

CLONED MOUSE INTERFERON- $\gamma$   
INHIBITS THE GROWTH OF *RICKETTSIA PROWAZEKII*  
IN CULTURED MOUSE FIBROBLASTS\*

BY JENIFER TURCO AND HERBERT H. WINKLER<sup>‡</sup>

*From the Laboratory of Molecular Biology, Department of Microbiology and Immunology,  
University of South Alabama College of Medicine, Mobile, Alabama 36688*

The bacterium *Rickettsia prowazekii* is the etiological agent of epidemic typhus, a disease of humans in which rickettsiae proliferate within the endothelial cells lining the capillaries. The rickettsiae grow freely in the cytoplasm of their host cells; they are not enclosed within vacuoles.

Antirickettsial lymphokines may represent a mechanism of host defense against rickettsiae present within nonprofessional phagocytes. Inhibition of the growth and survival of *R. prowazekii* in cultured mouse fibroblasts occurs when the fibroblasts are treated with lymphokines produced by antigen- or concanavalin A-stimulated mouse spleen cells (1). Rickettsial growth is also inhibited in cultured human fibroblasts, endothelial cells, and macrophages when these cells are treated with culture supernatants obtained from antigen- or lectin-stimulated human peripheral blood leukocytes (1, 2).

Several characteristics of the antirickettsial activity (ARA) in mouse lymphokines are consistent with the hypothesis that this activity is due to interferon- $\gamma$  (IFN- $\gamma$ ) (1, 3). Like IFN- $\gamma$ , ARA manifests species specificity or species preference, requires host cell protein synthesis for expression of activity, is relatively stable at 56°C, and is destroyed by exposure to pH 2, by trypsin, or by heating at 80°C. Furthermore, ARA is neutralized by antisera prepared against partially purified IFN- $\gamma$  (3). In the present study, we present definitive evidence that IFN- $\gamma$  has antirickettsial activity: cloned mouse IFN- $\gamma$  inhibits the growth of *R. prowazekii* in mouse L929 cells.

#### Materials and Methods

*Cell Cultures.* Mouse L929 cells were grown in Eagle's minimum essential medium supplemented with 10% calf serum or 10% fetal calf serum. Cultures of human foreskin fibroblasts were obtained from Mr. Frank Pindak, University of South Alabama, and were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum. All cells were grown in a CO<sub>2</sub> incubator at 34°C.

*Rickettsiae.* *Rickettsia prowazekii* E strain was prepared from yolk sacs as previously described (1).

*Cloned Mouse IFN- $\gamma$  and Control Medium for Cloned IFN- $\gamma$ .* Cloned mouse IFN- $\gamma$  produced by Chinese hamster ovary cells, control medium for this IFN- $\gamma$  (CM), and cloned mouse IFN- $\gamma$  produced by *Escherichia coli* were kindly provided by Dr. Patrick Gray and Dr. Gene Burton (Genentech, Inc., South San Francisco, CA).

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<sup>‡</sup> To whom requests for reprints should be addressed.

*Preparation of Lymphokines.* Lymphokines were prepared by culturing the spleen cells of *Listeria monocytogenes*-challenged mice in the presence of concanavalin A for 24 h according to a method modified from that of Havell et al. (4). Briefly, C57BL/6J mice (8–15 wk old) were inoculated intravenously with 0.1 ml of saline that contained  $1 \times 10^8$  *Listeria monocytogenes*. 7 d later, spleen cells were cultured at a concentration of  $1 \times 10^7$  cells/ml in serum-free RPMI 1640 medium to which 2  $\mu\text{g/ml}$  concanavalin A (Sigma Chemical Co., St. Louis, MO) had been added. Seven lymphokine preparations made according to this procedure contained 4,127–9,010 IU of interferon/ml ( $\bar{x} = 6,953$ ).

*Interferon Assay.* A cytopathic effect inhibition assay modified from that of Havell and Vilcek (5) as described previously (3) was used for measuring IFN. IFN activity was calibrated against a World Health Organization international reference preparation of mouse type I IFN (G-002-904-511) (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) and is expressed as international units.

