic surface by a 1-h treatment with ice-cold 0.3 N trichloroacetic acid. The fixed monolayers were rinsed overnight in cold running tap

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water to remove all remaining acid-soluble radioactivity. Control experiments showed that no acid-precipitable radioactive material was lost during this washing. The fixed monolayers in washed and dried well trays were dissolved by overnight treatment with 500 μ l of 0.1 NaOH at 37°C in a humid desiccator. The radioactivity in the NaOH solution was measured by a water-miscible scintillation fluid that contained sufficient acetic acid to make the mixture acidic. Preliminary experiments showed that this method of measuring the incorporation of [³H]uracil yielded the same data as the usual filtration method but with improved convenience and reproducibility. This method was also used to measure the incorporation of [³H]uridine, [³H]thymidine, and [³H]leucine into uninfected human fibroblast monolayer cultures.

Human recombinant gamma interferon was supplied by Genentech, Inc., at a concentration of 10⁷ U/ml in 0.5 M NaCl that contained 20 mM Tris-hydrochloride (pH 8.0) and 0.1% (vol/vol) beta-mercaptoethanol. We prepared a 10-fold dilution in the same solution and stored both at 4°C. On the day of use, a small volume of the 10⁶-U/ml solution was diluted to 10³ U/ml in medium that contained 3% fetal bovine serum, and this dilution was sterilized by filtration. Human recombinant alpha and beta interferons supplied by Genentech, Inc., were stored and diluted in the same way. All of the interferon concentrations reported here are based on the titrations carried out by Genentech, Inc., with vesicular stomatitis virus and HeLa cells. We titrated the antiviral activity of these interferons with the same virus but with our human fibroblast cells, with the endpoint being the cytopathic effect as determined microscopically.

[³H]uracil (20 Ci/mmol) was purchased from Moravak Biochemicals. [5,6-³H]uridine (52 Ci/mmol), [*methyl*-³H]thymidine (6 Ci/mmol), and L-[4,5-³H]leucine (40 Ci/mmol) were purchased from Amersham Corp.

RESULTS

One of the most common assays for the antiviral activity of interferon is the plaque reduction test. In this type of

assay, cultures are treated with serial dilutions of interferon for an appropriate interval and then challenged with sufficient virus to yield a countable number of plaques. The endpoint is the highest dilution of interferon that produces a specific reduction in the number of plaques. Since *T. gondii* forms plaques in human fibroblasts with high efficiency, we began our studies of recombinant human interferons with this assay. Figure 1 shows a typical plaque reduction assay of recombinant gamma interferon in which the cultures were treated for 24 h and then infected. An examination of stained cultures 1 week after infection showed that the formation of macroscopic plaques was totally suppressed by this interferon at 8 U/ml. The MIC of the human recombinant gamma interferon for *T. gondii* was measured by plaque reduction in parallel with each experiment reported below and always fell within the narrow range of 8 to 16 U/ml.

Although a plaque reduction assay is the definitive test for interferon activity, the fact that the results can only be read 1 week after infection does not permit a detailed examination of the early events that result in the inhibition of parasite growth. We therefore turned to an assay that measured *T. gondii* growth by the incorporation of [³H]uracil. We have previously shown that this precursor is specifically incorporated by the parasites in infected cultures because they, but not the host cells, contain the salvage enzyme uracil phosphoribosyltransferase (14). Uninfected cultures were also labeled in every experiment and consistently incorporated less than 1% of the radioactivity found in the control cultures that were infected but not treated with interferon. To demonstrate that the incorporation of [³H]uracil was a valid measure of *T. gondii* growth in interferon-treated cultures, we titrated gamma interferon in parallel by measuring both the incorporation of uracil and the production of viable parasites. For this latter assay, the intracellular parasites were released by artificial lysis of the host cells. The dilution required for the subsequent plaque assay reduced the interferon concentration at least 10-fold below the MIC. The assay of the interferon activity by the measurement of uracil incorporation and of viable parasites gave concordant results (Fig. 2).

