Susceptibility of *Rickettsia tsutsugamushi* Gilliam to Gamma Interferon in Cultured Mouse Cells

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Recombinant rodent gamma interferon (IFN-γ) inhibited the infection of cultured BALB/3T3 mouse fibroblasts by *Rickettsia tsutsugamushi* Gilliam, apparently mainly by clearance of intracellular rickettsiae. No significant effect on rickettsial entry into the cells was noted; IFN-γ was toxic to infected cells, as measured by the capacity of treated, infected cells to attach to the surfaces of culture vessels. In a small proportion of IFN-γ-treated cells, rickettsial replication appeared to persist at normal levels. A fraction (28%) of rickettsiae clonally isolated from cultures treated with IFN-γ was resistant to IFN-γ-mediated inhibition, but four serial passages of these resistant clones in the absence of additional IFN-γ resulted in the loss of resistance. In several respects, therefore, the IFN-γ-mediated inhibition of scrub typhus rickettsiae in cultured fibroblasts was similar to that reported for *Rickettsia prowazekii*.

Rickettsia tsutsugamushi, the causative agent of scrub typhus, is a small, obligately intracellular bacterium which replicates within the cytoplasm of eukaryotic cells. In tissue culture, a wide variety of cells can support rickettsial growth, while in natural infections of animals, the cells most prominently parasitized are vascular endothelial cells. Nonfatal rickettsial infections of humans are generally considered to be acute, self-limiting diseases, but much evidence points to the persistence of rickettsiae in infected hosts long after clinical recovery. Rickettsiae are maintained in nature in a variety of mammals and are transmitted by arthropod vectors. In all of these properties, *R. tsutsugamushi* is like other members of the genus *Rickettsia*. In contrast, scrub typhus rickettsiae have several characteristics which distinguish them from other *Rickettsia* species: cell wall ultrastructure (29) and chemical composition (1), staining properties, resistance to such antibiotics as penicillin and streptomycin, lack of T-cell-independent antibody induction (8, 13), and certain growth requirements (17). Thus, although definite similarities exist among all the rickettsiae, free extrapolation from *R. tsutsugamushi* to other *Rickettsia* species is unwarranted.

Rickettsiae of several species have been shown to induce interferons (IFNs). *Rickettsia prowazekii* and *Rickettsia typhii*, both in the typhus group, induced acid-stable, IFN-like viral inhibitors in mice (11) and in cultured chicken embryo cells (16), respectively. More recently, infection of cultured mouse fibroblast cells with *R. prowazekii* was found to stimulate the production of IFN-α/β (38, 39). In *R. tsutsugamushi*-infected primary chicken embryo cell cultures, the appearance of an acid-stable, virus-inhibitory protein followed increases in infectious rickettsiae (7). In addition, T-cell lines specific for scrub typhus rickettsial antigen which secrete IFN-γ upon stimulation have been established (6, 14). In vivo, primary infection of cynomolgus monkeys with *R. tsutsugamushi* resulted in the appearance of IFN-γ in plasma 2 weeks later (19), and the peritoneal cells of mice first contained detectable IFN-γ mRNA 5 to 7 days after intraperitoneal inoculation of *R. tsutsugamushi* (27). Mice immune to *R. tsutsugamushi* are capable of rapid IFN-γ production upon challenge with scrub typhus rickett-

sial antigen, with peak titers in serum occurring as soon as 4 h after antigenic stimulation (9, 26).

The contribution of IFN to recovery from acute infection or to prevention of recrudescence of persisting rickettsiae is not entirely clear, but several studies have pointed to its potential importance. In studies of rickettsiae of the spotted fever group, Nacy and Meltzer (24) correlated the genetically determined resistance of certain mouse strains to lethal infection by *Rickettsia akari* (spotted fever group) with the capacity of their macrophages to develop rickettsicidal capacity after stimulation by lymphokines; lymphokine treatment also resulted in decreased initial entry of rickettsiae into macrophages cultured from resistant mice. Modulation by IFN-γ of infection of mice with *Rickettsia conorii*, another spotted fever rickettsia, was suggested by the finding that treatment of the mice with monoclonal antibodies to IFN-γ prevented lethal infection (18). Mouse lymphokines containing IFN-γ inhibited *R. conorii* growth in L929 cells (32), and such inhibition was neutralized by anti-IFN-γ monoclonal antibody (10). However, in another study, IFN-γ treatment of HEp-2 cells was reported not to inhibit replication of *R. conorii*, although it did enhance the inhibitory effect of tumor necrosis factor alpha (20). Earlier work had shown that *R. akari* yields were inhibited by pretreatment of L cells with virus-induced IFN (12).

The role of IFN in *R. prowazekii* infections of cultured macrophages, fibroblasts, and endothelial cells has been studied fairly extensively. Treatment of cultured cells with IFN-γ may have three effects on *R. prowazekii* infections. (i) Unfractionated lymphokine preparations containing IFN-γ inhibited rickettsial entry into L929 cells; although recombinant IFN-γ did not inhibit rickettsial entry into L929 cells, it did inhibit rickettsial entry into mouse macrophagelike RAW264.7 cells, and antibody to IFN-γ reversed this inhibition (34, 36). (ii) Recombinant IFN-γ prevented rickettsial replication and led to the clearance of intracellular rickettsiae (31–33, 40). (iii) IFN-γ treatment resulted in the specific lysis of infected cells (30, 33, 40). In addition, *R. prowazekii* has been shown recently to be inhibited by either endogenous or added IFN-α/β (38, 39). The mechanism by which IFN inhibits rickettsial growth is not known, but the IFN-γ mediated inhibition of *R. prowazekii* apparently is neither

due to tryptophan (or other amino acid) degradation (35) nor to induction of a respiratory burst in macrophagelike cells (30).

The role of IFN in *R. tsutsugamushi* infections is not known, although several studies have examined the effect of crude and partially fractionated lymphokines prepared from cultured lymphocytes. IFN-γ-containing T-cell lymphokines have been shown to activate mouse peritoneal macrophages, leading to two effects on *R. tsutsugamushi* Gilliam: (i) decrease in initial entry into cultured macrophages and (ii) clearance (killing) of intracellular rickettsiae (21–23, 25). (Peritoneal macrophages from mice genetically resistant or susceptible to lethal infection with *R. tsutsugamushi* Gilliam were equally sensitive to activation by lymphokines to become rickettsicidal [21].) Furthermore, a lymphokine preparation from a scrub typhus rickettsial antigen-specific T-cell line was selectively cytolytic for *R. tsutsugamushi* Gilliam-infected BALB/c mouse macrophages (15). However, there have been no published reports of the susceptibility of *R. tsutsugamushi* to growth inhibition mediated by purified or recombinant IFN nor of its effect in cells other than macrophages. Here, I report that *R. tsutsugamushi* Gilliam infection of a continuous line of mouse fibroblast cells is susceptible to inhibition mediated by recombinant rodent IFN-γ and describe some of the manifestations of this inhibition.

