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Isolation of *Rickettsia prowazekii* with Reduced Sensitivity to Gamma Interferon

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The growth of *Rickettsia prowazekii* Madrid E was monitored in mouse L929 cells subcultured for several weeks in the presence of gamma interferon (IFN-γ) to determine whether the rickettsiae would be eliminated from or would persist in these cultures. *R. prowazekii* exhibited two distinct patterns in these IFN-γ-treated cultures. In some cases, IFN-γ-induced inhibition of rickettsial growth led to elimination of the rickettsiae from the L929 cell cultures; in other cases, the initial inhibition of rickettsial growth was followed by establishment of a persistent rickettsial infection in the IFN-γ-treated L929 cells. During the first 3 days after infection, the growth rate of the L929 cells was significantly lower and higher percentages of the cells were killed in the IFN-γ-treated, *R. prowazekii*-infected cultures than in the untreated, *R. prowazekii*-infected cultures or the mock-infected cultures, whether treated or untreated. This suppression of cell growth occurred in the infected, IFN-γ-treated cultures that eventually exhibited the elimination pattern as well as the IFN-γ-treated cultures that became persistently infected. It was not possible to predict the outcome of a particular infection from the early growth pattern of the culture. It was determined that the L929 cells in the persistently infected, IFN-γ-treated cultures had not lost the ability to respond to IFN-γ. These cells, after treatment with an antibiotic to eliminate the persistent rickettsiae, retained the ability to inhibit both the replication of vesicular stomatitis virus and the growth of *R. prowazekii* Madrid E after treatment with IFN-γ. In contrast, rickettsiae isolated from two persistently infected, IFN-γ-treated cultures were less sensitive than *R. prowazekii* Madrid E to the antirickettsial effects of IFN-γ in standard L929 cells. The maintenance of the phenotype of these altered rickettsiae during plaque purification and passage in the absence of IFN-γ suggests an alteration at the genetic level rather than phenotypic adaptation.

Rickettsia prowazekii is an obligate intracellular bacterium that grows in the cytoplasm of its host cells, unbounded by a phagosomal or phagolysosomal membrane (19). The etiological agent of epidemic typhus in humans, *R. prowazekii* proliferates in the endothelial cells that line the small blood vessels (27). This organism can also grow in macrophages (6).

The growth of *R. prowazekii* in cultured fibroblasts and macrophagelike cells is inhibited by gamma interferon (IFN-γ) (13, 16, 17, 25, 26), and this lymphokine has been shown to be important in host defense against *Rickettsia conorii* infection in mice (11). However, the mechanisms of action of IFN-γ against rickettsiae in vitro and in vivo have not been defined.

In previous studies with fibroblasts (mouse L929 cells and human fibroblasts), the growth of *R. prowazekii* was monitored in IFN-γ-treated cells during a 2-day period (13, 17). Rickettsial growth was dramatically inhibited in these experiments, but rickettsiae were still detectable by staining after 2 days. It is not known if IFN-γ can cure cultured fibroblasts of infection with *R. prowazekii*.

In the present study, the growth of *R. prowazekii* Madrid E was monitored in L929 cells subcultured for several weeks in the presence of IFN-γ to determine whether the rickettsiae would be eliminated from or would persist in these cultures. In addition, the growth and viability of the L929 cells after infection with *R. prowazekii* Madrid E and treatment with IFN-γ were examined. We report that *R. prowazekii* Madrid E exhibited two distinct patterns in cultures of L929 cells treated with IFN-γ for several weeks. In some cases, IFN-γ-induced inhibition of rickettsial growth led to elimination of the rickettsiae from the L929 cell cultures; in other cases, the initial inhibition of rickettsial growth was followed by establishment of a persistent rickettsial infection in the IFN-γ-treated L929 cells. Rickettsiae isolated from these persistently infected, IFN-γ-treated cultures had reduced sensitivity to the antirickettsial effect of IFN-γ.

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MATERIALS AND METHODS

Cell cultures. Mouse L929 cells were purchased from Flow Laboratories, Inc. (McLean, Va.) and grown in Eagle minimal essential medium supplemented with 10% newborn bovine serum. Mouse 3T3-A31 cells were purchased from the American Type Culture Collection, Rockville, Md., and grown in Dulbecco modified Eagle medium supplemented with 10% newborn bovine serum. Cultures of human foreskin fibroblasts were kind gifts from Frank Pindak, University of South Alabama, and were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum. All cells were grown as monolayers in a humidified atmosphere of 3% CO_2 in air at 34°C. The bovine sera were heated at 56°C for 30 min before use.

Rickettsiae. Six-day-old embryonated, antibiotic-free hen eggs (Truslow Farms, Chestertown, Md.) were inoculated with a seed pool of *R. prowazekii* Madrid E (yolk sac passage 280). Eight days later, the rickettsiae were harvested and purified from the infected yolk sacs as described previously (15). Viable rickettsiae were enumerated by the antibody hemolysis method of Walker and Winkler (18).

IFN-γ. Recombinant murine IFN-γ derived from *Escherichia coli* was generously provided by Genentech, Inc.

(South San Francisco, Calif.). IFN-γ was assayed by a cytopathic effect inhibition assay with vesicular stomatitis virus and L929 cells. The assay was modified from that of Havell and Vilcek (8) as previously described (14). IFN-γ activity was calibrated against a reference preparation of murine IFN-γ (Ga02-901-533; National Institute of Allergy and Infectious Diseases, Bethesda, Md.). One unit of IFN-γ in the present study is equivalent to 1.28 U of IFN-γ in our previous work (13, 17).

