Gamma-Interferon-Induced Inhibition of the Growth of *Rickettsia prowazekii* in Fibroblasts Cannot Be Explained by the Degradation of Tryptophan or Other Amino Acids

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We examined the role of amino acid deprivation in gamma-interferon-induced (IFN- γ) suppression of the growth of *Rickettsia prowazekii* in mouse L929 cells and human fibroblasts by measuring the amino acid pools in untreated and IFN- γ -treated cells. In recombinant IFN- γ -treated cultures of human fibroblasts, tryptophan was undetectable in both the intracellular pool and the extracellular medium. In contrast, tryptophan was not depleted from the intracellular pool or the extracellular medium of L929 cells treated with recombinant IFN- γ or crude mouse lymphokines. None of the other amino acids measured was severely depleted in IFN- γ -treated L929 cells and human fibroblasts. Extracts prepared from IFN- γ -treated human fibroblasts exhibited indoleamine 2,3-dioxygenase activity, converting tryptophan into products that cochromatographed with *N*-formylkynurenine and kynurenine; however, extracts prepared from untreated human fibroblasts, untreated L929 cells, recombinant IFN- γ -treated L929 cells, and mouse lymphokine-treated L929 cells did not degrade tryptophan. Human HeLa cells resembled the human fibroblasts in that they degraded tryptophan after IFN- γ treatment. Similarly, mouse 3T3-A31 cells and mouse embryo fibroblasts resembled mouse L929 cells in that they did not degrade tryptophan. Supplementation of the extracellular medium with additional tryptophan reconstituted the tryptophan pool in mock-infected and *R. prowazekii*-infected, X-irradiated, IFN- γ -treated human fibroblasts to values greater than those observed in untreated control cultures. However, reconstitution of the tryptophan pool did not relieve IFN- γ -induced inhibition of rickettsial growth. Addition of kynurenine or *N*-formylkynurenine to rickettsia-infected human fibroblasts at concentrations four times the usual tryptophan concentration did not inhibit growth of *R. prowazekii*. We conclude that neither tryptophan depletion nor depletion of the other amino acids studied explains the inhibitory effect of IFN- γ on rickettsial growth in mouse L929 cells. In IFN- γ -treated human fibroblasts, either tryptophan depletion is not involved in the inhibition of rickettsial growth or tryptophan depletion and some other mechanism(s) together contribute to the inhibition of rickettsial growth.

Rickettsia prowazekii is an obligate intracellular bacterium that grows directly in the cytoplasm of its eucaryotic host cells (29). The etiological agent of epidemic typhus in humans, *R. prowazekii* multiplies within the endothelial cells lining the capillaries (32) and can also multiply within human macrophages (3).

Gamma interferon (IFN- γ) induces fibroblasts and macrophages to express antimicrobial activity against both eucaryotic and procaryotic intracellular microorganisms, including *R. prowazekii* (1, 2, 11-14, 17, 23, 24, 27, 30). Treatment of cultured mouse fibroblasts and mouse macrophage-like cells with either crude lymphokine preparations (which contain IFN- γ) or recombinant IFN- γ inhibits the growth of *R. prowazekii* within these cells (24, 26, 27). Inhibition of the growth of *R. prowazekii* also occurs in cultured human fibroblasts (26, 31), human vascular endothelial cells (31), and human monocyte-derived macrophages (31) treated with crude lymphokine preparations. Hence, lymphokines (specifically IFN- γ) may play a role in host defense against *R. prowazekii*.

The mechanism(s) by which IFN- γ inhibits rickettsial growth in fibroblasts and macrophages is unknown. IFN- γ

may alter the host cells so that sufficient quantities of the metabolites needed by *R. prowazekii* for growth are not available. Pfefferkorn (13) demonstrated that IFN- γ inhibits the growth of *Toxoplasma gondii* in human fibroblasts by inducing the fibroblasts to degrade tryptophan to *N*-formylkynurenine and kynurenine. Conversion of tryptophan to *N*-formylkynurenine is catalyzed by indoleamine 2,3-dioxygenase, and this enzyme is thought to be induced by IFN- γ in human fibroblasts (13). Supplementation of the culture medium with additional tryptophan reverses IFN- γ -induced inhibition of the growth of *T. gondii* in human fibroblasts (13). In contrast, increasing the tryptophan concentration in the medium does not suppress IFN- γ -induced inhibition of the growth of *Chlamydia trachomatis* in murine McCoy cells (2).

In this study we analyzed the amino acid pools of untreated and IFN- γ -treated mouse and human fibroblasts so that amino acid deprivation could be evaluated as a mechanism for the antirickettsial effect of IFN- γ . The role of tryptophan degradation in IFN- γ -induced inhibition of the growth of *R. prowazekii* was examined in detail. We report that neither degradation of tryptophan nor depletion of the other amino acids studied satisfactorily explains the inhibitory effect of IFN- γ on the growth of *R. prowazekii* in fibroblasts.

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MATERIALS AND METHODS

Cell cultures. Mouse L929 cells were purchased from Flow Laboratories, McLean, Va., and grown in Eagle minimal essential medium supplemented with 10% newborn or fetal bovine serum. Mouse 3T3-A31 cells were purchased from the American Type Culture Collection, Rockville, Md., and grown in Dulbecco modified Eagle medium supplemented with 10% bovine calf serum. Cultures of human foreskin fibroblasts were obtained from Frank Pindak, University of South Alabama, grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum, and used between passages 2 and 18. Human HeLa cells were obtained from Joseph H. Coggin, University of South Alabama, and grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum. All cells were grown in a CO₂ incubator at 34°C. All sera were heated at 56°C for 30 min before use.

