INHIBITION OF THE GROWTH OF *RICKETTSIA PROWAZEKII* IN CULTURED FIBROBLASTS BY LYMPHOKINES*

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Epidemic typhus is an acute human disease caused by the obligate intracellular bacterium, *Rickettsia prowazekii. R. prowazekii* has a predilection for multiplication within the endothelial cells lining the capillaries (1) and an ability to multiply within the macrophages of the host (2). Thus cells that are not specially adapted for phagocytosis as well as professional phagocytes serve as sites of rickettsial multiplication. Rickettsiae multiply freely in the cytoplasm of their host cells; they do not grow within vacuoles.

The mechanisms of host defense against the etiological agents of epidemic typhus and other diseases caused by *Rickettsia* species are not clearly understood, but studies have indicated the importance of both humoral and cell-mediated immunity. The importance of specific antibody is suggested by the protective effect of the administration of immune serum in human cases of epidemic typhus (3). In addition, *R. prowazekii* treated with specific antiserum is destroyed within human monocyte-derived macrophages (4) and mouse macrophage-like cells (5). Specific antiserum also has been reported to inhibit the ability of *R. prowazekii* to infect L929 mouse fibroblasts (5).

A role for cell-mediated immunity in host defense against rickettsiae is indicated because spleen cells collected from guinea pigs that had recovered from *Rickettsia typhi* infection could transfer resistance to intradermal challenge to nonimmune recipients, whereas serum could not (6, 7). In addition, a T cell-mediated mechanism was responsible for the protection of mice immunized with the Gilliam strain of *Rickettsia tsutsugamushi* against subsequent challenge with the Karp strain of *R. tsutsugamushi* (8). T lymphocytes also function in host defense against *Rickettsia akari* and *Rickettsia conorii* infections in mice (9, 10). Other evidence for the importance of cell-mediated immunity derives from the fact that lymphokines produced by cultures of antigen-stimulated or concanavalin A-stimulated mouse spleen cells rendered mouse peritoneal macrophages capable of killing *R. tsutsugamushi* (11–13).

How the host copes with established rickettsial infection in nonprofessional phagocytes is unknown. We report here that lymphokines inhibit the growth of *R. prowazekii* in cultured fibroblasts in a species-specific manner and participate in the destruction of rickettsiae within these nonprofessional phagocytes.

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Materials and Methods

Cell Cultures. Mouse L929 cells and human Flow 5000 cells were purchased from Flow Laboratories, Inc., McLean, VA, and mouse 3T3-A31 cells were purchased from the American Type Culture Collection, Rockville, MD. Cultures of human foreskin fibroblasts were obtained from Mr. Frank Pindak, University of South Alabama.

L929 cells were grown in Eagle's minimum essential medium supplemented with 10% calf serum; 3T3-A31 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum; and Flow 5000 cells and human foreskin fibroblasts were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum. Calf and fetal calf sera were heat inactivated at 56°C before use. All cell cultures were grown in tissue culture dishes or flasks in a CO_2 incubator at 34°C.

Rickettsiae. *R. prowazekii* was grown in 6-d embryonated, antibiotic-free hen eggs (Truslow Farms, Chestertown, MD) inoculated from seed pools prepared from the E strain or the Breinl strain. Rickettsiae were harvested and purified from infected yolk sacs 8 d after inoculation by methods modified from those of Bovarnick and Snyder (14) and Wisseman et al. (15), as described previously (16). The rickettsiae were then passed through a type AP20 microfilter glass filter (Millipore Corp., Bedford, MA) (17, 18) and were stored at −70°C in 0.2-ml aliquots. Viable rickettsiae were enumerated by the antibody hemolysis method of Walker and Winkler (19).

Preparation of Lymphokines. Mouse lymphokines were prepared by three procedures. In the first procedure, spleen cells obtained from male BALB/c mice (16–32 wk old) were adjusted to a concentration of 5×10^6 cells/ml in RPMI 1640 medium supplemented with 5% fetal calf serum and incubated with 0.6–1.5 μg/ml concanavalin A (Sigma Chemical Co., St. Louis, MO). Alternatively, spleen cells were obtained from mice immunized 7 d previously by intraperitoneal injection of 1.4 mg heat-killed *Corynebacterium parvum* CN6134 (Burroughs Wellcome Co., Research Triangle Park, NC) and were incubated with 14 μg/ml (dry weight) heat-killed *C. parvum.* In the third procedure, spleen cells were obtained from mice immunized 7 d previously by intraperitoneal injection of 10^5–10^6 viable rickettsiae (E strain, *R. prowazekii*) and were incubated with heat-killed *R. prowazekii* E strain at a concentration of 14 μg of rickettsial protein/ml.

All spleen cell suspensions were prepared according to the method of Ruco and Meltzer (20). After incubation of the cultures at 34°C for 48 h, the culture fluids were centrifuged and sterilized by filtration (pore size, 0.2 μm). The culture fluids (crude mouse lymphokines) were stored at 4°C or −70°C.

For human lymphokine production, lymphocytes and monocytes were isolated from ethylenediamine tetraacetate-treated human blood samples by centrifugation of the diluted blood over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). The lymphocytes and monocytes were washed and resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 5% fetal calf serum. The cells were incubated with 1.5 μg/ml concanavalin A for 72 h at 34°C. Supernatant fluids (crude human lymphokines) were collected and stored at 4°C.

