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Cytokine Sensitivity and Methylation of Lysine in *Rickettsia prowazekii* EVir and Interferon-Resistant *R. prowazekii* Strains

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Modified *Rickettsia prowazekii* strains have been derived from the avirulent Madrid E strain by passage in the lungs of white mice (strain EVir) or by selection for resistance to gamma interferon (IFN-γ) (strains 427-19 and 87-17) or alpha/beta interferon (IFN-α/β) (strains 83-2P, 60P, 103-2P, and 110-1P). Compared with the Madrid E strain, strain EVir has increased virulence (N. M. Balayeva and V. N. Nikolskaya, J. Hyg. Epidemiol. Microbiol. Immunol. 17:11–20, 1973) and a different lysine methylation profile in its surface protein antigen (A. V. Rodionov, M. E. Eremeeva, and N. M. Balayeva, Acta Virol. 35:557–565, 1991). The other six strains differ from the Madrid E strain in their resistance to IFN and their ability to grow well in untreated macrophagelike RAW264.7 cells. In the present study, to determine which properties are shared by these strains, we examined *R. prowazekii* EVir for the following: (i) the sensitivity of its growth in L929 cells to the cytokines IFN-α/β, IFN-γ, tumor necrosis factor alpha (TNF-α), and IFN-γ plus TNF-α; (ii) the ability to grow in untreated RAW264.7 cells; and (iii) the ability to induce interferon in L929 cell cultures; we also evaluated strains 83-2P and 87-17 for lysine methylation. Multiplication of strain EVir in growing L929 cells was not markedly inhibited by either IFN-α/β or IFN-γ. In X-irradiated L929 cells, growth of strain EVir was slightly inhibited (11%) by TNF-α alone, somewhat inhibited (38%) by IFN-γ alone, and markedly inhibited (87%) by IFN-γ plus TNF-α. Nitrite production was induced in X-irradiated, strain EVir-infected L929 cell cultures treated with TNF-α alone or IFN-γ alone; however, more nitrite was produced in infected cultures treated with IFN-γ plus TNF-α. Nitrite production, the dramatic inhibitory effect of IFN-γ plus TNF-α, and the modest inhibitory effect of IFN-γ on the growth of strain EVir in X-irradiated L929 cells were all alleviated by the addition of the nitric oxide synthase inhibitor N^G-methyl-L-arginine. Strain EVir grew very well in untreated macrophagelike RAW264.7 cells and appeared defective in the ability to induce IFN in L929 cell cultures. All strains grown in L929 cells in the presence of radiolabeled lysine had similar percentages of their radioactivity as methylated lysines. However, strains EVir, 83-2P, and 87-17 differed significantly from the Madrid E strain in that they each had more radioactivity in the N^ϵ-trimethyl-lysine fraction, more radioactivity in the N^ϵ-dimethyl-lysine fraction, and less radioactivity in the N^ϵ-methyl-lysine fraction. The results indicate that strains EVir and 83-2P are similar to each other (and different from strain Madrid E) in cytokine sensitivity, ability to grow in RAW264.7 cells, and lysine methylation and that strain EVir differs from strains Madrid E and 83-2P by being a very poor inducer of IFN-α/β in L929 cell cultures.

Rickettsia prowazekii is the etiological agent of epidemic typhus, Brill-Zinsser disease, and flying squirrel-associated typhus in humans. This bacterium is an obligate intracellular microorganism which grows in the cytoplasm of its host cells unbounded by a phagosomal or phagolysosomal membrane.

An avirulent strain of *R. prowazekii* (the Madrid E strain) originated when the virulent Madrid strain spontaneously lost its virulence during passage in embryonated eggs (3, 13). Although the biochemical basis of virulence in *R. prowazekii* has not been defined, Gambrill and Wisseman (4) determined that the avirulent Madrid E strain grows poorly in human monocyte-derived macrophages whereas the virulent Breinl strain grows very well in these cells. The Madrid E strain also grows poorly in the mouse macrophagelike cell line RAW 264.7, which supports good growth of the Breinl strain (15).

The cytokines gamma interferon (IFN-γ), alpha/beta interferon (IFN-α/β), and tumor necrosis factor alpha (TNF-α) can induce antirickettsial activity (6, 7, 10, 12, 16, 19, 20, 22, 24). Experimental evidence has indicated that IFN-γ plays an important role in host defense against rickettsial infections (8, 11). Treatment of mouse L929 cells with IFN-γ inhibits growth of *R. prowazekii* Madrid E and Breinl in these cells (16, 21). IFN-α/β also suppresses growth of *R. prowazekii* Madrid E in L929 cells; however, its inhibitory effect is less dramatic than that of IFN-γ (19). The Breinl strain is resistant to IFN-α/β compared with the Madrid E strain (21). TNF-α inhibits growth of both the Madrid E and Breinl strains in L929 cells (22).

