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Comparative Susceptibility to Mouse Interferons of *Rickettsia tsutsugamushi* Strains with Different Virulence in Mice and of *Rickettsia rickettsii*

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Three strains of *Rickettsia tsutsugamushi* (Karp, Gilliam, and TA716, representing three virulence types in mice) were examined for their sensitivity to the inhibitory effects of recombinant gamma interferon (IFN-γ) and purified IFN-α/β in two cultured mouse fibroblast cell lines. The susceptibilities of another species, *Rickettsia rickettsii*, and of encephalomyocarditis virus (EMCV) were also tested for comparative purposes. IFN-γ inhibited rickettsial replication in only one of the six combinations of *R. tsutsugamushi* strains and mouse cells (strain Gilliam and the BALB/c mouse-derived cell line). In contrast, *R. rickettsii* and EMCV replication were markedly inhibited in both cell types, but to a greater extent in the BALB/c line than in the C3H cells. IFN-α/β (300 to 450 U/ml) was uniformly ineffective in three of the combinations of *R. tsutsugamushi* strains and mouse cells (Gilliam in C3H cells and Karp in both C3H and BALB/c cells); in the remaining sets, IFN-α/β-mediated inhibition of rickettsial replication was variable and in no case was it very pronounced. The tests with *R. rickettsii* in both cell types also indicated slight, variable sensitivity to IFN-α/β. EMCV, on the other hand, was very susceptible to IFN-α/β, confirming the potency of the preparation used; as with IFN-γ, virus replication was inhibited to a greater degree in the BALB/c cell line than in the C3H cultures. These results are discussed in terms of their relationship to the virulence properties of the *R. tsutsugamushi* strains in BALB/c and C3H mice and to the known IFN-sensitivities of the more widely studied *Rickettsia prowazekii*.

Rickettsia tsutsugamushi is an obligately intracellular bacterium which is maintained in nature in trombiculid mites and their rodent hosts and which causes scrub typhus in humans. Several strains have been identified by serological and cross-protection studies, and these antigenic analyses are being refined at the polypeptide and genome levels in a number of laboratories. Scrub typhus rickettsial strains also differ in their virulence for experimental animals, particularly mice. While *R. tsutsugamushi* does not kill mice infected via the skin (the natural route of infection), a single infectious organism from a virulent strain introduced intraperitoneally is capable of multiplying and ultimately killing a mouse. In contrast, mice can resist 10^4 to 10^5 infectious doses of nonvirulent rickettsiae administered by the same route. Although virulence in animal models has been associated with certain serological strains, one study found that different field isolates identified serologically as belonging to the Karp strain were not uniformly virulent in outbred mice (35). Rickettsia-associated factors which might contribute to virulence are completely unknown. Indeed, little is known about any biological properties which distinguish the scrub typhus rickettsial strains. Recently, one *R. tsutsugamushi* strain (Karp) was shown to form smaller plaques in mouse cell monolayers than the five other strains with which it was compared, and three strains (Karp, Gilliam, and TA716) were found to replicate at reproducibly different rates in mouse cell cultures (10).

The virulence of *R. tsutsugamushi* strains is also dependent on the host, even within a species (7). Their capacity to establish a lethal infection in mice after intraperitoneal inoculation is governed partially by the presence of the *Ric* gene on mouse chromosome 5 (and perhaps by other host factors) as well as by properties within the rickettsiae. Mice with the dominant Ric^r allele (e.g., BALB/c) survive large intraperitoneal doses of strain Gilliam but not similarly presented Karp rickettsiae (8, 9). In contrast, mice which are Ric^s (e.g., C3H/He) are killed equally effectively by low doses of Karp and Gilliam. A third pattern of scrub typhus rickettsial virulence is exemplified by strain TA716, which does not cause fatal disease in mice of either genotype (8). The C3HRV mouse strain, congenic with C3H/He mice and originally bred for resistance to lethal infections of flaviviruses (1, 6), was also found to survive infections of *R. tsutsugamushi* Gilliam, but not Karp, presumably also as a result of the Ric^r allele (16, 17).

Recently, the murine gene *Eta-1* (early T-lymphocyte activation 1), which maps near or at the *Ric* locus, has been isolated and characterized (31, 32, 36). Secreted by activated T lymphocytes, the *Eta-1*-encoded protein was shown to bind with high affinity to receptors on macrophages and to quickly recruit macrophages and, to a lesser extent, polymorphonuclear cells to the site of intradermal injection (36). A T-cell line (Ar5v) which expressed *Eta-1* even without activation also produced somewhat elevated levels of gamma interferon (IFN-γ) mRNA (32). Within hours after intraperitoneal infection of CBA/CaJ (Ric^r) mice with *R. tsutsugamushi* Gilliam, peritoneal cells contained *Eta-1* transcripts, and 5 days after infection, these cells contained very few rickettsiae (31). In contrast, peritoneal cells from similarly infected CBA/J (Ric^s) mice failed to produce detectable *Eta-1* mRNA until after the fourth day postinfection and contained large numbers of rickettsiae on the fifth day. Thus, activation of *Eta-1* in peritoneal cells by *R. tsutsugamushi* Gilliam infection appears to be a very early event in the establishment of the resistant state in experimentally infected mice. (This time frame is consistent with earlier studies of genetically related resistance of mice to *R. tsutsugamushi* Gilliam, which demonstrated differences in the

rickettsial content of peritoneal mesothelial cells by 2 days after intraperitoneal inoculation [26] or of peritoneal macrophages and spleen cells as early as 5 days after infection [16, 18].)