MATERIALS AND METHODS

Rickettsiae. The Gilliam strain of *R. tsutsugamushi* was obtained from C. L. Wisseman, Jr., as a yolk sac suspension which had undergone 103 passages in embryonated chicken eggs. After plaque purification in mouse embryo cells (see below), stocks were prepared in irradiated Vero cells. For this purpose, irradiated cells were incubated in suspension with enough rickettsiae to achieve infection of all cells within 1 h at 37°C (3). The infected cells were then diluted and distributed to tissue culture flasks for incubation as monolayer cultures at 37°C. Three to four days later, the heavily infected cells were scraped from the flasks with a rubber policeman, centrifuged, and resuspended in bovine serum albumin-containing attachment-penetration medium (BAP; 10 mM potassium phosphate buffer [pH 7.3], 5 mM potassium glutamate, 45 mM NaCl, 62.5 mM KCl, 10 mM $MgCl_2$, 0.05 mM $MnCl_2$, 0.3% [wt/vol] bovine serum albumin, 0.1% glucose) (3). The concentrated cells were disrupted by brief sonication and distributed to vials for freezing and storage at −80°C. The rickettsiae used in these experiments had been passaged 12 to 13 times in Vero cells. Production of rickettsial stocks in cells of primate origin ensured that they would contain no endogenous IFN capable of activity in the mouse cell cultures.

Cell culture. The BALB/c mouse embryo-derived cell line, BALB/3T3 clone A31, was obtained from American Type Culture Collection, Rockville, Md. The tissue culture medium used throughout, TCM, consists of antibiotic-free RPMI 1640 medium buffered by 15 mM N'-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and supplemented with 5% controlled process serum replacement-4 (CPSR-4; Sigma Chemical Co., St. Louis, Mo.). (CPSR-4 was used instead of the customary fetal bovine serum [FBS] [3] for the sake of economy.) Preliminary studies employing comparative growth curves (3) and plaque assays (4) demonstrated that 5% CPSR-4 supported cell growth and scrub typhus rickettsial replication and plaque formation as well as the 10% FBS used previously did (4). To inhibit host cell

replication during rickettsial growth studies, cells in suspension were irradiated with 3,000 rad with a ^{137}Cs irradiator.

Infection of suspended cells and rickettsial growth curves. The methods for infecting suspended cells and constructing rickettsial growth curves have been described in detail previously (3). Briefly, cells were removed from monolayers with trypsin, centrifuged, resuspended in TCM, irradiated, and mixed with rickettsiae which had been appropriately diluted in TCM. Tubes of rickettsia-cell mixtures were held in a 37°C water bath for 60 min with frequent gentle shaking and then washed by centrifugation, suspended in TCM to give 5×10^4 cells per ml, and distributed in 0.3-ml aliquots to each of several wells in 8-chamber tissue culture slides (LabTek Division of Miles Laboratories, Inc., Napierville, Ill.) which had been pretreated by overnight incubation in TCM. The chamber slides were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air to permit the attachment of cells and rickettsial replication. At appropriate intervals, slides were rinsed with saline, fixed with methanol, and stained with Giemsa stain for counting rickettsiae at a magnification of ×1,000 or for counting cells at a magnification of ×100. For rickettsiae counts, at least two sets of 100 cells each were counted for each condition, and for cell counts, at least four sets of counts were made. The results are presented as the mean numbers of cell-associated rickettsiae per cell (R/c) or the percentages of cells infected.

Alternatively, to quantitate infectious rickettsiae, irradiated cells infected in suspension as described above and thoroughly washed were distributed to 2-cm^2 wells in 24-well plates at a concentration of 10^5 cells per ml of TCM per well. These infected cultures also were held in the 37°C CO_2 incubator. At intervals, cells were scraped from the wells directly into the TCM, briefly sonicated under conditions which disrupted the cells without killing rickettsiae, and plaqued immediately. Duplicate wells were handled separately at each stage.

Plaque assays and rickettsial cloning. The microplaque assay for infectious rickettsiae in 2-day-old BALB/3T3 monolayer cultures in 2-cm^2 wells in 24-well plates has been described for a different cell line (4). In contrast to the original method, the use of BALB/3T3 cells required that they be irradiated before infection; otherwise, no plaques were detected. The only other deviation from the published technique was the replacement of 5% FBS in the overlaying medium with 2% CPSR-4, the concentration determined to be as efficient as 5% FBS (2). Inclusion of 5% chicken serum in the overlay medium containing CPSR-4 was required for optimum plaque formation by *R. tsutsugamushi*, just as it was needed in the FBS-containing overlay medium (2, 4).

Rickettsial clones were obtained by a combination of endpoint dilution and plaquing. Wells containing only one rickettsial plaque were identified microscopically without prior staining; the agarose from these wells was removed, and the cells were scraped into BAP, centrifuged, resuspended in BAP, and disrupted by sonication. The released rickettsiae were either immediately replaqued or stored frozen at −70°C. On average, about 10^4 PFU were recovered from a single plaque after incubation for 2 weeks.