Treatment of Cells with Lymphokines and Infection of Cells with Rickettsiae. All cells were x-irradiated with a General Electric Maximar 100 X-ray unit at a dosage adequate to prevent cell division (3,000–5,000 rad). After irradiation, the cell lines L929, 3T3-A31, Flow 5000, and human foreskin fibroblasts were adjusted to a density of $1.0–1.2 \times 10^5$ viable (trypan blue-excluding) cells/ml and were planted in eight-chambered slides (0.3 ml/chamber) (Lab-Tek Div., Miles Laboratories Inc., Naperville, IL). The cells were incubated overnight in a CO_2 incubator at 34°C. The culture medium was then removed and the cells were treated with medium alone or with various dilutions of lymphokines in tissue culture medium. After incubation for an additional 24 h, the cells were infected with rickettsiae.

Rickettsiae were diluted in Hanks' balanced salt solution supplemented with 0.1% gelatin and 4.9 mM L-glutamic acid (monopotassium salt). Rickettsiae were added to washed cells at multiplicities of 100–400 viable rickettsiae per cell. Cells and rickettsiae were incubated together for 1 h at 34°C. The cells were then washed twice and given fresh tissue culture medium or lymphokines diluted in tissue culture medium. Infected cells were incubated at 34°C for up to 48 h. At 0, 24, and 48 h after infection, chamber slides were washed gently in 0.85% NaCl and

were dried with a warm air stream. Slides were fixed in 1% formalin in 0.1 M sodium phosphate buffer, pH 6.8. The slides were washed in three changes of buffer and were then stained by a modification of the Gimenez method, as described by Wisseman et al. (21).

Similar experiments were conducted in 24-well plates (Flow Laboratories, Inc.) that contained cover glasses. In these experiments the tissue culture medium in each well (1 ml) was cytocentrifuged onto a microscope slide in a Cytospin SCA-0031 (Shandon Southern Instruments Inc., Sewickley, PA). The resultant slides as well as the cover glasses from the wells were fixed and stained as described above. A small number of cells was present in the smears prepared by cytocentrifugation of the tissue culture medium collected from both control and lymphokine-treated wells. The approximate number of cells in the medium was estimated by counting the number of cells in a measured area of each smear and comparing it with the numbers of cells present in the same areas of smears prepared from known numbers of cells.

All treatments were done in duplicate at each time point. 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 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),[1] the average number of rickettsiae per infected cell (RI), and the average number of rickettsiae per cell (NR), were determined. Many of the data were normalized to the zero time infection, e.g., RI at a given time divided by RI at zero time multiplied by 100 gives the average number of rickettsiae per infected cell as a percentage of that observed at zero time.

Experiments with Lymphokines and Inhibitors of Eukaryotic Protein Synthesis. Irradiated L929 cells were treated with tissue culture medium alone, medium plus lymphokines, medium plus lymphokines and inhibitor (cycloheximide, 1 μg/ml, or emetine, 0.5 μg/ml), or medium plus inhibitor. Rickettsial growth was monitored as described above. Percent inhibition of rickettsial growth in lymphokine-treated chambers (%LK) and percent inhibition of rickettsial growth in lymphokine plus inhibitor-treated chambers (%LKIN) were calculated as follows from the values of NR at 48 h after infection (expressed as percentages of the values of NR observed at 0 h): %LK = [(NR control chambers − NR lymphokine-treated chambers)/NR control chambers] × 100. %LKIN = [(NR inhibitor-treated chambers − NR lymphokine plus inhibitor-treated chambers)/NR inhibitor-treated chambers] × 100. Percent suppression of the anti-rickettsial effect of the lymphokines by the inhibitor was calculated from the difference between %LK and %LKIN.

Rapid Method for Assay of Anti-Rickettsial Activity (ARA) in Mouse Lymphokines. Irradiated L929 cells were treated before and after rickettsial infection with dilutions of the lymphokine preparation to be assayed and were examined 48 h after infection with rickettsiae. A cell was scored positive for rickettsial growth if it contained 10 or more rickettsiae, and negative if it contained 9 or fewer rickettsiae. At 48 h after infection, 60–90% of the cells in the control chambers were positive for rickettsial growth whereas at 0 h, <5% of the cells had been positive by this criterion. Percent inhibition of rickettsial growth was calculated from the difference between the percentages of positive cells in control and treated chambers. The amount of lymphokine that would cause 50% inhibition of rickettsial growth in a slide chamber (0.3 ml) was defined as 1 U of ARA.

Results

Effect of Mouse Lymphokine Treatment of L929 Cells on their Interaction with R. prowazekii (E Strain). The time at which lymphokines were presented was an important factor in determining the degree of rickettsial clearance and inhibition of rickettsial growth in lymphokine-treated L929 cells. Fig. 1 shows the results of several experiments in which %R and RI were monitored at 0, 24, and 48 h after rickettsial infection in L929 cells treated with lymphokines before infection, after infection, or before and after

[1] *Abbreviations used in this paper:* ARA, anti-rickettsial activity; %LK, percent inhibition of rickettsial growth in lymphokine-treated chambers; %LKIN, percent inhibition of rickettsial growth in lymphokine plus inhibitor-treated chambers; NR, average number of rickettsiae per cell; RI, average number of rickettsiae per infected cell; %R, percentage of cells infected with rickettsiae.

