INTERFERONLIKE FACTORS FROM ANTIGEN- AND MITOGEN-STIMULATED HUMAN LEUKOCYTES WITH ANTIRICKETTSIAL AND CYTOLYTIC ACTIONS ON *RICKETTSIA PROWAZEKII*

Infected Human Endothelial Cells, Fibroblasts, and Macrophages*

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The immunological response of Man to typhus fevers, caused by obligate intracellular parasitic bacteria of the genus *Rickettsia*, includes both antibody and cell-mediated components (CMI)[1] (1–3). The immune effector mechanisms that control intracellular rickettsial replication are unknown, but studies in animals indicate that they are mediated through immune T lymphocytes[2] (4). The relationship between *Rickettsia prowazekii*-infected cells to monocytes, macrophages, and lymphocytes in the classical typhus lesions (5) suggests the participation of CMI mechanisms other than phagocytosis and destruction by activated macrophages. Thus, the basic lesion, a vasculitis of small blood vessels, consists of focal areas of rickettsial infection of endothelial cells accompanied by perivascular accumulations of monocytes, macrophages, and lymphocytes. Some intravascular mononuclear cells, in contact with the luminal surface of endothelial cells, presumably pass across the infected endothelial cells to perivascular sites, which might activate specific T lymphocytes (6). Rickettsiae appear to be limited largely to the endothelial cells, despite their catholic in vitro host cell range (7).

Macrophages have been the main focus in several studies as putative final effectors of the destruction of various rickettsial species by both antibody-mediated and CMI mechanisms (2, 8–11). Both depend upon phagocytosis of extracellular rickettsiae, which is artificially facilitated by the in vitro and intraperitoneal test systems employed. Peritoneal macrophages from immune animals or those treated with lymphokines from stimulated immune spleen cells show increased capacity to destroy contained rickettsiae (8–11). However, in vivo studies raise questions of the relevance of these macrophage-dependent phenomena to control of rickettsial replication in tissues during infections induced by more natural peripheral routes. Opportunity for macrophages to interact in the lesions with extracellular rickettsiae in the classical

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[1] *Abbreviations used in this paper:* CM, control medium; CMI, cell-mediated immunity; CS, unstimulated leukocyte supernatant fluid; EDTA, ethylenediamine tetraacetate; ET, *R. prowazekii*; ET-S, supernatant fluid from ET antigen-stimulated typhus-immune leukocyte cultures; FCS, fetal calf serum; N, average number of rickettsiae per cell; N_i, average number of rickettsiae per infected cell; p_i, percent cells infected; PHA, phytohemagglutinin; PHA-S, supernatant fluid from PHA-stimulated leukocyte cultures.

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role of phagocytic cells may be limited (12). Indeed, passively transferred immune serum, which facilitates rickettsial destruction by macrophages in vitro, fails to restrict rickettsial replication in tissues in vivo[2] (4). Although *Rickettsia mooseri* (*R. typhi*) infection of mice activates liver and spleen macrophages for enhanced microbicidal action on *Listeria monocytogenes*, neither activation of fixed macrophages for enhanced microbicidal action with BCG or *Corynebacterium parvum*, nor their ablation by silica, influences rickettsial growth in the spleen of *R. mooseri*-infected mice. Nevertheless, adoptive transfer of immune T-lymphocytes markedly restricts rickettsial replication in this site.[2]

This initial study in the search for CMI effector mechanisms in typhus fevers describes the production in cultures of human blood leukocytes, upon immunologically specific stimulation with *R. prowazekii* antigen or nonspecific stimulation with the mitogen phytohemagglutinin, of a soluble host cell-specific factor(s) that causes the death in culture of *R. prowazekii* growing not only within human macrophages, but also within "somatic" target endothelial cells and in fibroblasts and that causes the specific lysis of *R. prowazekii*-infected, but not uninfected fibroblasts.

Materials and Methods

Cell Culture Media. Unless otherwise specified, the basic medium (CM) was half-strength Dulbecco's medium with Earle's salts containing 0.1% glucose (D/2 medium) (Gibco Laboratories, Grand Island, NY) and 10% fetal calf serum (FCS) (Gibco Laboratories). RPMI 1640 medium (Flow Laboratories, Inc., Rockville, MD) containing 10% heat-inactivated human AB serum was used for the production of human blood leukocyte supernatant fluids. Human umbilical cord endothelial cells were grown and tested either in M199 medium with Earle's salts (Gibco Laboratories) or in RPMI 1640 medium, each supplemented with 20% heat-inactivated human AB serum or FCS. Human blood monocyte-derived macrophages were allowed to develop, and were tested, in D/2 medium supplemented with 30% heat-inactivated human AB or autologous serum. Except for the initial isolation of endothelial cells, no antibiotics were added to any of the media.