IFN. Recombinant rat IFN-γ (10^7 IU/mg of protein) was purchased from Amgen Biologicals (Thousand Oaks, Calif.), reconstituted to 10^5 IU/ml in phosphate-buffered saline (pH 8.5) containing 1% FBS (Gibco, Grand Island, N.Y.) and frozen in small aliquots at −80°C. Activity was determined by AmGen in a vesicular stomatitis virus–L-cell assay. A plaque reduction assay of encephalomyocarditis virus in BALB/3T3 cells gave comparable titers. In the present
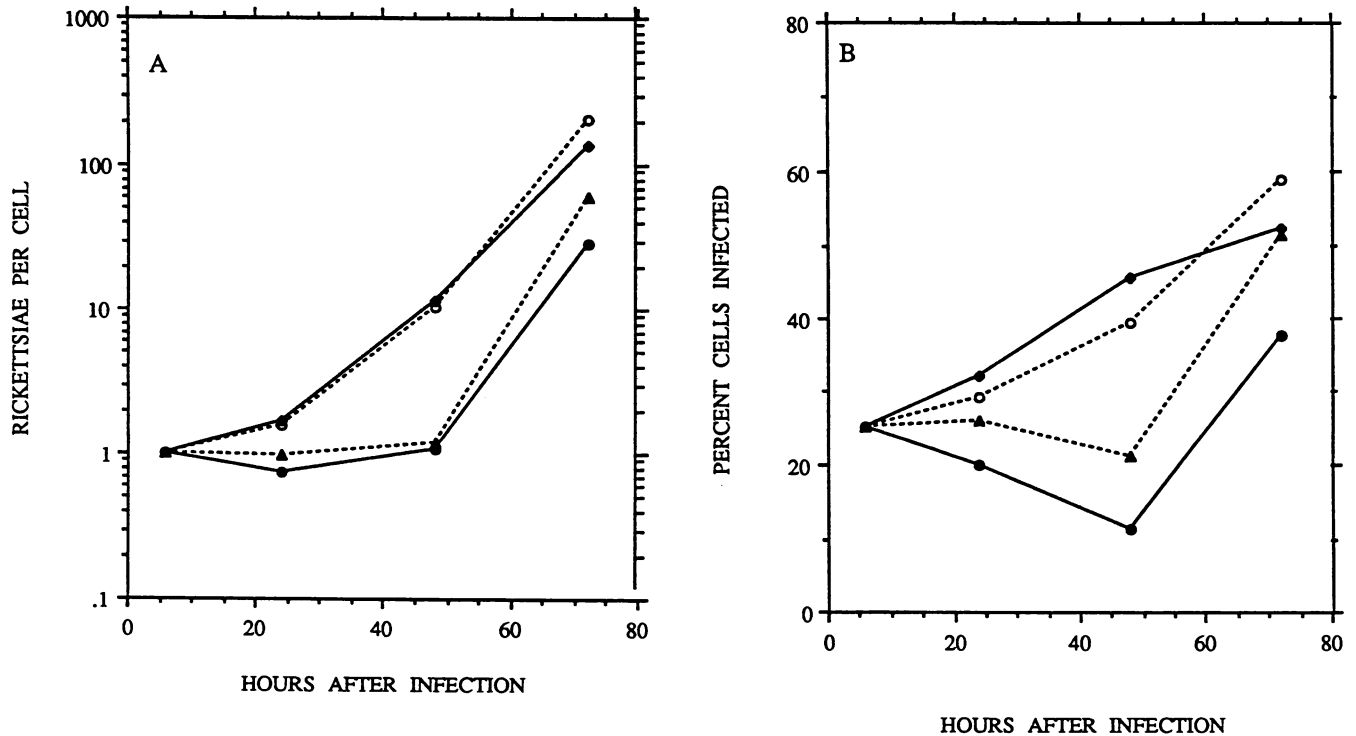
FIG. 1. Effect of recombinant IFN-γ treatment (500 IU/ml) on *R. tsutsugamushi* Gilliam replication in BALB/3T3 cells. Symbols: O, cultures given mock treatment; ●, cultures treated both 18 h before and 1 h after infection; ◆, cultures treated 1 h after infection only; ▲, cultures treated 18 h before infection only.

studies, unless otherwise noted, 1-day-old, just confluent monolayer cultures (which were in flasks when cells were to be infected in suspension or in 24-well plates for the plaque assay) were treated with IFN-γ approximately 18 h before infection by replacing the medium with TCM containing the appropriate concentration of IFN-γ. Controls were treated identically, except that the replacement medium contained no IFN-γ.

RESULTS

Effect of IFN-γ on *R. tsutsugamushi* replication. *R. tsutsugamushi* replication in BALB/3T3 cells was examined after a variety of IFN-γ treatments. Confluent monolayers of uninfected cells grown in 2-cm² wells were treated overnight with 500 IU of IFN-γ per ml of TCM or with TCM only. The next morning, the cells were trypsinized from the wells, washed, irradiated, and infected in suspensions containing 2×10^5 cells and 2×10^6 rickettsial PFU/ml. After 1-h incubation in suspension with rickettsiae to permit attachment and penetration of the organisms, the cells were again washed by centrifugation, suspended in TCM with or without additional IFN-γ (500 IU/ml), distributed to chamber slides, and incubated at 37°C. Slides were fixed at the indicated intervals and stained with Giemsa stain to monitor rickettsial growth.

Control cultures which received no IFN-γ supported rickettsial replication to a typical extent, with a rickettsial doubling time of 6.8 h between 24 and 72 h postinfection (Fig. 1A). As occasionally occurs in scrub typhus rickettsial infections, there was a continual spread of rickettsiae from cell to cell, demonstrated by the steady increase in the percentage of cells infected (Fig. 1B). In contrast, IFN-γ treatment of cells both before and just after infection re-

sulted in marked inhibition of rickettsial yields and percentages of cells infected during the first 48 h after infection. After this time point, rickettsial replication appeared to commence at an uninhibited rate; this was accompanied by rickettsial spread from cell to cell, which caused a gradual increase in the percentage of cells infected. The timing of IFN-γ treatment affected the results: if IFN-γ was added only on the day before infection, it was almost as inhibitory as when given both before and after. On the other hand, if IFN-γ treatment was withheld until just after infection, the average number of rickettsiae per cell and the percentage of cells infected was reduced only slightly and only after 48 h postinfection; no effect was seen at 6 or 24 h postinfection. Thus, pretreatment of cells was necessary to mediate optimal inhibition of rickettsial replication.