*Treatment of Cells with Cloned IFN- $\gamma$ , Infection of Cells with Rickettsiae, and Measurement of Rickettsial Growth.* Methods for x irradiation of cells, infection of cells, and measurement of rickettsial growth have been detailed previously (1). After x irradiation, L929 cells or human foreskin fibroblasts were adjusted to a density of  $1.2 \times 10^5$  viable cells/ml, planted in eight-chambered slides (0.3 ml/chamber) (Lab-Tek Div., Miles Laboratories, Inc., Naperville, IL), and incubated overnight. The culture medium was then removed and the cells were treated for 24 h with medium alone or with medium that contained cloned IFN- $\gamma$  or CM. Then, after the cells were infected with rickettsiae and washed, medium alone or medium that contained cloned IFN- $\gamma$  or CM was added. Infected cells were incubated at 34°C for up to 48 h. At 0, 24, and 48 h after infection, slides were dried, fixed, and stained by a modification of the Gimenez method, as previously described (1). Slides were examined microscopically with an oil immersion objective and the number of rickettsiae present in each of 100 cells was counted for each duplicate of each experimental treatment. When a cell contained >100 rickettsiae, it was assigned a value of 100; this practice causes an underestimate of the number of rickettsiae at later times. The percentage of cells infected with rickettsiae (%R), the average number of rickettsiae per infected cell (RI), and the average number of rickettsiae per cell (NR) were determined. The data were normalized to the zero time infection; e.g., RI at a given time divided by RI at 0 h multiplied by 100 gives the average number of rickettsiae per infected cell as a percentage of that observed at 0 h.

*Comparison of the Effect of Cloned IFN- $\gamma$  and Crude Mouse Lymphokines on Rickettsial Growth.* Dilutions of IFN- $\gamma$  or crude mouse lymphokines were made in eight-chambered Lab-Tek slides (150  $\mu\text{l}$ /chamber). A suspension of irradiated L929 cells was infected with *R. prowazekii*, and washed, and  $3.8 \times 10^4$  cells in 150  $\mu\text{l}$  of tissue culture medium were added per chamber. Samples of the infected cell suspension were immediately cytocentrifuged onto microscope slides, dried, fixed, and stained. After incubation at 34°C for 48 h, the chamber slides were also dried, fixed, and stained. Percent inhibition of rickettsial growth in the treated chambers was calculated from the values of NR at 48 h after infection (expressed as percentages of the values of NR observed at 0 h). At the time of each experiment, the IFN- $\gamma$  and crude lymphokines were assayed for IFN.

## Results

*ARA of Cloned Mouse IFN- $\gamma$ .* During the 48-h period after infection, dramatic effects on both %R and RI were observed in *R. prowazekii*-infected L929 cell cultures that were treated with cloned mouse IFN- $\gamma$  both 24 h before and after infection (Fig. 1). Only 63% of the L929 cells that were infected at 0 h remained infected at 48 h after infection in these IFN- $\gamma$ -treated cultures (%R). In contrast, in control cultures or in cultures treated with CM both before and after infection, all of the cells that were infected at 0 h remained infected at 48 h. RI underwent little change in these IFN- $\gamma$ -treated cultures during the 48 h period after infection; thus, rickettsial growth was almost completely inhibited in the IFN- $\gamma$ -treated cells that remained infected with rickettsiae. In contrast, rickettsial

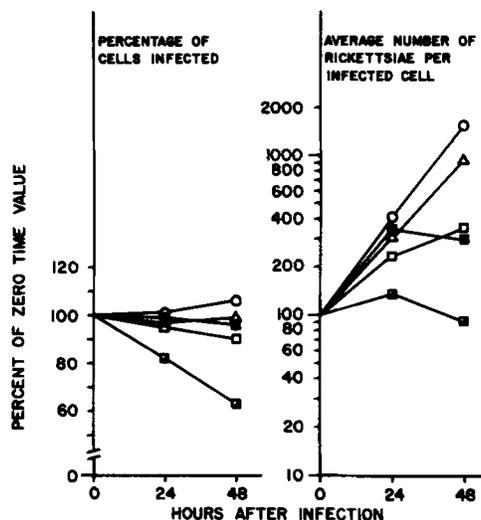


FIGURE 1. Effect of treatment of mouse L929 cells with cloned mouse IFN- $\gamma$  or control medium for this IFN (CM) on the growth of *R. prowazekii* in these cells. Cloned mouse IFN- $\gamma$  produced by Chinese hamster ovary cells and CM were diluted in Eagle's minimum essential medium supplemented with 10% fetal calf serum. L929 cells were treated with cloned mouse IFN- $\gamma$  (175 IU/0.3 ml) (□, ■, ■), CM ( $\Delta$ ), or tissue culture medium alone (O). The percentage of cells infected and the average number of rickettsiae per infected cell are expressed as percentages of the values observed at zero time. (□) Cells treated with IFN- $\gamma$  24 h before infection; (■) cells treated with IFN- $\gamma$  24 h before and after infection; (■) cells treated with IFN- $\gamma$  after infection; ( $\Delta$ ) cells treated with CM 24 h before and after infection. Each value represents the mean of two experiments. The percentage of cells infected at 0 h was 92 for control cells, 88 for cells treated with IFN- $\gamma$  before infection, and 99 for cells treated with CM before infection. The average number of rickettsiae per infected cell at 0 h was 4.7 for control cells, 3.9 for cells treated with IFN- $\gamma$  before infection, and 6.5 for cells treated with CM before infection.

growth occurred in the control and CM-treated cultures: RI increased  $\sim$ 15-fold in the former and 9-fold in the latter.