Primary cultures of mouse embryo cells were prepared from 16- to 18-day-old embryos removed from pregnant BALB/c mice. The cells were used between passages 2 and 6.

Rickettsiae. *R. prowazekii* strain E was harvested and purified from infected yolk sacs as described previously (26). Viable rickettsiae were enumerated by the antibody hemolysis method of Walker and Winkler (28).

Lymphokines and IFN- γ . Mouse lymphokines were prepared from concanavalin A-stimulated cultures of spleen cells from normal BALB/c mice or *Listeria monocytogenes*-challenged C57BL/6J mice by previously described procedures (24, 26).

Recombinant murine IFN- γ and recombinant human IFN- γ derived from *Escherichia coli* were generously provided by Genentech, Inc., South San Francisco, Calif.

IFN assay. IFN was assayed in samples of mouse lymphokines and recombinant murine IFN- γ by a cytopathic effect inhibition assay modified from that of Havell and Vilcek (4) as previously described (25). The concentrations of human recombinant IFN- γ given in this study are based on assays done at Genentech, Inc., with A549 cells and encephalomyocarditis virus.

Treatment of cells with lymphokines or IFN, infection of cells with rickettsiae, and measurement of rickettsial growth. In our previous studies of rickettsial growth in IFN- γ -treated host cells, the host cells were X-irradiated to inhibit their ability to divide (24-26). Inhibition of host cell division facilitated the measurement of rickettsial growth. Both nonirradiated and X-irradiated host cells were used in the present study. Nonirradiated cells were used for some of the amino acid pool measurements to eliminate any X-irradiation effects and to make our work of more general interest.

(i) Experiments with X-irradiated cells. Methods for X-irradiation of cells, treatment of cells with IFN- γ or crude lymphokines, infection of cells with rickettsiae, and measurement of rickettsial growth in chamber slide cultures have been detailed previously (24, 26).

Stained slides from *R. prowazekii*-infected cultures were examined with an oil immersion objective, and the number of rickettsiae present in each of 100 cells was counted for each duplicate of each experimental treatment. When a cell contained >100 rickettsiae, it was assigned a value of 100; this practice causes underestimation of the number of rickettsiae at later times. The percentage of cells infected with rickettsiae (%R) and the average number of rickettsiae per infected cell (RI) were determined.

(ii) Experiments with nonirradiated cells. Monolayer cultures of L929 cells or human foreskin fibroblasts were suspended in serum-supplemented medium (MS) at a concentration of 2×10^6 viable cells per ml. An equal volume of rickettsiae (5.7×10^7 rickettsiae per ml for human fibroblasts or 1.7×10^8 rickettsiae per ml for L929 cells) in Hanks balanced salt solution supplemented with 0.1% gelatin and 5 mM L-glutamic acid (monopotassium salt) was added to each cell suspension, and the cells and rickettsiae were incubated together for 1 h in a shaking water bath at 34°C. After being washed, a total of 1.5×10^5 L929 cells or 2.4×10^5 human fibroblasts was planted per well in 12-well plates (Costar, Cambridge, Mass.) that contained various concentrations of recombinant IFN- γ . Samples of the infected-cell suspensions were immediately centrifuged onto microscope slides, dried, fixed, and stained as previously described (25, 26). The plates were incubated at 34°C for 48 h. Both detached and attached cells were harvested, stained with trypan blue, and counted. Samples of the attached (monolayer) cells were cytocentrifuged onto microscope slides, fixed, and stained for rickettsiae as described above. Slides were examined for rickettsiae, and the total number of rickettsiae present in 100 cells on each slide was determined. The number of rickettsiae per well at a given time after infection was calculated from the average number of rickettsiae per attached cell and the number of attached cells per well.

Preparation of cell extracts for determination of amino acid pools. Nonirradiated cells (approximately 3×10^6) were planted in 100-mm dishes. After incubation overnight at 34°C, the medium was removed and the cells were treated with MS alone, MS that contained recombinant IFN- γ , or MS that contained crude lymphokines (10 ml per dish). The cells were then incubated for 44 to 48 h, and cell extracts were prepared.

Tryptophan pools were also determined in uninfected or *R. prowazekii*-infected, X-irradiated human fibroblasts after treatment with IFN- γ and additional tryptophan. These experiments were designed so that the tryptophan pools were measured under conditions comparable to those used to measure rickettsial growth in X-irradiated fibroblasts in chamber slide cultures. Since 0.3 ml of medium was used per slide chamber (1 cm²), 16.6 ml of medium was used per dish (55.4 cm²). When tryptophan pools were measured in *R. prowazekii*-infected cultures, the use of dishes that contained cover slips allowed the initial rickettsial infection and rickettsial growth to be monitored. In these experiments, X-irradiated human fibroblasts were adjusted to a density of 1.2×10^5 cells per ml in MS and planted in 100-mm dishes. After incubation overnight, the medium was removed and the cells were treated with MS, MS plus additional tryptophan (100 μ g/ml), MS plus recombinant human IFN- γ (188 U/ml), or MS plus IFN- γ and additional tryptophan. After incubation for 24 h, the washed cells were mock infected or infected with *R. prowazekii* and then washed twice. Fresh MS was added to cultures that had been incubated in MS or MS plus IFN- γ before infection, and MS plus additional tryptophan was added to the remaining cultures. Cultures were incubated for 2 or 48 h, and cell extracts were then prepared.