Other host factors which may determine virulence or susceptibility to scrub typhus infection are not understood entirely. Protection of mice from an otherwise lethal inoculation of *R. tsutsugamushi* has been achieved by transfer of either specific antibody (2, 3, 5, 33, 37) or T lymphocytes (19, 23, 24, 34), suggesting that both may play a role in recovery from natural infection. The possible involvement of IFN-γ and IFN-α/β in recovery from rickettsial infections has been suggested by in vitro and in vivo studies with various *Rickettsia* species (15, 40). IFN-γ-containing lymphokines, in some cases shown to be a T-cell product(s), have been demonstrated to activate mouse peritoneal macrophages, resulting in a decreased capacity to support growth of *R. tsutsugamushi* Gilliam (25, 27–30). Manifestations of rickettsial clearance were decreased entry of rickettsiae into the treated macrophages, loss of intracellular organisms (27–30), and cytolysis specific for treated, infected macrophages (25). Peritoneal macrophages cultured from genetically resistant BALB/c and susceptible C3H/He mice responded equally to lymphokines in their capacity to subsequently limit intracellular Gilliam growth (27).

Recently I reported the susceptibility of *R. tsutsugamushi* Gilliam to inhibition by recombinant IFN-γ in a BALB/c-derived 3T3 continuous mouse fibroblast cell line (13). The IFN-γ treatment had two effects: it was specifically cytotoxic to the Gilliam-infected fibroblasts, and it apparently led to the killing of intracellular rickettsiae. Here, I expand these results by comparing the IFN-γ susceptibility of Gilliam to that of two other strains, Karp and TA716, and by using a second cell line, derived from C3H mice, as well as BALB/3T3 cells. In addition, the effect of purified IFN-α/β in these rickettsia-cell combinations was examined. The results indicate that *R. tsutsugamushi* susceptibility to rodent interferons is strain dependent and that the cell line used is also important. The availability of scrub typhus rickettsial strains which differ in their sensitivity to IFN-mediated inhibition and of cell lines which differ in their capacity to support IFN-mediated inhibition should provide the means to examine the rickettsial and host cell factors which influence this phenomenon.

MATERIALS AND METHODS

Rickettsiae. Plaque-purified Karp and TA716 strains of *R. tsutsugamushi* were obtained as yolk sac suspensions from J. V. Osterman and T. R. Jerrells. The Gilliam strain was obtained from C. L. Wisseman, Jr., and plaque purified in our laboratory (10, 13). All were propagated in embryonated eggs and subsequently in irradiated Vero cells (13). A yolk sac suspension of plaque-purified *R. rickettsii* strain Sheila Smith was also donated by C. L. Wisseman, Jr., and subsequently passaged four times in irradiated Vero cells. Encephalomyocarditis virus (EMCV) was provided by R. Kamin-Lewis and was passaged by overnight culture in Vero cells.

Cell culture. The BALB/3T3 clone A31 (derived from BALB/c mouse embryos) and C3H/10T1/2 clone 8 (from C3H mouse embryos) fibroblast cell lines and Vero cells were purchased from the American Type Culture Collection (Rockville, Md.). The tissue culture medium used throughout was TCM (antibiotic-free RPMI 1640 supplemented with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] and 5% controlled process serum replacement-4 [CPSR-4; Sigma Chemical Co., St. Louis, Mo.]) (13). All cultures, infected or not, were incubated at 37°C in a humidified incubator with an atmosphere of 95% air and 5% CO_2.

Infection of suspended cells and rickettsial growth curves. The methods of infecting suspended cells and constructing rickettsial growth curves have been described in detail previously (11, 13). Briefly, cells were removed from monolayers with trypsin, centrifuged, resuspended in TCM, irradiated, and mixed in tubes with rickettsiae which had been appropriately diluted in TCM. After a 1-h absorption period at 37°C, the cells were washed by centrifugation, distributed to 8-chamber tissue culture slides (LabTek Division of Miles Laboratories, Inc., Napierville, Ill.), and incubated at 37°C. At appropriate intervals, slides were stained with Giemsa stain for counting of rickettsiae. At least three sets of 100 cells (each) were counted for each condition.

Plaque assays. The microplaque assay for infectious *R. tsutsugamushi* and *Rickettsia rickettsii* has been described previously (12, 13). All *R. tsutsugamushi* plaques were counted on day 14 after infection, and *R. rickettsii* plaques were counted on day 6 after infection. EMCV was plaqued similarly to *R. rickettsii* (no chicken serum in the overlay medium), but the plaques were visualized by staining methanol-fixed cultures with crystal violet, one day after infection.