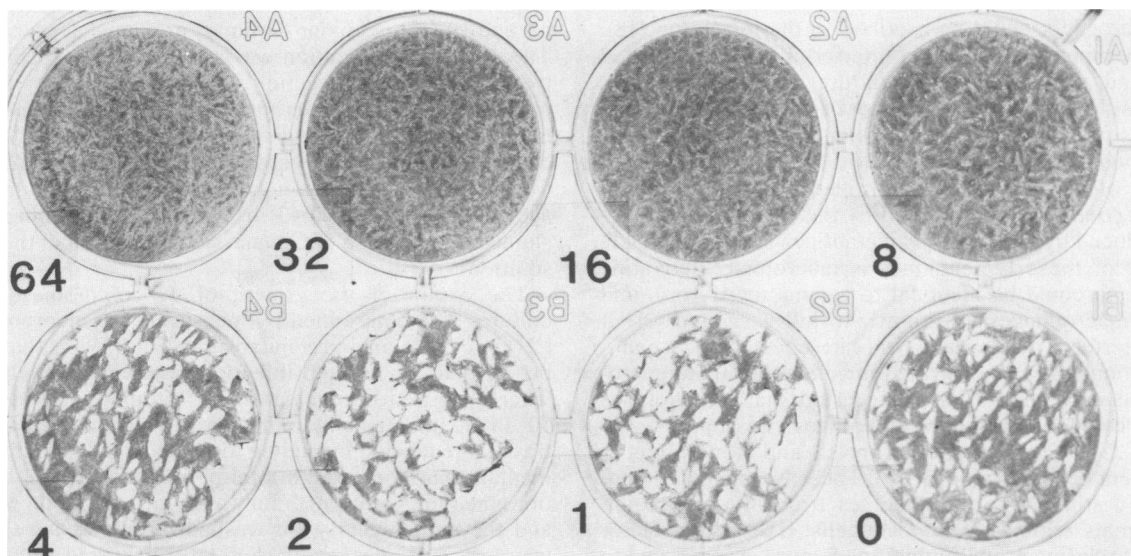


FIG. 1. Assay of human recombinant gamma interferon by its effect on the formation of plaques by *T. gondii*. Cultures were incubated in fresh medium with various dilutions of interferon ranging from 1 to 64 U/ml as indicated. After 1 day, the cultures were infected with ca. 50 PFU and incubated for an additional 7 days before fixing and staining.