The above experiments demonstrated that IFN-γ treatment can inhibit scrub typhus rickettsial replication in mouse BALB/3T3 cells and that even with a moderate initial rate of infection and with treatment 18 h before and 1 h after infection, the effect appeared to be transitory. The capacity of the cultures to support significant rickettsial replication and spread after 48 h postinfection could have been due to the depletion of the IFN-γ or IFN-γ-mediated effectors. To test this, cultures pretreated with 100 IU of IFN-γ per ml were infected as described above (but with fewer rickettsiae to permit a longer observation period), and more IFN-γ was added at regular intervals after infection. In control, untreated cultures, rickettsial replication began without a noticeable lag period and when the average number of rickettsiae per cell was plotted, the resulting line was logarithmic for 120 h, with a rickettsial doubling time of 9.7 h (Fig. 2A). As is usually seen in *R. tsutsugamushi* infections in cultured irradiated cells, there was a dramatic increase in the percent-
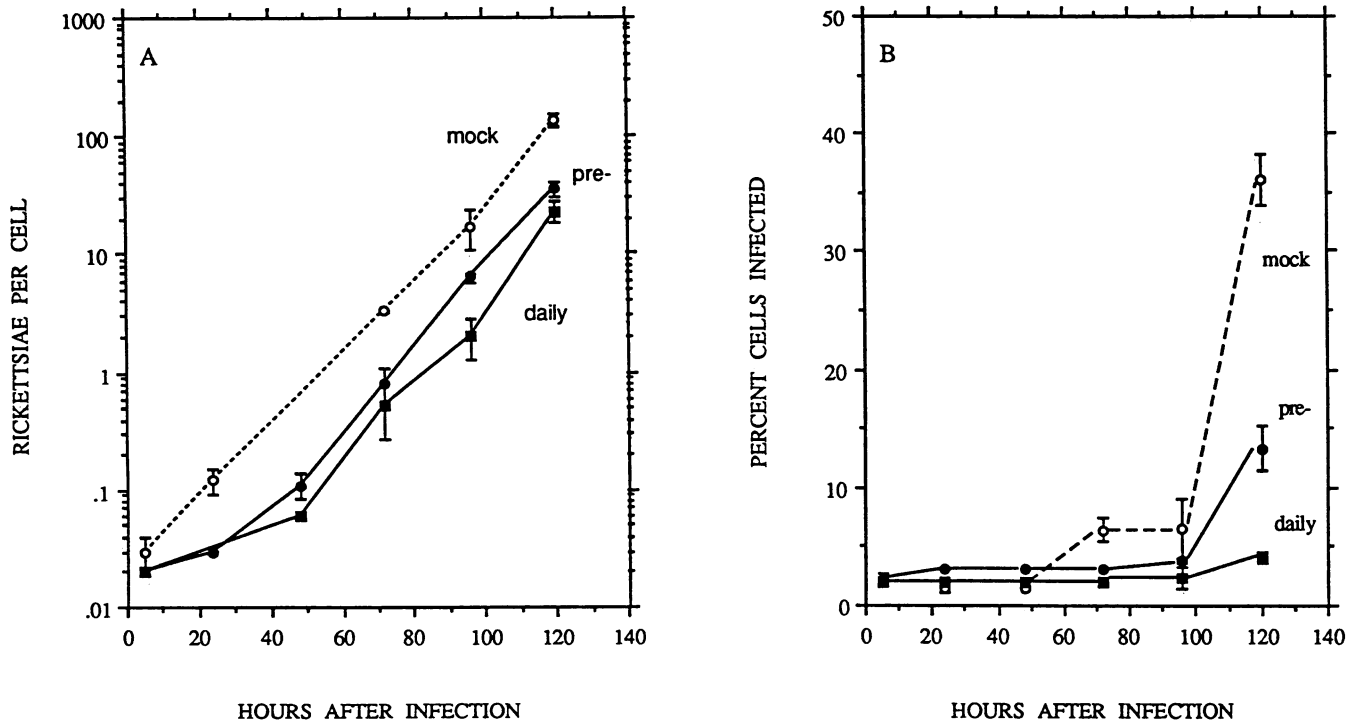
FIG. 2. Effect of recombinant IFN-γ treatment (100 IU/ml) on replication of *R. tsutsugamushi* Gilliam in infected BALB/3T3 cells. The treatments used were mock treatment, IFN-γ treatment 18 h before infection only (pre-), IFN-γ treatment 18 h before infection and daily thereafter (daily).

age of cells infected after 96 h, resulting from the bursting of heavily infected cells and spread of organisms to new cells (Fig. 2B).

In contrast to these controls, IFN-γ treatment of the cultures treated only once, 18 h before infection, resulted in an apparent lag period before logarithmic increase of rickettsiae began, agreeing with the results shown in Fig. 1 (Fig. 2A). The addition of 100 IU of IFN-γ per ml on a daily basis appeared to be slightly more effective than one treatment, but by no means did it completely prevent rickettsial replication. Thus, the lag period may have been somewhat prolonged, but at 120 h postinfection, the average R/c was reduced by 83% by daily treatment as opposed to 74% reduction by one treatment, which is not a statistically significant difference. The more-extensive treatment with IFN-γ apparently did reduce spread of rickettsiae from cell to cell, with the percentage of cells infected at 120 h being 13 versus 4 after one or daily treatments, respectively ($P <$ 0.001) (Fig. 2B). The same general pattern of results also was obtained with less-rigorous treatments, when the IFN-γ was added on alternate days (days -1, $+1$, and $+3$ relative to the day of infection) or when only 10 IU/ml were used throughout (not shown).

The cultures treated daily had relatively few cells containing rickettsiae, but these few cells were heavily infected. This is illustrated in Fig. 3, which shows the frequency of cells containing different numbers of rickettsiae 5 days after infection. Rickettsial infection in untreated, control cells showed a bimodal distribution of infected cells most typical of this stage of infection, with a large proportion of cells containing 50 or fewer rickettsiae, representing newly infected cells, and a normal distribution of cells containing more than 50 rickettsiae each, representing the population of
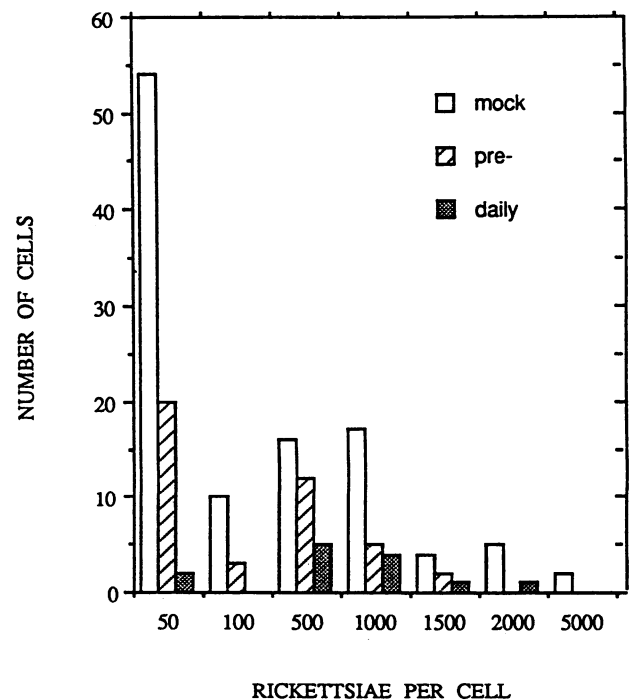

FIG. 3. Effect of IFN-γ treatment (100 IU/ml) on distribution of *R. tsutsugamushi* Gilliam in infected BALB/3T3 cells. The treatments used were mock treatment, cultures treated once 18 h before infection (pre-), and cultures treated 18 h before infection and daily thereafter (daily). The total number of cells counted in each sample was 300.