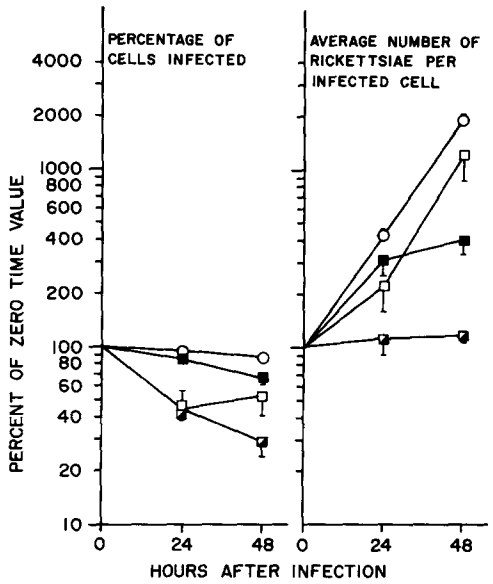

FIG. 1. Effect of mouse lymphokine treatment of mouse L929 cells on the growth of *R. prowazekii* E strain in these cells. Lymphokines were induced with the immunogen *C. parvum* or *R. prowazekii* or with concanavalin A. L929 cells were treated with 10–20% mouse spleen cell supernatant (lymphokines) (squares); control cultures received tissue culture medium alone (○). 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 lymphokines 24 h before infection; ◪, cells treated with lymphokines 24 h before infection and after infection; ■, cells treated with lymphokines after infection. Each value represents the mean ± the standard error of the mean for three or more experiments. The percentage of cells infected at 0 h was 71 ± 3 for control cells and 69 ± 3 for cells treated with lymphokines 24 h before infection. The average number of rickettsiae per infected cell at 0 h was 2.6 ± 0.1 for control cells and 2.4 ± 0.1 for cells treated with lymphokines 24 h before infection.

infection. Decreases in %R relative to the values observed at zero time indicate total clearance of rickettsiae from some of the cells. Treatment of L929 cells with lymphokines for 24 h before rickettsial infection resulted in complete clearance of rickettsiae from more than one-half of the infected cells by 24 h after infection. %R remained about the same during the next 24 h in cells treated with lymphokines only before infection, but this parameter continued to decrease in cells treated with lymphokines both before and after infection. When lymphokines were present only after rickettsial infection, there was a slower and less pronounced decrease in %R.

During the 48 h period after infection, RI, an index of rickettsial growth, increased ~19-fold in control cultures (Fig. 1). However, in cells treated with lymphokines both before and after infection, RI underwent little change. Thus rickettsial growth was almost completely inhibited in the lymphokine-treated cells that remained infected. Partial inhibition of rickettsial growth was observed when L929 cells were treated with lymphokines either only before or only after infection. In cells pretreated with lymphokines for 24 h before infection rickettsial growth was most suppressed during the first 24 h after infection. In contrast, in cells treated with lymphokines only after infection the most marked inhibition was apparent during the period from 24 to 48 hr after infection.

Examination of cytocentrifuged preparations from the tissue culture media revealed

the presence of small numbers of suspended L929 cells in both control and lymphokine-treated culture fluids. The cells present in the culture fluids represented 6–14% of the total cells in the cultures. Rickettsial growth was evident in many of the cells present in the smears prepared from control cultures but not in the cells present in the smears prepared from lymphokine-treated cultures.

The effect of pretreatment of L929 cells with lymphokines for 24 h on the ability of the cells to be initially infected with rickettsiae was variable. In 46 experiments the average %R at 0 h were 83 ± 2% and 71 ± 3% in control and lymphokine-treated cells, respectively. RI at 0 h were 3.7 ± 0.2 for the control cells and 2.9 ± 0.2 for the lymphokine-treated cells. Although these mean values did not reveal dramatic differences between the control and lymphokine-treated groups, the data from individual experiments showed that the RI in lymphokine-treated cultures was <75% of that in control cultures in 24 of the 46 experiments.

Inhibition of the Growth of R. prowazekii (Avirulent and Virulent Strains) by Various Concentrations of Mouse Lymphokines. Table I demonstrates the effect of three concentrations of mouse lymphokines on the growth of the avirulent E strain and the virulent Breinl strain of *R. prowazekii* in L929 cells. The L929 cells were treated with dilutions of lymphokines both before and after infection with rickettsiae, and the infection parameters, %R and RI, at 24 and 48 h after infection were expressed as percentages of the values observed at zero time. The controls indicate that the two rickettsial strains grew comparably in untreated L929 cells; %R underwent little change and RI increased ~18-fold during the 48 h period after infection. At the highest lymphokine concentration, %R and RI decreased for both strains of *R. prowazekii* during the 48 h

TABLE I

*Inhibition of the Growth of R. prowazekii (Avirulent and Virulent Strains) and R. conorii in L929 Cells Treated with Mouse Lymphokines**

Dilution of lymphokines	Hours after infection	R. prowazekii (E strain)		R. prowazekii (Breinl strain)		R. conorii	
		%R	RI	%R	RI	%R	RI
Control	0	(85 ± 7)	(4.7 ± 2.0)	(84 ± 11)	(4.0 ± 1.5)	(39 ± 4)	(1.7 ± 0.0)
	24	97 ± 2	387 ± 45	94 ± 2	410 ± 26	161 ± 16	2147 ± 147
	48	103 ± 3	1734 ± 533	108 ± 10	1873 ± 591		
1/20	0	(83 ± 7)	(4.0 ± 2.0)	(84 ± 9)	(3.5 ± 1.0)		
	24	80 ± 13	311 ± 122	65 ± 8	136 ± 16		
	48	77 ± 8	903 ± 306	54 ± 6	559 ± 247		
1/10	0	(81 ± 9)	(4.2 ± 2.0)	(89 ± 5)	(3.4 ± 0.7)		
	24	83 ± 13	129 ± 8	54 ± 8	78 ± 25		
	48	71 ± 6	224 ± 101	38 ± 9	102 ± 32		
1/5	0	(85 ± 8)	(4.8 ± 2.0)	(86 ± 7)	(3.9 ± 1.0)	(39 ± 3)	(1.5 ± 0.1)
	24	77 ± 13	86 ± 8	53 ± 1	61 ± 3	77 ± 2	325 ± 163
	48	51 ± 21	80 ± 6	39 ± 8	67 ± 6		