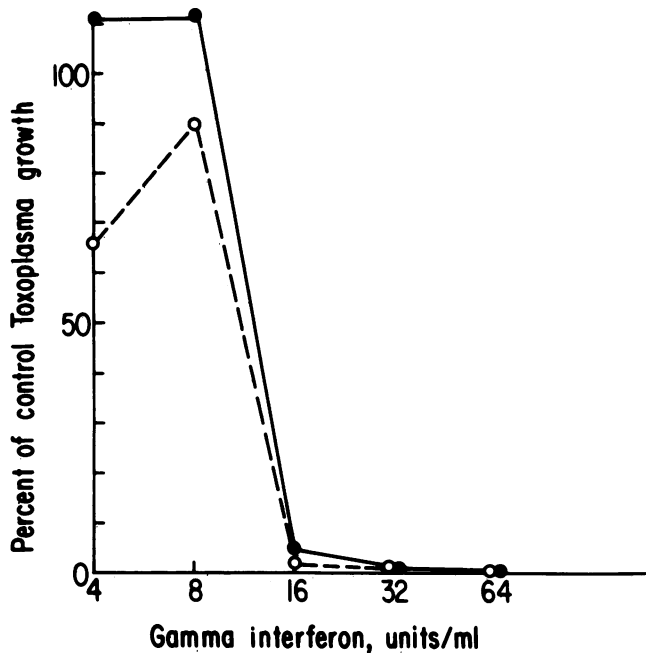


FIG. 2. Comparison of the inhibition of *T. gondii* by gamma interferon as measured by the incorporation of [³H]uracil and by the assay of viable parasites. A series of replicate cultures was treated with various concentrations of gamma interferon. One day later, the cultures were infected with 10⁴ PFU. Three days later, the growth of the parasites was measured by the incorporation of [³H]uracil during a 2-h pulse and by a plaque assay of the viable parasites released from the cells and diluted sufficiently to eliminate any effect of the interferon. The data from each method are plotted as the percentage of the control PFU or counts per minute observed in the absence of interferon. Symbols: ●, average of quadruplicate radioactivity determinations; ○, average of plaque assays from duplicate cultures.

The use of [³H]uracil incorporation allowed us to measure the kinetics of the induction of an antitoxoplasma state by gamma interferon. We measured the inhibitory effect of gamma interferon on the growth of *T. gondii* during the first 2 days of infection. As is customary when antiviral activity is measured, the interferon was added to the cultures for 1 day before infection. We then measured uracil incorporation both 1 and 2 days after infection. The inhibitory effect of the gamma interferon can be observed only 1 day after infection

TABLE 1. Inhibition of the growth of *T. gondii* by gamma interferon added to the human fibroblast host cells 24 h before infection

Interferon (U/ml)	% of control [³ H]uracil incorporation ^a at following time after infection:	
	24 h	48 h
64	18 ± 1 ^b	3 ± 0.2
32	20 ± 3	5 ± 0.5
16	23 ± 4	6 ± 1
8	78 ± 30	27 ± 8

^a The control incorporation in the absence of interferon was 7.6 × 10⁴ cpm per culture 24 h after infection and 4.4 × 10⁴ cpm per culture 48 h after infection.

^b Mean and standard deviation of quadruplicate samples.

TABLE 2. Inhibition of the growth of *T. gondii* by gamma interferon added at the time of infection of the human fibroblast host cells

Interferon (U/ml)	% of control [³ H]uracil incorporation ^a at following time after infection:	
	24 h	48 h
128	91 ± 4 ^b	12 ± 2
64	82 ± 3	9 ± 1
32	102 ± 3	11 ± 1
16	107 ± 3	16 ± 3

^a The control incorporation in the absence of interferon was 1.1 × 10⁵ cpm per culture 24 h after infection and 7.8 × 10⁴ cpm per culture 48 h after infection.

^b Mean and standard deviation of quadruplicate samples.

and is even more pronounced when measured after an additional day (Table 1). These data and those presented below are typical of many experiments in which the same pattern was observed, although the endpoint, represented by the MIC of gamma interferon, varied over a twofold range. This degree of variation is common in other serological assays. We next determined whether the inhibitory effect of gamma interferon could be observed when the parasites were added at the same time as the interferon. Although no inhibition was observed 1 day after infection, the growth of the parasites was reduced when measured 2 days after infection (Table 2).

Since cultures treated with gamma interferon required ca. 1 day to establish a parasite-resistant state, we next determined whether it was essential that the medium contain interferon once this resistant state was established. A series of cultures were treated with gamma interferon for 1 day. The medium from all cultures was then removed and saved. The cultures were washed repeatedly with medium that contained no interferon and then were reincubated either with the original interferon medium or with used medium to which interferon had not been added. The efficiency of the washing procedure was confirmed by testing the final wash of the cultures treated with the highest gamma interferon concentration, 128 U/ml. A plaque reduction assay of this final wash showed no inhibitory activity. The cultures were infected immediately after the washing procedure, and the incorporation of [³H]uracil was measured 2 days later. Although the interferon in the medium was removed by washing, 1 day of interferon treatment was still sufficient to establish resistance to *T. gondii* (Table 3).