IFN assays. Purified mouse IFN-α/β (4.7×10^5 IU/mg of protein) was obtained from ICN Biochemicals, Cleveland, Ohio. Recombinant rat IFN-γ (10^7 IU/mg of protein) was purchased from Amgen Biologicals (Thousand Oaks, Calif.). IFN-α/β was reconstituted in complete attachment-penetration buffer at pH 7.4 (AP; 11) and stored frozen in aliquots at −80°C; IFN-γ was reconstituted and stored as described previously (13).

IFN assays were performed as described previously (13). For growth curve assays in chamber slides, cell cultures just reaching confluency in flasks were pretreated with IFN 18 to 20 h before being trypsinized and infected in suspension (as described above). For plaque reduction assays, irradiated cells were seeded into 2-cm^2 wells 2 days before infection; the monolayers were treated with IFN the following day, 1 day before plaquing. In plaque reduction experiments, cultures were treated with one to three concentrations of IFN and infected with serial 10-fold dilutions of the infectious agent. The infectious titers (PFU/milliliter) in IFN-treated cultures were compared with those in the corresponding mock-treated cells, and the results are given as percentages of control PFU/milliliter. The statistical significance of the results was determined within each experiment by Student's *t* test, with $P < 0.05$ the cutoff for significant difference between the IFN-treated and mock-treated cultures. In comparative assays, the Gilliam strain was always included as a reference. In most comparative assays, all three *R. tsutsugamushi* strains or all five infectious agents were tested in both cell lines at once. IFN was added after infection only when indicated in the text.

RESULTS

Replication of Karp and Gilliam strains in cultured BALB/3T3 and C3H cell lines. Preliminary to experiments with interferon in cultured BALB/3T3 and C3H cells, it was important to establish the comparative replicative properties of Gilliam and Karp strains in the absence of added IFN. Previous studies had shown that Gilliam strain rickettsiae

TABLE 1. Replication of *R. tsutsugamushi* strains in untreated BALB/3T3 and C3H cell lines[a]

Rickettsial strain	Ratio (mean ± SE) of parameter in BALB/3T3 and C3H cells (*n*)[b] [statistical significance][c]		
	Generation time[d]	PFU/ml	Plaque size
Gilliam	1.06 ± 0.02 (3) [NS]	0.69 ± 0.12 (7) [NS]	0.80 ± 0.05 (7) [$P < 0.05$]
Karp	0.95 ± 0.06 (3) [NS]	1.55 ± 0.27 (6) [NS]	0.81 ± 0.11 (6) [NS]

[a] The ratios of a parameter in BALB/3T3 cells divided by the same parameter in C3H cells were determined directly in experiments using both types of cells at the same time.

[b] Number of experiments in which BALB/3T3 and C3H were compared directly.

[c] Statistical significance determined by paired *t* test, comparing values obtained in BALB/3T3 and C3H within the same experiments. NS, not statistically significant ($P > 0.05$).

[d] Generation time was calculated as rickettsial doubling time (in hours) by regression analysis of the growth curves during the logarithmic phase of growth (within 96 h after infection).

replicated slightly faster and formed larger plaques than did the Karp strain in the two mouse cell lines; also, at least in the C3H cells, both replicated faster than TA716, which formed the same-size plaques as Gilliam in BALB/3T3 and C3H cells (10, 12). The possible effect of cell type on rickettsial replication was determined directly either by counting stained, cell-associated rickettsiae at intervals after infection or by performing plaque assays in parallel cultures of irradiated BALB/3T3 and C3H cells. The resulting data are presented in Table 1 as the mean ratios obtained by comparing rickettsial generation time or plaque titer or size in BALB/3T3 and C3H cells in the same experiments; statistical significance was determined with paired *t* tests by considering each experiment individually. Such analysis of several experiments indicated that rickettsial replication could not be distinguished in these two cell types. Although in some experiments the plaque titer of Gilliam was significantly lower and that of Karp was significantly higher in BALB/3T3 than in C3H cells, these differences (reflected in the mean ratios in Table 1) were not found consistently. The slightly smaller plaque size in BALB/3T3 cultures, regardless of the rickettsial strain, likewise was not a consistent finding and may reflect observable differences in the density and morphology of the monolayers.

Effect of IFN-γ on replication of *R. tsutsugamushi* Karp in BALB/3T3 cells. In a growth curve type assay in chamber slides, the IFN-γ susceptibility of the Karp strain of *R. tsutsugamushi* was tested in irradiated BALB/3T3 cells which had been treated with 500 IU of IFN-γ per ml both 18 h before and immediately after infection (Fig. 1). Under these conditions, Karp appeared to be completely insensitive to the double treatment with IFN-γ, as measured by counting the number of cell-associated rickettsiae (Fig. 1) or by determining the percentage of cells infected (not shown) over a 72-h period. The Karp strain's apparent resistance to IFN-γ was in stark contrast to my earlier finding that the replication of strain Gilliam was markedly inhibited by similar treatment with IFN-γ in the same cell line (13). The lack of susceptibility of Karp to IFN-γ-mediated inhibition in BALB/3T3 cells was confirmed when the cells were pretreated with a wide range of IFN-γ concentrations (Fig. 2). Karp rickettsiae were not inhibited even by 1,000 IU/ml when either cell-associated rickettsiae per cell at 48 h postinfection or rickettsia-induced cytopathology at 96 h was assessed. Again, this differs from the finding that maximum inhibition of Gilliam replication and cytopathic effect was achieved by similar treatment with as low a dose as 33 IU of IFN-γ per ml (13).