Infection of L929 cells with rickettsiae, assessment of L929 cell growth and viability, and measurement of rickettsial growth. L929 cells were harvested from monolayer cultures by incubation with 0.5% trypsin and 0.02% disodium EDTA in a salt solution (Flow Laboratories, Inc.). After being washed, the cells were suspended in serum-supplemented medium (MS) at a concentration of 2×10^6 viable (trypan blue-excluding) cells per ml. Rickettsiae were diluted in Hanks balanced salt solution supplemented with 5 mM L-glutamic acid (monopotassium salt) and 0.1% gelatin (HBSSGG), and an equal volume of rickettsiae (approximately 2×10^8/ml) was added to each cell suspension. For mock infection, HBSSGG alone was added to the cells. After incubation in a shaking water bath at 34°C for 1 h, the cells were washed and planted into culture plates (diameter of 60 or 100 mm) that contained MS alone or MS plus IFN-γ (final concentration, 12 U/ml). Previous studies with both X-irradiated and nonirradiated L929 cells indicated that IFN-γ at this concentration markedly inhibits the growth of *R. prowazekii* in these cells (13, 17). For determination of the initial rickettsial infection, samples of the washed cell suspensions were immediately centrifuged onto microscope slides, dried, fixed, and stained as previously described (14, 15). The cultures were incubated at 34°C. After 3 days, the detached and attached cells were harvested separately, stained with trypan blue (for assessment of cell viability), and counted. The attached cells were also subcultured at that time and every 3 to 4 days thereafter. The number of cells planted per dish was adjusted so that uninfected, untreated L929 cells would grow to form confluent monolayers by the time of the next subculturing. For example, when the cells were to be subcultured in 3 days, approximately 4.5×10^5 viable (trypan blue-excluding) cells were planted per 60-mm-diameter dish and 1.3×10^6 viable cells were planted per 100-mm-diameter dish. The number of L929 cell doublings that occurred between subcultures was calculated from the number of viable cells planted and the total number of cells harvested at the time of the next subculture. It should be understood that in no case were additional cells added to the cultures.

At each time of subculturing, the cells were planted in fresh MS, and fresh IFN-γ (12 U/ml) was added to the treated cultures. Assay of MS that contained IFN-γ after 4 days of incubation at 34°C with or without L929 cells indicated that the IFN-γ was stable during this incubation period and that the cells did not deplete the IFN-γ from the medium.

In all experiments, samples of the attached cells were centrifuged onto microscope slides, fixed, and stained. The slides were examined for rickettsiae, and the percentage of cells infected and the average number of rickettsiae per infected cell were determined. A cell that contained more than 100 rickettsiae was assigned a value of 100. This practice results in underestimation of the numbers of rickettsiae present in heavily infected cells. A total of 100 cells from each culture were examined for rickettsiae, except that when very few rickettsiae were present, 500 cells were examined. The number of rickettsial doublings that occurred between subcultures was calculated from the number of rickettsiae present in the L929 cells that were planted and the number of rickettsiae per culture at the time of the next subculture. Unless noted otherwise, the number of rickettsiae per culture at the time of subculture was calculated from the number of attached cells and the average number of rickettsiae per attached cell. In some instances, the number of rickettsiae in the detached cells was determined and added to the number of rickettsiae in the attached cells to calculate the total number of rickettsiae per culture at the time of subculture.

Detection of viable rickettsiae in IFN-γ-treated L929 cells. To detect viable rickettsiae, samples of the infected, IFN-γ-treated cells from two cultures (approximately 3.5×10^5 cells in 0.2 ml of MS) were injected into 6-day-old embryonated, antibiotic-free hen eggs. L929 cells from each culture were inoculated into at least two eggs at each time point (egg passage 1). The eggs were candled daily, and yolk sac smears were prepared and stained from embryos that died within 8 or 9 days after inoculation. The yolk sacs were removed aseptically from the embryos that were viable after 8 or 9 days, and smears were prepared and stained from a small portion of each one. To the remaining yolk sac material, 4 ml of a sucrose phosphate glutamate solution (0.218 M sucrose, 3.76 mM KH_2PO_4, 7.1 mM K_2HPO_4, 4.9 mM potassium glutamate; pH 7.0) originally devised by Bovarnick et al. (4) was added per yolk sac, and the yolk sacs were homogenized in a Sorvall Omni-Mixer (Omni Corporation, Inc., Waterbury, Conn.). After centrifugation of the homogenate at $600 \times g$ for 10 min, 0.2 ml of the middle layer was reinoculated into 6-day-old embryonated hen eggs (egg passage 2). Yolk sac smears were prepared from these eggs as outlined above. To determine the limit of detection of viable rickettsiae by egg passage 1, L929 cells with a known rickettsial burden were titrated. Serial 10-fold dilutions of L929 cell suspensions infected with *R. prowazekii* Madrid E were inoculated into embryonated hen eggs in two independent titration experiments. From examination of stained yolk sac smears prepared within 9 days after inoculation, it was estimated that 50% of the inoculated eggs would be positive for rickettsiae when each egg was inoculated with approximately 21 viable rickettsiae (average of 33 and 9, the 50% endpoints of the two titration experiments).

Treatment of persistently infected IFN-γ-treated L929 cell cultures with antibiotics. To eliminate the rickettsiae from persistently infected, IFN-γ-treated L929 cells, cells derived from two independent experiments were subcultured for approximately 3 weeks with erythromycin (20 μg/ml in experiment 427) or chloramphenicol (10 μg/ml in experiment 87) in the presence or absence of IFN-γ. After additional subculture without antibiotics (but in the presence or absence of IFN-γ) for at least 1 week, the responsiveness of the cells to IFN-γ was evaluated in experiments with vesicular stomatitis virus and *R. prowazekii* Madrid E. Cells that had been treated with chloramphenicol were infected with rickettsiae approximately 1, 2, 5, and 8 weeks after removal of the chloramphenicol. Cells that had been treated with erythromycin were infected with rickettsiae approximately 2 and 5 weeks after removal of the erythromycin.