To prepare cell extracts, each dish of cells was quickly washed 10 times with Dulbecco phosphate-buffered saline at 34°C (total wash time was approximately 90 s per dish). Then 4 ml of ice-cold methanol-water (8:2) that contained norvaline as an internal standard was added per dish. The dishes were held on ice, and the cells in each dish were dislodged and transferred, along with the methanol-water,

to a centrifuge tube. Each dish was then washed with 1 ml of methanol-water (without norvaline), and the wash was transferred to the tube. After centrifugation at $1,800 \times g$ for 30 min, the supernatant fluids were evaporated under vacuum in a Speed Vac concentrator (Savant Instruments, Inc., Hicksville, N.Y.) and stored at -20°C for amino acid analysis. The pellets were washed once with methanol-water and stored at -20°C prior to determination of protein content by the method of Lowry et al. (10).

Samples of the extracellular medium in each experiment were prepared for amino acid analysis by the following procedure. Medium (60 μl) was added to 240 μl of cold (4°C) methanol; the mixture was vortexed, incubated at 0°C for 15 min, centrifuged at $8,700 \times g$ for 10 min, and analyzed for amino acid content.

It was important to establish that the extracellular medium had been efficiently removed from the cells and did not make a significant contribution to the measured intracellular pools. This was estimated as follows. One hour before cell extract preparation, 6 ml of medium was removed from each dish and added to 60 μl of [^{14}C]sucrose (25 $\mu\text{Ci/ml}$; 1 M) (ICN Pharmaceuticals, Inc., Irvine, Calif.). The remaining medium was removed from the dish and discarded, and the medium with [^{14}C]sucrose was added back to the dish. After incubation at 34°C for 1 h, the cells were washed and extracted. Based on the radioactivity in samples of the extracellular medium and the cell extract, the amount of extracellular medium which remained in the cell extracts was $0.6 \pm 0.1 \mu\text{l/mg}$ of protein. The amount of each intracellular amino acid pool contributed by the contaminating extracellular medium in a particular sample ranged from 0 to 6.8%, with 80% of the values being below 3%. The intracellular amino acid pool values have been corrected for these small contributions. The recovery of amino acids from the cell samples was based on the recovery of the internal standard, norvaline, which was $90 \pm 5\%$.

Analysis of amino acids by *o*-phthalaldehyde precolumn derivatization and reversed-phase HPLC. The analytical methods used in this study were modified from those of other investigators (5, 8, 15, 19) and are based on the reaction of primary amines with *o*-phthalaldehyde (OPA) and β -mercaptoethanol to form fluorescent products, followed by high-performance liquid chromatographic (HPLC) analysis of these products. Proline, since it is not a primary amine, does not react, and the derivative of cysteine has minimal fluorescence (16); therefore, these amino acids were not measured in this study. Appropriate standard curves were constructed to allow quantitation of the amino acids in the cell extracts.

The HPLC system consisted of a model 6000A/M-45 solvent delivery system with a model 680 gradient controller (Waters Associates, Milford, Mass.); a model 7125 syringe loading injector (Rheodyne, Inc., Cotati, Calif.); a guard column; a column (150 by 4.6 mm) packed with either Econosphere C18 (5 μm) or Adsorbosphere OPA-HR-C8 (5 μm) (Alltech Associates, Deerfield, Ill.); a fluorescence detector (RF-530; Shimadzu Corp., Columbia, Md.) set for excitation at 330 nm and emission at 450 nm; and an integrator (SP4270, Spectra-Physics, San Jose, Calif.).

Gradients were formed between two degassed eluents. With the Adsorbosphere OPA-HR-C8 column, eluent A consisted of 0.02 M sodium phosphate, pH 7.2, and eluent B consisted of methanol-eluent A (75:25). The flow rate was 1.5 ml/min. Figure 1 shows the gradient program used and the analysis of a standard mixture of amino acids on the Adsorbosphere OPA-HR-C8 column. The peaks were iden-

FIG. 1. Separation of OPA derivatives of amino acid standards (10 pmol each) by reversed phase HPLC on an Adsorbosphere OPA-HR-C8 column. Symbols: X, taurine; O, ornithine; *, norvaline. Other abbreviations are standard single-letter amino acid abbreviations (6).

tified by their coelution with the derivatives formed from individual amino acid standards. It is possible that some peaks in the extracts represented a mixture of an amino acid and an unknown primary amine which coeluted.

Assay of indoleamine 2,3-dioxygenase activity in cell extracts. To prepare extracts, either nonirradiated cells (3×10^6 or X-irradiated cells (1×10^7) were planted in 100-mm dishes, incubated overnight at 34°C , and then treated with MS alone (10 ml per dish), MS that contained recombinant IFN- γ , or MS that contained crude lymphokine for an additional 24 to 48 h. The cells were quickly washed four times with Dulbecco phosphate-buffered saline at 34°C . After removal of the last wash, 300 μl of hypotonic buffer (7.5 mM KH_2PO_4 , 14.2 mM K_2HPO_4 , pH 7.0) was added per dish, and the cells were dislodged with a rubber policeman. The cells were then broken at 4°C in a Dounce homogenizer or by four cycles of freezing and thawing. After cell breakage, each extract was centrifuged for 10 min at $1,000 \times g$ to remove the nuclei and any remaining intact cells.

