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# Comparison of Properties of Virulent, Avirulent, and Interferon-Resistant *Rickettsia prowazekii* Strains

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**Several properties of virulent, avirulent, and interferon-resistant *Rickettsia prowazekii* strains were compared. All of the interferon-resistant rickettsial strains (which were derived from the avirulent Madrid E strain) resembled the virulent Breinl strain in that they grew well in untreated mouse macrophagelike RAW264.7 cells. In contrast, the avirulent Madrid E strain grew poorly in untreated RAW264.7 cells. Pretreatment of interferon-resistant rickettsiae or *R. prowazekii* Breinl with antirickettsial serum or immunoglobulin G suppressed the ability of the rickettsiae to grow in untreated RAW264.7 cells. Interferon-resistant *R. prowazekii* strains, like the Madrid E and Breinl strains, rapidly killed a substantial proportion of RAW264.7 cells that had been treated with gamma interferon or very high concentrations of alpha/beta interferon. Untreated infected RAW264.7 cells and interferon-treated mock-infected RAW264.7 cells were not killed during the same period. In cultures of RAW264.7 cells treated with either alpha/beta interferon (120 to 1,200 U/ml) or a subsaturating concentration of gamma interferon (0.5 U/ml), *R. prowazekii* Breinl organisms killed a higher percentage of the cells than did comparable numbers of *R. prowazekii* Madrid E organisms or interferon-resistant rickettsiae. Although *R. prowazekii* Breinl (like *R. prowazekii* Madrid E) was quite sensitive to gamma interferon in mouse L929 cells, the Breinl strain was resistant to murine alpha/beta interferon compared with the Madrid E strain and the two strains selected for resistance to murine gamma interferon. One of the interferon-resistant strains (strain 60P, which was selected for resistance to murine alpha/beta interferon) differed from the other *R. prowazekii* strains in that it induced little or no detectable interferon in L929 cell cultures.**

Members of the genus *Rickettsia* are obligate intracellular bacteria that grow in the cytoplasm (and sometimes in the nucleus) of their host cell (33). Within their host cells, the rickettsiae are found free in the cytoplasm and are not surrounded by phagosomal or phagolysosomal membranes. *Rickettsia prowazekii*, which grows only in the cytoplasm, is the etiological agent of epidemic typhus in humans. An avirulent strain of *R. prowazekii* (the Madrid E strain) originated when the virulent Madrid strain spontaneously lost its virulence during egg passage (4, 18).

The biochemical basis for virulence in *R. prowazekii* has not been defined. However, Gambrill and Wisseman (6) found that the virulent Breinl strain of *R. prowazekii* grows well in human monocyte-derived macrophages, whereas the avirulent Madrid E strain grows poorly in these cells. Later work indicated that the Madrid E strain also grows poorly in several mouse macrophagelike cell lines that support good growth of the virulent Breinl strain (22). The ability of *R. prowazekii* Breinl to grow in human monocyte-derived macrophages or mouse macrophagelike cells is inhibited by pretreatment of the rickettsiae with antirickettsial serum, immunoglobulin G (IgG), or $F(ab')_2$ fragments derived from IgG (3, 14, 22).

Gamma interferon (IFN-γ) plays an important role in host defense against rickettsial infections. Mice that normally resist challenge with *Rickettsia conorii* become susceptible when given monoclonal antibody against IFN-γ (15). In addition, IFN-γ suppresses the growth of *R. conorii* in mouse macrophages in vitro (10). Murine IFN-γ also inhibits the growth of *R. prowazekii* Madrid E in mouse L929 cells and the growth of *R. prowazekii* Breinl in mouse macrophagelike cells (23, 26–28, 35). Similarly, in human fibroblasts, human IFN-γ inhibits the growth of *R. prowazekii* Madrid E (27) and *R. prowazekii* Breinl (37). However, there is no published information about the effect of murine IFN-γ on the growth of *R. prowazekii* Breinl in mouse L929 cells.

The combination of treating cells with IFN-γ and infecting them with *R. prowazekii* can damage the host cells (26, 28, 29, 37). When mouse macrophagelike RAW264.7 cells are treated with IFN-γ and infected with *R. prowazekii* Madrid E or Breinl, a substantial proportion of the host cells are killed within 4 to 6 h after infection (26). This rapid toxic effect is not observed in IFN-γ-treated mock-infected RAW264.7 cells or in untreated infected RAW264.7 cells. Mouse fibroblasts are also damaged by the combination of IFN-γ treatment and *R. prowazekii* Madrid E infection; however, host cell death occurs more slowly in L929 cell cultures than in RAW264.7 cell cultures (26, 28, 29).

Like IFN-γ, murine IFN-α/β alters the interaction of rickettsiae and cultured cells: growth of *Rickettsia akari* (13) and *R. prowazekii* Madrid E (30) in mouse L929 cells is suppressed by IFN-α/β. Comparison of the inhibitory effects of murine IFN-α/β and IFN-γ on *R. prowazekii* Madrid E indicated that IFN-α/β is less inhibitory than IFN-γ (30). It is not known if IFN-α/β can also inhibit the growth of *R. prowazekii* Breinl. The combination of treatment with IFN-α/β and infection with *R. prowazekii* Madrid E can also damage the L929 cells (30). In addition, *R. prowazekii* Madrid E induces the production of IFN-α and/or IFN-β in mouse L929 cells (30), and *R. prowazekii* Breinl induces the production of IFN-α and/or IFN-β in mice (11).

*R. prowazekii* organisms with reduced sensitivity to mu-

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TABLE 1. Properties of *R. prowazekii* strains

| Rickettsial strain | Parental strain | Selection method | Resistance to IFN in L929 cells (compared with Madrid E) | | Growth in untreated RAW264.7 cells | Suppression of growth in untreated RAW264.7 cells by antibody | Capacity for killing IFN-treated RAW264.7 cells | IFN induction in L929 cells (avg U/ml, day 4) | References |
|---|---|---|---|---|---|---|---|---|---|
| | | | IFN-α/β | IFN-γ | | | | | |
| Madrid E (avirule 't) | Madrid 1 | ? | NA[a] | NA | Poor | Yes | High | 239 | 22, 23, 26, 30; this study |
| Breinl (virulent) | NA | NA | Yes | No | Good | Yes | Very high | 152 | 14, 22, 26; this study |
| 427-19 | Madrid E | IFN-γ-treated L929 cells | Yes | Yes | Good | ND[b] | ND | 330 | 29, 31; this study |
| 87-17 | Madrid E | IFN-γ-treated L929 cells | No | Yes | Good | Yes | High | 93 | 29, 31; this study |
| 60P | Madrid E | L929 cells | Yes | Yes | Good | ND | High | 4 | 31; this study |
| 83-2P | Madrid E | L929 cells | Yes | Yes | Good | Yes | High | 48 | 31; this study |
| 103-2P | Madrid E, plaque purified | L929 cells | Yes | Yes | Good | ND | ND | 81 | 31; this study |
| 110-1P | Madrid E, plaque purified | L929 cells | Yes | Yes | Good | Yes | High | 184 | 31; this study |