Isolation and plaque purification of rickettsiae from persistently infected, IFN-γ-treated L929 cell cultures. Rickettsiae (*R. prowazekii* 427 and *R. prowazekii* 87) were isolated from cultures of persistently infected, IFN-γ-treated L929 cells by two methods. In the first method, infected monolayer cul-

tures of L929 cells in 100-mm-diameter tissue culture dishes were washed with Dulbecco phosphate-buffered saline, and 0.8 ml to 1.0 ml of HBSSGG was added per dish. The cells were scraped from each dish with a rubber policeman, transferred to a Dounce homogenizer, and broken at 4°C. After addition of an equal volume of a solution of 0.44 M sucrose, 0.2% glucose, 5 mM glutamic acid (monopotassium salt), and 20 mM $MgCl_2$, the homogenate was centrifuged at 600 × *g* for 10 min at 4°C to remove the cell nuclei and any remaining intact cells. Samples of the supernatant fluid were used immediately to infect L929 cells or were frozen in liquid nitrogen for later use. In the second method, infected L929 cells were inoculated into 6-day-old embryonated hen eggs (approximately 5×10^3 cells per egg). Seven to eight days later, the yolk sacs were removed aseptically from the viable embryos, and the rickettsiae were harvested.

Rickettsial suspensions prepared from infected yolk sacs were diluted and the rickettsiae were plaqued on 3T3-A31 cells or human foreskin fibroblasts by a method modified from that of Hanson (7). After two cycles of plaque purification, suspensions of the plaque-purified rickettsiae (*R. prowazekii* 427-19 and *R. prowazekii* 87-17) were prepared from infected L929 cell cultures. In addition, the rickettsial isolates were inoculated into embryonated hen eggs, and rickettsial suspensions were prepared from the infected yolk sacs approximately 1 week later.

Statistics. Data were analyzed by two-tailed Student's *t* test.

RESULTS

Fate of *R. prowazekii* in nonirradiated L929 cells subcultured with or without IFN-γ. The rickettsial infection was monitored in 16 IFN-γ-treated cultures and 16 untreated cultures afer nine independent infections of L929 cells with *R. prowazekii*. All of the IFN-γ-treated cultures were monitored for at least 3 weeks; 10 cultures were monitored for 4 weeks or longer. The untreated cultures were monitored for at least 3 days to ensure that the rickettsiae were viable and able to grow in the L929 cells. The growth and viability of the L929 cells were analyzed in detail for 10 4-week cultures of infected, IFN-γ-treated cells, 10 4-week cultures of mock-infected, IFN-γ-treated cells, 10 3-day cultures of infected, untreated cells, and 10 3-day cultures of mock-infected, untreated cells.

(i) Growth of *R. prowazekii*. Initially, the percentage of cells infected with rickettsiae was 84% ± 10% and the average number of rickettsiae per infected cell was 6.5 ± 2.2 (mean ± standard deviation). As expected, in the infected cultures that were not treated with IFN-γ, the rickettsiae grew well during the first 3 days; the percentage of cells infected increased to 96% ± 8% and the average number of rickettsiae per infected cell increased to 64 ± 15. These controls demonstrated that the rickettsial inocula were viable and capable of growth in these cultures. In contrast, during this period in the IFN-γ-treated cultures, the percentage of cells infected decreased, and the average number of rickettsiae per infected cell increased slightly or remained constant (Fig. 1).

During the next week, the percentage of cells infected in the IFN-γ-treated cultures continued to decrease and the average number of rickettsiae per infected cell did not increase. After 10 to 14 days of treatment with IFN-γ, a particular culture exhibited one of two distinct patterns. One pattern led to elimination of the rickettsiae from the treated cultures and was characterized by a decrease in the percentage of cells infected and the average number of rickettsiae per infected cell. The other pattern exhibited by the cultures after 10 to 14 days of treatment with IFN-γ led to persistence of the rickettsiae in the treated cultures and was characterized by marked increases in both the percentage of cells infected and the average number of rickettsiae per infected cell during the next 2 weeks. Of the 16 cultures of *R. prowazekii*-infected, IFN-γ-treated L929 cells, the rickettsiae were eliminated in 8 cultures and the rickettsiae persisted in 8 cultures. The 16 IFN-γ-treated cultures were derived from nine independent rickettsial infections as follows: two cultures each were derived from seven independent infections and a single culture was derived from each of the remaining two infections. In one of the infections with two cultures, there was one culture from which the rickettsiae were eliminated and one culture in which the rickettsiae persisted. In the other six infections with two cultures, the rickettsiae either were eliminated or persisted in both cultures. The infecting rickettsiae used in these experiments were all from the same seed pool.

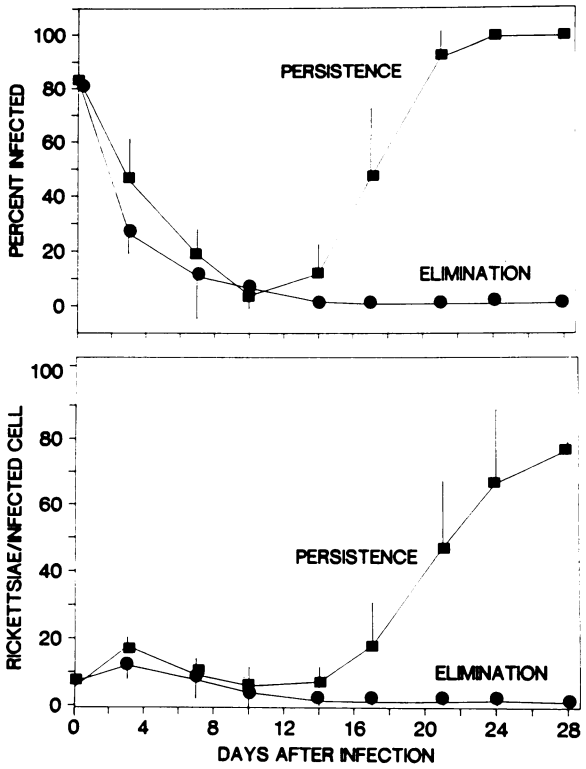

FIG. 1. *R. prowazekii* Madrid E infection in L929 cells maintained in the presence of IFN-γ for 4 weeks. At each time point, the attached cells were stained and examined for rickettsiae. The data obtained from 16 IFN-γ-treated cultures are shown. In eight of the IFN-γ-treated cultures, the rickettsiae were eliminated and in eight cultures, the rickettsiae persisted. Each point represents the mean ± the standard deviation.