Indoleamine 2,3-dioxygenase activity was assayed by methods modified from those described by other investigators (7, 13). The assay involved incubation of radioactive tryptophan with the extract followed by separation of tryptophan and the degradation products by cellulose thin-layer chromatography. L-[side chain-2,3- $^3\text{H}(\text{N})$]tryptophan (11.3 Ci/mmol) and L-[5- ^3H]tryptophan (28.7 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Inhibition of growth of *R. prowazekii* in nonirradiated fibroblasts by IFN- γ . In our previous work, recombinant mouse IFN- γ at a concentration of approximately 15 U/ml markedly inhibited the growth of *R. prowazekii* in X-irradiated L929 cells that were infected in suspension and treated with IFN- γ after rickettsial infection (24). Nondividing host cells were used to make the measurements of rickettsial growth easier. The amino acid pool studies described herein were done with nonirradiated cells; hence, we wished to establish the ability of IFN- γ to inhibit rickettsial growth in nonirradiated cells. Recombinant mouse IFN- γ at

TABLE 1. Inhibition of the growth of *R. prowazekii* in nonirradiated fibroblasts by IFN- γ ^a

Cell line (treatment)	Time postinfection (h)	No. of cells/well ^b		Cell viability (%)		No. of rickettsiae/well ^c
		Attached	Total	Attached	Total	
L929 (control)	24	137	139	>99	>99	263
	48	300	301	>99	>99	2,582
L929 (IFN- γ)	24	120	121	>99	>99	301
	48	125	155	>99	82	302
Human fibroblasts (control)	24	82	83	>99	98	272
	48	66	67	82	82	526
Human fibroblasts (IFN- γ)	24	61	69	>99	88	66
	48	91	95	73	70	148

^a Suspensions of nonirradiated L929 cells and human fibroblasts were infected with *R. prowazekii* and planted in MS alone (control) or MS with recombinant IFN- γ (15 U/ml). Detached and attached cells were harvested, stained with trypan blue, and counted. Each value represents the mean of duplicate determinations. The standard deviations averaged 20% of the mean values.

^b Expressed as a percentage of the number of cells planted per well at 0 h.

^c The number of rickettsiae per well at 24 and 48 h after infection is expressed as a percentage of the number present at time zero, which was calculated from the number of cells planted per well and the average number of rickettsiae per cell. The number of rickettsiae per well at 24 and 48 h after infection was calculated from the number of attached cells per well and the average number of rickettsiae per attached cell. In the L929 cell cultures, there were 7.9×10^5 rickettsiae per well at time zero; in the human fibroblast cultures, there were 3.7×10^5 rickettsiae per well at time zero.

a concentration of 15 U/ml inhibited the growth of *R. prowazekii* in nonirradiated L929 cells that were infected in suspension and treated with IFN- γ after infection (Table 1). Similarly, recombinant human IFN- γ suppressed the growth of *R. prowazekii* in both nonirradiated human fibroblasts (Table 1) and X-irradiated human fibroblasts (see Table 3).

Intracellular amino acid pools in untreated and IFN- γ -treated human fibroblasts and mouse L929 cells. The intracellular amino acid pools and the extracellular (culture medium) amino acid concentrations for untreated and IFN- γ -treated cultures of human foreskin fibroblasts and mouse L929 cells were compared so that amino acid deprivation could be evaluated as a mechanism for the antirickettsial effect of IFN- γ (Table 2). The percent change in the treated cultures relative to the control cultures is shown for each amino acid, and amino acids with statistically significant differences ($P \leq 0.1$) are shown in bold type. In addition, the mean concentration of each amino acid, its standard deviation (expressed as a percentage of that mean), and the value from the paired-sample *t* test are listed. The most striking decrease in an intracellular amino acid pool in the IFN- γ -treated human fibroblasts was in the tryptophan pool (Table 2). Tryptophan was not detected in either the intracellular pool or the extracellular medium of IFN- γ -treated human fibroblasts, when the limits of tryptophan detection were 0.06 nmol/mg of protein for the intracellular pool and 5 μ M for the extracellular medium. Therefore, the pool of tryptophan was at least 96% lower in IFN- γ -treated human fibroblasts than in untreated human fibroblasts, and the tryptophan concentration in the extracellular medium was at least 89% lower in IFN- γ -treated cultures of human fibroblasts than in untreated cultures. Other statistically significant differences between the human recombinant IFN- γ -treated and untreated human fibroblasts included a 37% decrease in the pool of aspartic acid, a 14% decrease in the pool of lysine, and a 28% increase in the pool of methionine (Table 2). Although no other major decreases in pool size resulting from IFN- γ treatment were seen, increases of 25% or greater in the average values for the pools of several other amino acids were observed, although these increases were not statistically significant. Thus, tryptophan was the only amino acid that was severely depleted in the IFN- γ -treated human fibroblasts.

The changes in the intracellular amino acid pools of mouse L929 cells after treatment with recombinant murine IFN- γ

contrasted with those observed in human recombinant IFN- γ -treated human fibroblasts. Several statistically significant decreases in the pools of IFN- γ -treated L929 cells relative to those of untreated L929 cells were noted; the largest change was a 41% decrease in the pool of glutamine in the IFN- γ -treated L929 cells. Other statistically significant changes included decreases of about 32% in the pools of arginine and lysine, a decrease of 25% in the pool of asparagine, and decreases of 22% or less in the pools of alanine, isoleucine, phenylalanine, tyrosine, and valine (Table 2). In striking contrast to the situation in human fibroblasts, the intracellular pools of tryptophan in the untreated and IFN- γ -treated cultures of L929 cells were similar. The average value for the tryptophan pool in the IFN- γ -treated cultures was only 13% lower than the average value for the untreated cultures, and this difference was not statistically significant. There was only a 4% decrease in the tryptophan concentration in the extracellular medium collected from the IFN- γ -treated (33 μ M) relative to the untreated (34 μ M) cultures of L929 cells (Table 2).