[a] NA, Not applicable.
[b] ND, Not determined.

rine IFN-γ have been isolated from infected L929 cells treated with IFN-γ for several weeks (29). In addition, rickettsiae resistant to both murine IFN-α/β and murine IFN-γ have been isolated after culture of *R. prowazekii* in untreated L929 cells for several weeks to several months (31). These IFN-resistant rickettsiae were all derived from the avirulent Madrid E strain of *R. prowazekii*. Since other researchers have noted changes in the virulence of the Madrid E strain after passage of the rickettsiae in animals or cell cultures (1, 2, 9, 12) and since the IFN-resistant rickettsiae were selected by passage in L929 cells, it would be most interesting to determine whether the virulence of the IFN-resistant rickettsiae differs from that of the Madrid E strain. In addition, comparison of other properties of the IFN-resistant and parental rickettsial strains will be necessary in order to understand the basis for IFN resistance.

In the present study, several properties of the IFN-resistant *R. prowazekii* isolates were examined and compared with the properties of the virulent Breinl and avirulent Madrid E strains. We report that (i) all of the IFN-resistant strains are similar to the virulent Breinl strain (and dissimilar to their parental Madrid E strain) in that they grow well in untreated macrophagelike cells; (ii) infection of IFN-α/β-treated or IFN-γ-treated macrophagelike cells with the Breinl, Madrid E, or some IFN-resistant rickettsial strain is cytotoxic for a substantial proportion of the cells, but the Breinl strain has a greater capacity than the other strains for killing macrophagelike cells treated with IFN-α/β or with a subsaturating concentration of IFN-γ; (iii) the Breinl strain (although it is very sensitive to IFN-γ treatment in L929 cells) is less sensitive to IFN-α/β treatment in L929 cells than the Madrid E strain; and (iv) one of the IFN-resistant strains (60P) differs from *R. prowazekii* Madrid E, *R. prowazekii* Breinl, and the other IFN-resistant strains in that it induces little or no detectable IFN-α/β in L929 cells.

## MATERIALS AND METHODS

**Cell cultures.** Mouse L929 cells and mouse macrophage-like RAW264.7 cells were obtained and cultured as previously described (21). The culture media for these cell lines were, respectively, Eagle minimal essential medium supplemented with 10% newborn bovine serum and Dulbecco's modified Eagle medium supplemented with 10% newborn bovine serum. Both cell lines were grown in a humidified atmosphere of 3% $CO_2$ in air at 34°C.

**Rickettsiae and antirickettsial antibody.** The parental rickettsial strains and the selection methods previously employed to isolate the plaque-purified, IFN-resistant *R. prowazekii* strains are given in the first three columns of Table 1 (29, 31). Rickettsial suspensions for use in experiments were prepared from the yolk sacs of embryonated hen eggs inoculated with *R. prowazekii* Madrid E, *R. prowazekii* Breinl, or IFN-resistant *R. prowazekii* strains, as previously described (25, 29, 31). The numbers of viable rickettsiae were estimated from their hemolytic activity (32).

For preparation of antirickettsial serum, a rabbit was injected intradermally at five sites with heat-killed *R. prowazekii* Breinl in complete Freund adjuvant (total rickettsial protein, 250 μg). The rabbit was then given weekly subcutaneous injections of killed *R. prowazekii* Breinl in incomplete Freund adjuvant for 3 weeks (250 μg of rickettsial protein per week divided among five injection sites). Two weeks later, the rabbit was injected subcutaneously at five sites with viable *R. prowazekii* Madrid E (without adjuvant) (total rickettsial protein, 500 μg). Serum was collected from the rabbit 3 weeks later, heated at 56°C, and stored at −80°C. The titer of the serum as determined by an indirect enzyme-linked immunosorbent assay which used *R. prowazekii* Madrid E as antigen was $10^5$. IgG was purified from the rabbit serum as previously described (21), and the yield of IgG from the serum was 13.7 mg/ml. To calculate the concentration of IgG present when serum (rather than IgG) was used, the serum was assumed to contain 13.7 mg of IgG per ml.

**IFNs and IFN assay.** Recombinant murine IFN-γ derived from *Escherichia coli* (IFN-γ; $1.9 \times 10^7$ U/mg) was the generous gift of Genentech, Inc., South San Francisco, Calif. A mixture of virus-induced murine IFN-α and IFN-β (IFN-α/β; $4.7 \times 10^5$ U/mg) was purchased from Lee BioMo-

lecular Research Laboratories, Inc., San Diego, Calif. IFN was assayed by a cytopathic effect inhibition assay with L929 cells and vesicular stomatitis virus as previously described (24, 31).

**Evaluation of growth of rickettsiae in untreated RAW264.7 cells and ability of rickettsiae to kill IFN-α/β-treated or IFN-γ-treated RAW264.7 cells.** RAW264.7 cells were exposed to 3,000 rads of X-irradiation to prevent cell division and therefore simplify the evaluation of rickettsial growth (26). The cells were suspended in serum-supplemented medium (MS) at a concentration of $2 \times 10^6$ cells per ml and incubated at 34°C for 1 h with an equal volume of rickettsiae diluted in Hanks balanced salt solution supplemented with 0.1% gelatin and 5 mM potassium glutamate (HBSSGG). The multiplicity of infection was approximately 40 rickettsiae per cell. After being washed, the cells were planted in 24-well plates that contained cover slips ($4 \times 10^5$ cells per well in 0.8 ml of MS), and portions of each cell suspension were immediately centrifuged onto microscope slides and stained for rickettsiae (7). After 48 h, the cover slips were also stained for rickettsiae. Slides and cover slips were examined with an oil immersion objective, and the number of rickettsiae present in each of 100 cells was determined for each duplicate culture. A cell that contained more than 100 rickettsiae was assigned a value of 100; it should be noted that this practice results in underestimation of the numbers of rickettsiae present in heavily infected cultures. The number of rickettsial doublings was calculated from the initial number of rickettsiae per cell and the number of rickettsiae per cell at 48 h.