Exogenous IFN-γ or endogenously produced IFN-α/β has been used to select interferon-resistant *R. prowazekii* organisms in L929 cell cultures originally infected with the IFN-sensitive Madrid E strain (18, 20). All four strains selected for IFN-α/β resistance (including strains 83-2P and 60P) are also resistant to IFN-γ. However, of the two strains selected for resistance to IFN-γ (strains 87-17 and 427-19), only strain 427-19 is significantly resistant to IFN-α/β compared with the parental Madrid E strain (20). Like the virulent Breinl strain (and unlike the parental Madrid E strain), all six IFN-resistant strains have the ability to grow well in macrophagelike RAW264.7 cells (21). Compared with the parental Madrid E strain, one of the IFN-resistant strains (*R. prowazekii* 60P) is highly defective in its ability to induce IFN-α/β in L929 cell cultures (21). Examination of IFN-resistant *R. prowazekii* 83-2P indicated that it is resistant to TNF-α (22). Although

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growth of strain 83-2P is not markedly inhibited in L929 cells treated with IFN-γ alone or TNF-α alone, it is dramatically inhibited in L929 cells treated with IFN-γ plus TNF-α (22). Inhibition of growth of strain 83-2P in L929 cells treated with IFN-γ plus TNF-α is alleviated by the inhibition of nitric oxide synthase (22).

Balayeva and Nikolskaya (1) obtained *R. prowazekii* organisms with increased virulence for mice and guinea pigs after repeatedly passaging the avirulent Madrid E strain in the lungs of white mice. This strain, designated *R. prowazekii* EVir, was later compared with the Madrid E and Breinl strains in a study of the amino acid composition of the *R. prowazekii* surface protein antigen (14). Rodionov et al. (14) determined that the respective amounts of lysine and methylated lysine (N^{ϵ}-Me-lysine plus N^{ϵ}-Me_3-lysine) in the surface protein antigens of all three strains (EVir, Madrid E, and Breinl) are similar. However, the surface protein antigens of strains Breinl and EVir contained more N^{ϵ}-Me_3-lysine and less N^{ϵ}-Me-lysine when compared with the surface protein antigen of strain Madrid E. The cytokine sensitivity and ability of *R. prowazekii* EVir to grow in macrophagelike RAW264.7 cells have not been evaluated.

In the present study, we evaluated *R. prowazekii* EVir for the sensitivity of its growth in L929 cells to cytokines (IFN-α/β, IFN-γ, TNF-α, and IFN-γ plus TNF-α), its ability to grow in untreated macrophagelike RAW264.7 cells, and its ability to induce IFN in L929 cells, and we examined the lysine methylation profile in IFN-resistant *R. prowazekii* strains with the aim of determining the properties shared by these strains. We report that *R. prowazekii* EVir (i) is resistant to IFN-α/β, IFN-γ, and TNF-α compared with the parental Madrid E strain, (ii) has its growth inhibited by a nitric oxide synthase-dependent mechanism in L929 cells treated with IFN-γ plus TNF-α, (iii) grows well in untreated macrophagelike RAW 264.7 cells, and (iv) is defective in its ability to induce IFN-α/β in L929 cells. In addition, we report that lysine methylation in IFN-resistant *R. prowazekii* 83-2P and 87-17 is similar to that in strain EVir.

MATERIALS AND METHODS

Cell cultures. L929 cells (originally purchased from Flow Laboratories, Inc.) were grown as monolayers in Eagle minimal essential medium supplemented with 10% newborn bovine serum. RAW264.7 cells (originally obtained from the Cell Distribution Center, Salk Institute) were grown in Dulbecco modified Eagle medium supplemented with 10% defined, iron-supplemented bovine calf serum (Hyclone Laboratories, Inc., Logan, Utah). Cell cultures were kept in an atmosphere of 3% CO_2 in air at a temperature of 34°C.

Rickettsiae. *R. prowazekii* EVir was a gift from N. M. Balayeva, Gamaleya Research Institute of Epidemiology and Microbiology, Academy of Medical Sciences, Moscow, Russia. Rickettsiae to be used for infecting cell cultures were purified from the yolk sacs of embryonated chicken eggs inoculated with *R. prowazekii* Madrid E (yolk sac passage 281), Breinl, 83-2P, 87-17, and EVir as previously described (17, 20). The numbers of viable rickettsiae were estimated by assaying their hemolytic activity (23).

Cytokines. Recombinant murine IFN-γ derived from *Escherichia coli* (1.9×10^7 U/mg) and recombinant murine TNF-α derived from *E. coli* (1.2×10^7 U/mg) were generously provided by Genentech, Inc., South San Francisco, Calif. A mixture of virus-induced murine IFN-α and IFN-β (IFN-α/β; 4.7×10^5 U/mg) was purchased from Lee BioMolecular Research Laboratories, Inc., San Diego, Calif. The antiviral activity of the IFNs and the ability of TNF-α to kill actinomycin D-treated L929 cells were assayed as previously described (22).