Cells. Three types of cells were routinely employed: (*a*) chicken embryo (CE) cells obtained from embryonated specific pathogen-free eggs (SPAFAS, Norwich, CT) as previously described (13); (*b*) WI-38 diploid human fibroblastic cells; and (*c*) a continuous line of human embryonic skin and muscle fibroblasts (F-1000 line from Flow Laboratories).

Endothelial cells from human umbilical cord veins (14) were plated ($0.5–1.0 \times 10^5$ viable cells per cm^2) in 35- or 60-mm plastic tissue culture dishes (Falcon Plastics, Div. of Becton, Dickinson & Co., Cockeysville, MD) or in 12-well plastic plates (Linbro, Div. of Flow Laboratories, Hamden, CT), incubated to confluence at 37°C, removed with trypsin-EDTA and either used directly or split 1:2 for further propagation.

Macrophage cultures from human peripheral blood monocytes were prepared by modification of our basic method (2). Briefly, mononuclear cells, isolated from venous blood by the Ficoll-Hypaque method (15) and suspended at a concentration of 1×10^6 mononuclear cells per ml in D/2 medium containing 30% heat-inactivated autologous or human AB serum, were dispensed in 0.6-ml volumes to each chamber of four-chamber culture slides (Lab-Tek Div., Miles Laboratories Inc., Naperville, IL) and were incubated at 37°C for 6 d, with medium changes at 2 h and 3 d, to allow monocytes to develop into macrophages.

General Cell Culture Methods. Cells were trypsinized with 0.1% trypsin (Gibco Laboratories) and 0.02% Na-EDTA (ethylene diaminotetra-acetic acid) in phosphate-buffered physiological saline (PBS), pH 7.2. Total and viable cell hemocytometer counts were made by trypan blue exclusion. Cultures were routinely incubated in humid air containing 5% CO_2 and, unless otherwise specified, at 32°C. Irradiated cells received 3,000 rad x-irradiation as previously described (13).

Rickettsia. A plaque-purified line of *Rickettsia prowazekii* (Breinl strain) (ET) of human origin with a history of 155 egg, 3 tissue culture, and 3 egg passages was propagated in the yolk sac of

embryonated specific pathogen-free chicken eggs (SPAFAS). Ordinary seeds were prepared as 20% (wt/vol) infected yolk sac homogenates in 3.7% brain heart infusion broth (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, MD). Highly purified seeds enriched in viable rickettsiae were prepared from the light bands from Renografin gradients (16). Convenient volumes were stored at −70°C.

General Rickettsial Methods. R. prowazekii (Breinl) antigen for stimulating leukocytes was prepared from a highly purified suspension (16) that was killed by 0.1% formalin at 4°C, washed by centrifugation, and resuspended in PBS, pH 7.0, to a concentration of 1 mg/ml (dry wt) determined optically. Rickettsial plaque counts were made in monolayers of primary CE cells in 60-mm plastic petri dishes (Falcon Plastics) by modifications of the method of Wike et al. (17). Cells were infected with rickettsiae either in suspension or in cultures of attached cells (13). Rickettsial growth was measured by microscopic observation of slide chamber cultures (13). The Gimenez stain (18) was usually employed but, in some instances, methanol-fixed smears were stained by the Giemsa method or acetone-fixed smears were stained with fluorescein-conjugated human typhus immune serum.

Preparation of Stimulated Leukocyte Supernatant Fluids. Stimulated supernatant fluids were produced from leukocytes (19) obtained from healthy adult donors who either (*a*) had no history and no serological evidence of previous typhus infection or vaccination, or (*b*) had recovered from an infection with *R. prowazekii* or, in some instances, *R. mooseri.* Venous blood was drawn into containers containing 10 U heparin (Upjohn Co., Kalamazoo, MI) per 1 ml blood; 1 ml 6% dextran (Dextran T-250, Pharmacia, Uppsala, Sweden) in physiological saline was added for each 10 ml of blood; and, after ~40 min at 37°C, leukocytes were harvested from the leukoplasma and washed three times in RPMI 1640 medium by centrifugation at 4°C. The cells were adjusted to the desired concentration in RPMI 1640 medium containing 10% heat-inactivated human AB serum or FCS. For the production of stimulated leukocyte supernatant fluids, 1 ml of suspension containing 1×10^6 leukocytes and 1 μg ET antigen or purified phytohemagglutinin (Wellcome Reagents, Ltd., Beckenham, England) (PHA) per milliliter was added to each of a series of tissue culture tubes (No. 3033, Falcon Plastics) or 20 ml of suspension containing 1×10^8 leukocytes and 3 μg ET antigen or PHA per ml was added to each 75-cm^2 plastic tissue culture flask (Costar, Data Packaging, Cambridge, MA). Incubation was at 37°C for 18–24 h. The centrifuged supernatant fluid was cultured, passed through a 0.22-μm Millipore filter (Millipore/Continental Water Systems, Bedford, MA) and stored at −20°C or −70°C in small volumes. Control supernatant fluids were collected from similar, but unstimulated, cultures.