TABLE 3. Inhibition of the growth of *T. gondii* when the interferon was added 24 h before infection

Interferon (U/ml)	% of control [³ H]uracil incorporation 24 h after infection ^a	
	Interferon washed out at the time of infection	Interferon present throughout
128	23 ± 2 ^b	16 ± 2
64	23 ± 2	15 ± 3
32	40 ± 1	20 ± 1
16	117 ± 18	27 ± 3
8	107 ± 18	104 ± 6

^a The control incorporation in the absence of interferon was 6.4 × 10⁴ cpm per culture.

^b Mean and standard deviation of quadruplicate samples.

Before we considered the mechanism by which gamma interferon blocks the growth of *T. gondii*, it was first necessary to exclude the possibility of a direct parasitocidal effect on extracellular parasites. To examine this possibility, we released the parasites from their host cells and incubated 10^7 parasites per ml at 37°C in fresh medium that contained 128 U of interferon per ml or no interferon. Viable parasites were assayed at intervals by plaque formation, with dilutions that reduced the interferon concentration well below the MIC. Both the interferon-treated and control extracellular parasites died at roughly the same rate (Fig. 3).

Since a host cell is essential for the growth of *T. gondii*, we considered the possibility that the interferon so severely affected the host cell that the growth of the parasites was compromised. A series of replicate human fibroblast cultures were treated with 32 U of gamma interferon per ml, whereas others received control medium. At daily intervals we measured macromolecular synthesis in these uninfected cultures by the incorporation of labeled uridine, thymidine, and leucine. RNA and DNA synthesis showed a slight initial increase followed by a decline (Fig. 4). Protein synthesis declined progressively. These data show the most dramatic effect of gamma interferon on the host cells that was observed. Other experiments showed no significant inhibition of the incorporation of thymidine. As argued below,

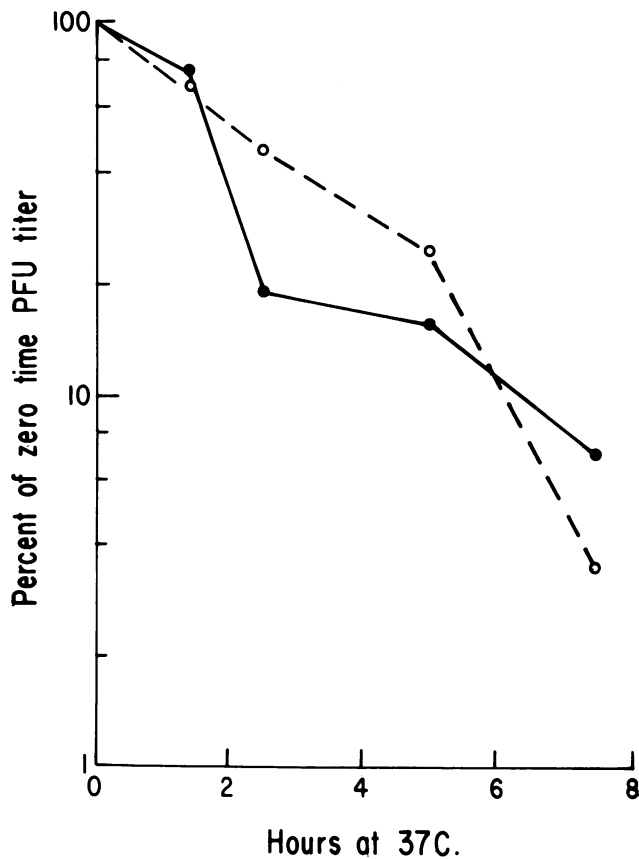


FIG. 3. Comparison of the viability of extracellular *T. gondii* incubated in the presence (●) or absence (○) of 128 U of gamma interferon per ml. The incubation was done at 37°C in medium that contained 3% serum. Samples were removed at intervals to measure the PFU titer at dilutions that precluded any action of residual interferon.