Evaluation of rickettsiae for cytokine sensitivity, ability to grow in untreated RAW264.7 cells, and ability to induce IFN-α/β in L929 cells. Experiments to evaluate the sensitivity of rickettsiae to cytokines in L929 cells and the ability of rickettsiae to grow in untreated RAW264.7 cells were conducted essentially as previously described (20–22). X-irradiated host cells were used in this study whenever possible because inhibition of the division of the host cells simplifies the measurement of rickettsial growth. It was necessary to use nonirradiated L929 cells in the experiments with IFN-α/β because the inhibition of rickettsial growth by this cytokine is modest and is best observed in dividing rather than X-irradiated L929 cells (19). To evaluate the sensitivity of rickettsiae to IFN-α/β and IFN-γ in growing L929 cells, we planted the cells (approximately 5.5×10^4 cells per well in a total volume of 1.2 ml) in 12-well plates containing Eagle minimal essential medium supplemented with 10% newborn bovine serum and IFN-α/β (100 U/ml) or IFN-γ (100 U/ml) as required. After 2 days, the cells were washed and infected. After additional washing, the cultures were given fresh serum-supplemented culture medium that contained IFN-α/β or IFN-γ as required. Immediately after infection and washing and again after another 2 days duplicate wells were harvested and the cells were counted and stained for rickettsiae (5). The rickettsial infection was monitored by microscopically counting the number of rickettsiae present in each of 100 cells from each culture. The number of rickettsiae in a cell that contained more than 100 rickettsiae was estimated. The numbers of rickettsial doublings was calculated from the numbers of rickettsiae per culture on the day of infection and after incubation for 2 days. To evaluate the sensitivity of rickettsiae to IFN-γ, TNF-α, or IFN-γ plus TNF-α in X-irradiated L929 cells, the cells were infected and planted in eight-chambered glass slides (Nunc, Inc., Naperville, Ill.) (approximately 6.6×10^4 cells per well in a total volume of 300 μl) that contained cytokines and/or the nitric oxide synthase inhibitor N^G-methyl-L-arginine (0.5 mM) as required. Arginine-deficient Eagle minimal essential medium supplemented with 30 μM arginine and 5% newborn bovine serum was used in these experiments. (This reduced concentration of arginine was used to increase the effectiveness of N^G-methyl-L-arginine in inhibiting nitric oxide synthase.) Immediately after infection, samples of the cell suspensions were stained for rickettsiae. After incubation of the cultures for 44 h, the culture fluids were collected, centrifuged, and assayed for nitrite. The cells were stained so that rickettsial growth in the attached cells could be monitored. The rickettsial infection was determined as described above, except that 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 in heavily infected cells. The number of rickettsiae per cell at 44 h was expressed as a percentage of the number per cell immediately after infection, and the values for the treated cultures were then expressed as percentages of the respective control values. The ability of rickettsiae to grow in untreated, X-irradiated RAW264.7 cells was evaluated as previously described (21).

The ability of rickettsiae to induce IFN-α/β in L929 cells was monitored by methods similar to those previously described (21). Briefly, suspensions of nonirradiated L929 cells were mock infected or infected with rickettsiae at various concentrations for 1 h. The cells were then washed and planted in Eagle minimal essential medium supplemented with 10% newborn bovine serum (4.5×10^4 L929 cells in 600 μl of serum-supplemented medium in each well of 24-well plates).

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. After incubation of the cultures for 1 to 4 days, the media were collected and assayed for IFN (antiviral activity) as previously described (21). Each sample was prepared by pooling the media from at least two wells.