In one experiment, two infected, IFN-γ-treated cultures that became persistently infected with rickettsiae were monitored for over 8 weeks (Fig. 2). The percentage of cells infected and the average number of rickettsiae per infected cell in these cultures increased between days 14 and 24. Then these parameters, particularly the average number of rickettsiae per infected cell, fluctuated in a cyclic manner (Fig. 2).

To calculate the growth rates of rickettsiae in cultures of

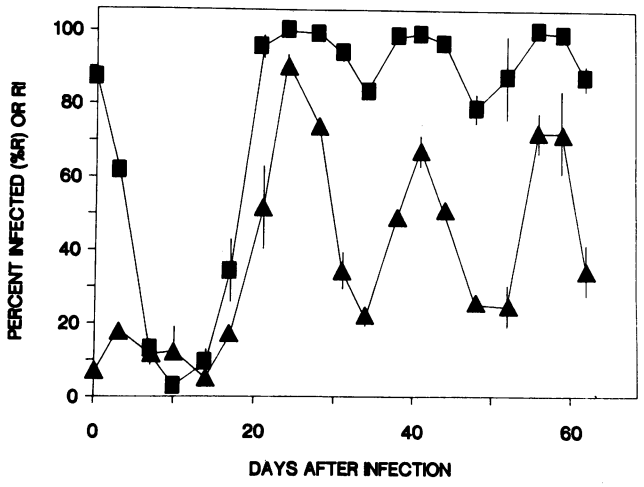

FIG. 2. Rickettsial infection in persistently infected, IFN-γ-treated L929 cells maintained for 8 weeks. At each time point, the attached cells were harvested and subcultured, and samples of the cells were stained and examined for rickettsiae. Each point represents the mean ± standard deviation for duplicate cultures derived from one infection. ■, %R (percent infected); ▲, RI (average number of rickettsiae per infected cell).

growing L929 cells, consideration must be given to the number of L929 cells per culture as well as the parameters of rickettsial infection discussed above. In the 10 cultures in which L929 cell growth was monitored, the rickettsiae in the attached cells in the untreated cultures doubled 2.0 ± 0.2 times per day from days 0 to 3, and the rickettsiae in the attached cells in the IFN-γ-treated cultures doubled 0.3 ± 0.5 times per day during this time interval. From days 3 to 7, the number of doublings of rickettsiae per day in the IFN-γ-treated cultures was −0.4 ± 0.5, indicating that the numbers of rickettsiae in the attached cells decreased during this time interval. However, in the IFN-γ-treated cultures in which the rickettsiae established persistent infections, the rickettsiae doubled 2.2 ± 0.6 times per day between days 14 and 17, indicating that their growth returned to the control rate and was no longer being inhibited by IFN-γ. When very small percentages of cells were infected (as in the infected, IFN-γ-treated cultures from which the rickettsiae were eliminated), the calculated numbers of rickettsial doublings at later times would have had a large error due to the small numbers of cells examined for rickettsiae. Therefore, these values are not given.

(ii) Growth and viability of L929 cells. Growth and viability of the L929 cells were examined to determine whether early differences in these parameters might be related to the outcome of the rickettsial infection in the IFN-γ-treated cultures. Between 0 and 3 days after infection, the growth of the infected, IFN-γ-treated L929 cell cultures was suppressed relative to all other cultures (mock-infected, untreated cultures; mock-infected, IFN-γ-treated cultures; and infected, untreated cultures) ($P < 0.001$ [Fig. 3]). Furthermore, on day 3, in these infected, IFN-γ-treated cultures, substantial percentages of the cells were dead, whereas very few cells were dead in the other cultures. Thus, the rickettsial infection and the treatment with IFN-γ were harmful for the L929 cells. This early suppression of L929 cell growth and killing of some of the L929 cells occurred in the IFN-γ-treated cultures from which the rickettsiae were eliminated, as well as the IFN-γ-treated cultures that eventually became persistently infected. It was not possible to

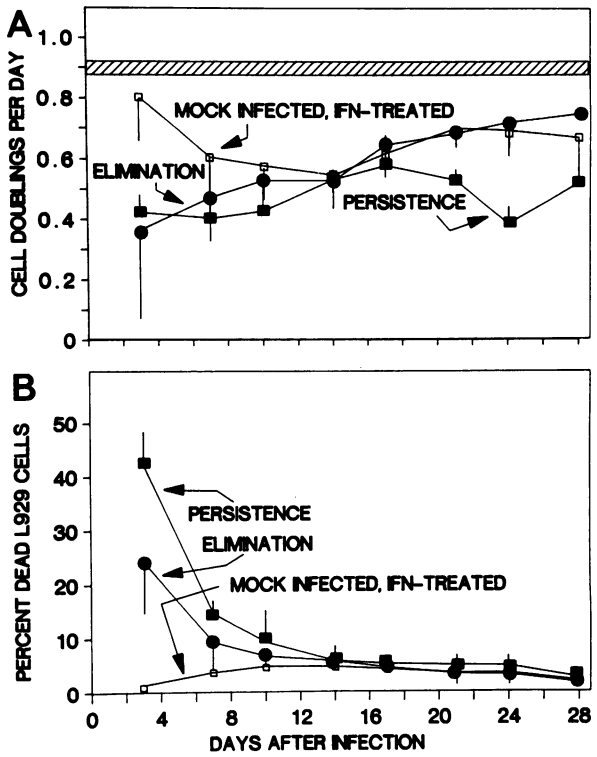

FIG. 3. L929 cell growth and percent dead cells in mock-infected or *R. prowazekii* Madrid E-infected L929 cell cultures maintained in the presence of IFN-γ for 4 weeks. Data for 10 mock-infected, IFN-γ-treated cultures, six infected, IFN-γ-treated cultures from which rickettsiae were eliminated, and four infected, IFN-γ-treated cultures in which rickettsiae persisted are shown. Each point represents the mean ± standard deviation. (A) Growth of L929 cells. The first point on each curve represents the number of doublings of L929 cells per day for the time interval beginning with the initial planting and ending on day 3. Later points represent subsequent time intervals. The average number of doublings that occurred per day in the mock-infected, untreated cultures ($n = 10$) throughout the 4-week period is shown (▨). The number of doublings that occurred per day in the infected, untreated cultures ($n = 10$) between days 0 and 3 was 0.8 ± 0.2. (B) Percent dead L929 cells. At each time point, the attached and detached cells were harvested and stained with trypan blue for determination of cell viability. Less than 6% of the cells were dead in the mock-infected, untreated cultures at each time and in the infected, untreated cultures on day 3.

predict the outcome of a particular infection from the early growth pattern of the culture.