For experiments with antirickettsial antibody, untreated, X-irradiated RAW264.7 cells were planted in 24-well plates that contained cover slips ($4 \times 10^5$ cells per well) and the cells were incubated overnight. Rickettsiae were diluted in HBSSGG with or without antirickettsial antibody (5 pg of IgG per rickettsia) and incubated at room temperature for 20 min before being further diluted with 2 volumes of HBSSGG and added to the cells at a multiplicity of approximately 2 to 5 viable rickettsiae per cell (0.3 ml per well). The plates were then centrifuged at 500 × g for 15 min at room temperature and incubated at 34°C for an additional 45 min. After being washed, cover slips were immediately removed from duplicate cultures and stained for rickettsiae. Additional cover slips were removed and stained after incubation of the cells for 28 h.

Experiments designed to evaluate the killing of IFN-treated RAW264.7 cells by rickettsiae were performed as previously described (21). Briefly, X-irradiated RAW264.7 cells incubated in MS, MS plus various concentrations of IFN-γ, or MS plus various concentrations of IFN-α/β for 24 h were mock infected or infected with rickettsiae. After being washed, cover slips were immediately removed from duplicate cultures and stained for rickettsiae. Additional duplicate or triplicate wells were incubated for 4 h and then stained with trypan blue for determination of cell viability.

In these experiments, the parameters given for the initial infections with the various strains (percent infected and rickettsiae per infected cell) were averaged from the values obtained in untreated cultures and cultures treated with various concentrations of IFN. It was shown previously that treatment of RAW264.7 cells with IFN-γ at concentrations of 10 U/ml or lower had little or no effect on the ability of the cells to be initially infected with *R. prowazekii* (26). In addition, treatment of the RAW264.7 cells with a wide range of concentrations of IFN-α/β also had little or no effect on the ability of the cells to be initially infected with the various *R. prowazekii* strains (data not shown).

**Comparison of growth of various *R. prowazekii* strains in IFN-treated L929 cells.** Experiments with growing (nonirradiated) L929 cells were performed as previously described (31). IFN-α/β and IFN-γ were used at concentrations which cause maximal inhibition of rickettsial growth in L929 cells (30). Briefly, L929 cells incubated for 2 days in MS, MS plus IFN-α/β (60 U/ml), or MS plus IFN-γ (60 U/ml) were washed and infected with rickettsiae. After additional washing, some cultures were immediately harvested, and the cells were counted and stained for rickettsiae. Additional cultures were harvested, counted, and stained for rickettsiae after incubation with fresh MS, MS plus IFN-α/β, or MS plus IFN-γ for an additional 2 to 3 days. The number of rickettsial doublings was calculated from the number of rickettsiae per culture on the day of infection and the number of rickettsiae per culture after the additional 2 to 3 days. In each experiment, duplicate cultures were harvested for each treatment at each time.

For experiments with X-irradiated L929 cells, cell suspensions were infected with various rickettsial strains, washed, and then planted in eight-chambered glass slides (Nunc, Inc., Naperville, Ill.) as follows. Samples of the infected cells ($3.8 \times 10^4$ cells in 150 μl of MS) were added to wells that contained 150 μl of MS (controls) or 150 μl of MS plus IFN-γ (final IFN-γ concentration, 60 U/ml). Cells were stained for rickettsiae immediately after infection and after incubation for an additional 2 days. Duplicate wells were evaluated, and the number of rickettsial doublings was calculated from the initial number of rickettsiae per cell and the number of rickettsiae per cell after 2 days.

Rickettsial growth rates in the IFN-α/β- and IFN-γ-treated cultures were expressed as percentages of the rates observed in the untreated control cultures, and these percent control values were analyzed by the two-tailed Student's *t* test.

**Evaluation of IFN production in L929 cell cultures infected with various *R. prowazekii* strains.** Suspensions of nonirradiated L929 cells were mock infected or infected with rickettsiae, washed, and planted in MS ($9 \times 10^4$ L929 cells in 1.2 ml of MS in each well of 12-well plates). The cell density used allowed the mock-infected cells to become confluent in about 3 to 4 days. Samples of the cells were stained for rickettsiae immediately after infection and at later times to check the condition of the L929 cells and to ensure that rickettsial growth had occurred. The culture media were collected after 1 to 4 days and assayed for IFN (antiviral activity). In some experiments, the media were filtered (filter pore size, 0.2 μm) before being assayed. Alternatively, unfiltered media were assayed for antiviral activity in the presence of gentamicin (100 μg/ml) and erythromycin (25 μg/ml) to inhibit possible rickettsial infection and growth in the IFN assay. The IFN assay itself was not affected by the addition of these antibiotics to the culture medium.

## RESULTS

**Growth of IFN-resistant *R. prowazekii* strains in untreated macrophagelike RAW264.7 cells.** Because the virulence of IFN-resistant rickettsial strains may differ from that of the Madrid E strain and because virulence in *R. prowazekii* is associated with an ability to grow in macrophages and macrophagelike cells (6, 22), the IFN-resistant strains were evaluated for their abilities to grow in untreated macrophagelike RAW264.7 cells (Table 2). All of the IFN-resistant strains resembled the virulent Breinl strain of *R. prowazekii* in that they grew well in untreated macrophagelike

TABLE 2. Growth of *R. prowazekii* strains in untreated X-irradiated RAW264.7 cells

| Strain | n[a] | Infection[b] at: | | | | No. of doublings[c] |
|---|---|---|---|---|---|---|
| | | Day 0 | | Day 2 | | |
| | | %R | RI | %R | RI | |
| Madrid E | 8 | 67 ± 6 | 5.9 ± 0.7 | 53 ± 10 | 10 ± 2 | 0.0 ± 0.4 |
| Breinl | 5 | 60 ± 8 | 6.0 ± 1.1 | 84 ± 9 | 43 ± 8 | 3.3 ± 0.5*** |
| 427-19 | 2 | 33 ± 3 | 2.4 ± 0.6 | 61 ± 17 | 22 ± 3 | 4.1 ± 0.9** |
| 87-17 | 2 | 53 ± 6 | 3.9 ± 0.7 | 65 ± 18 | 31 ± 9 | 3.1 ± 1.0* |
| 60P | 2 | 44 ± 5 | 3.0 ± 0.5 | 67 ± 11 | 19 ± 2 | 3.3 ± 0.5* |
| 83-2P | 3 | 72 ± 4 | 5.3 ± 1.1 | 99 ± 1 | 73 ± 9 | 4.4 ± 0.2*** |
| 103-2P | 2 | 61 ± 2 | 3.2 ± 0.3 | 100 ± 0 | 66 ± 1 | 5.1 ± 0.2*** |
| 110-1P | 2 | 53 ± 9 | 2.9 ± 0.1 | 98 ± 2 | 58 ± 1 | 5.2 ± 0.3*** |

[a] *n*, Number of experiments with duplicate cultures.
[b] %R, Percentage of cells infected; RI, average number of rickettsiae per infected cell.
[c] Data were analyzed by Student's two-tailed *t* test. A significant difference between a particular rickettsial strain and the Madrid E Strain is indicated by * ($P < 0.025$), ** ($P < 0.01$), or *** ($P < 0.001$).