Determination of lysine methylation in *R. prowazekii* strains. To evaluate lysine methylation, the rickettsiae were labeled with L-[4,5-^{3}H(N)]lysine (87.5 Ci/mmol, 1 mCi/ml; Du Pont Co., NEN Research Products, Boston, Mass.) by being grown in L929 cells in the presence of radiolabeled lysine and emetine (an inhibitor of eucaryotic protein synthesis). Suspensions of nonirradiated L929 cells were mock infected or infected with rickettsiae, washed, and resuspended in lysine-free Eagle minimal essential medium supplemented with 5% newborn bovine serum. The L929 cells were then planted in 100-mm tissue culture dishes that contained coverslips (1.2×10^7 cells per dish in a volume of 10 ml) and incubated overnight at 34°C. After addition of emetine (1.2 μg/ml) and incubation of the cells for approximately another 45 min, 2 ml of L-[4,5-^{3}H(N)]-lysine (15 μCi/ml) in lysine-free Eagle minimal essential medium supplemented with 5% newborn bovine serum was added to each dish. The cells were then incubated at 34°C for approximately 36 h and harvested. Initially, the average percentages of cells infected were 98, 93, 100, and 100% and the average numbers of rickettsiae per infected cell were 9, 7, 22, and 12, for strains Madrid E, EVir, 83-2P, and 87-17, respectively. Before the addition of radiolabeled lysine, the percentages of cells infected were 99, 91, 100, and 100% and the average numbers of rickettsiae per infected cell were 13, 13, 32, and 29, respectively. At the time of harvest, substantial numbers of cells were detached in some of the cultures, 99 to 100% of the attached cells were infected, and the average numbers of rickettsiae per infected cell (in the attached cells) were 74, 77, 97, and 96, respectively, for strains Madrid E, EVir, 83-2P, and 87-17. In these experiments, a cell that contained more than 100 rickettsiae was assigned a value of 100. To harvest the rickettsiae from each dish, we scraped the cells into 1.5 ml of sucrose-phosphate-glutamate solution (SPG; 0.218 M sucrose, 3.76 mM KH_2PO_4, 7.1 mM K_2HPO_4, 4.9 mM potassium glutamate) (2) and combined them with the medium, together with an additional SPG (1.5 ml) rinse of the dish. After centrifugation (27,200 × *g* for 15 min at 4°C), the pellets were frozen in liquid nitrogen and stored at −80°C for later processing. The pellets were thawed at 34°C, resuspended in 1.5 ml of SPG, vortexed for 1 min to disrupt the cells, and centrifuged at 250 × *g* for 7 min at 4°C. Each supernatant fluid was then layered onto 30 ml of 26% Renografin in SPG and centrifuged at 27,200 × *g* for 45 min to sediment the rickettsiae. Each pellet was resuspended in 1,050 μl of SPG, and in two experiments 3 μl of each suspension was removed to monitor the radioactivity. Expression of the radioactivity in the mock-infected samples as a percentage of the radioactivity in the least radioactive infected sample gave values of 0.6 and 2.4% in the two experiments. These data suggested that most of the radioactivity in the infected samples was associated with the rickettsiae. After centrifugation of the suspensions (9,500 × *g* for 20 min), the pellets were frozen in liquid nitrogen and stored at −80°C for further processing.

Each sample was hydrolyzed in approximately 200 μl of 6 N HCl in a sealed ampule at 110°C for at least 24 h. A standard mixture (which contained 342 nmol of L-lysine, 342 nmol of N^{ϵ}-Me_3-lysine, 342 nmol of N^{ϵ}-Me_2-lysine, and 342 nmol of N^{ϵ}-Me-lysine [Bachem Bioscience Inc., Philadelphia, Pa.]) was added to the samples either before or after hydrolysis. After hydrolysis, the acid was evaporated with a stream of N_2, the samples were dissolved in distilled water, the insoluble residue was removed by centrifugation (9,500 × *g* for 10 min), and the samples were analyzed by thin-layer chromatography on silica gel GHLF (Analtech, Inc., Newark, Del.), with *n*-butanol–glacial acetic acid–water (1:1:1) as the mobile phase. The spots were visualized with ninhydrin, and the R_f values for N^{ϵ}-Me_3-lysine, N^{ϵ}-Me_2-lysine, N^{ϵ}-Me-lysine, and lysine were 0.25, 0.37, 0.48, and 0.59, respectively. The chromatograms were similar whether the standards were added before or after the acid hydrolysis step. Each lane was divided into 10 fractions, the silica gel in each fraction was scraped, the labeled compounds were eluted with 0.1 N HCl, and the radioactivity in each fraction was determined by liquid scintillation techniques.

Statistical analysis. A one-sample or two-sample *t* test was used for statistical analysis. For analysis of the growth of the rickettsiae or L929 cells, the percent control values were used. For the various treatments, a one-sample *t* test was used to test the null hypothesis that the mean was equal to 100. In addition, a two-sample *t* test was used to compare cytokine-treated cultures with and without N^G-methyl-L-arginine. The two-sample *t* test was also used to compare nitrite production in the various cultures and to compare lysine methylation in the different rickettsial strains.

RESULTS

Effect of cytokines on growth of *R. prowazekii* EVir in L929 cells. The effects of IFN-α/β and IFN-γ on the growth of strains EVir and Madrid E were evaluated by using growing L929 cells (Table 1). As previously demonstrated (19, 20), growth of the Madrid E strain in these cells was significantly inhibited by either IFN-α/β or IFN-γ and the inhibitory effect of IFN-γ was more dramatic than that of IFN-α/β. In contrast, growth of strain EVir in L929 cells was not markedly inhibited by either IFN-α/β or IFN-γ (Table 1). Compared with the Madrid E strain, strain EVir was resistant to IFN-α/β and IFN-γ.