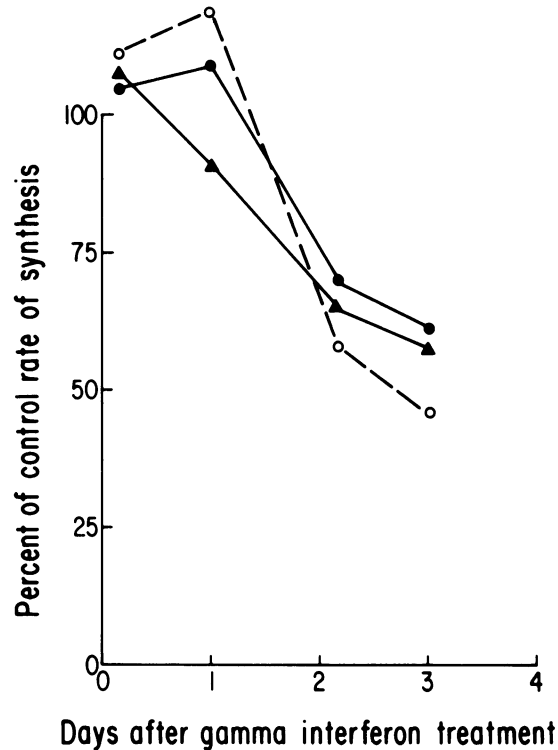


FIG. 4. Effect of 32 U of gamma interferon per ml on the incorporation of $[^3\text{H}]$ leucine (▲), $[^3\text{H}]$ thymidine (○), and $[^3\text{H}]$ uridine (●) into acid-precipitable material by uninfected fibroblasts. Quadruplicate control and interferon-treated cultures were labeled in 4-h pulses with $5 \mu\text{Ci/ml}$. The data from the interferon-treated cultures are plotted as a percentage of the control value for each time.

even the greatest observed effects on macromolecule synthesis by the host cells are unlikely to block the growth of the parasite.

We also examined the antitoxoplasma effect of human recombinant alpha and beta interferons by both the $[^3\text{H}]$ uracil incorporation assay and the plaque reduction test. Neither of these interferons had any effect at 1,024 U/ml, the highest concentration tested (data not shown). In our human fibroblasts, both of these interferons inhibited vesicular stomatitis virus at a concentration of 2 U/ml. As noted below, these observations excluded some mechanisms for the antitoxoplasma activity of gamma interferon.

DISCUSSION

The human recombinant gamma interferon used in these studies differs in at least one significant way from the natural substance. The recombinant gamma interferon, like all bacterial proteins, is unglycosylated. Despite this difference, the recombinant gamma interferon had a high antiviral activity in the titration carried out at Genentech, Inc., with vesicular stomatitis virus and HeLa cells. Recombinant gamma interferon also inhibited the growth of *T. gondii* in cultured fibroblasts. This observation proves that at least some of the antitoxoplasma activity observed by others in crude lymphokines can be ascribed to gamma interferon. It supports the tentative conclusion of Shirahata and Shimizu (20) that a murine lymphokine that blocked the growth of *T. gondii* in mouse L-cells was gamma interferon. Their conclusion was based on several properties of the lymphokine, including