TABLE 1. Effect of serial passage of *R. tsutsugamushi* Gilliam
in IFN-γ-treated BALB/3T3 cells on sensitivity
of selected rickettsial clones to IFN-γ

No. of passages in IFN-γ	PFU/ml (% of untreated control)[a]						
	Resistant plaque:					Sensitive plaque[b]:	
	3	4	9	14	18	5	13
1	79.0	?[c]	70.8	103.8	58.3	15.7*	16.8*
2	ND[d]	63.2	63.6	88.5	125.6	27.5*	8.4*
			79.5	131.6			6.8*
3	ND	96.9	ND	97.3	ND	ND	ND
		86.0		129.0			

[a] Rickettsiae were plaqued in irradiated BALB/3T3 cell cultures, some of which had been treated with 200 to 500 IU of IFN-γ per ml the day before. Results are given as: (PFU/milliliter in IFN-γ-treated cultures divided by PFU/milliliter in untreated cultures) × 100. In some cases, two plaques from the same group were tested. In each experiment, control plates indicated significant IFN-γ-mediated inhibition of plaque formation by Gilliam which had not been passaged previously in the presence of IFN-γ. The mean value ± standard error from unpassaged Gilliam used as controls in each of the experiments was 24.3 ± 5.3 ($n = 8$).
[b] An asterisk indicates a statistically significant difference from the control PFU/ml, i.e., significantly inhibited by IFN-γ ($P < 0.001$); the remaining values do not differ significantly from control PFU/ml ($P > 0.01$), as determined by Student's *t* test. (Although the results are listed as percentages, the *t* tests were done on the raw data.)
[c] Not enough control plaques to determine sensitivity to IFN-γ.
[d] ND, not done.

cells initially infected. In cultures treated once with IFN-γ, 18 h before infection, the distribution was similar, although the total number of infected cells was lower. In contrast, daily treatment with IFN-γ, in addition to reducing the number of infected cells even more, also seemed to prevent the appearance of a population of newly infected cells. Thus, the few infected cells mostly contained very large numbers of rickettsiae, explaining why the average number of rickettsiae per cell was not reduced by more than 85%. Thus, even when infected initially with a very small number of rickettsiae (average R/c at 0 h = 0.02) and when treated before and every day after infection, some rickettsial replication persisted.

IFN-γ sensitivity of rickettsiae after passage in IFN-γ-treated cells. The apparent capacity of some rickettsiae to escape IFN-γ-mediated inhibition suggested the possibilities that either a few cells in the culture were refractory to IFN-γ, a few resistant rickettsiae were selected, or both. To see whether rickettsiae which replicated in the face of IFN-γ treatment were more resistant to IFN-γ-mediated inhibition than those not previously exposed to IFN-γ, rickettsial clones (isolated plaques) obtained from IFN-γ-treated cultures were replaqued in IFN-γ-treated and untreated cells (Table 1). The IFN-γ doses used in these experiments (200 to 500 IU/ml) inhibited overall plaque formation by 70 to 80%. Of 18 plaques isolated after one passage in IFN-γ-treated cultures, four (plaques 3, 9, 14, and 18) were not subsequently inhibited by IFN-γ; one (plaque 4) did not yield enough plaques on which to base a conclusion, but it did yield progeny plaques which were resistant (Table 1). Further serial passages of some of the resistant plaques in the presence of IFN-γ indicated that they retained their IFN-γ resistance under these conditions (Table 1). Of the 18 plaques picked from one passage in IFN-γ-treated cultures, the remaining 13 proved to be as sensitive to IFN-γ as the

control rickettsiae not previously passaged in the presence of IFN-γ; two of these (plaques 5 and 13) were replaqued in IFN-γ-treated cells and all of the progeny plaques which were tested also were sensitive (Table 1). Thus, with a limited number of plaques tested, 28% (5 of 18) of the rickettsiae isolated after one passage in IFN-γ-treated cultures were resistant to inhibition by IFN-γ, and those that were resistant passed the resistance to their progeny.

Attempts were made to produce bulk stocks of the IFN-resistant plaque progeny by serial passage in increasingly larger cultures of IFN-γ-treated BALB/3T3 cells. In all cases, the cultures died before substantial numbers of rickettsiae could be harvested. Therefore, stocks were prepared in untreated BALB/3T3 cultures. Progeny from single plaques 4 and 18 (starting with the equivalent of 1/3 plaque) were serially passaged in succeedingly larger culture vessels (total surface areas of 12, 75, 300, and finally 750 cm^2), and rickettsiae from the fourth serial passage were stored at −80°C for future analysis. Thawed aliquots were tested in three replicate experiments for their IFN-γ sensitivity in the plaque reduction assay, using 400 to 450 IU of IFN-γ per ml. After just these few serial passages in untreated BALB/3T3 cultures, the progeny of plaques 4 and 18 appeared to have lost their IFN-γ resistance, because the mean percentages of untreated control PFU/ml for plaque 4, 18, and unpassaged Gilliam were 41.3 ± 8.2 ($P < 0.05$), 33.2 ± 10.6 ($P < 0.05$), and 26.7 ± 3.9 ($P < 0.01$), respectively. Moreover, the sensitivity of the progeny from clones 4 and 18 to IFN-γ was not significantly different from that of unpassaged Gilliam ($P > 0.05$, paired *t* test).

Relative sensitivity of Gilliam to IFN-γ-mediated inhibition. In dose-response experiments, the sensitivity of Gilliam to IFN-γ closely paralleled that of the commercial (Amgen) test virus system; maximum inhibition was caused by as few as 33 antiviral IU per ml and 50% inhibition was achieved by 2 to 3 IU/ml (Fig. 4). One standard procedure for assessing IFN-γ-mediated inhibition of rickettsial replication, described above, was to count cell-associated rickettsiae (R/c) at the indicated times after infection of IFN-γ-treated and control cells. Examination of other manifestations of infection in the same experiments indicated that neither simply counting the percentage of cells infected nor measuring protection of cells from rickettsia-induced cytopathology was as sensitive as counting R/c (Fig. 4).