* L929 cells were treated with tissue culture medium alone or with dilutions of concanavalin A-induced mouse lymphokines for 24 h before infection and after infection with rickettsiae. The actual values of %R and RI at 0 h are in parentheses. %R and RI at 24 and 48 h after infection are expressed as percentages of those values observed at 0 h. Each value represents the mean ± standard error for two or three experiments.

period after infection; hence, inhibition of growth and even clearance of a substantial proportion of the rickettsiae from the infected fibroblasts occurred. At the lower lymphokine concentrations, the changes in %R and RI with either strain were less dramatic than those observed at the highest lymphokine concentration. The data indicate that lymphokine treatment of L929 cells had similar dose-dependent anti-rickettsial effects on both the avirulent E and virulent Breinl strains of *R. prowazekii* within the L929 cells.

R. prowazekii was not unique among *Rickettsia* species in its sensitivity to the effects of lymphokines: lymphokine treatment of L929 cells also suppressed the growth of *Rickettsia conorii*, a member of the spotted fever rickettsial group (Table I).

Effect of Inhibitors of Eukaryotic Protein Synthesis on the Inhibition of Rickettsial Growth in L929 Cells by Mouse Lymphokines. Addition of cycloheximide (1 μg/ml) to L929 cells at the same time as lymphokines markedly suppressed the inhibition of the growth of *R. prowazekii* by mouse lymphokines. This suppressive effect of cycloheximide on the anti-rickettsial effect of the lymphokines was observed when lymphokines and cycloheximide were incubated with L929 cells only before rickettsial infection, only after infection, or both before and after infection (Table II). Addition of cycloheximide alone to L929 cells was sometimes associated with slight inhibition of rickettsial growth in the cells.

Percent suppression of the anti-rickettsial effect of mouse lymphokines by cyclohex-

TABLE II

Effect of Cycloheximide or Emetine on the Inhibition of Rickettsial Growth in L929 cells by Mouse Lymphokines

Treatment of L929 cells*	Hours after infection	Time of treatment of L929 cells with lymphokines and/or inhibitor‡					
		24 h before infection		24 h before infection and after infection		After infection	
		%R	RI	%R	RI	%R	RI
Control	0	(90 ± 8)	(5.4 ± 1.8)	(77 ± 9)	(3.8 ± 1.2)	(79 ± 4)	(3.4 ± 0.5)
	48	97 ± 3	1097 ± 153	98 ± 2	1508 ± 257	105 ± 6	1772 ± 271
Lymphokines	0	(65 ± 25)	(2.7 ± 1.2)	(67 ± 11)	(2.5 ± 0.5)	(79 ± 4)	(3.4 ± 0.5)
	48	64 ± 1	175 ± 81	38 ± 11	103 ± 14	83 ± 7	325 ± 68
Lymphokines plus 1 μg/ml cycloheximide	0	(88 ± 6)	(3.5 ± 1.0)	(68 ± 12)	(2.6 ± 0.7)	(79 ± 4)	(3.4 ± 0.5)
	48	89 ± 1	958 ± 2	79 ± 5	1060 ± 567	98 ± 4	1221 ± 304
1 μg/ml cycloheximide	0	(90 ± 3)	(3.7 ± 0.6)	(73 ± 10)	(2.8 ± 0.6)	(79 ± 4)	(3.4 ± 0.5)
	48	93 ± 3	1235 ± 137	81 ± 8	1261 ± 278	105 ± 5	1576 ± 326
Control	0					(87 ± 4)	(4.2 ± 0.7)
	48					103 ± 6	1581 ± 410
Lymphokines	48					86 ± 1	178 ± 21
Lymphokines plus 0.5 μg/ml emetine	48					102 ± 1	933 ± 336
0.5 μg/ml emetine	48					99 ± 4	1166 ± 425

* Lymphokines were the supernatant fluids collected from mouse spleen cells cultured in the presence of concanavalin A or antigen (*C. parvum* or *R. prowazekii*) for 48 h. Lymphokines were used at a concentration of 10–20%.

‡ %R and RI at 0 h are shown in parentheses. %R and RI at 48 h after rickettsial infection are expressed as percentages of the values observed at 0 h. Each value represents the mean ± the standard error of the mean. Treatment with lymphokines and/or cycloheximide before infection, $n = 2$ experiments; treatment before and after infection, $n = 4$ experiments; treatment after infection, $n = 11$ experiments; treatment with lymphokines and/or emetine after infection, $n = 4$ experiments.

imide varied in different experiments. For example, in L929 cells treated with lymphokines and/or cycloheximide only after rickettsial infection, percent suppression of the anti-rickettsial effect by cycloheximide was >96% in 3 of 11 experiments, between 45 and 75% in 7 experiments, and 20% in the remaining experiment. When lymphokines and/or cycloheximide were added to L929 cells both before and after infection, percent suppression of the anti-rickettsial effect of the lymphokines by cycloheximide was >90% in two of four experiments and was 18% in the remaining two experiments. Some of this variability may be due to the toxicity of certain lymphokine-cycloheximide combinations for L929 cells, especially when cells were treated both before and after infection. Emetine, another inhibitor of eukaryotic protein synthesis, also suppressed the inhibition of rickettsial growth in L929 cells by mouse lymphokines (Table II).