molecular weight, lability at pH 2, and susceptibility only to proteolytic enzymes. Our observations are also consistent with the conclusion that the antitoxoplasma lymphokine was not interleukin-2 (3). However, gamma interferon is clearly distinct from a hamster antiparasitic lymphokine described by Chinchilla and Frenkel (5). These authors found that their active material had a much lower molecular weight of 4,000 to 5,000 and specifically protected hamster kidney cells only against the parasite (*T. gondii* or *Besnoitia jellisoni*) that was used to sensitize the lymphocytes. There is no evidence that interferon has such specificity for infectious agents and, indeed, recombinant gamma interferon protected almost equally well against *T. gondii* and vesicular stomatitis virus. There is reason to believe that the gamma interferon restricted the growth of *T. gondii* by a direct cytotoxic effect on the human fibroblast host cells. All interferons have an antiproliferative effect on their homologous cells. Some evidence of this can be seen in our measurements of DNA, RNA, and protein synthesis in human fibroblasts treated with gamma interferon (Fig. 4). Although the cells showed no cytopathic effect by phase-contrast microscopy, 3 days after treatment the synthesis of each of these macromolecules was reduced to 50 to 60% of normal. This reduced rate of synthesis is unlikely to compromise the growth of *T. gondii*. It is known that the parasite does not require host protein synthesis (16) and that enucleated cells, which can only have mitochondrial RNA and DNA synthesis, support the growth of *T. gondii* (10, 19). We can also exclude a direct effect of gamma interferon on extracellular *T. gondii*. Although free parasites die rapidly in our medium, there was no increase in the rate of death in the presence of gamma interferon (Fig. 3). An effect on extracellular parasites is also excluded by the experiment in which host cells restricted the growth of the parasite even though the gamma interferon had been removed by washing the monolayer before infection (Table 3).

The inhibition of *T. gondii* by human recombinant gamma interferon shares some features with the antiviral activity of interferon (11). We found that the antitoxoplasma state was not established immediately upon contact with interferon but, rather, required some time to develop. Once the inhibitory state was developed, however, it was no longer necessary for the interferon to remain in the medium. All interferons are thought to exert their antiviral effect by binding to specific receptors and then inducing the synthesis of new proteins. Thus, the establishment of the antiviral state can be blocked by inhibiting host cellular protein or RNA synthesis. Two of the proteins induced by all three interferons have been implicated in their antiviral activity (reviewed in reference 11). One is a protein kinase that phosphorylates and hence inactivates the elongation factor eIF2. The other is an enzyme that synthesizes the polynucleotide 2-5A that then serves to activate an endonuclease that attacks RNA. Both of these interferon-induced enzymes are activated by double-stranded RNA that is thought to be a by-product of viral replication. Thus, protein synthesis is compromised in a cell that was pretreated with interferon and then infected with a virus.

The antiviral mechanisms thought to be shared by all three kinds of interferon are quite unlikely to account for the antitoxoplasma activity of gamma interferon. The growing intracellular parasite is separated from the cytoplasm of its host cell by two membranes, the membrane of the parasite itself and that of its parasitophorous vacuole. It would not be expected that the interferon-induced enzymes that exert the antiviral effect could cross these membranes. Similarly, these barriers should be impermeable to the effector mole-

cule 2-5A. The conclusion that the antitoxoplasma activity induced by gamma interferon cannot be mediated by the same biochemical mechanisms that inhibit viral growth is strongly supported by our demonstration that human recombinant alpha and beta interferons had no effect on the growth of *T. gondii*, although they are presumed to induce the same antiviral proteins as gamma interferon.

It is likely that some additional protein(s) induced by gamma interferon is responsible for its antitoxoplasma effect. In this context, it is of interest that recent reports have shown that gamma interferon induced the synthesis of at least three mRNAs (6) and six proteins (22) in addition to those that were also induced by alpha or beta interferons. One or more of these host cell proteins peculiar to gamma interferon treatment may be responsible for the antitoxoplasma effect. The observed suppression of host cell RNA and protein synthesis by gamma interferon (Fig. 4) need not compromise the synthesis of putative proteins that inhibit the growth of *T. gondii*. We have shown that the antiparasite state was largely established during day 1 of treatment with gamma interferon (Tables 2 and 3), a period during which the synthesis of host cell macromolecules was not affected. During the subsequent days, proteins induced by gamma interferon could be preferentially synthesized even in the face of some inhibition of total RNA and protein synthesis. We have suggested an antiparasitic mechanism in which gamma interferon induces an enzyme(s) that degrades tryptophan (E. R. Pfefferkorn, submitted for publication).

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