RAW264.7 cells. In contrast, the Madrid E strain (from which the IFN-resistant *R. prowazekii* strains were derived) grew poorly in untreated RAW264.7 cells.

The effect of pretreatment with antirickettsial serum or IgG on the growth of three of the IFN-resistant *R. prowazekii* strains in untreated RAW264.7 cells was also evaluated (Table 3). In each instance, treatment of IFN-resistant rickettsiae with antirickettsial antibody suppressed their growth in RAW264.7 cells, as did similar treatment of *R. prowazekii* Breinl.

**Killing of IFN-α/β- or IFN-γ-treated RAW264.7 cells by various *R. prowazekii* strains.** Treatment of RAW264.7 cells with high concentrations of IFN-α/β and infection of the cells with similar numbers of *R. prowazekii* organisms of the Breinl, Madrid E, or some IFN-resistant strain (87-17, 60P, 83-2P, or 110-1P) killed substantial numbers of RAW264.7 cells within 4 to 6 h after infection (Fig. 1). In contrast, mock-infected IFN-α/β-treated cells were not killed. Much higher concentrations of IFN-α/β than IFN-γ (measured as antiviral activity) were required for killing of the RAW264.7 cells to occur. Treatment of the RAW264.7 cells with 100 times more IFN-α/β (1,200 U/ml) was required for the percentage of cells killed by *R. prowazekii* Breinl (mean ± standard error, 84% ± 2%) to approach the percentage of cells killed (by *R. prowazekii* Breinl) in cultures treated with 12 U of IFN-γ per ml (92% ± 1%) (Fig. 1). Less than 10% of the RAW264.7 cells were killed in cultures that had been treated with 12 U of IFN-α/β per ml and infected with similar numbers of the various rickettsial strains (Fig. 1). In cultures treated with IFN-α/β at concentrations of 120 to 1,200 U/ml, the mean percentages of cells killed by *R. prowazekii* Breinl were greater than the corresponding percentages of cells killed by *R. prowazekii* Madrid E and the IFN-resistant strains (Fig. 1). Although similar percentages of cells were killed by all of the tested rickettsial strains in cultures treated with 12 U of IFN-γ per ml (Fig. 1), the Breinl strain killed a higher percentage of RAW264.7 cells than at least two of the other rickettsial strains in cultures treated with a subsaturating concentration of IFN-γ (0.5 U/ml). In cultures treated with 0.5 U of IFN-γ per ml, the percentages of cells killed by strains Breinl, Madrid E, and 110-1P were, respectively, 70% ± 5%, 42% ± 3%, and 46% ± 5% (means ± standard

TABLE 3. Inhibition of the growth of IFN-resistant rickettsiae in untreated X-irradiated RAW264.7 cells by pretreatment of the rickettsiae with antirickettsial antibody[a]

| Strain and treatment | Infection[b] | | | | Growth (no. of doublings) |
|---|---|---|---|---|---|
| | Initial | | At 28 h | | |
| | %R | RI | %R | RI | |
| Breinl | | | | | |
| Control | 77 ± 1 | 3.2 ± 0.0 | 74 ± 1 | 11.4 ± 1.7 | 1.8 ± 0.2 |
| Antibody | 81 ± 1 | 3.3 ± 0.1 | 40 ± 3 | 2.5 ± 0.0 | −1.4 ± 0.1 |
| 87-17 | | | | | |
| Control | 84 ± 4 | 4.4 ± 0.7 | 93 ± 0 | 18.2 ± 1.1 | 2.2 ± 0.2 |
| Antibody | 94 ± 3 | 6.0 ± 1.1 | 86 ± 1 | 10.3 ± 1.4 | 0.7 ± 0.1 |
| 83-2P | | | | | |
| Control | 86 ± 1 | 4.6 ± 0.2 | 87 ± 2 | 18.5 ± 0.5 | 2.0 ± 0.1 |
| Antibody | 84 ± 3 | 4.5 ± 0.2 | 67 ± 2 | 5.9 ± 0.1 | 0.1 ± 0.2 |
| 110-1P | | | | | |
| Control | 90 ± 0 | 5.1 ± 0.1 | 85 ± 9 | 16.1 ± 3.0 | 1.5 ± 0.4 |
| Antibody | 92 ± 2 | 7.0 ± 0.5 | 66 ± 1 | 4.5 ± 0.6 | −1.1 ± 0.2 |

[a] Each value represents the mean ± standard error of the mean for two experiments (one with serum and one with IgG).
[b] %R, Percentage of cell infected; RI, average number of rickettsiae per infected cell.

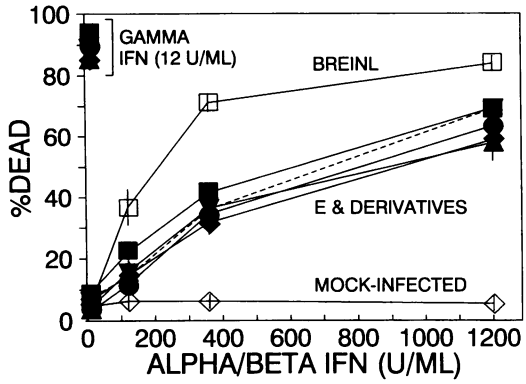


FIG. 1. Killing of IFN-α/β- or IFN-γ-treated RAW264.7 cells by various *R. prowazekii* strains. RAW264.7 cells that had been X-irradiated and pretreated with IFN-α/β or IFN-γ were infected with rickettsiae, and cell viability was determined 4 to 6 h after infection. Immediately after infection, the percentages of cells infected (means ± standard errors) were 89% ± 2%, 82% ± 2%, 87% ± 2%, 93% ± 1%, 92% ± 1%, and 85% ± 3% for strains Breinl, Madrid E, 87-17, 60P, 83-2P, and 110-1P, respectively, and the numbers of rickettsiae per infected cell were 4.8 ± 0.5, 3.1 ± 0.2, 4.0 ± 0.4, 7.2 ± 0.8, 4.5 ± 0.3, and 3.9 ± 0.3, respectively. The numbers of rickettsiae per infected cell ranged from 1.6 to 14.4 in these experiments. In the figure, the standard error of the mean is either within the symbol or shown by a bar. The dashed line represents the Madrid E strain.