The data in Table 2 represent the results of three experiments in which L929 cells were treated with recombinant IFN- γ at a concentration of 430 U/ml. We also measured the pools of tryptophan in L929 cells treated with recombinant IFN- γ at concentrations of 108 and 1,720 U/ml, and in both cases the tryptophan pools in the IFN- γ -treated cells were similar to those in the untreated cells (data not shown). In addition, we measured tryptophan pools in L929 cells treated with crude mouse lymphokines diluted to contain native murine IFN- γ at concentrations between 35 and 70 U/ml. The pool of tryptophan in the lymphokine-treated cultures of L929 cells was only 16% lower than the pool of tryptophan in the untreated control cultures (data not shown).

Indoleamine 2,3-dioxygenase activity in extracts prepared from untreated and IFN- γ -treated human fibroblasts and mouse L929 cells. Extracts prepared from human foreskin fibroblasts treated with recombinant human IFN- γ for 24 h (data not shown) or 48 h (Fig. 2) converted radiolabeled tryptophan into compounds that cochromatographed with *N*-formylkynurenine and kynurenine. This indoleamine 2,3-dioxygenase activity was present in extracts prepared by freezing and thawing the IFN- γ -treated human fibroblasts (Fig. 2) or by rupturing the IFN- γ -treated human fibroblasts in a Dounce homogenizer (data not shown). In contrast, such

TABLE 2. Effect of IFN on amino acid pools in human foreskin fibroblasts and mouse L929 cells^a

Cells and amino acid	Intracellular pool (nmol/mg of protein)						Extracellular concn (μM)					
	Control		IFN-γ treated		<i>t</i> ^b	% Change	Control		IFN-γ treated		<i>t</i>	% Change
	Avg	% SD	Avg	% SD			Avg	% SD	Avg	% SD		
Human fibroblasts												
Ala	16.8	16	17.1	24	0.2	2	227	16	192	13	3.8	-15
Arg	8.1	36	7.1	29	1.4	-12	435	0	467	3	3.5	7
Asn	5.8	13	6.7	25	0.8	14	54	19	51	21	0.9	-5
Asp	33.8	38	21.5	43	4.3	-37	10	42	8	56	7.5	-19
Gln	137.9	19	197.8	25	2.0	43	3,341	4	3,730	7	4.8	12
Glu	193.3	21	194.6	21	0.3	1	160	19	109	30	6.0	-32
Gly	23.3	10	38.0	14	2.9	63	73	17	80	15	2.9	9
His	11.2	45	11.2	56	0.0	0	159	4	170	4	2.1	7
Ile	7.1	15	9.3	6	2.0	30	285	7	312	5	3.5	9
Leu	7.8	16	10.5	4	2.2	34	302	8	336	5	3.4	11
Lys	4.9	8	4.2	2	3.2	-14	374	8	400	1	1.5	7
Met	2.5	8	3.2	11	3.4	28	94	4	100	6	3.1	7
Orn	0.9	37	0.8	24	0.8	-9	58	30	61	21	1.1	7
Phe	5.4	12	7.1	8	2.0	30	180	6	194	2	2.1	8
Ser	2.3	21	2.5	31	0.9	9	22	6	23	15	0.4	4
Tau	32.7	6	37.0	18	0.9	13						
Thr	16.7	12	21.7	14	1.4	30	263	9	293	7	10.1	11
Trp	1.4	36	<0.1	0	3.7	-96	46	8	<5	0	15.4	-89
Tyr	6.8	24	8.4	11	2.2	23	169	2	188	5	2.3	12
Val	7.4	10	9.2	7	1.7	23	336	9	366	5	2.8	9
Mouse L929 fibroblasts												
Ala	45.1	30	38.0	40	5.4	-16	454	12	471	11	9.8	4
Arg	4.8	11	3.2	35	3.7	-33	389	7	366	11	2.0	-6
Asn	6.1	28	4.6	35	6.7	-25	74	7	63	5	6.3	-15
Asp	29.3	26	28.7	26	1.6	-2	81	41	76	42	3.6	-7
Gln	21.6	16	12.8	24	5.0	-41	730	32	674	52	0.5	-8
Glu	136.5	17	122.8	12	1.5	-10	571	17	510	20	21.9	-11
Gly	54.2	10	45.0	20	2.5	-17	255	9	242	10	47.9	-5
His	8.1	44	6.4	40	2.4	-21	158	7	146	3	1.8	-8
Ile	4.2	12	3.3	23	4.9	-22	156	24	159	23	10.6	2
Leu	3.7	16	3.0	23	2.8	-20	140	34	146	33	56.3	4
Lys	2.2	16	1.5	39	4.2	-32	239	16	241	15	0.9	1
Met	1.0	33	0.8	19	1.1	-13	72	16	73	18	0.5	2
Orn	2.0	7	1.6	28	1.8	-20	157	5	156	4	0.4	-1
Phe	4.4	17	3.5	28	4.9	-20	125	14	121	13	3.1	-3
Ser	3.8	28	2.9	17	2.1	-23	36	22	37	33	0.3	4
Tau	56.4	28	53.2	31	2.0	-6						
Thr	16.7	10	13.3	28	2.4	-21	204	16	199	15	1.3	-2
Trp	1.1	15	1.0	23	0.9	-13	34	14	33	15	28.6	-4
Tyr	5.7	15	4.5	28	3.4	-21	137	3	127	8	1.3	-7
Val	5.4	8	4.3	19	3.9	-21	196	21	199	21	42.9	2

^a Cells were treated with MS alone or with recombinant human IFN-γ (188 U/ml) or recombinant mouse IFN-γ (430 U/ml) for 44 to 48 h. Each value represents the mean of three experiments, except for the extracellular media collected from the L929 cell cultures, where each value represents the mean of two experiments. % SD, Standard deviation expressed as a percentage of the mean.