Assay Methods for Antirickettsial and Cytolytic Actions of Leukocyte Supernatant Fluids. Initial observations were made at intervals over 48 h incubation at 32°C by microscopic examination of slide chamber cultures to which ET-S or PHA-S diluted in CM was added after infection. Separate assay methods subsequently were developed for the antirickettsial and cytolytic actions.

Intracellular antirickettsial action, devoid of cytolytic action, was measured by a modification of the slide chamber method. The medium over confluent cultures of host cells (F-1000 human fibroblasts unless otherwise indicated) growing in plastic vessels was replaced with CM or CS or with ET-S or PHA-S diluted in CM. After an 18-h incubation at 32°C, the cells were washed three times with CM, removed with trypsin-EDTA, centrifuged, resuspended in CM, infected in suspension, washed in CM, and distributed to four-chamber culture slides, 2.5×10^4 cells per chamber in 0.5 ml CM. Slides were removed at intervals during a 40–48-h incubation period, heat fixed, and stained with Gimenez stain. The rickettsiae in 300 cells per condition were counted; percent cells infected (p_i), average number of rickettsiae per cell (N), and average number of rickettsiae per infected cell (N_i) were calculated (13).

The cytolytic action of stimulated supernatant fluids was measured by a modified microtiter method (20), which depends on the direct counting of crystal violet-stained residual cells in flat bottom 96 well microtiter plates (Linbro). A washed suspension of trypsinized infected F-1000 cells (4×10^3 cells/ml CM) was distributed to the microtiter plates, 0.1 ml per well. After a 6-h incubation, the CM was replaced with 0.1 ml fresh CM, CS, ET-S, or PHA-S, usually diluted 1:2 or 1:10 in CM. At intervals during the subsequent 40–48-h incubation, the culture fluid was removed from the wells and the cells were fixed for 10 min in methanol, stained for 5 min

with crystal violet (0.05% in physiological saline), washed in tap water, and dried. The cells remaining attached in each of six wells per condition were counted under a dissecting microscope. Uninfected cell controls accompanied each condition. Rickettsial growth was monitored in parallel slide chamber cultures.

The term "pretreatment" designates the treatment of host cells before infection, usually with a leukocyte supernatant fluid for 18–20 h, and "posttreatment" designates treatment of host cells after infection, usually starting at ~6 h and continuing throughout the experiment.

Radioisotope Uptake Methods. To verify the action of inhibitors of protein, RNA, and DNA synthesis, F-1000 cells cultured in 12-well plastic Linbro plates were pulsed at the desired time for 4 h with CM containing [^{3}H]leucine (Amersham Corp., Arlington Heights, IL) (20–50 μCi/ml), [^{3}H]uridine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY; 10–15 μCi/ml), or [^{3}H]thymidine (Amersham Corp.; 10 μCi/ml). After discarding the medium, the cells were washed three times with D/2 medium and lysed with 0.5 ml 2% sodium dodecyl sulfate. A 50-μl aliquot of each sample was dried on a small piece of Whatman No. 1 filter paper, extracted with trichloroacetic acid, rinsed in two changes of 95% ethanol, dried, and counted in OCS (Amersham Corp.) scintillation fluid.

Special Reagents. The sources of some special reagents were as follows: emetine HCl (Boehringer Mannheim, Federal Republic of Germany); cycloheximide (Gibco Laboratories); actinomycin D (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, CA); mitomycin C (Sigma Chemical Co., St. Louis, MO); and chloramphenicol (Schering Corp., Bloomfield, NJ).