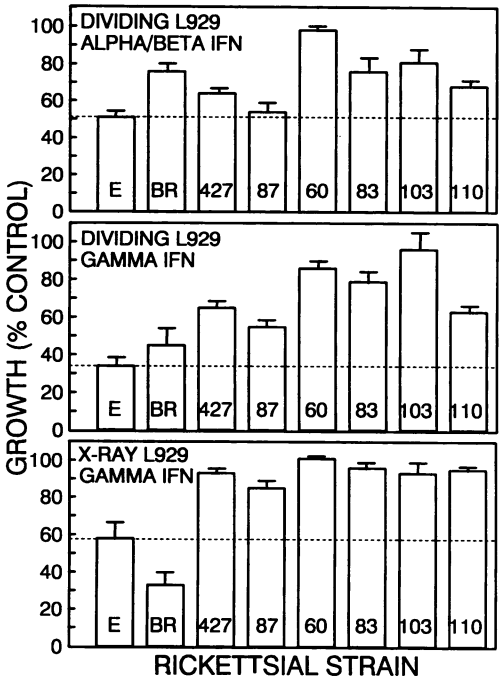


FIG. 2. Growth of various *R. prowazekii* strains in IFN-α/β- or IFN-γ-treated L929 cells. Dividing L929 cells were treated with 60 U of IFN-α/β or IFN-γ per ml both before and after infection. X-irradiated L929 cells were treated with IFN-γ (60 U/ml) after infection only. Rickettsial growth (doublings per day) in the IFN-treated cultures is expressed as a percentage of that observed in the untreated control cultures. Each value represents the mean ± standard error of the mean. The numbers of experiments with dividing L929 cells were 15 for Madrid E, 4 for Breinl and strain 427-19, and 3 for the remaining strains. The numbers of experiments with X-irradiated L929 cells were 5 for Madrid E, 4 for Breinl, and 3 for the remaining strains. The average number of rickettsial doublings per day in the untreated control cultures of dividing L929 cells ranged from 2.0 to 2.7 for the different strains. With the exception of the data for *R. prowazekii* Breinl, the data for the experiments with dividing L929 cells were previously published (31). In the untreated X-irradiated L929 cells, the average numbers of rickettsial doublings per day for the various strains ranged from 2.0 to 2.5. Immediately after infection, the average percentages of X-irradiated cells infected ranged from 68 to 82% with the various strains, and the numbers of rickettsiae per infected cell ranged from 3.1 to 5.2.

errors; $n = 4$ experiments each). The percentages of cells infected with the different strains in these experiments were, respectively, 88% ± 4%, 82% ± 5%, and 85% ± 5%, and the numbers of rickettsiae per infected cell were, respectively, 3.3 ± 0.3, 2.9 ± 0.3, and 4.1 ± 0.8. As demonstrated in earlier studies with the Madrid E and Breinl strains (26), only RAW264.7 cells that were both IFN-γ treated and infected with rickettsiae were killed within 4 to 6 h after infection.

**Evaluation of IFN-α/β- and IFN-γ-induced inhibition of growth of *R. prowazekii* strains in dividing and X-irradiated L929 cells.** The sensitivity of the virulent Breinl strain of *R. prowazekii* to IFN-α/β and IFN-γ was evaluated to determine whether resistance to these IFNs was correlated with virulence. Since previous work with IFN-γ utilized both dividing host cells and host cells that were X-irradiated, the sensitivities of the rickettsial strains to IFN-γ were evaluated in both types of cells (Fig. 2). However, since X-irradiated (nondividing) L929 cells were not suitable for evaluating the modest inhibitory effect of IFN-α/β (30), the sensitivities of the various rickettsial strains to IFN-α/β were evaluated only in dividing L929 cells.

In dividing L929 cell cultures treated with IFN-α/β or IFN-γ for 2 days before infection and for the entire period after infection (2 to 3 days), *R. prowazekii* Breinl grew at rates that were 76% ± 4% and 45% ± 9%, respectively, of the rate observed in untreated control cultures (2.5 ± 0.2 doublings per day) (mean ± standard error; $n = 4$ experiments) (Fig. 2). The growth rates of the L929 cells and the parameters of rickettsial infection were required to calculate the growth rates of *R. prowazekii* Breinl in each experiment. In untreated, IFN-α/β-treated, and IFN-γ-treated cultures infected with *R. prowazekii* Breinl, the growth rates of the L929 cells were, respectively, 1.0 ± 0.1, 0.7 ± 0.0, and 0.8 ± 0.1 doubling per day. Immediately after infection, the percentages of cells infected in the untreated, IFN-α/β-treated, and IFN-γ-treated cultures were 71% ± 9%, 89% ± 4%, and 80% ± 9%, respectively, and the numbers of rickettsiae per infected cell were 2.9 ± 0.4, 5.1 ± 1.0, and 4.1 ± 0.7, respectively. After incubation of these cultures for 2 to 3 days, the percentages of cells infected were 89% ± 10%, 94% ± 4%, and 46% ± 6%, respectively, and the numbers of rickettsiae per infected cell were 46 ± 11, 51 ± 13, and 16 ± 4, respectively. IFN-γ-induced suppression of the growth of *R. prowazekii* Breinl in dividing L929 cells was similar to that observed previously with *R. prowazekii* Madrid E (31) ($P > 0.2$; Fig. 2). However, the Breinl strain was less sensitive to IFN-α/β than the Madrid E strain ($P < 0.005$).