Effect of IFN-γ on adhesion of infected cells. A cytotoxic effect of IFN-γ pretreatment on Gilliam-infected BALB/3T3 cells has been described previously (13). This was shown by the failure of cells which had been pretreated with IFN-γ and then infected in suspension with Gilliam to attach to the surface of the chamber slide as well as did untreated, Gilliam-infected cells. To see whether this cytotoxic effect of IFN-γ also occurred in Karp-infected BALB/3T3 cells, monolayer flask cultures were pretreated with half-log dilutions of IFN-γ for 18 h and then the cells were trypsinized and suspended in TCM. Rickettsiae were added and permitted to infect the suspended cells for 1 h, after which the cells were washed to remove unadsorbed rickettsiae and distributed to slide chambers. Twenty-four hours later, the cells which had attached to the slides were counted, and the results are shown in Fig. 3. Attachment of Karp-infected BALB/3T3 cells was not inhibited by prior exposure to IFN-γ; if anything, high concentrations (333 to 1,000 IU/ml) slightly increased the number of adhering cells.

Effect of IFN-γ on replication of *R. tsutsugamushi* strains, *R. rickettsii*, and EMCV in cultured mouse cells. Further studies of comparative IFN-γ susceptibility were done by the plaque reduction assay, which also is capable of demon-

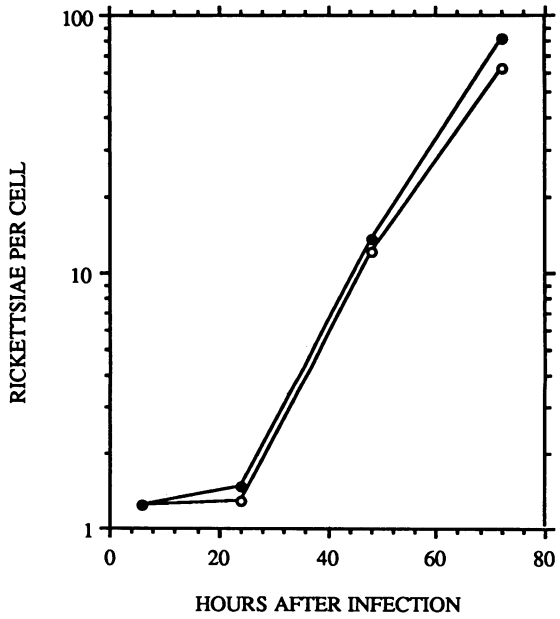

FIG. 1. Replication of *R. tsutsugamushi* Karp in mock-treated (○) or IFN-γ-treated (●) BALB/3T3 cells. The IFN treatment was 500 IU/ml 18 h before infection and 500 IU ml immediately after infection. Each point is the mean number of cell-associated rickettsiae per cell.

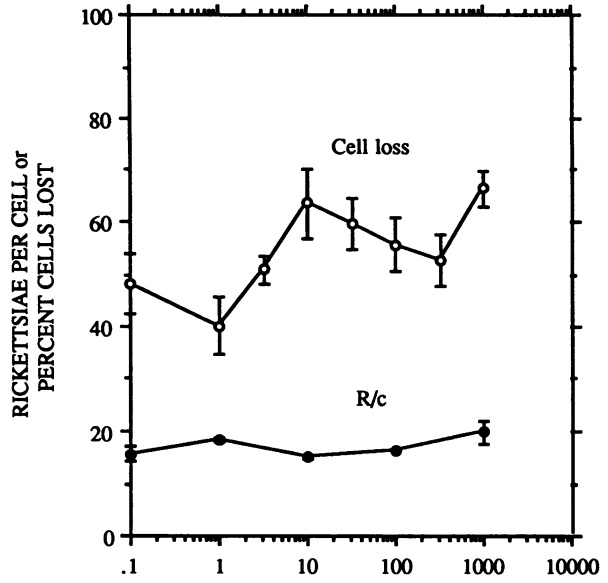

FIG. 2. Effect of IFN-γ pretreatment on replication of *R. tsutsugamushi* Karp in BALB/3T3 cells. For each IFN-γ concentration, the values for cell-associated rickettsiae per cell (*R/c*) at 48 h after infection and cell loss at 96 h after infection were determined. The percentages of cells detached from the cultures were determined after subtracting the numbers of cells remaining at 96 h from those present at 24 h.