^b Paired-sample *t*-test values. Bold type indicates a significant difference between the control and treated cells ($P \leq 0.1$).

activity was not detected in extracts prepared from untreated human foreskin fibroblasts or any L929 cells whether they were untreated or treated (Fig. 2). L929 cells were treated with recombinant IFN-γ at concentrations of 430 (Fig. 2) or 1,303 U/ml (data not shown) and with crude mouse lymphokines diluted to contain IFN at a concentration of 35 U/ml (Fig. 2), and in no case was tryptophan-degrading activity observed in the cell extracts. Extracts prepared from control and treated X-irradiated fibroblasts, both human and mouse, had indoleamine 2,3-dioxygenase

activities similar to those of the corresponding nonirradiated fibroblasts (data not shown).

Indoleamine 2,3-dioxygenase activities and tryptophan pools in other human and mouse cells. Extracts prepared from IFN-γ-treated human HeLa cells, like those prepared from IFN-γ-treated human foreskin fibroblasts, converted tryptophan into compounds that cochromatographed with *N*-formylkynurenine and kynurenine but extracts prepared from untreated HeLa cells did not degrade tryptophan (Fig. 2). Tryptophan was not degraded by extracts prepared from

FIG. 2. Degradation of tryptophan by extracts of human IFN- γ -treated human fibroblasts and HeLa cells, and lack of tryptophan degradation by extracts of murine IFN- γ -treated L929 cells, 3T3-A31 cells, and mouse embryo cells. Human fibroblasts (HF) and HeLa cells were incubated for 44 to 48 h in MS alone or MS plus recombinant human IFN- γ (188 U/ml). Mouse L929 cells, mouse embryo cells (ME), and mouse 3T3-A31 cells were incubated for 44 to 48 h in MS alone, MS plus recombinant murine IFN- γ (430 U/ml), or MS plus crude mouse lymphokines (L929 cells and mouse embryo cells, 36 U of IFN per ml; 3T3-A31 cells, 160 U of IFN per ml). Cell extracts were prepared, assayed as described in the Materials and Methods, and analyzed by cellulose thin-layer chromatography as shown in this figure. The tryptophan (T), kynurenine (K), and *N*-formylkynurenine (N) spots were located with UV illumination. Symbols: ○, untreated control cells; □, recombinant IFN- γ -treated cells; △, mouse lymphokine-treated cells.

untreated, IFN- γ -treated, or mouse lymphokine-treated cultures of mouse 3T3-A31 cells or mouse embryo cells (Fig. 2). In experiments with mouse embryo cells, cell extracts were prepared after 24 h (data not shown) or 48 h (Fig. 2) of treatment with IFN- γ (215 or 430 U/ml) or crude

lymphokines (35 or 160 U/ml), and the cells were broken by freezing and thawing (data not shown) or with a Dounce homogenizer (Fig. 2). Furthermore, in some experiments, the length of incubation of the cell extracts with radiolabeled tryptophan was increased to 6 h (data not shown). However, in no case did the extracts prepared from untreated, IFN- γ -treated, or mouse lymphokine-treated mouse embryo cells demonstrate detectable degradation of tryptophan.

The tryptophan pool in IFN- γ -treated HeLa cells was less than 10% of the tryptophan pool in untreated HeLa cells, which was 1.1 nmol/mg of protein. In contrast, the tryptophan pools in recombinant IFN- γ -treated (430 U/ml) or crude lymphokine-treated (35 or 160 U/ml) mouse embryo cells ranged from 44 to 100% of the tryptophan pools in untreated mouse embryo cells, which averaged 0.7 nmol/mg of protein. Similar concentrations of tryptophan were present in the medium removed from untreated, IFN- γ -treated, and mouse lymphokine-treated cultures of mouse embryo cells after 48 h of incubation (71, 69, and 67 μ M, respectively).

Reconstitution of the tryptophan pool in IFN- γ -treated human fibroblasts did not relieve the inhibition of rickettsial growth. Supplementation of the medium with additional tryptophan (100 μ g/ml) reconstituted the tryptophan pool in IFN- γ -treated, X-irradiated human fibroblasts to values greater than those observed in untreated control cultures (Table 3). In cultures treated with IFN- γ (188 U/ml) and additional tryptophan before mock infection and with additional tryptophan after mock infection, the tryptophan pool at 48 h after mock infection was almost five times the pool in the mock-infected control cultures. It was important to determine whether the tryptophan pool was similarly reconstituted in *R. prowazekii*-infected cultures and whether such reconstitution occurred soon after rickettsial infection. The tryptophan pools in *R. prowazekii*-infected cultures that had been treated with IFN- γ and additional tryptophan before infection and with additional tryptophan after infection were approximately six times the tryptophan pools in the infected control cultures at 2 and 48 h after infection (Table 3). Examination of rickettsial growth in these cultures indicated that reconstitution of the tryptophan pool did not suppress IFN- γ -induced inhibition of the growth of *R. prowazekii* in X-irradiated human fibroblasts (Table 3). Additional tryptophan also did not suppress the inhibition of rickettsial growth in human fibroblasts treated with IFN- γ after infection (data not shown). Experiments were performed with a wide range of IFN- γ concentrations and at various tryptophan concentrations, and in no case did additional tryptophan suppress IFN- γ -induced inhibition of the growth of *R. prowazekii* (data not shown).