In a limited number of plaque reduction assays done with low concentrations of IFN-γ, 50% inhibition was also achieved by about 1 IU of IFN-γ per ml. Rickettsial plaque size was not affected by pretreatment of the cultures with IFN-γ. In 13 separate experiments in which a mean of 30 plaques each were microscopically measured (4), plaques in IFN-treated cultures were 92.6% (±3.4%) the size of those in control, untreated cultures.

Effect of IFN-γ on infected BALB/3T3 cells. A possible cytotoxic effect of IFN-γ treatment was looked for by two methods: (i) an increase in trypan blue-staining BALB/3T3 cells immediately before and after infection and (ii) a decrease in the number of cells which attached to slide chambers 6 and 24 h after inoculation (Fig. 5). Treatment of uninfected BALB/3T3 cell monolayers with IFN-γ for 18 h (followed by irradiation) did not adversely affect recovery of trypan blue-excluding cells from the culture wells. However, subsequent infection of these cells in suspension did show a toxic effect of IFN-γ, manifested slightly by counts of trypan blue-excluding cells immediately after 1-h incubation with rickettsiae and, more significantly, by counts of cells attached to chamber slides after 6- and 24-h incubation at 37°C.
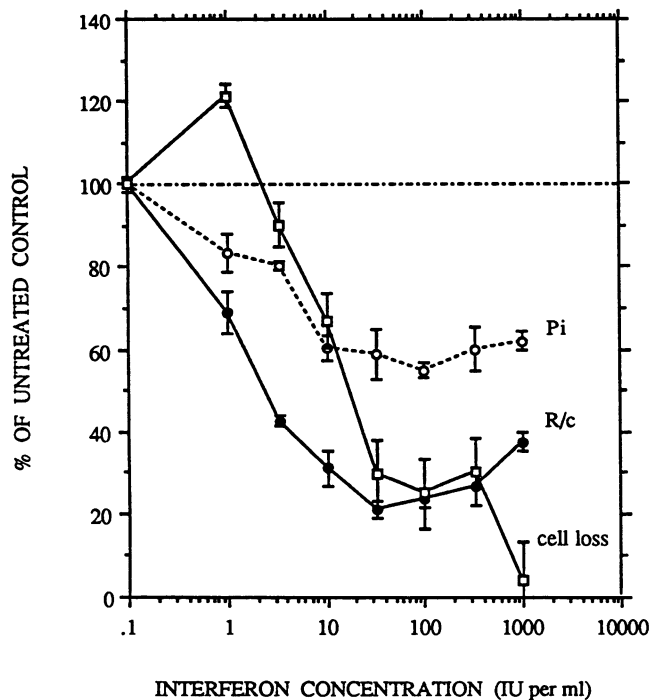
FIG. 4. Relative sensitivity of *R. tsutsugamushi* Gilliam to IFN-γ-mediated inhibition in BALB/3T3 cells. Cultures were treated with various concentrations of IFN-γ 18 h before infection. Data are from 72 h postinfection. Results in mock-treated cultures (receiving no IFN-γ) were set to 100%. The percentage of cells infected (Pi), R/c, and percentage of cells per chamber which detached (cell loss) are shown.

FIG. 5. Cytotoxicity of IFN-γ to infected and uninfected BALB/3T3 cells. Cultures were mock treated or treated with various concentrations of IFN-γ for 18 h. After suspension and irradiation, a portion of the cells was mixed with trypan blue to determine the number of stain-excluding cells in each culture (0 h). The remaining cells were mixed with *R. tsutsugamushi* Gilliam for 1 h to permit infection, and additional aliquots were counted in the presence of trypan blue to determine the number of stain-excluding cells in each culture (Gil. 0 h). The mock-treated and IFN-γ-treated, infected cells were distributed to chamber slides for incubation at 37°C; 6 and 24 h later, slides were fixed and the numbers of attached cells were counted (Gil. 6 and 24 h, respectively).

Those infected cells which did attach to the chamber slide surface were spread well and grew large in size, i.e., they appeared to be healthy. This apparent cytotoxicity of IFN-γ seen immediately after rickettsial infection should not be confused with the later protection from rickettsia-induced cytopathology described above.

Effect of IFN-γ pretreatment on attachment and penetration of rickettsiae. BALB/3T3 cells pretreated for 18 h with IFN-γ (33 to 500 IU/ml) and then irradiated were as readily invaded by *R. tsutsugamushi* under standard conditions of infection as were untreated controls. This was determined by comparing R/c 4 to 6 h after infection and assessing the results from several experiments by the paired *t* test (86.0% ± 10.5% of control, $P > 0.05$). Rickettsial replication, however, was inhibited in these experiments. Thus, inhibition of rickettsial growth cannot be attributed to direct prevention of rickettsial entry into cells in the cultures.

Effect of IFN-γ on recovery of infectious rickettsiae early after infection. Assessment of rickettsial replication by counting stained organisms is somewhat limited because the method does not distinguish infectious rickettsiae from those which have lost their capacity to replicate but which still are recognizable microscopically. To obtain a more accurate view of the effect of IFN-γ on *R. tsutsugamushi* and to try to distinguish inhibition of replication from rickettsial killing, the infection was monitored by scraping cells from infected-cell monolayers into TCM at intervals, disrupting both the adhered and floating cells by brief sonication, and comparing the total number of PFU in each suspension. During the first 12 h after infection, control cultures supported a nearly threefold increase in PFU recovered, while in cultures
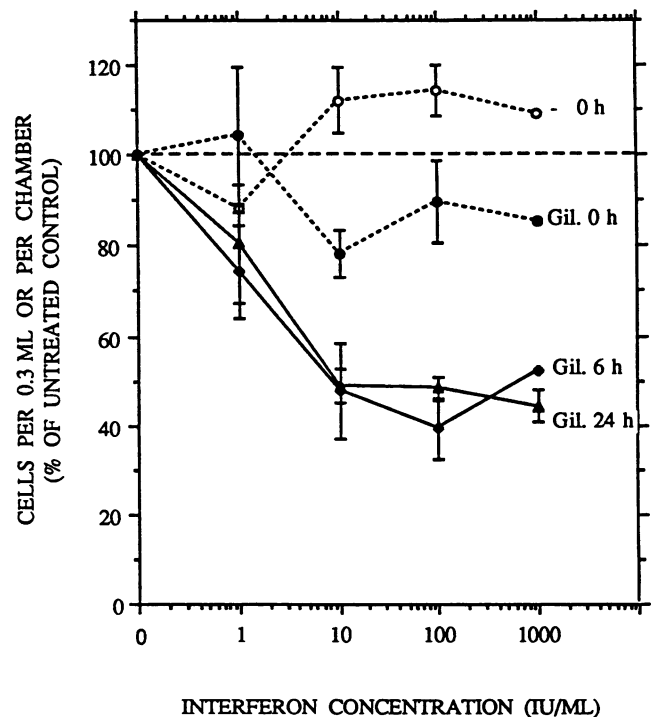
pretreated with 100 IU of IFN-γ per ml, there was a twofold decrease during the same time period, resulting in a 90% reduction of infectious rickettsiae overall (Fig. 6). Sometime after 12 h, rickettsial replication in IFN-γ-pretreated cultures appeared to recover, so that by 24 h postinfection, the infectious rickettsial yield was 42% of that from untreated wells. The initial decrease in infectious rickettsiae recovered from IFN-γ-treated cells suggests that some killing occurred under conditions in which no killing was apparent in control cells.