Suppression of cell growth in the mock-infected, IFN-γ-treated cultures was less precipitous and was first detected between days 3 and 7. After day 7, the growth of the mock-infected, IFN-γ-treated cultures was similar to the growth of the infected, IFN-γ-treated cultures from which the rickettsiae were eliminated (Fig. 3). Between days 17 and 28, the growth of the IFN-γ-treated, persistently infected cultures was suppressed relative to the growth of the IFN-γ-treated cultures from which the rickettsiae were eliminated (Fig. 3). This suppression of cell growth may be related to the rickettsial burden in these persistently infected cells.

Detachment of cells in the various cultures was also evaluated to determine whether there were any differences between the IFN-γ-treated cultures from which the rickettsiae were eliminated and the IFN-γ-treated cultures that became persistently infected. Significantly, during the entire 28-day period, cell detachment was similar in the cultures

from which the rickettsiae were eliminated and the cultures that became persistently infected. The percentages (mean ± standard deviation) of cells that were detached on days 3, 7, 10, 14, 17, 21, 24, and 28 were, respectively, 11 ± 5, 8 ± 6, 6 ± 2, 5 ± 1, 3 ± 1, 2 ± 0, 3 ± 1, and 2 ± 0 in the cultures from which the rickettsiae were eliminated and 10 ± 2, 9 ± 3, 8 ± 7, 5 ± 3, 4 ± 2, 3 ± 1, 4 ± 3, and 2 ± 1 in the cultures that became persistently infected. Less than 5% of the cells were detached in the mock-infected cultures at each time of cell harvest. In the infected, untreated cultures, less than 5% of the cells were detached on day 3, but as the infection progressed more cells usually became detached (data not shown).

(iii) Assay of IFN-γ-treated L929 cells for viable rickettsiae. Four IFN-γ-treated cultures that exhibited the elimination pattern and appeared to be free of rickettsiae were further evaluated by subculturing them for 2 weeks in the absence of IFN-γ and staining them for rickettsiae. Subculture of the treated cells in medium without IFN-γ was initiated on day 38 for two cultures derived from one infection and on day 42 for two cultures derived from another infection. Rickettsiae were not detected in these subcultures in any case.

Two IFN-γ-treated cultures that were derived from a third infection and that exhibited rickettsial elimination were assayed for viable rickettsiae after treatment with IFN-γ for 7, 14, 21, 28, or 35 days. Inoculation of the L929 cells into embryonated hen eggs was the method used for detection of viable rickettsiae in the cultures because it allowed the assay of large numbers of L929 cells. As anticipated on the basis of stains of the L929 cell cultures, after egg passage 1, all yolk sac smears prepared from eggs inoculated with infected L929 cells that had been treated with IFN-γ for 7 days contained rickettsiae. In contrast, smears prepared from eggs inoculated with approximately 3.5×10^5 IFN-γ-treated L929 cells from day 14, 21, 28, or 35 were negative for rickettsiae after egg passage 1. After egg passage 2, rickettsiae were detected in the L929 cell cultures at day 14 (five of six eggs) and day 21 (two of four eggs) but were not detected in the L929 cell cultures at day 28 and day 35. Assuming that inoculation of 42 rickettsiae per egg would have been detected in egg passage 1, one can conservatively estimate from these data that there was less than one viable rickettsia per 8,000 L929 cells on day 14 and thereafter. Because the L929 cells at day 28 and day 35 were negative for rickettsiae even after egg passage 2, there was much less than one rickettsia per 8,000 cells at those times. These data provide additional evidence for the elimination of the rickettsiae from the IFN-γ-treated cultures that appeared to be free of rickettsiae as judged by staining.

Responsiveness of antibiotic-cured L929 cells derived from persistently infected, IFN-γ-treated cultures to IFN-γ. To determine whether the L929 cells in the persistently infected, IFN-γ-treated cultures had lost the ability to respond to IFN-γ, two cultures derived from different infections (experiments 427 and 87) were cured of their rickettsial infection and the ability of these cells to mount both IFN-γ-induced antiviral and antirickettsial responses was evaluated. The rickettsiae were eliminated from the cells by treatment with erythromycin (experiment 427) or chloramphenicol (experiment 87) for 3 weeks with or without IFN-γ.

Concentrations of IFN-γ that protected normal L929 cells from the cytopathic effects of vesicular stomatis virus also protected the antibiotic-cured cells derived from the persistently infected, IFN-γ-treated cultures (data not shown). IFN-γ (12 U/ml) also inhibited the growth of *R. prowazekii* Madrid E in these cured cells (Table 1). Cells that had been cultured without IFN-γ during the antibiotic treatment and before the experiments supported rickettsial growth, except when IFN-γ was present during the experiments (Table 1). However, even when IFN-γ was not added during the experiments, the rickettsiae did not grow (or grew very poorly) in cells that had been continuously maintained in the presence of IFN-γ during the antibiotic treatment and before the experiments. These data indicate that the persistently infected, IFN-γ-treated L929 cells, whether they were cured of their rickettsial infection with or without IFN-γ, retained the ability to inhibit rickettsial growth when they were treated with IFN-γ. The failure of *R. prowazekii* Madrid E to grow in the cells that had been cured in the presence of IFN-γ, even when IFN-γ was not added after infection, probably reflects the antirickettsial effects of the IFN-γ that was present in these cultures before infection. Residual effects of the antibiotics used to cure the cells were not responsible for the lack of rickettsial growth in these cells because the cells supported rickettsial growth when they were infected with the IFN-γ-resistant rickettsiae described below (data not shown). The parameters of rickettsial infection in antibiotic-cured cells that were infected with *R. prowazekii* Madrid E and subcultured in the presence of IFN-γ for 10 days exhibited patterns similar to those observed in standard L929 cells that were infected and treated with IFN-γ (Fig. 4). The percentage of cells infected decreased in all cases, and the average number of rickettsiae per infected cell did not increase markedly in any case. Taken together, these data indicate that L929 cells derived from the persistently infected cultures and cured of their rickettsial infection retained the ability to respond to IFN-γ.