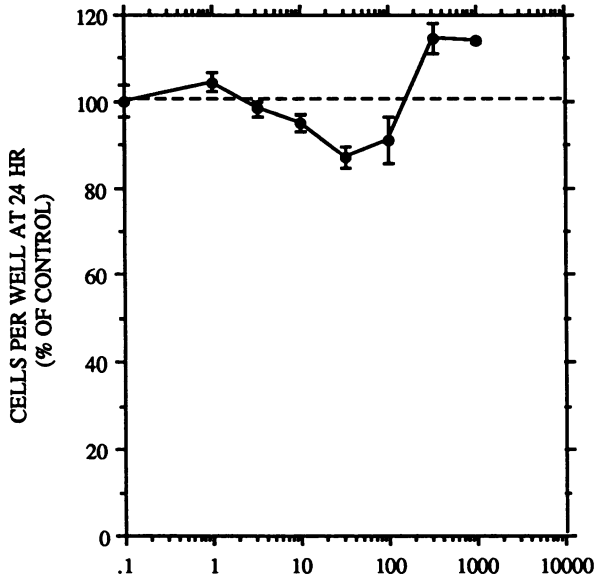

FIG. 3. Cytotoxicity of IFN-γ in rickettsia-infected cultures. Effect of pretreatment of BALB/3T3 cells with IFN-γ and infection with *R. tsutsugamushi* Karp on the capacity of the cells to adhere to glass surfaces under standard tissue culture conditions. Pretreated cells were infected in suspension and immediately distributed to chamber slides and incubated at 37°C. Adhered cells were counted 24 h later, and results are given as percentages of untreated control cells which adhered.

strating the IFN-γ-mediated inhibition of Gilliam in BALB/3T3 cells (13). The *R. tsutsugamushi* strains were tested with 300 to 400 IU of IFN-γ per ml, about 10 times the amount needed for maximum inhibition of Gilliam rickettsiae in BALB/3T3 cells. Lower doses were used for *R. rickettsii* and EMCV when it was found that they were more sensitive to IFN-γ (Table 2). Of the six scrub typhus rickettsial strain-cell combinations, IFN-γ was effective only in Gilliam-infected BALB/3T3 cells: IFN-γ treatment did not affect Gilliam replication in C3H cells, nor did it alter the replication of Karp or TA716 in either cell type. To determine whether scrub typhus rickettsiae were unique in their resistance to IFN-γ-mediated inhibition in C3H cells, another species, *R. rickettsii*, and a virus, EMCV, were tested (Table 2). The plaque titers of these infectious agents were reduced to a much greater extent than were those of the scrub typhus rickettsiae; the doses (300 to 400 IU/ml) which inhibited Gilliam plaque formation by about 67% inhibited both *R. rickettsii* and EMCV by more than 99.9%. This was true in both cell types and in irradiated as well as nonirradiated cultures. Treatment with lower IFN-γ concentrations revealed that both *R. rickettsii* and EMCV were more sensitive to IFN-γ in BALB/3T3 cells than in C3H cells: 3 to 10 times more IFN-γ was required to effect the same degree of inhibition of these agents in BALB/3T3 cells than in C3H cultures.

Gilliam plaques formed in IFN-γ-treated BALB/3T3 cells were no smaller than those in untreated cultures (13). This lack of effect on plaque size extended to the other two *R. tsutsugamushi* strains and to both cell types. In contrast, EMCV and *R. rickettsii* plaque sizes were reduced in IFN-γ-treated BALB/3T3 and C3H cells (59 to 86% of the plaque diameters in parallel, untreated cultures [$P < 0.05$ to $P < 0.001$]).

Effect of IFN-α/β on replication of *R. tsutsugamushi* strains, *R. rickettsii*, and EMCV in cultured mouse cells. The effect of various concentrations of purified mouse IFN-α/β on *R. tsutsugamushi* Gilliam and on EMCV plaque formation was tested in BALB/3T3 and C3H cell monolayers, respectively (Fig. 4). EMCV, as expected, was inhibited by very low concentrations of the IFN-α/β, with 1 IU/ml resulting in 60% inhibition in this experiment. In contrast, a 50% decrease of plaque inhibition of Gilliam in BALB/3T3 cultures was achieved only with IFN-α/β concentrations over 300 IU/ml. Thus, the sensitivity of Gilliam plaque formation to IFN-α/β was at least 300-fold less than that for EMCV.

Comparative plaque reduction assays were performed with purified mouse IFN-α/β as had been done with the IFN-γ (Table 3). For the sake of economy, doses of 300 to 450 IU/ml were used rather than the more strongly inhibitory 1,000 IU/ml (Fig. 4). Karp in BALB/3T3 and C3H cells and Gilliam in C3H cells always gave negative results. In contrast, the remaining combinations, Gilliam in BALB/3T3 and TA716 in either cell type, were significantly ($P < 0.05$) inhibited in some experiments but not in others. Likewise, *R. rickettsii* was slightly inhibited by 300 to 450 IU/ml in half the experiments, but not in the other half. In none of these cases of rickettsial inhibition was the degree of inhibition very great, even with the large doses of IFN-α/β used. The inconsistencies in these experiments probably reflect the fact that the IFN-α/β concentrations used were at the borderline of the MIC (Fig. 4). Still, these results do suggest the greater sensitivity of some rickettsial strain-cell line combinations to IFN-α/β than of others. EMCV, on the other hand, was very

TABLE 2. Plaque formation of *R. tsutsugamushi* Gilliam, Karp, and TA716 and of *R. rickettsii* and EMCV in IFN-γ-treated mouse cells