Four of the strains which were resistant to IFN-γ compared with the Madrid E strain (31) were also significantly resistant to IFN-γ compared with the Breinl strain in dividing L929 cells (strain 427-19, $P < 0.1$; strains 60P, 83-2P, and 103-2P, $P < 0.05$) (Fig. 2). However, the sensitivity of the remaining two IFN-resistant strains to IFN-γ did not differ significantly from that of the Breinl strain in dividing L929

TABLE 4. Production of IFN by L929 cell cultures infected with *R. prowazekii* strains

| Strain[a] | Results on[b]: Day 1, positive cultures (%)[c] | Day 2 | | | Day 3 | | | Day 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Positive cultures (%) | IFN detected (U/ml) | | Positive cultures (%) | IFN detected (U/ml) | | Positive cultures (%) | IFN detected (U/ml) | |
| | | | Mean ± SD | Median | | Mean ± SD | Median | | Mean ± SD | Median |
| Madrid E | 0/12 (0) | 3/16 (19) | 11 ± 21 | <2 | 61/66 (92) | 231 ± 335 | 42 | 65/65 (100) | 239 ± 311 | 132 |
| Breinl | 0/6 (0) | 0/6 (0) | <2 | <2 | 12/20 (60) | 42 ± 64 | 7 | 17/20 (85) | 152 ± 245 | 51 |
| 427-19 | NT | NT | NT | NT | 18/18 (100) | 285 ± 124 | 301 | 12/12 (100) | 330 ± 119 | 332 |
| 87-17 | NT | 0/4 (0) | <2 | <2 | 8/8 (100) | 131 ± 116 | 81 | 14/14 (100) | 93 ± 110 | 30 |
| 60P | NT | 0/4 (0) | <2 | <2 | 4/6 (67) | 4 ± 2 | 4 | 5/12 (42) | 4 ± 6 | <2 |
| 83-2P | 0/6 (0) | 0/6 (0) | <2 | <2 | 4/14 (29) | 40 ± 101 | <2 | 12/14 (86) | 48 ± 67 | 15 |
| 103-2P | NT | NT | NT | NT | 5/16 (31) | 7 ± 9 | <2 | 20/22 (91) | 81 ± 83 | 61 |
| 110-1P | NT | NT | NT | NT | 10/16 (63) | 15 ± 22 | 7 | 12/12 (100) | 184 ± 126 | 164 |

[a] Immediately after infection and washing, the average numbers of rickettsiae per L929 cell (ranges) were 1.0 to 13.9 for Madrid E, 0.9 to 14.8 for Breinl, 1.2 to 8.0 for isolate 427-19, 0.8 to 12.0 for isolate 87-17, 2.6 to 9.8 for isolate 60P, 1.0 to 12.7 for isolate 83-2P, 2.1 to 10.2 for isolate 103-2P, and 3.3 to 14.6 for isolate 110-1P.

[b] When IFN was not detected, the limit of detection of the IFN assay (approximately 2 U/ml) was used in calculating the mean values of IFN induced. NT, No cultures were tested.

[c] Number of positive cultures/total number of cultures.

cell cultures (strain 87-17, $P > 0.4$; strain 110-1P, $P > 0.1$). The extent of inhibition of the growth of the Breinl strain in IFN-α/β-treated L929 cells was similar to the inhibition observed with strains 83-2P, 103-2P, and 110-1P ($P > 0.5$, $P > 0.4$, and $P > 0.1$, respectively). *R. prowazekii* Breinl, however, was more sensitive to IFN-α/β than *R. prowazekii* 60P ($P < 0.01$) and less sensitive to IFN-α/β than strains 427-19 and 87-17 ($P < 0.05$).

In X-irradiated L929 cells, the Breinl strain appeared somewhat more sensitive to IFN-γ than the Madrid E strain ($P < 0.1$). In addition, all six of the IFN-resistant strains were significantly resistant to IFN-γ compared with either the Madrid E or Breinl strains in X-irradiated L929 cells (strains 427-19, 60P, 83-2P, 103-2P, and 110-1P, $P < 0.05$ versus Madrid E; strain 87-17, $P < 0.1$ versus Madrid E; all strains, $P < 0.005$ versus Breinl).

**Induction of IFN production in L929 cell cultures infected with various *R. prowazekii* strains.** The Madrid E and Breinl strains, as well as most of the IFN-resistant strains, were able to induce IFN (antiviral activity) in L929 cell cultures (Table 4 and Fig. 3). The IFN produced in these cultures is likely to be IFN-α and/or IFN-β, since the IFN produced in L929 cell cultures infected with *R. prowazekii* Madrid E was neutralized by anti-mouse IFN (α + β) serum but not by antibodies against murine IFN-γ (30, 31). IFN was never detected in culture media collected from mock-infected L929 cells.

Most of the infected L929 cell cultures tested did not contain detectable IFN on the first and second days after infection. However, by the third day after infection, all cultures infected with strain 427-19 or strain 87-17 contained IFN, 92% of the cultures infected with the Madrid E strain contained IFN, and lower percentages of the cultures infected with the remaining strains contained IFN (Table 4). The percentages of cultures that contained IFN on day 4 were higher than the corresponding percentages on day 3 for strains Madrid E, Breinl, 83-2P, 103-2P, and 110-1P. In addition, for cultures infected with strains Breinl, 103-2P, and 110-1P, the average amount of IFN per milliliter on day 4 was greater than that on day 3. Compared with the other rickettsial strains, IFN-resistant strain 60P appeared highly defective in its ability to induce IFN in L929 cell cultures. IFN was detected in only half of all of the strain 60P-infected

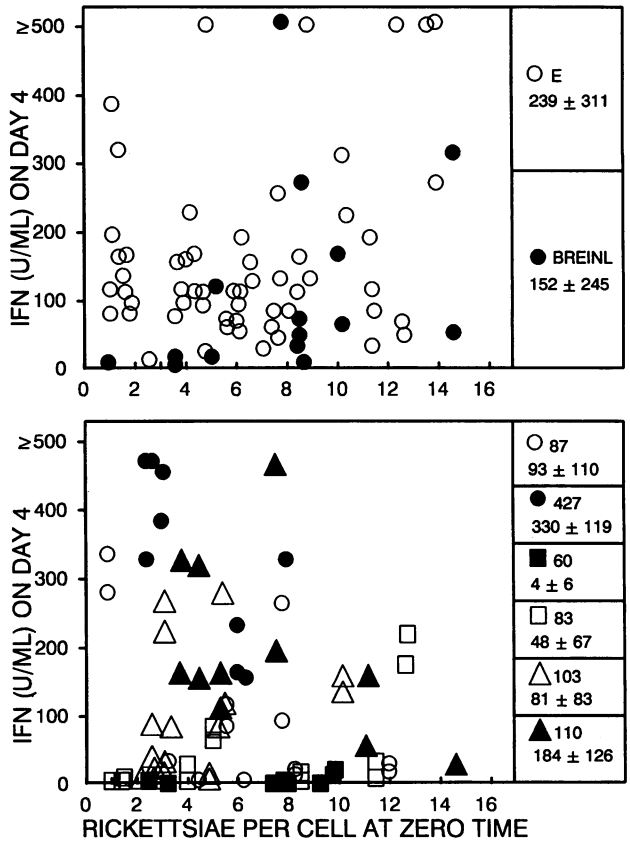


FIG. 3. Induction of IFN in L929 cells by *R. prowazekii* strains. Culture media were assayed for IFN 4 days after infection. Each point represents one culture, and some points are superimposed. The amounts of IFN (means ± standard deviations) induced in cultures infected with the various rickettsial strains are given on the right. For cultures in which IFN was not detected, the limit of detection of the IFN assay (approximately 2 U/ml) was plotted and used in calculating the mean values of IFN induced.

cultures tested on day 3 or day 4 (9 of 18 cultures), and the average amount of IFN detected in the cultures on either day was only 4 U/ml.