Less dramatic ARA was observed when L929 cells were treated with cloned mouse IFN- $\gamma$  either only before or only after infection with *R. prowazekii* (Fig. 1). In these cultures, rickettsial growth was markedly inhibited, but most of the cells that were infected with rickettsiae at 0 h remained infected at 48 h after infection. In cultures treated with IFN- $\gamma$  only after infection, there was complete inhibition of rickettsial growth between 24 and 48 h after infection but inhibition was minimal or absent between 0 and 24 h after infection.

*Cycloheximide Suppression and Species Specificity of the ARA of Cloned Mouse IFN- $\gamma$ .* Cycloheximide (1  $\mu$ g/ml) markedly suppressed the inhibition of the growth of *R. prowazekii* by IFN- $\gamma$  (produced by Chinese hamster ovary cells). During the 48 h period after infection, RI increased 15.5-fold in untreated cultures, 3.5-fold in cultures treated with IFN- $\gamma$  (175 IU/0.3 ml) 24 h before infection, 13.0-fold in cultures treated with IFN- $\gamma$  and cycloheximide 24 h before infection, and 16.4-fold in cultures treated with cycloheximide before infection (two experiments each). The values of %R in these cultures at 48 h after infection were 106, 90, 99, and 109%, respectively, of the values of %R observed at 0 h after infection.

Treatment of human foreskin fibroblasts with cloned mouse IFN- $\gamma$  (produced

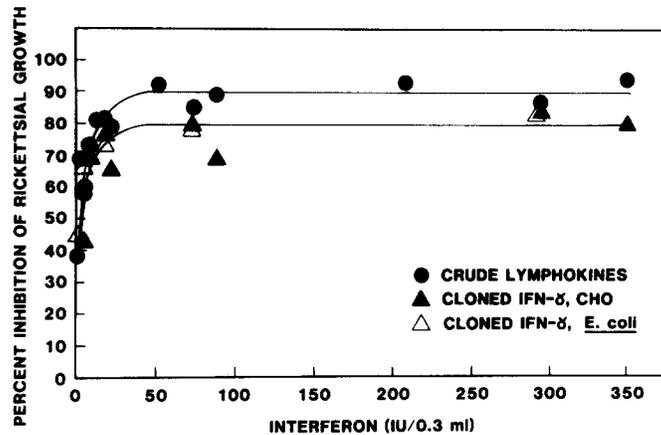


FIGURE 2. Inhibition of the growth of *R. prowazekii* in mouse L929 cells by various concentrations of cloned mouse IFN- $\gamma$  or crude mouse lymphokines. L929 cells infected with *R. prowazekii* were added to chamber slides that contained tissue culture medium alone, dilutions of cloned mouse IFN- $\gamma$  produced by Chinese hamster ovary cells ( $\blacktriangle$ ), dilutions of cloned mouse IFN- $\gamma$  produced by *E. coli* ( $\triangle$ ), or dilutions of crude mouse lymphokines ( $\bullet$ ). After incubation for 48 h, the slides were fixed and stained. Rickettsial growth was monitored and percent inhibition of rickettsial growth was calculated as described in Materials and Methods. Lymphokines and cloned IFN- $\gamma$  were assayed for IFN (antiviral activity) at the time of each experiment. Results of three experiments are shown. At 0 h after infection, the average values for %R and RI were  $65 \pm 8$  and  $4.5 \pm 0.8$ , respectively. In the control cultures, NR increased  $15.1 \pm 4.2$ -fold during the 48-h period after infection.

by Chinese hamster ovary cells; 175 IU/0.3 ml), both before and after infection with *R. prowazekii*, failed to inhibit rickettsial survival and growth in these cells. During the 48-h period after infection, %R increased slightly in all cultures and RI increased 16.3-fold in untreated cultures, 14.0-fold in IFN- $\gamma$ -treated cultures, and 12.6-fold in CM-treated cultures (two experiments each).

*Comparison of the ARA of Cloned Mouse IFN- $\gamma$  and Crude Mouse Lymphokines.* Marked inhibition of rickettsial growth was apparent in chambers that contained as few as 5 IU of IFN as cloned mouse IFN- $\gamma$  or crude mouse lymphokines (Fig. 2). Cloned mouse IFN- $\gamma$  produced by either Chinese hamster ovary cells or *E. coli* had identical dose-response curves. The percent inhibition of rickettsial growth in cultures treated with various concentrations of crude mouse lymphokines plateaued at a slightly higher value ( $\sim 90\%$ ) than that observed in cultures treated with various concentrations of cloned mouse IFN- $\gamma$  ( $\sim 80\%$ ). Most of the ARA of the crude lymphokines can be accounted for by the IFN- $\gamma$  that is present in the crude lymphokines. However, some other minor antirickettsial factor(s) may also be present in the crude lymphokines.