Kynurenine and *N*-formylkynurenine did not inhibit rickettsial growth in human fibroblasts. Because reconstitution of the tryptophan pool did not relieve the inhibition of rickettsial growth in human fibroblasts, we tested the ability of the products of tryptophan degradation to inhibit rickettsial growth. Neither kynurenine nor *N*-formylkynurenine inhibited the growth of *R. prowazekii* when added to human fibroblast cultures at a concentration four times the usual tryptophan concentration in Eagle minimal essential medium (50 μ M) (Table 4). With either compound, however, there was a slight inhibitory effect on rickettsial growth at a concentration of 500 μ M.

DISCUSSION

There are at least three general hypotheses to account for the inhibitory effect of IFN- γ on the growth of *R. prowazekii*

TABLE 3. Failure of reconstitution of the tryptophan pool to relieve inhibition of rickettsial growth in human fibroblasts treated with recombinant human IFN- γ

Cell treatment		Tryptophan pool (% of control) ^a			Rickettsial growth 48 h after infection (% of zero-time value) ^b	
Before infection	After infection	Mock-infected (48 h)	Infected		%R	RI
			2 h	48 h		
Control	Control	100	100	100	118 $\pm$ 1	1,519 $\pm$ 66
Trp	Trp	597 $\pm$ 80	1,962 $\pm$ 946	1,500	109 $\pm$ 2	1,588 $\pm$ 188
IFN- γ	Control	<9 $\pm$ 7	<2 $\pm$ 1	<8	74 $\pm$ 21	479 $\pm$ 112
IFN- γ plus Trp	Trp	480 $\pm$ 160	596 $\pm$ 23	611	65 $\pm$ 11	434 $\pm$ 69

^a For measurement of the tryptophan pools, X-irradiated human fibroblasts were treated as indicated (Trp, 100 μ g/ml; IFN- γ , 188 U/ml) and extracted at 2 or 48 h after mock or rickettsial infection. The tryptophan pools in the treated cultures are expressed as percentages of the tryptophan pool in the untreated control cultures. The tryptophan pool in the untreated control cultures averaged 1.24 nmol/mg of protein. For mock-infected cultures at 48 h, $n = 3$; for infected cultures at 2 h, $n = 2$; and for infected cultures at 48 h, $n = 1$. Where $n > 1$, each value represents the mean $\pm$ standard deviation.

^b For measurement of rickettsial growth, X-irradiated human fibroblasts were treated as indicated and stained at 0 and 48 h after rickettsial infection. The percentage of cells infected (%R) and the average number of rickettsiae per infected cell (RI) at 48 h after infection are expressed as percentages of the values observed at 0 h. Each value represents the mean $\pm$ standard error of the mean for two experiments. At 0 h after infection, 84 $\pm$ 1% of the cells were infected, and there were 5.1 $\pm$ 0.5 rickettsiae per infected cell. The various cell treatments had no effect on this initial rickettsial infection.

in fibroblasts. First, the IFN- γ could enter the host cell and act directly on the rickettsiae. This hypothesis is unlikely because IFN- γ does not inhibit rickettsial growth when an inhibitor of eucaryotic protein synthesis is present (24, 30). Second, the IFN- γ could induce production of a factor by the host cell which acts directly on the rickettsiae to inhibit their growth. Finally, the IFN- γ itself or factors induced in response to the IFN- γ could modify the host cell in such a way that the requirements of the rickettsiae for growth are not met.

This study was designed to determine whether treatment of fibroblasts with IFN- γ leads to alterations in the intracellular amino acid pools which are related to the ability of IFN- γ to inhibit the growth of *R. prowazekii*. In a thought-provoking study, Pfefferkorn (13) had shown that inhibition of the growth of *T. gondii* in human fibroblasts treated with IFN- γ could be explained by degradation of the tryptophan in the IFN- γ -treated host cells so that this amino acid was unavailable to satisfy the nutritional needs of the parasite.

In our study, the most dramatic change in the intracellular amino acid pools of recombinant IFN- γ -treated human fibroblasts was depletion of the amino acid tryptophan. Tryptophan was undetectable in the intracellular pool and the extracellular medium of the IFN- γ -treated human fibroblasts. Extracts prepared from IFN- γ -treated human fibroblasts degraded tryptophan to products that cochroma-

tographed with kynurenine and *N*-formylkynurenine. This suggests that activation or induction of indoleamine 2,3-dioxygenase is responsible for the absence of intracellular tryptophan in the IFN- γ -treated human fibroblasts.

Surprisingly, tryptophan was not depleted from the intracellular pool or the extracellular medium of mouse L929 cell cultures treated with recombinant IFN- γ or crude lymphokine preparations, and extracts prepared from these cells did not degrade tryptophan. Human HeLa cells (a continuous cell line) resembled the human fibroblasts (derived from primary cultures) in that they degraded tryptophan after IFN- γ treatment, and mouse 3T3-A31 cells (a continuous cell line) and mouse embryo cells (derived from primary cultures) resembled the L929 cells (a continuous cell line) in that they did not. These data suggest that degradation of tryptophan after treatment with IFN- γ does not occur (or occurs at a much lower level) in mouse cells and that indoleamine 2,3-dioxygenase is not induced. Curiously, high concentrations of murine type I IFN induce indoleamine 2,3-dioxygenase in mouse lung slices *in vitro* (33), and this induction is suppressed by inhibitors of prostaglandin synthesis (18).