Species Specificity of the ARA in Mouse and Human Lymphokines. Clearance of *R. prowazekii* and inhibition of rickettsial growth occurred in mouse lymphokine-treated 3T3-A31 mouse fibroblasts as well as in mouse lymphokine-treated mouse L929 cells (Table III). However, rickettsial survival and growth were not inhibited in mouse lymphokine-treated human foreskin fibroblasts (Table III) or in mouse lymphokine-treated human Flow 5000 cells (data not shown). Similarly, human lymphokine treatment of human foreskin fibroblasts resulted in rickettsial clearance and inhibition of rickettsial growth in these cells, but human lymphokine treatment of mouse L929 cells had no effect on rickettsial survival and growth (Table III).

Assay of ARA in Mouse Lymphokines. As demonstrated in Table I, it was possible to measure the anti-rickettsial potency of a given mouse lymphokine preparation by determining the effects of dilutions of the preparation on rickettsial survival and growth as measured by %R and RI. This method was very time-consuming; hence we developed a more rapid and convenient scoring method for determining the titer of ARA in lymphokine preparations when the details of the parameters of rickettsial growth were not of special interest (details in Materials and Methods). Fig. 2 shows

TABLE III

*Effect of Mouse and Human Lymphokines on the Growth of R. prowazekii in Mouse and Human Cell Lines**

Treatment before and after infection	Hours after infection	Cell line					
		Mouse L929		Mouse 3T3-A31		Human foreskin fibroblasts	
		%R	RI	%R	RI	%R	RI
Control	0	(90 ± 1)	(4.6 ± 0.5)	(47 ± 11)	(2.7 ± 0.4)	(55 ± 8)	(2.7 ± 0.4)
	24	94 ± 3	531 ± 49	104 ± 20	361 ± 74	112 ± 9	603 ± 107
	48	98 ± 5	1632 ± 131	94 ± 20	1415 ± 201	143 ± 12	2,285 ± 459
Mouse lymphokines	0	(77 ± 4)	(3.5 ± 0.4)	(28 ± 7)	(2.0 ± 0.1)	(35 ± 10)	(2.9 ± 0.5)
	24	66 ± 8	131 ± 20	39 ± 8	95 ± 0	176 ± 48	562 ± 72
	48	47 ± 6	207 ± 32	54 ± 14	81 ± 13	215 ± 34	2,101 ± 225
Human lymphokines	0	(87 ± 6)	(4.5 ± 1.1)	ND‡	ND	(38 ± 8)	(2.1 ± 0.1)
	24	97 ± 1	425 ± 64	ND	ND	59 ± 8	138 ± 30
	48	93 ± 2	1430 ± 359	ND	ND	65 ± 22	374 ± 144

* Cells were treated with tissue culture medium alone or with 20% mouse or human lymphokines in tissue culture medium. Values in parentheses represent %R and RI at 0 h. %R and RI at 24 and 48 h after infection are expressed as percentages of the values observed at 0 h. Each value represents the mean ± standard error for two or more experiments.

‡ Not determined.

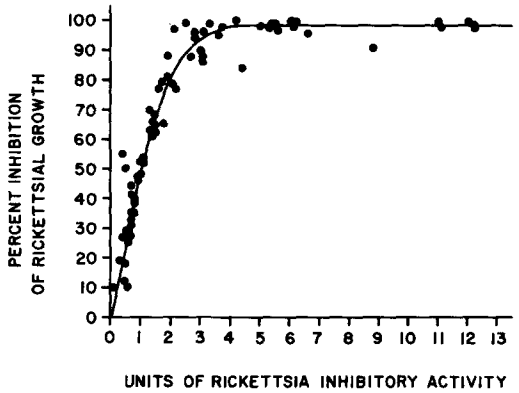

FIG. 2. Dose-response curve for the inhibition of rickettsial growth by mouse lymphokines. L929 cells were treated with dilutions of lymphokines both 24 h before infection and after infection with *R. prowazekii* E strain. Infected cultures were incubated for 48 h, stained, and examined microscopically. The data shown were collected in 18 experiments. The number of units of ARA per 0.3 ml of a given lymphokine preparation was the reciprocal of that dilution which by interpolation gave 50% inhibition. The units of ARA present and percent inhibition for all of the dilutions of each experiment were plotted. For example, if a 1:40 dilution caused 60% inhibition and a 1:80 dilution caused 30% inhibition, by interpolation a 1:50 dilution would cause 50% inhibition (1 U/0.3 ml). Hence there would be 50 U/0.3 ml in the undiluted lymphokine and 1.25 U caused 60% inhibition, whereas 0.63 U caused 30% inhibition. This assay provided a relatively rapid and standardized evaluation of the potency of particular lymphokine preparations.

a dose-response curve obtained by plotting percent inhibition of rickettsial growth against calculated units of ARA. Data shown were pooled from 18 experiments.

During these studies we assayed the ARA in 24 preparations of concanavalin A-induced mouse lymphokines. Four of these preparations contained <10 U of ARA in 0.3 ml and were therefore not used in experiments. The remaining 20 preparations contained between 18 and 238 U of ARA in 0.3 ml (mean = 68 U). Two control culture supernatants obtained from mouse spleen cells incubated for 48 h without concanavalin A and one control supernatant prepared from spleen cells incubated with concanavalin A for 1 h all contained <3 U of ARA in 0.3 ml.