Rickettsial growth in X-irradiated L929 cells infected with strain EVir and treated with IFN-γ, TNF-α, or IFN-γ plus TNF-α was also evaluated (Table 2). Growth of strain EVir in L929 cells was slightly inhibited (11%) by TNF-α alone and modestly inhibited (38%) by IFN-γ alone; however, it was markedly suppressed (87%) in L929 cells treated with IFN-γ plus TNF-α. Nitrite production was induced in strain EVir-infected cultures treated with IFN-γ alone or TNF-α alone; however, more nitrite was produced in strain EVir-infected cultures treated with IFN-γ plus TNF-α. In all instances, nitrite production was inhibited in cultures treated with the nitric oxide synthase inhibitor N^G-methyl-L-arginine (Table 2). The dramatic inhibitory effect of IFN-γ plus TNF-α on the growth of strain EVir was markedly alleviated by N^G-methyl-L-arginine, and the modest inhibitory effect of IFN-γ (and TNF-α) on the growth of strain EVir was also alleviated by N^G-methyl-L-arginine. Strain EVir was resistant to IFN-γ alone and TNF-α alone compared with strain Madrid E ($P < 0.05$ and $P < 0.001$, respectively) and strain Breinl ($P < 0.025$ and $P < 0.001$, respectively) (Fig. 1). In contrast, strains EVir and 83-2P did not differ significantly in sensitivity to IFN-γ alone and TNF-α alone ($P > 0.5$). Strain EVir was also similar to strain 83-2P (and different from strains Madrid E and Breinl) in that the inhibition of nitric oxide synthase alleviated the dramatic inhibitory effect of IFN-γ plus TNF-α (Table 2; Fig. 1).

Ability of *R. prowazekii* EVir to grow in macrophagelike RAW264.7 cells. *R. prowazekii* EVir was similar to *R. prowazekii* Breinl (15) (Table 3) and all of the IFN-resistant *R.*

TABLE 1. Resistance of *R. prowazekii* EVir to IFN-α/β and IFN-γ in nonirradiated L929 cell cultures

Rickettsial strain	Treatment[a]	Growth (% of control)[b]		Rickettsial infection[c]			
		L929 cells	Rickettsiae	Initial (day 0)		Day 2	
				%R	RI	%R	RI
Madrid E	Control	{1.0 ± 0.0}	{3.0 ± 0.1}	74 ± 7	4.6 ± 0.2	86 ± 9	64 ± 14
Madrid E	IFN-α/β	50 ± 11*	67 ± 6**	87 ± 6	6.9 ± 1.4	84 ± 5	55 ± 11
Madrid E	IFN-γ	59 ± 7**	35 ± 7**	81 ± 7	5.7 ± 0.7	54 ± 6	15 ± 1
EVir	Control	{1.1 ± 0.0}	{3.2 ± 0.1}	72 ± 9	3.2 ± 0.5	71 ± 13	64 ± 14
EVir	IFN-α/β	77 ± 7	90 ± 3†	85 ± 6	5.3 ± 1.0	84 ± 7	96 ± 23
EVir	IFN-γ	76 ± 7	88 ± 4††	74 ± 8	4.0 ± 1.0	76 ± 9	61 ± 13

[a] Untreated, IFN-α/β-treated, and IFN-γ-treated L929 cells were infected with rickettsiae on day 0. Cells were treated with IFN before and after infection.

[b] The numbers of doublings per day in the untreated control cultures are given in braces. The values for the treated cultures are expressed as percentages of the values in the respective control cultures. Each value represents the mean ± standard error of the mean (SEM) for three experiments. The percent control values were used for statistical analysis. Each value that was significantly different from 100 (as determined by a one-sample *t* test) is indicated as follows: *, $P < 0.1$; **, $P < 0.05$. Strain EVir-infected cultures which differed significantly from the corresponding strain Madrid E-infected cultures (as determined by the two-sample *t* test) are indicated as follows: †, $P < 0.05$; ††, $P < 0.01$).

[c] %R, percentage of cells infected; RI, average number of rickettsiae per infected cell. Each value represents the mean ± SEM for three experiments.

prowazekii strains (21) in that it grew very well in macrophagelike RAW264.7 cells (Table 3). In contrast, the Madrid E strain failed to grow (or grew poorly) in macrophagelike RAW264.7 cells (15) (Table 3).