### Discussion

The effects of cloned mouse IFN- $\gamma$  on the survival and growth of *R. prowazekii* in mouse fibroblasts were similar to the effects of crude mouse lymphokines (1). In mouse L929 cells treated with either material 24 h before and after infection, the rickettsiae were cleared from some of the infected cells and rickettsial growth was suppressed in the cells that remained infected. Time was required for the

establishment of antirickettsial conditions within L929 cells by either material because rickettsial growth was more strongly inhibited during the first 24 h after infection in cells pretreated for 24 h than in cells treated only after infection. Continued expression of the antirickettsial conditions induced by either material may require the continued presence of the material since the antirickettsial effects observed in cells treated before and after infection were more pronounced than those observed in cells treated only before infection. Induction of antirickettsial conditions by either material was suppressed by cycloheximide, and neither the cloned mouse IFN- $\gamma$  nor the crude mouse lymphokines induced antirickettsial conditions in human foreskin fibroblasts.

Our previous work indicated that ARA and IFN- $\gamma$  have several characteristics in common (1, 3). We have shown here that cloned mouse IFN- $\gamma$  induces antirickettsial conditions in mouse fibroblasts. Inhibition of the growth of a protozoan parasite, *Toxoplasma gondii*, within human fibroblasts treated with cloned human IFN- $\gamma$  was recently reported (6). Thus, IFN- $\gamma$  can induce antimicrobial activity against either prokaryotic or eukaryotic microorganisms that grow within nonprofessional phagocytes.

The effectiveness of IFN- $\gamma$  against *R. prowazekii* in vivo remains to be determined, but we speculate that production of IFN- $\gamma$  by sensitized T lymphocytes may explain (in large part) the protective effect of adoptively transferred T lymphocytes or unfractionated spleen cells from immune animals against various rickettsial infections in animals (7-11). Production of IFN- $\gamma$  has been demonstrated in immunized mice after challenge with viable *R. tsutsugamushi* (12). The induction of ARA in nonprofessional phagocytes (fibroblasts) by cloned IFN- $\gamma$  has been documented in this study, and provides a mechanism for the control and elimination of *R. prowazekii* sequestered within their principal host cells in vivo. The participation of cloned IFN- $\gamma$  in macrophage activation has been demonstrated by other investigators (13), and we have found that treatment of mouse macrophage-like cells with cloned mouse IFN- $\gamma$  makes these cells unable to support the growth of *R. prowazekii* (J. Turco, and H. H. Winkler, manuscript in preparation). It seems likely that IFN- $\gamma$  has a significant role in host defense against *R. prowazekii* infections.

### Summary

The effect of treating cultured mouse fibroblasts (L929 cells) with cloned mouse interferon- $\gamma$  on the growth of *Rickettsia prowazekii* within the fibroblasts was studied. Within 48 h after infection, rickettsiae were cleared from a substantial proportion of the initially infected cells and rickettsial growth was inhibited in those cells that remained infected, when L929 cells were treated with cloned mouse interferon- $\gamma$  both before and after infection. When L929 cells were treated with cloned mouse interferon- $\gamma$  either only before or only after infection with rickettsiae, rickettsial growth was markedly inhibited but rickettsiae were not cleared from many cells. Addition of cycloheximide to L929 cells markedly suppressed the antirickettsial activity of the interferon, and cloned mouse interferon- $\gamma$  did not induce antirickettsial activity in human foreskin fibroblasts. The antirickettsial effects of cloned mouse interferon- $\gamma$  were similar to those induced by crude mouse lymphokines prepared from concanavalin A-stimulated mouse

spleen cells. Equivalent amounts (units) of cloned mouse interferon- $\gamma$  produced by Chinese hamster ovary cells or by *Escherichia coli* caused equivalent inhibition of rickettsial growth in mouse fibroblasts. However, at high concentrations of interferon- $\gamma$ , treatment of rickettsia-infected fibroblasts with equivalent amounts (units) of interferon- $\gamma$ , as crude mouse lymphokines or cloned mouse interferon- $\gamma$ , resulted in slightly greater inhibition of rickettsial growth by the crude lymphokines. Most of the antirickettsial activity of crude mouse lymphokines can be explained by the interferon- $\gamma$  that is present in these preparations. Interferon- $\gamma$ , by virtue of its ability to inhibit rickettsial growth and effect the clearance of rickettsiae from nonprofessional phagocytes, may play a crucial role in the elimination of rickettsiae from the infected host.

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