Four *C. parvum*-induced mouse lymphokine preparations contained ARA ranging from 21 to 76 U/0.3 ml (mean, 40 U); and two *R. prowazekii*-induced lymphokine preparations contained 38 and 103 U in 0.3 ml. One supernatant obtained after incubation of spleen cells of *C. parvum*-immunized mice for 48 h without additional immunogen contained 16 U of ARA in 0.3 ml, and one preparation obtained after incubation of spleen cells of *R. prowazekii*-immunized mice for 48 h without additional immunogen contained <3 U in 0.3 ml.

In Table I, the concentration of ARA at the highest dilution of mouse lymphokines (1:20) was ~1 U/0.3 ml. In Fig. 1 and Tables II and III, mouse lymphokine-treated cells were treated with at least 3 U of ARA/0.3 ml.

Preliminary Characterization of the ARA in Mouse Lymphokines. Table IV shows the results of several experiments in which the ARA in crude mouse lymphokines induced with concanavalin A was assayed after the lymphokines were subjected to various treatments. Substantial amounts of the ARA remained after heating the lymphokines at 56°C for 30 min. However, heating the lymphokines at 80°C for 10 min destroyed

TABLE IV

*Effect of Various Treatments on ARA in Mouse Lymphokines**

Lymphokine treatment	Experiment	ARA (U/0.3 ml)‡	
Untreated	1	28	(100)§
	2	35	
	3	61	
56°C, 30 min	1	24	(86)
	2	23	(66)
	3	38	(62)
80°C, 10 min	1	<2.5	(<9)
	2	<2.5	(<8)
	3	<2.5	(<4)
Untreated	4	31	(100)
	5	46	
Dialyzed	4	17	(55)
	5	18	(39)
pH 2, 24 h; dialyzed‖	4	<4	(<13)
	5	<2.5	(<6)

* Mouse lymphokines were the supernatant fluids collected from mouse spleen cells cultured in the presence of concanavalin A for 48 h.

‡ ARA was assayed as defined in Materials and Methods.

§ Numbers in parentheses express the titer of the treated lymphokine sample as a percentage of the titer of the corresponding untreated lymphokine preparation.

‖ Samples of lymphokines were adjusted to pH 2 with 1 N HCl and were allowed to stand at 4°C for 24 h. Lymphokines were then dialyzed against Eagle's minimum essential medium and sterilized by filtration.

the ARA. Treatment of the lymphokines at pH 2 for 24 h followed by dialysis of the lymphokines also resulted in destruction of the ARA.

Discussion

Roles for both specific antibody and sensitized T lymphocytes in anti-rickettsial immunity have been indicated (3, 4, 6–13), but the mechanisms of protection have not been well defined. Destruction of antibody-treated rickettsiae occurs within macrophages (4), and destruction of rickettsiae by lymphokine-activated macrophages has also been noted (11–13). However, destruction of *R. prowazekii* by macrophages would seem to be insufficient for eliminating rickettsiae in the host because the endothelial cells (nonprofessional phagocytes) lining the capillaries are major sites of rickettsial multiplication (1). Destruction of infected cells by sensitized T cells and/or establishment of conditions unsuitable for rickettsial survival and growth in non-professional phagocytes would constitute important mechanisms of host defense against *R. prowazekii* and other *Rickettsia* species. The former mechanism would require alteration of the membranes of the infected host cells and a cytotoxic mechanism that would not only lyse the altered host cells but also destroy the rickettsiae within these cells. No evidence exists to support such a mechanism of defense in rickettsial infections. The latter mechanism would involve less damage to host tissues, and in

vitro correlates of this possible host defense mechanism are provided by the present study. The lymphokines used in this study induced anti-rickettsial conditions in fibroblasts. Thus mediators produced by sensitized cells may function in controlling rickettsial survival and growth in nonprofessional phagocytes in the infected host.

Several features of the lymphokine-rickettsia-host cell interaction deserve further emphasis. First, the lymphokines themselves, which were produced by spleen cells after stimulation with mitogen or antigen, were probably not directly toxic for the rickettsiae: not only were the lymphokines species specific, but the anti-rickettsial effect was suppressed when lymphokines were added to fibroblasts along with an inhibitor of eukaryotic protein synthesis. Second, the development of anti-rickettsial conditions within mouse fibroblasts did not occur immediately after lymphokine addition because rickettsial growth during the first 24 h after infection was more strongly inhibited in cells pretreated with lymphokines for 24 h than in cells treated with lymphokines only after infection. The presence of the lymphokines may be required for continued expression of ARA because the inhibition of rickettsial growth in L929 cells treated with lymphokines both before and after infection was greater than the inhibition observed in cells treated with lymphokines only before infection. Finally, the avirulent E strain of *R. prowazekii* was not more susceptible to the anti-rickettsial conditions induced by lymphokines in fibroblasts than was the virulent Breinl strain.

Induction of microbistatic or microbicidal activity in macrophages or monocytes by lymphokines has been noted by many researchers (12, 22–30). In contrast, there have been very few reports of the induction of such activity in nonprofessional phagocytes by lymphokines. One report described the inhibition of the growth of *Toxoplasma* and *Besnoitia* in hamster fibroblasts and kidney cells treated with supernatants derived from cultures of antigen-stimulated hamster lymphocytes (31). Inhibition of the growth of *Toxoplasma* in mouse L929 cells treated with supernatants obtained from cultures of antigen-stimulated mouse spleen cells has also been noted (32). The anti-*Toxoplasma* factor in this system had certain properties in common with interferon-γ (32).