There are several possible explanations for these dichotomies. The failure of crude mouse lymphokine preparations to induce tryptophan-degrading activity in mouse cells weakens the hypothesis that native murine IFN- γ and recombinant murine IFN- γ differ in their ability to induce indoleamine 2,3-dioxygenase. Another possibility is that virus-induced murine IFN induces indoleamine 2,3-dioxygenase in mouse cells, but murine IFN- γ does not. However, we were unable to detect tryptophan-degrading activity in extracts prepared from mouse embryo cells treated for 24 or 48 h with a crude preparation of mouse type I IFN (100 U/ml) (data not shown). A third possibility is that murine IFN- γ , as well as virus-induced murine IFN, induces indoleamine 2,3-dioxygenase, but induction of this enzyme by murine IFN requires mediators which are produced efficiently in mouse lung slices but not in these cultured cells. Alternatively, it may be that the enzyme indoleamine 2,3-dioxygenase is induced in IFN- γ -treated mouse cells, but tryptophan is not degraded substantially because insufficient O₂⁻ is present for this O₂⁻-requiring reaction (22) to occur.

IFN- γ inhibited the growth of *R. prowazekii* in both mouse fibroblasts and human fibroblasts, yet degradation of tryptophan occurred only in IFN- γ -treated human fibroblasts. The absence of tryptophan depletion after IFN- γ -treatment of mouse cells indicates that tryptophan degrada-

TABLE 4. Rickettsial growth in human fibroblasts maintained in medium with added kynurenine or *N*-formylkynurenine^a

Treatment after infection	Conc (μ M)	Rickettsial growth 48 h after infection	
		%R	RI
Control		130 $\pm$ 14	1,587 $\pm$ 279
Kynurenine	50	125 $\pm$ 6	1,736 $\pm$ 497
	200	128 $\pm$ 12	1,674 $\pm$ 331
	500	112 $\pm$ 13	1,092 $\pm$ 171
<i>N</i> -Formylkynurenine	50	144 $\pm$ 14	1,776 $\pm$ 462
	200	136 $\pm$ 18	1,714 $\pm$ 316
	500	107 $\pm$ 10	1,093 $\pm$ 195

^a Irradiated human foreskin fibroblasts were infected with *R. prowazekii* and treated as indicated. Slides were stained at 0 and 48 h after rickettsial infection. %R and RI at 48 h after infection are expressed as percentages of the values observed at 0 h. The average values for %R and RI at 0 h were 53 $\pm$ 14 and 2.6 $\pm$ 0.6, respectively. Each value represents the mean $\pm$ standard error of the mean for two experiments.

tion is not the mechanism for inhibition of the growth of *R. prowazekii* (or any other intracellular microorganism) in these cells. In IFN- γ -treated human fibroblasts, reconstitution of the intracellular tryptophan pool did not relieve the inhibition of rickettsial growth. Therefore, either tryptophan depletion is not involved in inhibition of rickettsial growth in IFN- γ -treated human fibroblasts, or tryptophan degradation and some other mechanism(s) contribute to the inhibition of growth of *R. prowazekii* in these cells. *R. prowazekii* can transport tryptophan (H. H. Winkler and R. M. Daugherty, unpublished data), but whether the organism can also synthesize this amino acid is not known. The intracellular pools of the other amino acids studied were not severely depleted after IFN- γ treatment of human fibroblasts or mouse L929 cells, and no common denominator of IFN- γ treatment could be seen in comparing the depletions that occurred in human and murine cells.

Inhibition of the growth of *T. gondii* in IFN- γ -treated human fibroblasts is clearly a result of tryptophan degradation (13). However, recombinant murine IFN- γ inhibits the growth of *T. gondii* in BALB/c mouse embryo cells (21), and we have now demonstrated that tryptophan depletion does not occur after IFN- γ treatment of BALB/c mouse embryo cells. Therefore, murine IFN- γ must inhibit the growth of *T. gondii* in these mouse cells by a mechanism other than tryptophan depletion. Moreover, the murine effect must not occur in human fibroblasts since tryptophan is sufficient to restore toxoplasma growth in these host cells. It also seems likely that IFN- γ -induced inhibition of the growth of *C. trachomatis* in murine cells occurs by a mechanism other than tryptophan deprivation. De la Maza et al. (2) showed that increasing the tryptophan concentration in the medium has no effect on IFN- γ -induced inhibition of the growth of *C. trachomatis* in McCoy cells. Since these McCoy cells are mouse cells which are likely to have been derived from mouse L cells (2) and L929 cells represent a subclone of mouse L cells, it seems likely that McCoy cells do not degrade tryptophan after treatment with IFN- γ . On the other hand, the inhibitory effect of IFN- γ on the growth of *Chlamydia psittaci* in human T24 cells (bladder carcinoma cells) is reversed by supplementation of the medium with additional tryptophan (G. I. Byrne, personal communication). It thus appears that the mechanisms involved in IFN- γ -induced inhibition of the multiplication of intracellular microorganisms, like the mechanisms involved in IFN-induced inhibition of viral replication (9, 20), may vary with the host cell and with the parasite.

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