Results

A Host Cell-specific Complex Action of Antigen-stimulated Human Immune Leukocyte Supernatant Fluids on Rickettsia-infected Cells In Vitro. The supernatant fluids from typhus-immune human leukocytes that had been stimulated with killed *R. prowazekii* antigen (ET-S) induced, after a lag period of a few hours, a complex set of effects when added to *R. prowazekii*-infected human (WI-38), but not chicken embryo fibroblasts (Fig. 1): (*a*) an *antirickettsial* action on intracellular rickettsiae, as evidenced by declines in percent cells infected (p_i) and average number of rickettsiae per cell (N), and (*b*) a *cytolytic* action on infected cultures, as indicated by a progressive, visible cell loss. In a minority of infected and treated cells, however, rickettsial growth appeared to be relatively uninhibited. Fig. 1 is representative of the results obtained in many experiments with the same lot of ET-S, different lots from the same individual and lots from different immune subjects. Identical results were obtained with supernatant fluids from leukocytes from either nonimmune or typhus-immune donors following stimulation with PHA. Supernatant fluids from unstimulated immune human leukocytes (CS) or from ET antigen-stimulated leukocytes from nonimmune donors failed to show either antirickettsial or cytolytic actions.

Dissociation of Intracellular Antirickettsial Action from Cytolytic Action. Results similar to those described above theoretically could have been obtained by selective loss of infected cells. However, intracellular *antirickettsial* action of ET-S or PHA-S in the absence of complicating cytolysis was demonstrable when uninfected F-1000 human fibroblast cultures were pretreated with ET-S or PHA-S before infection and subsequently incubated in CM and was evidenced by a progressive decline in p_i and N over a 48-h period without detectable damage or loss of host cells (Fig. 2). Nevertheless, rickettsiae continued to grow in a small minority of cells. In contrast, cytolytic action measured in microtiter plates caused a progressive loss of posttreated cells over a 48-period (Fig. 3).

Actions of ET-S on Rickettsial Infection of Human Macrophages and Umbilical Vein Endothe-

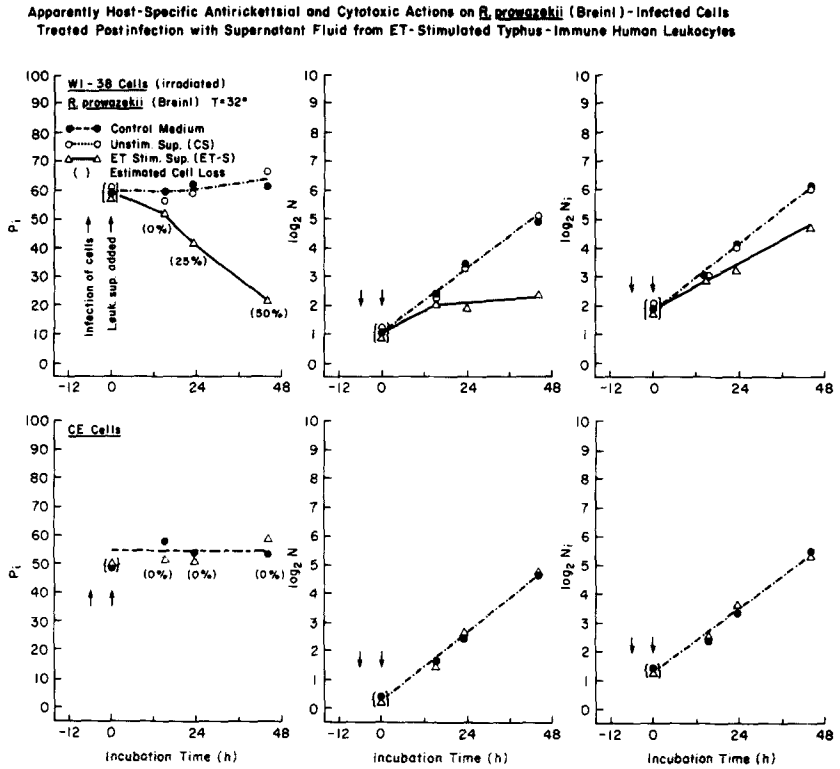

FIG. 1. Apparent antirickettsial and cytolytic actions of supernatant fluid from ET-antigen stimulated typhus-immune human leukocytes (ET-S) on slide chamber cultures of *R. prowazekii* (Breinl)-infected *irradiated* WI-38, but not CE cells. Percentages in parentheses in left panels refer to estimated cell loss. ET-S was added 6 h after infection and was continuously present thereafter.

lial Cells. As with human fibroblasts, growth of *R. prowazekii* (Breinl) was markedly restricted in cultured macrophages pretreated with ET-S (Fig. 4). Similarly, after the initial loss of incompetent cells, *R. prowazekii* (Breinl) multiplied in untreated endothelial cells but declined progressively in endothelial cells pretreated with ET-S (Fig. 5).