Reduced sensitivity of rickettsiae from persistently infected, IFN-γ-treated cultures to the antirickettsial effect of IFN-γ. The original *R. prowazekii* Madrid E and rickettsiae isolated from persistently infected IFN-γ-treated L929 cells differed greatly in their responses to IFN-γ treatment in standard L929 cells. These modified responses to IFN-γ were observed with rickettsiae harvested by breaking persistently

TABLE 1. IFN-γ-induced inhibition of the growth of *R. prowazekii* Madrid E in antibiotic-cured L929 cells derived from persistently infected, IFN-γ-treated cultures[a]

Host cell type or source	Presence of IFN-γ during antibiotic treatment	Treatment after infection	Initial rickettsial infection[b]		Rickettsial growth (no. of doublings/day, mean ± SEM)
			%R	RI	
Standard L929 cells	NA[c]	Control	84 ± 4	6.4 ± 1.9	1.5 ± 0.2
		IFN-γ	84 ± 4	6.4 ± 1.9	0.1 ± 0.2
Expt. 427, cured	No	Control	81 ± 2	9.7 ± 3.1	0.9 ± 0.1
		IFN-γ	81 ± 2	9.7 ± 3.1	0.1 ± 0.0
Expt. 427, cured	Yes	Control	85 ± 2	11.3 ± 4.0	−0.4 ± 0.0
		IFN-γ	85 ± 2	11.3 ± 4.0	−0.7 ± 0.0
Expt. 87, cured	No	Control	74 ± 7	8.2 ± 2.5	1.1 ± 0.3
		IFN-γ	74 ± 7	8.2 ± 2.5	0.1 ± 0.2
Expt. 87, cured	Yes	Control	81 ± 5	8.9 ± 2.9	0.1 ± 0.2
		IFN-γ	81 ± 5	8.9 ± 2.9	−0.1 ± 0.1

[a] For details, see Materials and Methods. Four experiments were done for standard L929 cells and cells cured with chloramphenicol; two experiments were done for cells cured with erythromycin.

[b] %R, Percentage of cells infected; RI, average number of rickettsiae per infected cell.

[c] NA, Not applicable.

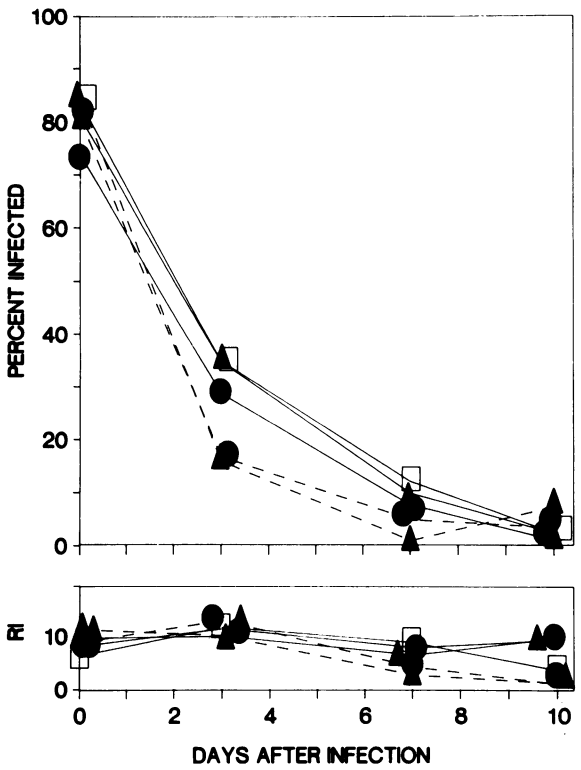

FIG. 4. Parameters of *R. prowazekii* Madrid E infection in IFN-γ-treated cultures of antibiotic-cured L929 cells derived from persistently infected, IFN-γ-treated cultures. Persistently infected, IFN-γ-treated cultures from two independent infections were cured of their rickettsial infections by treatment with an antibiotic in the presence (-----) or absence (——) of IFN-γ. After subculture without the antibiotic, these cells and standard L929 cells were infected with *R. prowazekii* Madrid E and treated with IFN-γ. At each time point, the attached cells were subcultured and samples were stained and examined for rickettsiae. The data were averaged from the following experiments: ●, cells from experiment 87, cured with chloramphenicol with or without IFN-γ, $n = 4$; ▲, cells from experiment 427, cured with erythromycin with or without IFN-γ, $n = 2$; □, standard L929 cells, $n = 4$. RI is the average number of rickettsiae per infected cell.

infected, IFN-γ-treated L929 cells (data not shown) and were retained after the rickettsiae were grown in embryonated hen eggs (*R. prowazekii* 427 and *R. prowazekii* 87 [Table 2]). Furthermore, *R. prowazekii* 427 and *R. prowazekii* 87 that were plaque purified two times and subsequently grown again in embryonated hen eggs also had modified responses to IFN-γ treatment in standard L929 cells (*R. prowazekii* 427-19 and *R. prowazekii* 87-17 [Table 2]). The percentage of cells infected increased or remained high in IFN-γ-treated L929 cells infected with *R. prowazekii* from experiments 427 and 87 but decreased in IFN-γ-treated cultures infected with *R. prowazekii* Madrid E. Similarly, although the average number of rickettsiae per infected cell increased markedly in IFN-γ-treated cultures that were infected with rickettsiae isolated from the persistently infected, IFN-γ-treated cultures, this parameter changed very little in the IFN-γ-treated cultures that were infected with *R. prowazekii* Madrid E.