Effect of added L-tryptophan on IFN-γ-mediated inhibition. To see whether increasing the L-tryptophan concentration of the TCM could reverse the IFN-γ-mediated inhibition of Gilliam plaque formation in BALB/3T3 cells, a standard plaque reduction assay in which some of the cultures were treated with an additional 100 μg of L-tryptophan per ml during IFN-γ pretreatment was done. IFN-γ was equally effective in reducing plaque formation in the presence of the additional L-tryptophan (Table 2).

DISCUSSION

In the experimental system used here, *R. tsutsugamushi* Gilliam in BALB/3T3 cells, pretreatment of cells with recombinant IFN-γ resulted in marked inhibition of rickettsial growth during the first 48 h of the infection. This was
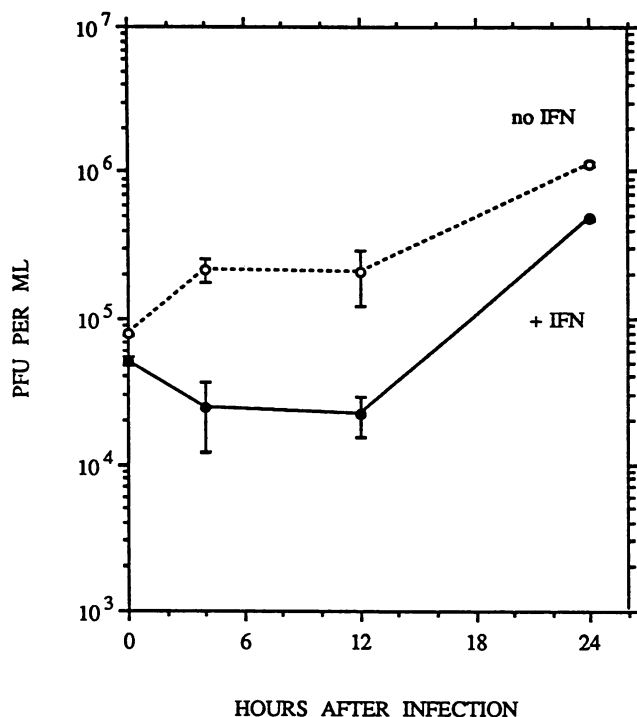
FIG. 6. Effect of IFN-γ treatment on recovery of infectious *R. tsutsugamushi* Gilliam early after infection. BALB/3T3 cultures were mock treated or treated with IFN-γ (100 IU/ml) 18 h before infection. Suspended, irradiated cells were mixed for 1 h with rickettsiae to permit infection, washed twice, and distributed to 2-cm² tissue culture wells for incubation at 37°C. At the indicated intervals, the attached cells were scraped into TCM (containing any unattached cells) and disrupted by sonication, and the samples were immediately plaqued.

manifested by a decreased *R/c* value and a reduction during this period of the number of infected cells. After 48 h postinfection, rickettsial replication appeared to recover; although the total yield of rickettsiae was still lower than that in untreated controls, the rate of rickettsial increase was comparable to that in the control cultures.

Examination of the distribution of rickettsiae in individual, pretreated cells revealed that a few heavily infected cells accounted for most of the rickettsial replication after 48 h postinfection. To see whether the apparent IFN-γ resistance

TABLE 2. Effect of additional tryptophan on IFN-γ-mediated inhibition of *R. tsutsugamushi* Gilliam plaque formation in BALB/3T3 monolayer cultures

Medium[a]	PFU/ml (mean ± SE)		P[b]
	Untreated	IFN-γ-treated	
TCM	$(2.0 \pm 0.3) \times 10^7$	$(4.3 \pm 0.9) \times 10^6$	<0.001
TCM + tryptophan	$(6.2 \pm 2.0) \times 10^7$	$(4.3 \pm 1.2) \times 10^6$	<0.001

[a] TCM contained RPMI 1640 with 5 μg of L-tryptophan per ml; TCM + tryptophan contained an additional 100 μg/ml, which was present during the 18 h immediately prior to infection, concurrent with IFN-γ treatment. IFN treatment was with 300 IU/ml.

[b] Statistical significance by Student's *t* test, comparing rickettsial yields in cultures treated or not treated with IFN-γ. When rickettsial yields in cultures treated or not treated with additional tryptophan were compared, no statistically significant differences were found (*P* > 0.01).

to inhibition of rickettsiae in these cells could be due to inherent properties of the organisms themselves, rickettsial plaques formed in pretreated BALB/3T3 cells were isolated and compared with the parental Gilliam rickettsiae for their sensitivity to IFN-γ-mediated inhibition. About a quarter (28%) of the plaques were found to contain rickettsiae resistant to IFN-γ, and one or two subsequent plaque purifications of these clones in the presence of IFN-γ yielded all resistant progeny. On the other hand, when some of the remaining 72% of plaques which yielded IFN-γ-sensitive rickettsiae were replaqued, the progeny all were as sensitive to inhibition as control Gilliam. These experiments suggested that a relatively large proportion of the rickettsiae were or became inherently resistant to IFN-γ. This property apparently was not stable, because the trait was lost from both clones tested during minimal passage in the absence of IFN-γ. Whether testing of more clones would yield stably resistant progeny is uncertain. In comparable work, Turco and Winkler found that *R. prowazekii* isolated in the presence of IFN-γ or IFN-α/β retained their resistance to IFN relative to that of the parental isolates during subsequent passage in the absence of added IFN; however, some absolute sensitivity to IFN-mediated inhibition was evident (37–39).