Lack of Direct Action of Stimulated Leukocyte Supernatant Fluids on Extracellular Rickettsiae. No direct rickettsiacidal action of unstimulated or stimulated leukocyte supernatant fluids was observed in two kinds of experiments (data not shown). (*a*) There was no loss of recoverable PFU from a dilute yolk sac suspension of *R. prowazekii* (Breinl) incubated with ET-S and then assayed in nonresponder CE cell monolayers. (*b*) Direct microscopic measurement failed to reveal any effect on either uptake or growth of highly purified *R. prowazekii* (Breinl) pretreated with ET-S during the early stages of the first infection cycle in responder F-1000 fibroblasts.

Some Actions of Stimulated Leukocyte Supernatant Fluids on Uninfected Host Cells. F-1000 human fibroblasts, either pretreated with ET-S before trypsinization from flasks or in suspensions incubated in the presence of ET-S, showed an enhanced tendency to aggregate, suggesting induction of some change in host cell surface. In the microtiter cytolysis assay system, pretreatment of uninfected F-1000 cells with ET-S or PHA-S followed by subsequent incubation in CM often resulted in significant enhancement

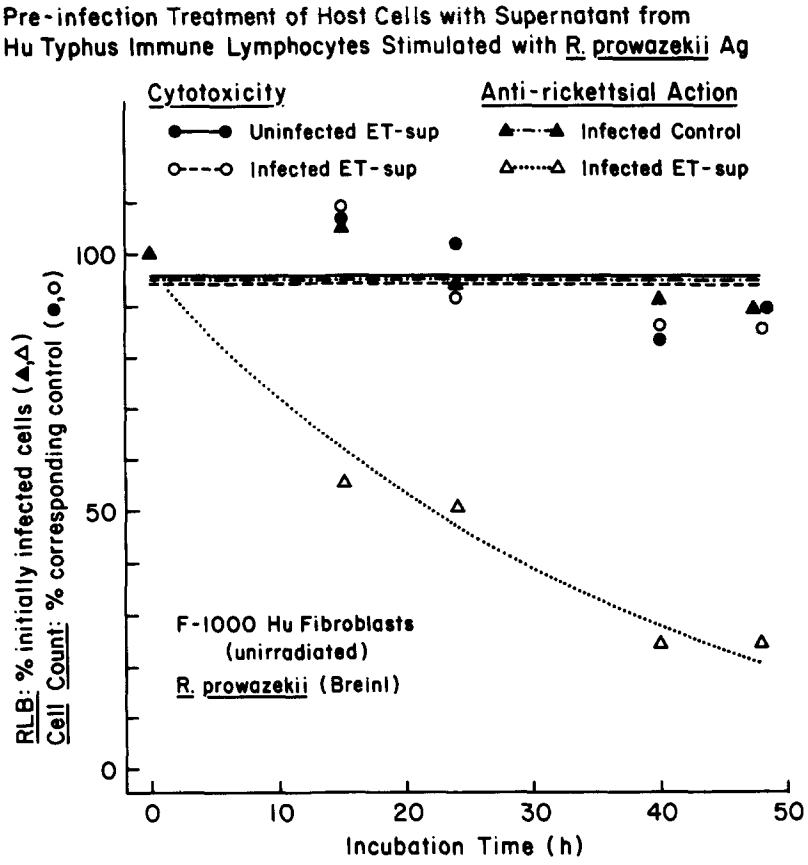

FIG. 2. Antirickettsial action (slide chamber) without cytolytic action (microtiter) in unirradiated F-1000 human fibroblasts pretreated for 18 h with ET-S and then infected *R. prowazekii* (Breinl). Postinfection incubation was in CM in the absence of ET-S. *RLB,* Cells with intracellular Gimenez-positive rickettsia-like bodies.

of cell growth, whereas continuous incubation in the presence of either ET-S or PHA-S resulted in reversible cytostasis, but never cytolysis (data not shown). On two separate occasions in which great care was taken to assure maximal dispersion of host cells, no effect of pretreatment of F-1000 cells with leukocyte supernatants was seen on the rate of uptake of untreated *R. prowazekii* in the suspended cell system (data not shown).

Fate of rickettsiae in ET-S Pretreated F-1000 Cells. The loss of Gimenez-stainable organisms from ET-S pretreated F-1000 cells was found to reflect loss of both morphological and infectious units.

Replicates of pretreated, infected F-1000 human fibroblast cultures showing characteristic loss of rickettsiae by Gimenez stain were stained with (*a*) Giemsa stain or (*b*) fluorescein-labeled typhus convalescent serum. Neither method revealed the presence of organisms in numbers or distribution not detected by the Gimenez stain. Hence, the disappearance of visible organisms from Gimenez-stained cell cultures was not likely an artifact of the Gimenez stain.