Rickettsial growth was assessed between days 0 and 3 in untreated and IFN-γ-treated, standard L929 cells infected with *R. prowazekii* isolates 427, 87, 427-19, and 87-17 to determine whether these rickettsiae were resistant to the antirickettsial effect of IFN-γ. The growth rates of all four rickettsial isolates were significantly lower in L929 cells treated with IFN-γ (12 U/ml) than in untreated L929 cells (Table 2). However, although the growth rates of the four rickettsial isolates and *R. prowazekii* Madrid E were similar in untreated L929 cells, the growth rates of the four rickettsial isolates in IFN-γ-treated L929 cells were significantly higher than the growth rate of *R. prowazekii* Madrid E in IFN-γ-treated L929 cells (Table 2). A higher concentration of IFN-γ (100 U/ml) did not cause a further reduction in the growth rates of the Madrid E strain or the rickettsiae isolated from persistently infected, IFN-γ-treated L929 cells (data not shown). These data indicate that the rickettsiae isolated from persistently infected, IFN-γ-treated L929 cells were less sensitive to the antirickettsial effect of IFN-γ than *R. prowazekii* Madrid E; however, these rickettsiae were not completely resistant to IFN-γ.

Examination of L929 cell growth in untreated and IFN-γ-treated L929 cells infected with *R. prowazekii* Madrid E or *R. prowazekii* 427, 87, 427-19, or 87-17 revealed that cell growth between days 0 and 3 was significantly lower in the infected, IFN-γ-treated cultures than in the infected control cultures, except in cultures infected with *R. prowazekii* 427-19 (Table 2). Interestingly, between days 3 and 7, cell growth was significantly lower in the IFN-γ-treated cultures infected with *R. prowazekii* 427, 87, 427-19, or 87-17 than in the IFN-γ-treated cultures infected with *R. prowazekii* Madrid E (Table 2). During this time, the parameters of rickettsial infection were also much higher in the IFN-γ-treated cultures infected with *R. prowazekii* 427, 87,427-19, or 87-17 than in the IFN-γ-treated cultures infected with *R. prowazekii* Madrid E (Table 2). Rickettsial growth was not determined between days 3 and 7 because on day 7, there was evidence of substantial host cell damage in cultures infected with rickettsiae isolated from persistently infected, IFN-γ-treated L929 cells.

DISCUSSION

Rickettsial growth was inhibited during the first 10 days when *R. prowazekii* Madrid E-infected L929 cells were cultured in the presence of IFN-γ. This initial period of inhibition of rickettsial growth was followed by either elimination or persistence of the rickettsiae.

One hypothesis to explain the elimination of *R. prowazekii* from some cultures of infected, IFN-γ-treated L929 cells is that the nongrowing rickettsiae were simply diluted by the growing L929 cells. One can explore this hypothesis by examining certain parameters of the rickettsial infection. On day 3, there were approximately 22,240 rickettsiae per 8,000 L929 cells in two infected, IFN-γ-treated cultures derived from one infection. Between days 3 and 14, the infected IFN-γ-treated L929 cells in these cultures increased about 105-fold. On day 14, when one might have expected (by dilution) to find approximately 212 rickettsiae per 8,000 cells, there was actually less than 1 rickettsia per 8,000 cells by the egg inoculation assay. These data suggest that dilution of the nongrowing rickettsiae is not an adequate explanation for the elimination of *R. prowazekii* from IFN-γ-treated cultures of L929 cells. Other factors that might have contributed to the elimination of *R. prowazekii* from some cultures of infected, IFN-γ-treated L929 cells include the following: i) some of the rickettsiae could have been killed in the IFN-γ-treated cells, ii) rickettsiae released from infected cells in the cultures could have been lost in the supernatant fluids during

TABLE 2. Rickettsial growth and L929 cell growth in untreated and IFN-γ-treated cultures of standard L929 cells infected with rickettsiae isolated from persistently infected, IFN-γ-treated L929 cells

Rickettsial strain	Cell treatment after infection	Interval (days)	No. of expts	Growth[a] (no. of doublings/day, mean ± SEM)		Rickettsial infection at end of interval[c]	
				L929 cells	Rickettsiae[b]	%R (mean ± SEM)	RI (mean ± SEM)
Madrid E	NA[d]	0	6			60 ± 8	3.6 ± 0.4
	None (control)	0–3	6	0.9 ± 0.1	2.1 ± 0.1	78 ± 9	35 ± 3
	IFN-γ	0–3	6	0.5 ± 0.1**	0.8 ± 0.1**	33 ± 5	10 ± 1
	IFN-γ	3–7	6	0.6 ± 0.0	ND[e]	14 ± 2	8 ± 1
427	NA	0	4			38 ± 7	2.3 ± 0.3
	None (control)	0–3	4	0.7 ± 0.1	2.5 ± 0.2	95 ± 3	39 ± 8
	IFN-γ	0–3	4	0.4 ± 0.1*	2.0 ± 0.1*‡‡‡	91 ± 4	25 ± 3
	IFN-γ	3–7	4	0.1 ± 0.1††	ND	98 ± 1	51 ± 6
427-19	NA	0	2			49 ± 4	2.4 ± 0.2
	None (control)	0–3	2	0.6 ± 0.0	2.5 ± 0.1	99 ± 1	64 ± 3
	IFN-γ	0–3	2	0.3 ± 0.1	1.9 ± 0.1**‡‡‡	98 ± 0	38 ± 1
	IFN-γ	3–7	2	0.2 ± 0.1††	ND	87 ± 7	32 ± 5
87	NA	0	4			76 ± 3	3.9 ± 0.7
	None (control)	0–3	4	0.7 ± 0.1	2.3 ± 0.1	100 ± 0	74 ± 3
	IFN-γ	0–3	4	0.3 ± 0.1**	1.3 ± 0.2**‡	93 ± 2	20 ± 3
	IFN-γ	3–7	4	0.3 ± 0.1††	ND	93 ± 5	44 ± 7
87-17	NA	0	2			67 ± 1	3.4 ± 0.1
	None (control)	0–3	2	0.6 ± 0.0	2.3 ± 0.0	100 ± 0	81 ± 1
	IFN-γ	0–3	2	0.5 ± 0.0**	1.8 ± 0.1**‡‡‡	99 ± 1	45 ± 5
	IFN-γ	3–7	2	0.3 ± 0.1††	ND	98 ± 1	53 ± 9