Figure 3 shows the amounts of IFN present in the individual cultures on day 4. The amount of IFN produced in cultures infected with the Madrid E strain was highly variable, and the reasons for this variability are unknown. For the range of initial rickettsial infections tested, the data failed to define a relationship between the initial rickettsial infection and the amount of IFN produced. Although smaller numbers of cultures infected with the IFN-resistant *R. prowazekii* strains were assayed for IFN, the amounts of IFN produced in these cultures also appeared variable (except in the case of strain 60P, which was a poor inducer of IFN) and precluded a definition of the relationship between the initial rickettsial infection and the amount of IFN produced.

To determine whether differences in the growth rates of the L929 cells or rickettsiae might explain the variability in the amount of IFN produced, IFN was assayed on day 4 in *R. prowazekii* 103-2P-infected cultures in which the growth rate of the L929 cells, L929 cell viability, and growth rate of the rickettsiae were also determined. Five independent experiments with duplicate cultures were performed, and the average amounts of IFN produced in these experiments were 5, ≤2, 244, 81, and 8 U/ml. The average initial numbers of rickettsiae per cell were, respectively, 2.2, 2.8, 3.1, 3.4, and 4.8, and the growth rate of the infected L929 cells ranged from 0.7 to 0.8 doubling per day in these experiments. (Mock-infected L929 cells grow at a slightly faster rate [0.9 doubling per day] under similar experimental conditions [30].) The growth rates of the rickettsiae were, respectively, 2.0, 1.9, 1.6, 1.8, and 1.9 doublings per day, and the percentages of dead (trypan blue-permeable) L929 cells in the cultures on day 4 were 13, 6, 20, 11, and 8%, respectively. These data indicate that differences in the growth rates of the L929 cells or rickettsiae do not adequately explain the variability in the amount of IFN produced in cultures initially infected with moderate numbers of *R. prowazekii* 103-2P organisms. It is interesting that the rickettsial growth rate was lowest and the percentage of dead cells was highest in the experiment in which the greatest amount of IFN was produced; however, it should be noted that cell death would adversely affect rickettsial multiplication and that the IFN produced may itself have contributed to the cell death observed in these infected cultures (30, 31).

For cultures infected with different rickettsial strains, differences in the growth of the rickettsiae and the growth of the L929 cells also represent potential sources of variability. To assess the growth of the rickettsiae and the condition of the L929 cells, cover slips removed from replicate cultures at the time of collection of the media for IFN assay were stained and examined in each experiment. These cover slips indicated that rickettsial growth had occurred in all cases. On days 3 and 4, the density of the L929 cells was lower in some of the infected cultures than in the mock-infected cultures. In addition, some infected cultures showed evidence of cell death, particularly on day 4. Although the growth rates of the L929 cells and rickettsiae were not determined in the present study in all cultures in which IFN was assayed, both parameters were previously evaluated in similar experiments, and some of the data have been published (29–31). L929 cells were infected with moderate numbers of rickettsiae (average initial number of rickettsiae per cell, ≤7), and growth rates of the L929 cells and rickettsiae were evaluated 3 days after infection in 23 experiments with *R. prowazekii* Madrid E, 12 experiments with *R. prowazekii* Breinl, and 2 experiments with each of the remaining rickettsial strains. The average rickettsial growth rates for the various strains ranged from 2.0 to 2.6 doublings per day, and the average growth rates of the L929 cells were 0.6 doubling per day for cultures infected with strain 427-19 or 87-17; 0.8 doubling per day for cultures infected with strain Madrid E, 60P, or 83-2P; and 0.9 doubling per day for cultures infected with strain Breinl, 103-2P, or 110-1P. These data indicate that both L929 cells and rickettsiae grew well in cultures infected with any of the rickettsial strains and that the poor IFN production in cultures infected with strain 60P was not due to inability of the infected L929 cells to grow.

## DISCUSSION

The properties of the rickettsial strains compared in this study are summarized in Table 1. The Breinl and Madrid E strains of *R. prowazekii* differed in their capacity for killing macrophagelike RAW264.7 cells that had been treated with either IFN-α/β or a subsaturating concentration of IFN-γ (0.5 U/ml); the Breinl strain had a somewhat higher capacity for killing the cells than the Madrid E strain. Although the IFN-resistant strains resembled the Breinl strain in their ability to grow in untreated macrophagelike cells, the IFN-resistant strains tested resembled the Madrid E strain (from which they were derived) in their capacity for killing IFN-treated RAW264.7 cells. A striking difference was observed in the concentrations of IFN-α/β and IFN-γ required to prepare RAW264.7 cells for rapid killing by *R. prowazekii*: much higher concentrations of IFN-α/β than IFN-γ were needed. Other researchers have likewise demonstrated that IFN-γ is much more potent than either IFN-α or IFN-β at priming mouse macrophages for killing tumor cells (17).

How the IFN-resistant rickettsiae and *R. prowazekii* Breinl (in contrast to the Madrid E strain) survive and grow in untreated macrophagelike cells is an interesting question. Neither the Breinl strain nor the Madrid E strain of *R. prowazekii* induces a respiratory burst in RAW264.7 cells (14, 21); hence, differential induction or sensitivity to the products of the respiratory burst is not an adequate explanation. Winkler and Daugherty (34) provided evidence that in RAW264.7 cells, the poor growth of the Madrid E strain relative to the Breinl strain is a function of the cytoplasm of the macrophagelike cells. Perhaps the Madrid E strain is compromised in its ability to obtain the nutrients required to support its growth in RAW264.7 cells. Another possibility (one that is germane to the present study) is that cytokines produced by the RAW264.7 cells in response to the rickettsiae play a role in determining the survival and growth of *R. prowazekii* strains in these cells. Studies are in progress to examine these possibilities.