Rickettsia strain or virus	Amt (IU/ml) of IFN-γ	BALB/3T3 cells			C3H cells			*P* (3T3 vs. C3H)
		No. of positive expts[a]/total no. of expts	PFU/ml (% of mock-treated control, mean ± SE)	*P*[b]	No. of positive expts/total no. of expts	PFU/ml (% of mock-treated control, mean ± SE)	*P*	
Gilliam	300–400	5/5	33.4 ± 6.1	<0.001	0/4	112.0 ± 27.6	NS	<0.01
Karp	300–400	0/4	80.9 ± 9.4	NS	0/3	121.9 ± 14.4	NS	NS
TA716	300–400	1/5	98.1 ± 32.2	NS	0/3	100.6 ± 5.5	NS	NS
R. rickettsii	1.0	1/1	25.8	<0.01	1/2	92.1 ± 14.1	NS	
	3.0	2/2	9.8 ± 5.8	<0.001	3/3	44.0 ± 7.0	<0.01	
	10.0	2/2	0.4 ± 0.0	<0.001	3/3	25.2 ± 5.8	<0.001	
	≥30.0	4/4	<0.5	<0.001	3/3	20.7 ± 9.4	<0.001	
	Total	8/8			8/8			<0.01
EMCV	0.1	1/1	50.7	<0.05	0/1	94.2	NS	
	0.3	1/1	8.9	<0.001	1/1	69.8	<0.05	
	1.0	3/3	<0.1	<0.001	3/3	26.6 ± 4.7	<0.001	
	≥3.0	4/4	<0.5	<0.001	4/4	<0.5	<0.001	
	Total	5/5			4/5			<0.02

[a] Number of positive experiments showing significant inhibition by IFN-γ.

[b] Statistical significance determined by paired *t* tests, comparing IFN-γ with mock treatment or C3H cells with BALB/3T3 cells (compared in the same experiment). NS, differences not significant ($P > 0.05$). (Although the results are listed as percentages, the paired *t* tests were done on the raw data [PFU/milliliter].)

susceptible to inhibition by IFN-α/β in all of the experiments and more so in the BALB/3T3 cultures than in the C3H cultures. As with IFN-γ, IFN-α/β did not affect the plaque size of *R. tsutsugamushi* in either cell type, while it did reduce the plaque sizes of *R. rickettsii* and EMCV in BALB/3T3 and C3H cultures (53 to 86% of the plaque diameters in parallel, untreated cultures [$P < 0.01$ or $P < 0.001$]).

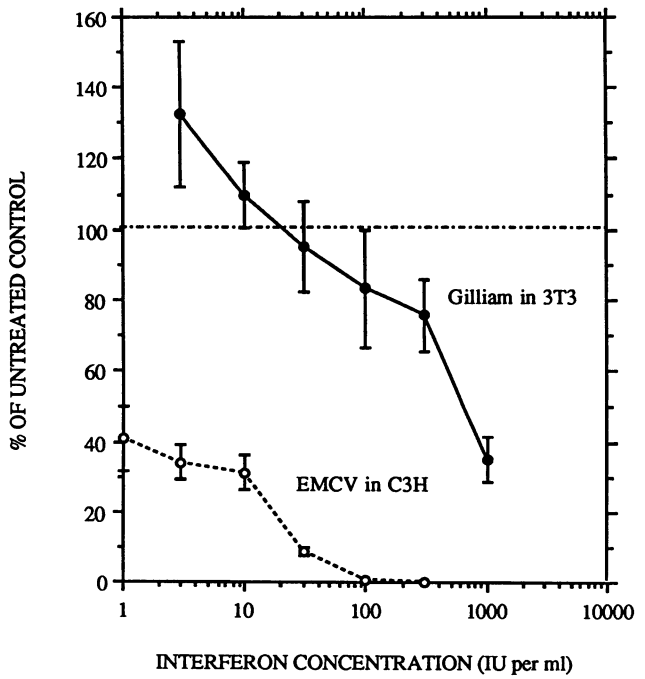

FIG. 4. IFN-α/β-mediated inhibition of *R. tsutsugamushi* Gilliam and EMCV plaque formation in BALB/3T3 and C3H mouse cell lines, respectively. Results are given as percentages of PFU/milliliter in mock-treated control cultures.

DISCUSSION

In contrast to the previously demonstrated IFN-γ-mediated inhibition of *R. tsutsugamushi* Gilliam growth in BALB/3T3 cells (13), similar infection with Karp strain completely resisted the effects of as large a dose as 1,000 IU of IFN-γ per ml. Both manifestations of Gilliam inhibition, clearance of intracellular rickettsiae and decreased initial adherence of the treated cells infected in suspension, were absent in the Karp infections. This striking difference between Karp and Gilliam strains was also found in the second type of IFN-γ assay, reduction of plaque formation in mouse fibroblast monolayers. In fact, the combination of Gilliam rickettsiae and BALB/3T3 cells was the only *R. tsutsugamushi*-cell line pair in which IFN-γ did affect plaque formation: Gilliam in the C3H-derived cell line and Karp and TA716 in either cell line were not inhibited at all. The somewhat surprising insensitivity of the scrub typhus rickettsiae to IFN-γ led us to examine another species, *R. rickettsii*, as well as EMCV. Both of these infectious agents were much more sensitive to IFN-γ-mediated plaque reduction in either cell line than was Gilliam in BALB/3T3 cells. Comparable inhibition was attained with over 100-fold-less IFN-γ than was required for inhibition of Gilliam. Moreover, IFN-γ inhibited both *R. rickettsii* and EMCV to a greater degree in the BALB/3T3 than in the C3H cells.