Poor induction of IFN in cultures of L929 cells infected with *R. prowazekii* EVir. In four independent experiments L929 cells were mock infected or infected with various concentrations of *R. prowazekii* EVir or Madrid E. In three additional experiments L929 cells were mock infected or infected with various concentrations of *R. prowazekii* EVir. Culture media were tested for antiviral activity at various times after infection. The numbers of rickettsiae per cell immediately after infection ranged from 4.0 to 39 for strain EVir and from 9.8 to 52 for strain Madrid E; in all cases, examination of stained cells from the infected cultures at later times indicated that rickettsial growth had occurred. Antiviral activity was not detected in the media collected from the mock-infected cultures at any time after infection or in the media collected from any infected cultures on days 1 and 2 after infection. For the EVir-infected cultures, a low level of antiviral activity (approximately 2 U/ml) was detected in 1 of the 58 media samples (2%) collected on day 3 after infection and in 1 of the 58 media samples collected on day 4 after infection. In contrast, for the Madrid E-infected cultures, antiviral activity was detected in 41 of the 74 media samples (55%) collected on day 3 after infection and in 34 of the 74 media samples (46%) collected on day 4 after infection. The amount of IFN detected in the Madrid E-infected cultures

TABLE 2. Nitrite production by and growth of *R. prowazekii* EVir in cultures of X-irradiated L929 cells treated with IFN-γ and/or TNF-α[a]

Cytokine	NGMA[b] concn (mM)	Rickettsial growth (% control)[c]	Nitrite concn (μM)[d]
None	0	{1,499 ± 58}	1.6 ± 0.5
None	0.5	98 ± 3	1.6 ± 0.3
IFN-γ (100 U/ml)	0	62 ± 9*	8.2 ± 1.9‡
IFN-γ (100 U/ml)	0.5	89 ± 4†	1.5 ± 0.5
TNF-α (100 U/ml)	0	89 ± 1**	5.6 ± 1.2‡
TNF-α (100 U/ml)	0.5	97 ± 3	1.7 ± 0.5
IFN-γ + TNF-α	0	13 ± 0***	21.2 ± 1.6‡‡
IFN-γ + TNF-α	0.5	64 ± 3***††	2.5 ± 0.8

[a] Rickettsial growth in the attached cells and nitrite production were monitored 44 h after infection.

[b] NGMA, N^G-methyl-L-arginine.

[c] In each experiment, the average number of rickettsiae per cell in the untreated control cultures 44 h after infection was expressed as a percentage of the number per cell immediately after infection, and the mean ± standard error of the mean (SEM) for three experiments is given in braces. The numbers of rickettsiae per cell in the treated cultures (expressed as percentages of the number immediately after infection) were expressed as percentages of the control values, and each value given represents the mean ± SEM for three experiments. Immediately after infection, 86% ± 3% of the cells were infected, there were 6.3 ± 0.3 rickettsiae per infected cell, and there were 5.4 ± 0.3 rickettsiae per cell. The percent control values were used for statistical analysis. Each mean that was significantly different from 100 as determined by a one-sample *t* test is indicated as follows: *, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.01$. Cytokine-plus-N^G-methyl-L-arginine-treated cultures which differed significantly from the corresponding cultures treated with cytokine alone (as determined by the two-sample *t* test) are indicated as follows: †, $P < 0.1$; ††, $P < 0.01$.

[d] Each value represents the mean ± SEM for three experiments. Treated cultures which differed significantly from the untreated control cultures are indicated as follows: ‡, $P < 0.1$; ‡‡, $P < 0.01$.

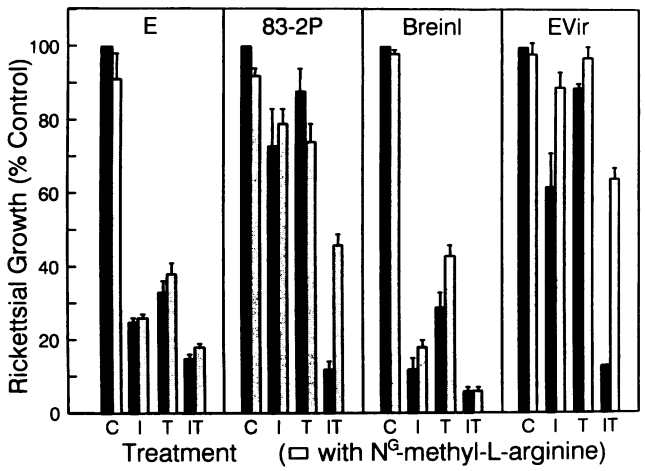

FIG. 1. Growth of *R. prowazekii* strains in X-irradiated L929 cells treated with IFN-γ and/or TNF-α. Rickettsial growth was monitored in the attached cells. The numbers of rickettsiae per cell in the treated cultures at 44 h after infection (expressed as percentages of the values of NR immediately after infection) are shown as percentages of the respective control values. Each value represents the mean ± standard error of the mean (SEM) for at least three experiments. The data for strains Madrid E, 83-2P, and BreinI were previously published (22). Nitrite production and details of the experiments with strain EVir are given in Table 2. N^G-Methyl-L-arginine was used at a concentration of 0.5 mM. C, control; I, IFN-γ (100 U/ml); T, TNF-α (100 U/ml); IT, IFN-γ plus TNF-α.