Because the induction of ARA in mouse fibroblasts by mouse lymphokines was inhibited by cycloheximide or emetine, and because mouse and human lymphokines induced ARA in fibroblasts of the homologous species but not in fibroblasts of the heterologous species, the anti-rickettsial factor (or one of the anti-rickettsial factors) in our lymphokine preparations might be an interferon. Inhibition of the growth of *R. akari* in mouse fibroblasts by virus-induced, pH 2-stable interferon was described over 10 years ago (33). However, it seems highly unlikely that mouse interferons β and α are responsible for the ARA of our concanavalin A-induced mouse lymphokine preparations because these interferons are stable at pH 2 (34, 35), whereas the ARA in our concanavalin A-induced mouse lymphokines was destroyed by exposure to pH 2. The relative stability of the ARA at 56°C also contrasts with the instability of such interferons at 56°C (34).

Interferon-γ, however, remains a possible candidate for all or some of the ARA because it is labile at pH 2 and stable at 56°C (34). Interferon-γ is produced in spleen cell cultures after stimulation with antigens or mitogens (reviewed in 36), and our concanavalin A-induced mouse lymphokine preparations contain antiviral activity that is labile at pH 2 (data not shown). Experiments are currently being conducted

in our laboratory to determine the nature of the lymphokines responsible for inhibiting the survival and growth of *R. prowazekii* in fibroblasts.

Summary

The effect of lymphokine treatment of mouse and human fibroblast cell lines on the growth of *Rickettsia prowazekii* within the fibroblasts was studied. Treatment of mouse L929 cells with concanavalin A- or antigen-induced mouse lymphokines both before and after infection with *R. prowazekii* led to clearance of the rickettsiae from a substantial proportion of the cells and suppression of rickettsial growth in those cells which remained infected. Similar but less dramatic anti-rickettsial effects were observed in L929 cells treated with mouse lymphokines either only before or after infection with rickettsiae. Mouse lymphokine treatment of L929 cells had similar anti-rickettsial effects on the avirulent E strain and the virulent Breinl strain of *R. prowazekii*. Addition of cycloheximide or emetine to L929 cells at the same time as the lymphokines markedly suppressed the inhibition of rickettsial growth by the lymphokines. Mouse lymphokine treatment inhibited rickettsial survival and growth in mouse 3T3-A31 cells as well as in mouse L929 cells, but had no effect on rickettsial survival and growth in human foreskin fibroblasts. Conversely, concanavalin A-induced human lymphokines inhibited rickettsial survival and growth in human foreskin fibroblasts but had no effect on rickettsial survival and growth in mouse L929 cells. The rickettsia inhibitory activity in concanavalin A-induced mouse lymphokines was destroyed by heating the lymphokines at 80°C for 10 min or by holding the lymphokines at pH 2 for 24 h but was retained after heating at 56°C for 30 min.

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References

1. Wolbach, S. B., J. C. Todd, and F. W. Palfrey. 1922. The Etiology and Pathology of Typhus. Harvard University Press, Cambridge, MA. 222.
2. Gambrill, M. R., and C. L. Wisseman, Jr. 1973. Mechanisms of immunity in typhus infections. II. Multiplication of typhus rickettsiae in human macrophage cell cultures in the nonimmune system: influence of virulence of rickettsial strains and of chloramphenicol. *Infect. Immun.* **8:**519.
3. Yeomans, A., J. C. Snyder, and A. G. Gilliam. 1945. The effects of concentrated hyperimmune rabbit serum in louse borne typhus. *JAMA (J. Am. Med. Assoc.)*. **129:**19.
4. Beaman, L., and C. L. Wisseman, Jr. 1976. Mechanisms of immunity in typhus infections. VI. Differential opsonizing and neutralizing action of human typhus rickettsia-specific cytophilic antibodies in cultures of human macrophages. *Infect. Immun.* **14:**1071.
5. Turco, J., and H. H. Winkler. 1982. Differentiation between virulent and avirulent strains of *Rickettsia prowazekii* by macrophage-like cell lines. *Infect. Immun.* **35:**783.
6. Murphy, J. R., C. L. Wisseman, Jr., and P. Fiset. 1979. Mechanisms of immunity in typhus infection: adoptive transfer of immunity to *Rickettsia mooseri*. *Infect. Immun.* **24:**387.
7. Murphy, J. R., C. L. Wisseman, Jr., and P. Fiset. 1980. Mechanisms of immunity in typhus infections: analysis of immunity to *Rickettsia mooseri* infection of guinea pigs. *Infect. Immun.* **27:**730.
8. Shirai, A., P. J. Catanzaro, S. M. Phillips, and J. V. Osterman. 1976. Host defenses in

experimental scrub typhus: role of cellular immunity in heterologous protection. *Infect. Immun.* **14:**39.