In two additional experiments (Table I), replicate cultures of *R. prowazekii*-infected

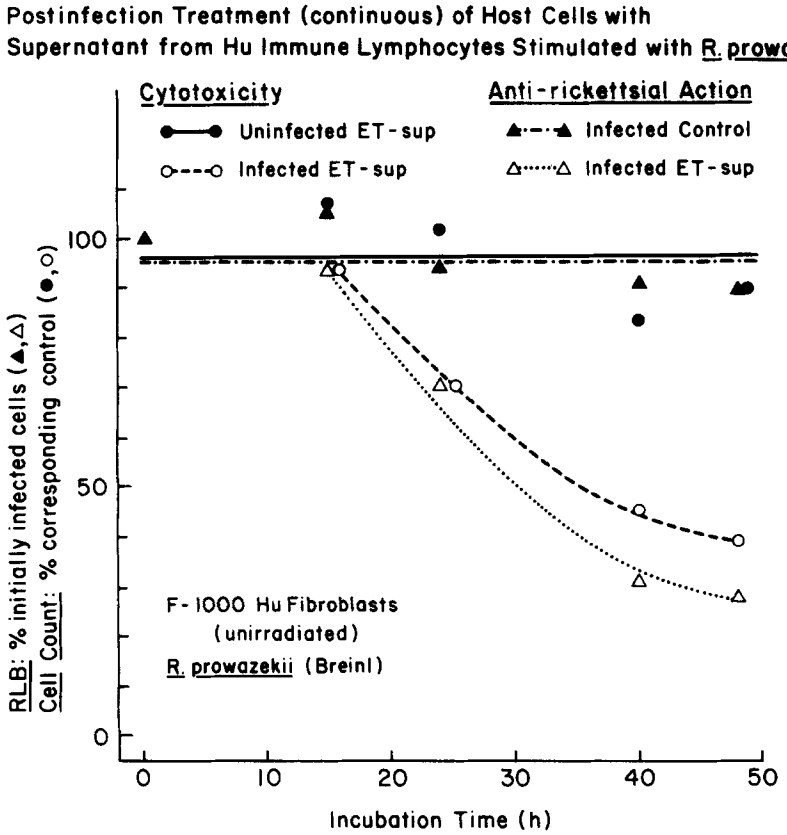

FIG. 3. Cytolytic (microtiter) and apparent antirickettsial (slide chamber) actions of ET-S added to unirradiated F-1000 human fibroblasts 6 h after infection with *R. prowazekii* (Breinl) and continuously present thereafter. Note that when ET-S was added after infection both cytolytic and antirickettsial actions were usually expressed after a lag period of several hours.

F-1000 human fibroblast cells pretreated with CM, CS, or ET-S, the last showing the characteristic decline of rickettsiae in Gimenez-stained preparations, were disrupted at 48 h and the viable rickettsial content was determined by plaque count. In one experiment, the plaque count from ET-S-treated cells was $<10^{-2}$ of that of untreated cells. In the other, in which plaque counts were made at 0 h as well as 48 h, untreated cells showed about a 40-fold increase in titer, whereas the ET-S-treated cells showed a 69% decline. Concurrent microscopic examination revealed that the majority of cells lost microscopically detectable rickettsiae, whereas a minority permitted relatively normal rickettsial growth.

Some Factors Influencing Antirickettsial and Cytolytic Actions of ET-S. The antirickettsial and cytolytic actions of ET-S on F-1000 cells infected with *R. prowazekii* (Breinl) at 32°C were essentially the same at 35°C, the optimal growth temperature for *R. prowazekii* (7; Wisseman, C. L. Jr., et al., unpublished observations), and at 37°C, a more physiologic temperature for F-1000 cells and for the action of lymphotoxins (21) (data not shown). Uninfected F-1000 cells were not lysed at 37°C.