TABLE 3. Growth of *R. prowazekii* strains in untreated, X-irradiated macrophagelike RAW264.7 cells[a]

Strain	Rickettsial growth[b]	Rickettsial infection[c]					
		Initial (day 0)			Day 2		
		%R	RI	NR	%R	RI	NR
Madrid E	52 ± 28	53 ± 4	3.9 ± 0.7	2.2 ± 0.5	19 ± 5	4.0 ± 0.9	0.8 ± 0.3
EVir	2,998 ± 700*	44 ± 6	3.1 ± 0.5	1.5 ± 0.4	88 ± 2	40 ± 3	36 ± 3
Breinl	2,266 ± 600*	52 ± 6	4.4 ± 1.0	2.5 ± 0.8	92 ± 7	47 ± 2	43 ± 5

[a] X-irradiated RAW264.7 cells were infected with rickettsiae on day 0.
[b] The numbers of rickettsiae per cell on day 2 were expressed as percentages of the respective numbers on day 0, and then the data from three experiments were averaged for each strain. Each value represents the mean ± standard error of the mean (SEM). Values which differ significantly from the value for the Madrid E strain ($P < 0.05$, as determined by the two-sample t test) are indicated by *.
[c] %R, percentage of cells infected; RI, rickettsiae per infected cell; NR, number of rickettsiae per cell. Each value represents the mean ± SEM for three experiments.

ranged from 3 to 105 U/ml. For the Madrid E-infected samples in which IFN was detected, the mean and median values were, respectively, 22 and 32 U/ml on day 3 and 26 and 25 U/ml on day 4. Thus strain EVir appeared to be a very poor inducer of IFN in L929 cell cultures compared with the Madrid E strain.

Lysine methylation in *R. prowazekii* strains. Hydrolysis of the purified rickettsiae and analysis of the samples by thin-layer chromatography showed that at least 98% of the radioactivity was present in the lysine and methylated lysine fractions (Table 4). Most of this radioactivity (93.7 to 94.9%) comigrated with lysine, and the remaining 5.1 to 6.3% comigrated with the methylated lysines. Thus all of the *R. prowazekii* strains tested had similar percentages of their radioactivity in the lysine fraction and the combined fractions that contained the methylated lysines. However, strains EVir, 83-2P, and 87-17 differed significantly from the Madrid E strain in having more radioactivity in the N^{ϵ}-Me_3-lysine fraction, more radioactivity in the N^{ϵ}-Me_2-lysine fraction, and less radioactivity in the N^{ϵ}-Me-lysine fraction. Thus lysine methylation in strains 83-2P and 87-17 was similar to that in strain EVir.

DISCUSSION

In the present study, *R. prowazekii* EVir (a virulent strain derived from the avirulent Madrid E strain by passage in mice [1]) was found to be resistant to IFN-α/β, IFN-γ, and TNF-α compared with the parental Madrid E strain. *R. prowazekii* 83-2P, which was selected for resistance to IFN-α/β by passage in L929 cells, is also resistant to these three cytokines (20–22). Thus passage of the Madrid E strain in the mouse lung or in mouse L929 cells resulted in the selection of cytokine-resistant *R. prowazekii* organisms. Since IFN-α/β is produced in mice after intravenous injection of *R. prowazekii* (9), and since mouse L929 cells produce IFN-α/β in response to *R. prowazekii* (19, 21), the two selection strategies may have been similar.

The virulence of strain 83-2P has not been evaluated; however, both strains 83-2P and EVir are similar to the virulent Breinl strain of *R. prowazekii* (and different from the avirulent, parental Madrid E strain) in their lysine methylation profiles (14; also see above) and ability to grow in macrophagelike RAW264.7 cells (21; also see above). The Breinl strain of *R. prowazekii* is resistant to IFN-α/β compared with the Madrid E strain (21); however, it is very sensitive to IFN-γ (21) and TNF-α (22). *R. prowazekii* 87-17, which can grow in macrophagelike RAW264.7 cells (21) and has a lysine methylation profile similar to that of strains 83-2P and EVir, is sensitive to IFN-α/β (20) but resistant to IFN-γ (18). Although sensitivity to cytokines (IFN-α/β, IFN-γ, and TNF-α) is not constant among the *R. prowazekii* strains that share similar lysine methylation profiles and ability to grow in RAW264.7 cells, it may be that resistance to one or more of these three cytokines is associated with these properties (and, possibly, with virulence).

Although compared with the Madrid E strain, EVir was resistant to IFN-γ in both X-irradiated and nonirradiated L929 cells, IFN-γ suppressed the growth of strain EVir in X-irradiated L929 cells but did not restrict it in nonirradiated L929 cells. Inhibition of the growth of strain EVir in X-irradiated L929 cells was dependent on the nitric oxide synthase pathway, since the inhibition was prevented by the addition of N^{G}-methyl-L-arginine. Although nitrite production was not measured in the experiments with nonirradiated L929 cells, it seems likely that the growth of strain EVir was not inhibited by IFN-γ in nonirradiated L929 cells because the activity of nitric oxide synthase was insufficient for restriction of rickettsial growth to occur.