[a] For the time interval beginning on day 0 and ending on day 3, a significant difference between IFN-γ-treated cultures and untreated control cultures infected with the same rickettsial strain is indicated by ** ($P \leq 0.05$) or * ($0.05 < P \leq 0.1$) and a significant difference between IFN-γ-treated cultures infected with *R. prowazekii* Madrid E and IFN-γ-treated cultures infected with rickettsiae isolated from persistently infected, IFN-γ-treated L929 cells is indicated by ‡‡‡ ($P \leq 0.005$) or ‡ ($0.05 < P \leq 0.1$). For the time interval beginning on day 3 and ending on day 7, a significant difference between IFN-γ-treated cultures infected with *R. prowazekii* Madrid E and IFN-γ-treated cultures infected with rickettsiae isolated from persistently infected, IFN-γ-treated L929 cells is indicated by †† ($P \leq 0.05$).

[b] In each instance, the number of rickettsial doublings that occurred between days 0 and 3 was calculated from the number of rickettsiae present in the L929 cells that were planted on day 0 and the number of rickettsiae per culture on day 3. The number of rickettsiae per culture on day 3 was calculated by adding the number of rickettsiae in the detached cells to the number of rickettsiae in the attached cells.

[c] %R (percentage of cells infected) and RI (average number of rickettsiae per infected cell) for the intervals ending on days 3 and 7 represent the attached cells only.

[d] NA, Not applicable.

[e] ND, Not determined.

subculturing, and iii) infected cells could have been selectively lost from the cultures.

The selective toxicity of IFN-γ for *R. prowazekii*-infected L929 cells in contrast to uninfected cells was a significant feature of these experiments. Wisseman and Waddell (26) found that supernatant fluids collected from stimulated cultures of human leukocytes had a cytolytic effect on *R. prowazekii*-infected human fibroblasts and suggested that IFN-γ was responsible for the cytolytic effect of the supernatant fluids. The present study indicates that IFN-γ, in the absence of other lymphokines, has a toxic effect on *R. prowazekii*-infected mouse L929 cells. Macrophagelike RAW264.7 cells are also killed by the combination of IFN-γ treatment and infection with *R. prowazekii* (16). However, for IFN-γ-treated RAW264.7 cells, cell death occurs more rapidly (within 4 to 6 h after infection), when similarly treated and infected L929 cells are still viable (16). Whether similar mechanisms are involved in the killing of both fibroblasts and macrophagelike cells by *R. prowazekii* and IFN-γ is not known.

How, or even if, the toxic effect of IFN-γ on *R. prowazekii*-infected cells relates to the ability of IFN-γ to inhibit rickettsial growth is not known. It is also not known whether the rickettsiae within the IFN-γ-treated cells are killed as the cells die. Clearly, the most beneficial antirickettsial activity (from the standpoint of the host) would result in destruction of the rickettsiae without damage to the host cells. If IFN-γ-mediated killing of *R. prowazekii*-infected cells occurs in vivo, it could contribute to pathology in the infected host.

The emergence of cultures in which the rickettsiae grew well in the presence of IFN-γ could have been due to modification of either the host cells or the rickettsiae. Since persistently infected, IFN-γ-treated L929 cells that were cured of their rickettsial infection retained the ability to respond to IFN-γ, but rickettsiae isolated from the persistently infected, IFN-γ-treated cultures had modified responses to IFN-γ treatment in standard L929 cells, the establishment of persistent infections in IFN-γ-treated L929 cells reflects an alteration(s) in the rickettsiae. The maintenance of the phenotype of these altered rickettsiae during plaque purification and passage in the absence of IFN-γ suggests an alteration at the genetic level rather than phenotypic adaptation. Experiments are currently in progress to determine whether strains of *R. prowazekii* other than the Madrid E strain can also establish persistent infections in IFN-γ-treated L929 cells.

Interestingly, the Madrid E strain itself arose when the virulent Madrid strain of *R. prowazekii* spontaneously lost virulence during egg passage (5, 12). Several studies suggest

that changes occur in the Madrid E strain after passage in animals or cell cultures. For example, enhancement of the virulence of the Madrid E strain after passage in mice or guinea pigs has been reported (3, 10). Furthermore, the virulence of the Madrid E strain has been reported to increase (9) or decrease (2) after passage in cell cultures. In addition, several drug-resistant or antibiotic-resistant substrains of *R. prowazekii* Madrid E have been isolated after laboratory passage of rickettsiae in the presence of *p*-aminobenzoic acid, 2,3-dimethyl quinoxaline-1,4-dioxide, acetylsalicylic acid, chloramphenicol, or erythromycin (20–24). Mutants resistant to erythromycin or rifampin have also been selected after exposure of *R. prowazekii* Madrid E to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1). The molecular basis of these variations in the Madrid E strain has not been determined.

Because the Madrid E strain of *R. prowazekii* used in the present study had not been plaque purified, variant rickettsiae present at the time of infection may have been selected by subculturing the infected cells in the presence of IFN-γ. Alternatively, the occurrence of a mutation sometime after infection may have been followed by selection of the altered rickettsiae. Regardless of the time at which mutation of the rickettsiae occurred, characterization of these altered rickettsiae should facilitate determination of the mechanisms by which IFN-γ inhibits rickettsial growth in cultured cells.

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