Host cell (F-1000) protein synthesis from newly transcribed messenger RNA was required for the expression of the antirickettsial action, but not for the cytolytic

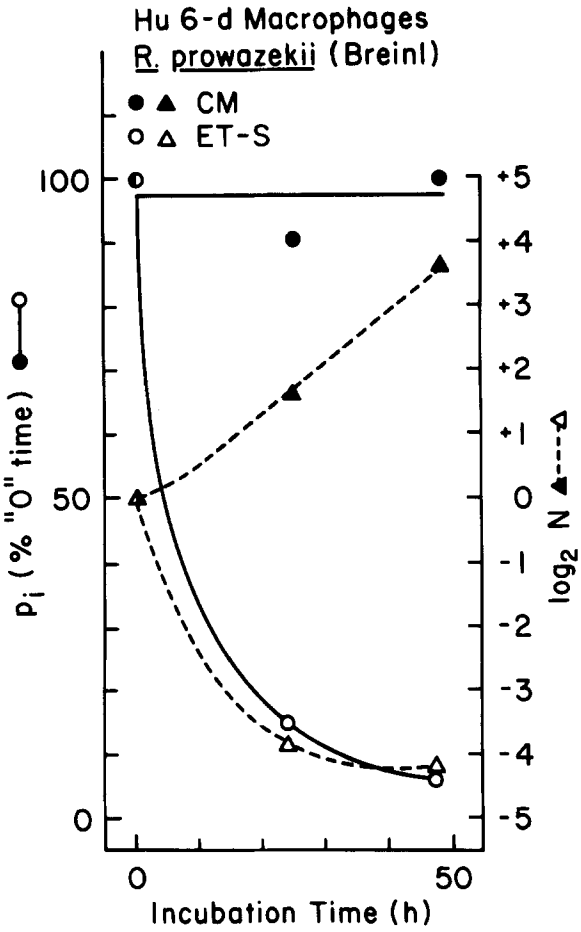

FIG. 4. Antirickettsial action induced by pretreatment (18 h) of cultured human 6-d macrophages with ET-S and then infected with *R. prowazekii* (Breinl). Note that antirickettsial action is reflected by reduction in both percent cells infected (p_i) and average number of rickettsiae per cell (N). In contrast, *R. prowazekii* grew as expected (2) in untreated macrophages.

action, of ET-S (data not shown). Thus, inhibition of host cell transcription with mitomycin C (50 μg/ml) (22) or actinomycin D (1 μg/ml) (22) or translation with cycloheximide (1 μg/ml) or emetine (1 μg/ml) (23) during pre-infection exposure of host cells to ET-S prevented expression of antirickettsial action. Appropriate inhibition patterns of host cell protein and nucleic acid synthesis were confirmed by measuring the uptake of [^{3}H]leucine, [^{3}H]thymidine, and [^{3}H]uridine. None of the inhibitor treatments interfered with rickettsial growth. In contrast, none of these treatments interfered with the cytolytic action of ET-S on *R. prowazekii*-infected F-1000 cells.

Relationship between Rickettsial Protein Synthesis and Replication and Induction of Susceptibility of Host Cells to Cytolytic Action. R. prowazekii protein synthesis or replication was not required for the infection to induce susceptibility to ET-S cytolysis in F-1000 fibroblasts. Microtiter cytolysis assays were performed with and without chloramphenicol (10 μg/ml). Chloramphenicol inhibits *R. prowazekii* growth in culture at about 1.0 μg/ml (24). It inhibits procaryotic protein synthesis at the translational

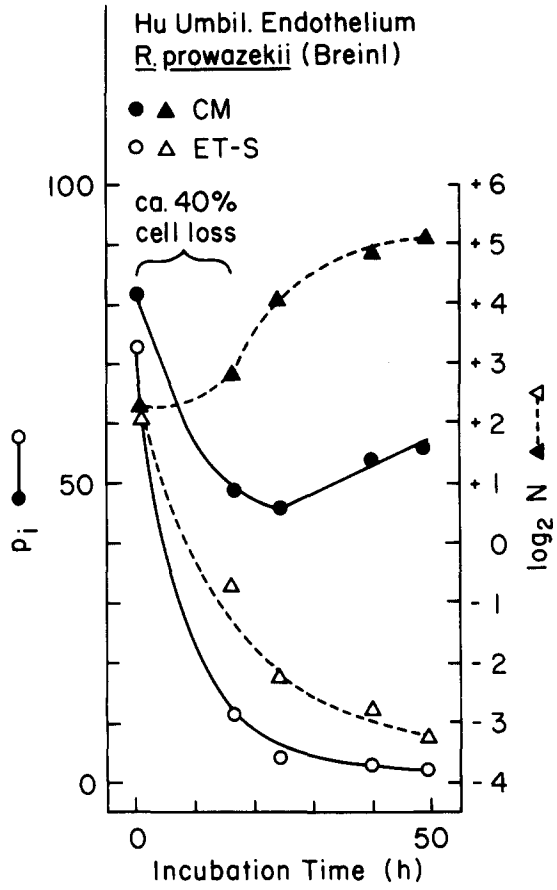

FIG. 5. Antirickettsial action induced by pretreatment (18 h) of cultured human umbilical cord endothelial cells with ET-S and then infected with *R. prowazekii* (Breinl). Note the initial loss or detachment of infected cells which produced an early distortion of the curves depicting p_i and N in control (CM) cultures, with subsequent recovery. In contrast, in ET-S treated cultures, both p_i and N showed the characteristic progressive decline.