9. Kenyon, R. H., and C. E. Pedersen, Jr. 1980. Immune responses to *Rickettsia akari* infection in congenitally athymic nude mice. *Infect. Immun.* **28:**310.
10. Kokorin, I. N., E. A. Kabanova, E. M. Shirokova, G. E. Abrosimova, N. N. Rybkina, and V. I. Pushkareva. 1982. Role of T lymphocytes in *Rickettsia conorii* infection. *Acta Virol. (Prague) (Engl. Ed.).* **26:**91.
11. Nacy, C. A., and J. V. Osterman. 1979. Host defenses in experimental scrub typhus: role of normal and activated macrophages. *Infect. Immun.* **26:**744.
12. Nacy, C. A., and M. S. Meltzer. 1979. Macrophages in resistance to rickettsial infection: macrophage activation *in vitro* for killing of *Rickettsia tsutsugamushi. J. Immunol.* **123:**2544.
13. Nacy, C. A., E. J. Leonard, and M. S. Meltzer. 1981. Macrophages in resistance to rickettsial infections: characterization of lymphokines that induce rickettsiacidal activity in macrophages. *J. Immunol.* **126:**204.
14. Bovarnick, M. R., and J. C. Snyder. 1949. Respiration of typhus rickettsiae. *J. Exp. Med.* **89:**561.
15. Wisseman, C. L., Jr., E. B. Jackson, F. E. Hahn, A. C. Ley, and J. E. Smadel. 1951. Metabolic studies of rickettsiae. I. The effects of antimicrobial substances and enzyme inhibitors on the oxidation of glutamate by purified rickettsiae. *J. Immunol.* **67:**123.
16. Winkler, H. H. 1974. Inhibitory and restorative effects of adenine nucleotides on rickettsial adsorption and hemolysis. *Infect. Immun.* **9:**119.
17. Weiss, E., J. C. Coolbaugh, and J. C. Williams. 1975. Separation of viable *Rickettsia typhi* from yolk sac and L cell host components by Renografin density gradient centrifugation. *Appl. Microbiol.* **30:**456.
18. Phibbs, P. V., Jr., and H. H. Winkler. 1982. Regulatory properties of citrate synthase from *Rickettsia prowazekii. J. Bacteriol.* **149:**718.
19. Walker, T. S., and H. H. Winkler. 1979. Rickettsial hemolysis: rapid method for enumeration of metabolically active typhus rickettsiae. *J. Clin. Microbiol.* **9:**645.
20. Ruco, L. P., and M. S. Meltzer. 1977. Macrophage activation for tumor cytotoxicity: induction of tumoricidal macrophages by supernatants of PPD-stimulated bacillus Calmette-Guerin-immune spleen cell cultures. *J. Immunol.* **119:**889.
21. Wisseman, C. L., Jr., A. D. Waddell, and W. T. Walsh. 1974. *In vitro* studies of the action of antibiotics on *Rickettsia prowazekii* by two basic methods of cell culture. *J. Infect. Dis.* **130:**564.
22. Hinrichs, D. J., and T. R. Jerrells. 1976. *In vitro* evaluation of immunity to *Coxiella burnetii. J. Immunol.* **117:**996.
23. Patterson, R. J., and G. P. Youmans. 1970. Demonstration in tissue culture of lymphocyte-mediated immunity to tuberculosis. *Infect. Immun.* **1:**600.
24. Klun, C. L., and G. P. Youmans. 1973. The induction by *Listeria monocytogenes* and plant mitogens of lymphocyte supernatant fluids which inhibit the growth of *Mycobacterium tuberculosis* within macrophages *in vitro. J. Reticuloendothel. Soc.* **13:**275.
25. Horwitz, M. A., and S. C. Silverstein. 1981. Activated human monocytes inhibit the intracellular multiplication of Legionnaires' disease bacteria. *J. Exp. Med.* **154:**1618.
26. Jones, T. C., L. Len, and J. G. Hirsch. 1975. Assessment *in vitro* of immunity against *Toxoplasma gondii. J. Exp. Med.* **141:**466.
27. Borges, J. S., and W. D. Johnson, Jr. 1975. Inhibition of multiplication of *Toxoplasma gondii* by human monocytes exposed to T-lymphocyte products. *J. Exp. Med.* **141:**483.
28. Fowles, R. E., I. M. Fajardo, J. L. Leibowitch, and J. R. David. 1973. The enhancement of macrophage bacteriostasis by products of activated lymphocytes. *J. Exp. Med.* **138:**952.
29. Cole, P. 1975. Activation of mouse peritoneal cells to kill *Listeria monocytogens* by T-lymphocyte products. *Infect. Immun.* **12:**36.

30. Nogueira, N., and Z. A. Cohn. 1978. *Trypanosoma cruzi: in vitro* induction of macrophage microbicidal activity. *J. Exp. Med.* **148:**288.
31. Chinchilla, M., and J. K. Frenkel. 1978. Mediation of immunity to intracellular infection (*Toxoplasma* and *Besnoitia*) within somatic cells. *Infect. Immun.* **19:**999.
32. Shirahata, T., and K. Shimizu. 1980. Production and properties of immune interferon from spleen cell cultures of *Toxoplasma*-infected mice. *Microbiol. Immunol.* **24:**1109.
33. Kazar, J., P. A. Krautwurst, and F. B. Gordon. 1971. Effect of interferon and interferon inducers on infections with a nonviral intracellular microorganism, *Rickettsia akari*. *Infect. Immun.* **3:**819.
34. Youngner, J. S., and S. B. Salvin. 1973. Production and properties of migration inhibitory factor and interferon in the circulation of mice with delayed hypersensitivity. *J. Immunol.* **111:**1914.
35. Stewart, W. E., J. E. Blalock, D. C. Burke, C. Chany, J. K. Dunnick, E. Falcoff, R. M. Friedman, G. J. Galasso, W. K. Joklik, J. T. Vilcek, J. S. Youngner, and K. C. Zoon. 1980. Interferon nomenclature. *Nature* (*Lond.*). **286:**110.
36. Epstein, L. B. 1979. The comparative biology of immune and classical interferons. *In* Biology of the Lymphokines. S. Cohen, E. Pick, and J. J. Oppenheim, editors. Academic Press, Inc., New York. 443–514.