Similar comparative plaque reduction assays done with 300 to 450 IU of purified mouse IFN-α/β per ml demonstrated minimal sensitivity of *R. tsutsugamushi* to this cytokine as well. Three scrub typhus rickettsiae-cell line pairs were consistently negative: Gilliam in C3H and Karp in either BALB/3T3 or C3H. In the other combinations, half or fewer of the experiments demonstrated IFN-α/β-mediated reduction in plaque number. The response of Gilliam to treatment of BALB/3T3 cells with increasing doses of IFN-

TABLE 3. Plaque formation of *R. tsutsugamushi* Gilliam, Karp, and TA716 and of *R. rickettsii* and EMCV in IFN-α/β-treated mouse cells

Rickettsia strain or virus	Amt (IU/ml) of IFN-α/β	BALB/3T3 cells			C3H cells			P (3T3 vs. C3H)
		No. of positive expts[a]/total no. of expts	PFU/ml (% of mock-treated control, mean ± SE [range])	P[b]	No. of positive expts/total no. of expts	PFU/ml (% of mock-treated control, mean ± SE [range])	P	
Gilliam	300–450	2/5	51.4 ± 7.2 (35.5–75.3)	<0.01	0/3	87.4 ± 10.3 (62.1–100.0)	NS	NS
Karp	300–450	0/4	76.5 ± 8.4 (50.0–92.5)	NS	0/3	123.6 ± 10.8 (104.5–149.1)	NS	NS
TA716	300–450	1/4	67.3 ± 11.5 (42.4–100.9)	NS	1/3	47.0 ± 3.3 (41.2–54.7)	<0.01	NS
R. rickettsii	300–450	2/4	52.8 ± 9.9 (23.7–78.7)	<0.02	2/4	64.3 ± 8.7 (45.9–90.9)	<0.05	NS
EMCV	10	4/4	8.3 ± 3.4 (2.3–19.5)	<0.001	5/5	49.5 ± 4.5 (31.1–61.0)	<0.01	<0.01
	300	ND[c]	ND	ND	1/1	0.03	<0.001	

[a] Number of positive experiments showing significant inhibition by IFN-α/β.
[b] Statistical significance determined by paired *t* tests, comparing IFN-α/β treatment with mock treatment or C3H cells with BALB/3T3 cells (compared in the same experiment). NS, differences not significant ($P > 0.05$). (Although the results are listed as percentages, the paired *t* tests were done on the raw data [PFU/milliliter].)
[c] ND, not done.

α/β (Fig. 4) suggests that more consistent inhibition would have been attained in these experiments with higher IFN-α/β concentrations. The results with *R. rickettsii* were similar to those with *R. tsutsugamushi*, in that 300 to 450 IU of IFN-α/β per ml inhibited plaque formation in only half the experiments, and this was true in both cell types. The marked sensitivity of EMCV to this inhibitor served as a control for the integrity of the IFN-α/β preparations used: as low a dose as 10 IU/ml inhibited EMCV plaque formation consistently in both cell types, and as with IFN-γ, the BALB/3T3 cells were more sensitive than the C3H cells.

The mean inhibition by high doses of IFN-α/β of Gilliam in BALB/3T3 cells and of TA716 and *R. rickettsii* in both cell lines was about 40 to 50%. This is comparable to the maximum inhibition reported by Turco and Winkler for *Rickettsia prowazekii* in L929 cells treated with IFN-α/β (41) and can be contrasted to the roughly 80% maximum inhibition of Gilliam in BALB/3T3 cells and *R. prowazekii* in L929 cells by recombinant rodent IFN-γ (13, 39, 41). (EMCV, on the other hand, was inhibited by 99.97% or greater by 300 IU of IFN-α/β per ml.) To my knowledge, the present study is the first to examine the effects of IFN-γ and IFN-α/β on the replication of *R. rickettsii*, although other members of the spotted fever group have been shown to be sensitive to IFN-γ (20, 38). In addition, *Rickettsia akari*, also a spotted fever rickettsia, was inhibited by virus-induced IFN in L cells; similar to our results with IFN-α/β, more than 200 times the IFN dose required for inhibition of vesicular stomatitis virus was needed to inhibit *R. akari* (22). From these experiments, it appears that IFN-α/β in itself is not a very potent inhibitor of rickettsiae from the typhus, spotted fever, and scrub typhus groups, at least when tested in fibroblast cell lines. This is not to say that IFN-α/β cannot significantly affect the outcome of rickettsial infections. In fact, endogenous IFN-α/β was shown to influence *R. prowazekii* yields in bulk L929 cell cultures, and persistently infected L929 cultures gave rise to *R. prowazekii* variants that were resistant to IFN-α/β (as well as to IFN-γ) (42, 43).