The growth of strain EVir in L929 cells treated with IFN-γ plus TNF-α (like that of strain 83-2P) was dramatically suppressed, and the suppression was alleviated by the nitric oxide synthase inhibitor, N^{G}-methyl-L-arginine. Thus, both strains were sensitive to the antirickettsial effect mediated by the nitric oxide synthase pathway. Although nitrite production was effectively inhibited by N^{G}-methyl-L-arginine, this inhibitor did not completely prevent the inhibition of the growth of strain

TABLE 4. Lysine methylation in rickettsial strains[a]

Rickettsial strain	% of total radioactivity as lysine and methylated lysine	% of lysine and methylated lysine radioactivity as:				
		Lysine	Methylated lysine	N^{ϵ}-Me_3-lysine	N^{ϵ}-Me_2-lysine	N^{ϵ}-Me-lysine
Madrid E	98.4 ± 0.8	94.3 ± 0.7	5.7 ± 0.7	0.5 ± 0.1	0.5 ± 0.1	4.8 ± 0.6
EVir	98.0 ± 1.1	93.7 ± 1.2	6.3 ± 1.2	2.6 ± 0.5***	1.1 ± 0.2**	2.5 ± 0.5**
83-2P	98.8 ± 0.5	94.0 ± 1.1	6.0 ± 1.1	2.7 ± 0.6***	1.1 ± 0.3*	2.2 ± 0.2***
87-17	98.2 ± 0.2	94.9 ± 0.1	5.1 ± 0.1	2.2 ± 0.0***	1.0 ± 0.0**	1.9 ± 0.1**

[a] For all strains except 87-17, each value represents the mean ± standard deviation (SD) for three experiments. For strain 87-17, each value represents the mean ± SD for duplicate cultures in a single experiment. Values for any rickettsial strain that differ significantly from the respective values for the Madrid E strain (as determined by the two-sample t test) are indicated as follows: *, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.01$.

EVir in L929 cells treated with IFN-γ plus TNF-α. Similar results were obtained with *R. prowazekii* 83-2P in experiments in which either N^G-methyl-L-arginine or aminoguanidine was used to inhibit nitric oxide synthase (22). Thus, it seems likely that some mechanism(s) other than the production of nitric oxide plays a role in inhibiting the growth of these *R. prowazekii* strains in L929 cells treated with IFN-γ plus TNF-α.

A high degree of variability was observed in some of the experiments reported in the present study (e.g., Table 3). Some of the variability in the growth of strains EVir and Breinl in RAW264.7 cells is probably a consequence of (i) underestimation of the numbers of rickettsiae in heavily infected cultures (which resulted from the practice of assigning a value of 100 to a cell that contained more than 100 rickettsiae) and (ii) variability in the initial rickettsial infections among the individual experiments. Since RAW264.7 cells are very sensitive to bacterial lipopolysaccharide, variation in the levels of lipopolysaccharide present in the cultures might also have influenced the ability of the various rickettsial strains to grow in the RAW264.7 cells in the three experiments.

Our data indicate that, compared with strain Madrid E, strain EVir is defective in its ability to induce IFN-α/β in L929 cell cultures. Only 2% of the media samples collected from strain EVir-infected L929 cell cultures on days 3 and 4 after infection contained IFN, and the levels of IFN detected were very low (about 2 U/ml). In contrast, 51% of the media samples collected from strain Madrid E-infected L929 cell cultures on days 3 and 4 after infection contained IFN, and the average amount of IFN detected was 29 U/ml. In an earlier study we found that the two *R. prowazekii* strains selected for resistance to IFN-γ (strains 427-19 and 87-17) and three of the four strains selected for resistance to IFN-α/β (strains 83-2P, 103-2P, and 110-1P) induced interferon in L929 cell cultures (21). However, like strain EVir, strain 60P (which had been selected for resistance to IFN-α/β) was highly defective in its ability to induce IFN in L929 cells (21).

Unexpectedly, we found that the percentages of Madrid E-infected cultures that exhibited IFN activity in the present study were much lower than the corresponding percentages observed in the earlier study. In the earlier study, 92 and 100% of Madrid E-infected L929 cell cultures contained IFN on days 3 and 4 after infection (respectively) and the average amounts of IFN detected were 42 and 239 U/ml, respectively (21). The difference between the data in the two studies did not appear to be due to differences in the Madrid E rickettsiae used in the two studies, since our current experiments used frozen Madrid E rickettsial suspensions prepared in 1988, 1989, 1992, and 1993. However, because different lots of newborn bovine serum were used for culturing the L929 cells in the two studies, differences in these sera might have affected our results.

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