The inhibitory effect of IFN-γ was demonstrated in two types of assays: (i) direct counting of Giemsa-stained, infected cultures for the number of cell-associated rickettsiae over a span of several days and (ii) observing development of rickettsial plaques after a 2-week period of successive rounds of rickettsial replication. In each assay, the antiviral units (as indicated by the manufacturer) of the recombinant IFN-γ required for 50% inhibition was the same, 1 to 3 IU/ml; maximum inhibition required about 33 IU/ml. This is similar to the sensitivity of *R. prowazekii* to recombinant mouse IFN-γ in L929 fibroblasts or macrophagelike RAW264.7 cells (31, 33). In our experiments with *R. tsutsugamushi*, maximum inhibition of about 80% was achieved, again comparable to that reported for *R. prowazekii* in L929 or RAW264.7 cells (31, 33). The recombinant rodent IFN-γ used in our experiments did not affect Gilliam replication in Vero cells from monkeys.

Pretreatment of BALB/3T3 cells with recombinant IFN-γ and subsequent infection with *R. tsutsugamushi* Gilliam was cytotoxic, as measured by counting the cells which attached to the culture slides 6 to 24 h after infection. A maximum cytotoxic effect was found by pretreatment with 10 IU of IFN-γ per ml, which resulted in about a 50% decrease in attached cells. IFN-γ pretreatment reduced the number of trypan blue-excluding cells immediately after infection by no more than 20%. Likewise, lysis of IFN-γ-treated, *R. prowazekii*-infected fibroblasts and macrophagelike cells has been reported, and IFN-γ treatment in the absence of infection with viable rickettsiae was not cytotoxic (33, 37, 40).

Pretreatment of BALB/3T3 cells with recombinant IFN-γ apparently did not affect the degree to which they were subsequently invaded by *R. tsutsugamushi*. This is consistent with the findings of Turco and Winkler, who noticed a slight inhibitory effect of IFN-γ-containing lymphokines (32) but not of recombinant IFN-γ on L929 fibroblast cell invasion by *R. prowazekii* (34, 36) and a more pronounced effect of recombinant IFN-γ on initial infection of macrophagelike RAW264.7 cells (33). A loss of infectious scrub typhus rickettsiae from IFN-γ-treated BALB/3T3 cultures during the first 12 h after infection and a decrease in the percentage of cells infected with microscopically recognizable organ-

isms during the first 48 h after infection suggested that enhanced rickettsial killing, rather than inhibition of replication, could explain the observed diminution of rickettsial yields. These observations again are similar to the initial decreases in the percentages of infected cells which were noted in the IFN-mediated inhibition of *R. prowazekii* (31, 32, 40). Lysis of IFN-treated, infected cells may also contribute to decreases in overall rickettsial yield (33, 37, 40). In both types of the experiments reported here, the rickettsiae which survived these early periods went on to replicate at normal rates.

Finally, because tryptophan degradation has been shown to be involved in the inhibitory effect of IFN-γ on *Toxoplasma gondii* (28), the effect of additional tryptophan in TCM on the IFN-γ-mediated inhibition of *R. tsutsugamushi* growth was determined. Not surprisingly, since Turco and Winkler showed that IFN-γ did not lead to the degradation of tryptophan in the same BALB/3T3 cell line we used (although in a different tissue culture medium) (35), the addition of 100 μg of tryptophan per ml to the TCM did not alter the IFN-γ sensitivity of Gilliam.

The earliest studies of the role of lymphokines in rickettsial infections were done by Nacy and colleagues, who described the interaction of *R. tsutsugamushi* Gilliam with mouse peritoneal macrophages activated either in vivo or in vitro by spleen cell-derived lymphokines (21–23, 25). Although they did not directly look for possible effects of IFN-γ, it is likely that the spleen cell lymphokines did contain IFN-γ, and their results resemble those reported here for the interaction of *R. tsutsugamushi* Gilliam in BALB/c mouse-derived fibroblasts. Thus, they also noted a persistent fraction of replicating rickettsiae in a small proportion of the infected cells and apparent rickettsial killing in activated macrophages. In addition, these researchers found a decrease in the initial infection of the activated peritoneal macrophages after lymphokine treatment, similar to that reported for *R. prowazekii* entry into lymphokine- or recombinant IFN-γ-treated macrophagelike cells (33). Moreover, Kodama et al. (15) recently described the cytotoxicity of combined lymphokine treatment and scrub typhus rickettsial infection of cultured macrophages.

It is likely that more than one mechanism is involved in maintaining the delicate balance of rickettsial survival without killing their mammalian hosts and because IFN-γ has many effects on various types of cells, the mechanisms by which it may inhibit rickettsiae in vivo may also be numerous. However, the present studies and some preceding ones indicate that IFN-γ can provide a means of rickettsial destruction while they are sequestered in nonphagocytic cells. The undiminished replication of rickettsiae in a few cells after treatment with IFN-γ (as described above; 25, 40) and the ready isolation of rickettsial clones which are resistant to this inhibition (as described above; 37–39) are interesting observations in light of the known persistence of rickettsiae in infected hosts. This escape by a few rickettsiae from IFN-γ-mediated degradation may be important in providing a means by which these microorganisms ensure their survival in their mammalian reservoirs.

The results of these experiments are not presented to serve as a model for what occurs in infected animals but rather to understand the interaction of rickettsiae and nonphagocytic target cells in the presence of IFN-γ. Additional effects of IFN-γ on the immune response are assumed, and different effects on infection in macrophages may also be anticipated. These studies, done with only one scrub typhus rickettsial strain and with one cell line, have been useful in

showing that *R. tsutsugamushi* replication can be inhibited by IFN-γ, in characterizing the inhibition, and in establishing methods for showing this inhibition. The existence of scrub typhus rickettsial strains with different virulence in particular mouse strains has led us to widen our examination of the sensitivity of *R. tsutsugamushi* to IFN, and in the following report, it will be demonstrated that the combination of Gilliam and BALB/3T3 cells is particularly sensitive to IFN-γ-mediated inhibition (5).

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