In the present study, the ability of IFN-γ to inhibit the growth of *R. prowazekii* strains was evaluated in both dividing and nondividing (X-irradiated) L929 cells in order to facilitate comparisons with our previous studies, which have utilized both types of cells. It should be noted that comparison of the percent control values for growth of a particular rickettsial strain in IFN-γ-treated dividing L929 cells and IFN-γ-treated X-irradiated L929 cells is complicated by at least two factors. First, the time of treatment of the cells with IFN-γ differed in the experiments with dividing cells and those with X-irradiated cells. (The dividing cells were treated with IFN-γ both before and after infection, whereas the X-irradiated cells were treated with IFN-γ only after rickettsial infection.) Previous work with X-irradiated L929

cells indicated that treatment of the cells with IFN-γ both before and after infection results in greater inhibition of growth of *R. prowazekii* Madrid E than does treatment of the cells with IFN-γ only before infection or only after infection (23). Second, the microscopic counting method used to assess rickettsial growth may have influenced the results, because no attempt was made to quantitate rickettsiae in excess of 100 per cell. Untreated X-irradiated L929 cells generally become more massively infected than untreated dividing L929 cells, because cell division decreases the number of rickettsiae per infected cell. Hence, differences between the actual numbers of rickettsiae per culture and the numbers of rickettsiae determined by counting were probably greater in untreated X-irradiated L929 cell cultures than in untreated dividing L929 cell cultures, and these differences would affect the percent control values for the IFN-γ-treated cultures. In a previous study with IFN-α/β (30), a difference in the results with dividing and X-irradiated L929 cells was noted and found to be due to the microscopic counting method employed. In light of these two factors (the time of treatment of the cells with IFN-γ and the microscopic counting method used to collect the data), it is not surprising that for most of the rickettsial strains, the average percent control values for the IFN-γ-treated X-irradiated cultures were higher than the corresponding average percent control values for the IFN-γ-treated dividing cultures. The data indicate that in dividing L929 cells, the Breinl and Madrid E strains had similar sensitivities to IFN-γ; however, in nondividing (X-irradiated) L929 cells, the Breinl strain appeared somewhat more sensitive to IFN-γ than the Madrid E strain. Whether the apparently greater sensitivity of the Breinl strain to IFN-γ treatment in X-irradiated cells is related to the X-irradiation itself or to the fact that the X-irradiated cells do not divide is unknown. Although X-irradiation does not affect the ability of untreated L929 cells to support rickettsial growth, it is possible that nondividing cells have a greater capacity than rapidly dividing cells for suppressing the growth of *R. prowazekii* Breinl in the presence of IFN-γ. Such a difference in the responsiveness of various cells to IFN-γ might be important in vivo.

Resistance to IFN-γ, IFN-α/β, or both IFNs was not consistently associated with the ability of *R. prowazekii* strains to grow in untreated macrophagelike RAW264.7 cells. The Breinl strain, which grew very well in RAW264.7 cells, was somewhat more sensitive to IFN-γ in X-irradiated L929 cells than either the Madrid E strain (which did not grow in RAW264.7 cells) or the IFN-resistant strains (which did grow in RAW264.7 cells). Moreover, the average percent control value for the growth rate of the Breinl strain in IFN-γ-treated dividing L929 cells was between the value for the Madrid E strain and the values for strains 87-17 and 110-1P and did not differ significantly from them. Resistance to IFN-α/β was not consistently associated with the ability of *R. prowazekii* strains to grow in untreated RAW264.7 cells, since IFN-γ-resistant strain 87-17 (which grew very well in RAW264.7 cells) was not resistant to IFN-α/β treatment in L929 cells compared with the Madrid E strain. However, it remains possible that resistance to IFN-α/β is associated with virulence in *R. prowazekii*, since the virulent Breinl strain was resistant to IFN-α/β compared with the avirulent Madrid E strain.

Although virulence in *R. prowazekii* is associated with the ability to grow in macrophages and macrophagelike cells (6, 22), it has not been determined if this ability is sufficient (or even necessary) for *R. prowazekii* to be virulent. Nevertheless, it seems reasonable to hypothesize that this ability is important to virulent *R. prowazekii* organisms, since studies with other *Rickettsia* species underscore the importance of macrophages in host defense against rickettsial infections (reviewed in references 16 and 36). Clearly, studies in experimental animals will be required in order to evaluate the virulence of the IFN-resistant strains. Animal models which reproduce many of the features of *R. prowazekii* infection in humans are unfortunately limited to the cotton rat (5, 19) and the cynomolgus monkey (8). The potential usefulness of the flying squirrel (which serves as a reservoir of *R. prowazekii* [20]) as an animal model for *R. prowazekii* infection has not been adequately evaluated. Although the febrile response of guinea pigs after intraperitoneal inoculation with rickettsiae can distinguish the virulent Breinl and avirulent Madrid E strains (12, 18), these studies suffer from the same uncertainties as those with macrophages.

As shown in this study, both murine IFN-α/β and murine IFN-γ inhibit the growth of the Breinl and Madrid E strains of *R. prowazekii*, although the Breinl strain is less sensitive to IFN-α/β than the Madrid E strain. Host cells treated with IFN-α/β or IFN-γ are also damaged by infection with these *R. prowazekii* strains. Moreover, IFN-α/β is induced in L929 cell cultures by the Breinl and Madrid E strains, and it is produced after injection of mice with *R. prowazekii* Breinl (11). The exact mechanism(s) by which IFNs exert their antirickettsial effects in vitro, much less in vivo, has not been proven. However, the IFN-resistant *R. prowazekii* strains may represent tools to define the role of IFN in host defense against rickettsiae. Mice are relatively insusceptible to infection with *R. prowazekii* (5), but the reasons for this insusceptibility are unknown. If mice were found to be more susceptible to infection with the IFN-resistant strains, this finding would suggest that the inhibitory effect of IFN against rickettsiae growing within their host cells is a significant host defense in vivo. Interestingly, IFN-resistant strain 60P (which was selected for resistance to IFN-α/β by culturing the Madrid E strain in L929 cells for 8 months [31]) differed from both the Madrid E and Breinl strains in that it induced little or no IFN in L929 cells. Hence, this strain may be useful not only in determining the molecular basis for IFN induction by *R. prowazekii* but also in evaluating the importance of IFN-α/β as a host defense against *R. prowazekii*.

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