The two mouse cell lines tested, BALB/3T3 clone 31 and C3H clone 8, differed in their responses to both IFN-γ and IFN-α/β, regardless of the infecting agent. Thus, BALB/3T3 cells were more sensitive to both cytokines in limiting EMCV infections, and they also supported greater inhibition of R. rickettsii and Gilliam by IFN-γ. Differences in sensitivity to IFNs among cell lines are not uncommon (4). In light of this and other observations outlined below, the relevance of the greater sensitivity of Gilliam to IFN-γ-mediated inhibition in the BALB/3T3 cells to the genetically determined resistance of BALB/c mice to this strain is dubious. If there were a definitive correlation between sensitivity to IFN-γ and nonvirulence in mice, then one would expect that TA716 would be inhibited by IFN-γ in both cell lines, and the opposite was found. Moreover, treatment of C3HRV and C3H/He macrophage cultures with mouse lymphokines containing IFN-γ inhibited Gilliam growth equally (27), and the lack of a role of IFN-γ in the *Ric* gene-associated resistance to scrub typhus rickettsial infection in mice was also indicated by the failure to detect IFN-γ mRNA in peritoneal cells until the fifth or sixth day after intraperitoneal infection of either *Ric*^{s} or *Ric*^{r} mice with Gilliam, long after the resistant state had been manifested (31). As far as it goes, all the existing data suggest that IFN-γ per se does not play the determining role in *R. tsutsugamushi* virulence in mice, although they do not preclude the possibility of an accessory or protective role for IFN-γ in later stages of primary or secondary infections.

The consistent insensitivity to IFN-α/β of Gilliam in C3H cultures and of Karp in BALB/3T3 and C3H cultures does correlate with the virulence of these scrub typhus rickettsial strains in BALB/c and C3H mice. Likewise, the sometime inhibition by IFN-α/β of Gilliam in BALB/3T3 and TA716 in both BALB/3T3 and C3H cells correlates with the lack of virulence of Gilliam and TA716 in BALB/c and in BALB/c and C3H mice, respectively. Nonetheless, conclusions about the biological relevance of these observations would be very premature. Continuous cell lines were used in these initial studies for the sake of convenience, particularly in the plaque assays. Obviously, a more direct assessment of the role of host cell genotype on IFN-mediated inhibition of scrub typhus rickettsial replication requires the use of cell cultures derived directly from genetically resistant and susceptible mice, including macrophages, which feature prominently in natural infections. Acid-stable IFN was reported to appear in serum 1 to 3 h after intravenous infection of mice with *R. prowazekii* (21); this time frame is more compatible with the resistance of naive mice to certain *R. tsutsugamushi* strains than is the apparently much longer lag in IFN-γ production.

When *R. tsutsugamushi* replication in untreated BALB/3T3 and C3H cells was compared during 4-day growth curves, the derived generation times were indistinguishable. (Although Gilliam replicated faster than Karp, it did so to the same extent in both types of cells [Table 1; 10].) This agrees with earlier reports that in short-term experiments, Gilliam grew equally in untreated mouse embryo fibroblast cultures derived from BALB/c and C3H/He mice (8) and in macrophages cultured from BALB/c and C3H/He mice (27). On the other hand, when long-term replication was assessed, i.e., in the 2-week plaque assays, the plaque titer of Gilliam was sometimes significantly lower and the plaque titer of Karp was sometimes significantly higher in BALB/3T3 cells than in C3H cultures (Table 1). This trend correlates with the also inconsistent reduction in Gilliam, but not Karp, plaque numbers in IFN-α/β-treated BALB/3T3 cultures. Thus, it is conceivable that the sometimes reduced number of plaques formed by Gilliam in BALB/3T3 cultures relative to those in C3H cells is due to the effects of endogenous IFN-α/β. Further, if endogenous IFN-α/β were affecting *R. tsutsugamushi* plaque formation during the 2-week incubation, this might minimize the effects of any added IFN-α/β. (Note, however, that *R. rickettsii* plaque assays were incubated for only 6 days.) This question might be clarified by determining whether the addition of antibody to IFN-α/β increased the plaque titers of scrub typhus rickettsiae (and *R. rickettsii*) in mouse cultures, as it increased the yields of *R. prowazekii* in L929 cell cultures (42). *R. tsutsugamushi* Karp has been shown to induce an acid-stable IFN-like viral inhibitor in cultured chicken embryo cells (14).

These studies have shown a remarkable difference between Karp and TA716, on one hand, and Gilliam, on the other, in their sensitivity to IFN-γ in the BALB/3T3 cell line. Although differences in the antigens and proteins of these strains are readily demonstrable and although *R. tsutsugamushi* strains have long been known to differ in their virulence in mice, nothing is understood about the physiological differences among them. The difference in sensitivity to IFN-mediated inhibition reported here is the only biological parameter other than growth rate and plaque size which has been observed. This knowledge should provide a valuable tool for exploring *R. tsutsugamushi* strain variation at the level of the organisms' interaction with host cells. Such a handle has been missing heretofore. Regardless of the ultimate effect of the mouse *Ric* gene or of other host components leading to survival of scrub typhus rickettsial infections, the rickettsial factors which contribute to virulence remain an open but intriguing question.

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