level with little effect on eucaryotic protein synthesis except in mitochondria (25). Both rickettsiae and host cells were separately pre-incubated with chloramphenicol and maintained continuously in its presence during infection and cytolytic assay. Parallel slide chamber cultures confirmed that 71–80% of the cells were infected with an average of 3–4.6 rickettsiae per cell at the beginning of the tests, that no rickettsial replication occurred during the 40-h incubation period in the presence of chloramphenicol, and that the expected rickettsial replication occurred in its absence. The curves depicting the course of ET-S-induced cytolysis in the presence or absence of chloramphenicol were indistinguishable from one another (data not shown).

Thermal and pH Stability of Antirickettsial and Cytolytic Factors. Both antirickettsial and cytolytic actions of ET-S and PHA-S measured in the F-1000 cell-*R. prowazekii* (Breinl) system were destroyed either by heating for 1 h at 56°C or by exposure to pH 2 for 24 h (data not shown).

TABLE I

Intracellular Antirickettsial Action of ET-S and PHA-S on R. prowazekii (Breinl) in Human F-1000 Fibroblastic Cells: Comparison of Microscopic Slide Chamber Methods with Recovery of Plaque-forming Units from Parallel Cultures

Treatment	Slide chamber counts					Viable rickettsia recovery		
	P_i		N			PFU/flask ($\times 10^{-4}$)		
	0 h	48 h	0 h	48 h	N_{48}/N_0	0 h	48 h	N_{48}/N_0
*Experiment 1**								
CM	53	52	1.6	25.7	16.1	—	1,120	—
CS	56	51	1.5	24.5	16.3	—	1,180	—
ET-S	42	3.7	0.89	0.21	0.24	—	17.4	—
PHA-S	43	10.7	0.84	3.9	4.6	—	318	—
*Experiment 2**								
CM	64	64	2.2	41.8	*19*	4.4	*145*	*33*
CS (ET-S)	51	45	1.6	22.7	14	4.1	110	27
ET-S	49	3.3	1.6	0.48	*0.31*	4.1	*0.8*	*0.2*
CS (PHA-S)	52	52	1.7	28.8	17	4.0	118	30
PHA-S	62	26	2.2	12.2	*5.6*	4.4	*26*	*6*

In Experiment no. *1*, the various supernatant fluids were prepared from aliquots of the leukocytes obtained from a single large bleeding of a typhus-immune subject. In Experiment no. *2*, the ET-S and its corresponding CS were obtained from the leukocytes of a typhus-immune individual whereas the PHA-S and its corresponding CS were obtained from a subject not immune to typhus.

Note that, by both microscopic and plaque count methods, supernatants from unstimulated leukocytes had no detectable effect on rickettsial growth, whereas supernatants from both antigen- and mitogen-stimulated leukocytes strongly inhibited or killed intracellular rickettsiae.

Discussion

Supernatant fluids from cultures of peripheral blood leukocytes from typhus-immune human subjects, after immunologically specific stimulation with *R. prowazekii* antigen or nonspecific stimulation with the mitogen PHA, exhibit two distinct kinds of action on *R. prowazekii*-infected human but not chicken embryo, fibroblasts ("somatic" cells) and on human endothelial cells ("target" cells) as well as macrophages ("professional phagocytes"): (*a*) a microbicidal action on intracellular, but not extracellular rickettsiae and (*b*) lysis of infected, but not uninfected cells.

The data presented do not reveal whether the two apparently distinct actions are induced by two different components in the leukocyte supernatant fluids or whether a single molecular species is responsible for both. Both methods of stimulating the leukocytes, i.e., antigen and mitogen, are known to induce the production of a variety of lymphokines, including immune interferon (IFN-γ) and lymphotoxins (21, 26). Both types of action were always present in the supernatant fluids regardless of the method of stimulation and both were equally susceptible to inactivation by heating to 56°C for 1 h or exposure to pH 2.

The *antirickettsial* action exhibited certain features in common with interferon action in viral infections (27): (*a*) no direct action upon the microbial agent; (*b*) host cell species specificity (this study; and Wisseman, C. L., Jr., and A. Waddell, manuscript in preparation); (*c*) antimicrobial action induced by pre-infection treatment of host cell; and (*d*) antimicrobial action dependent upon host cell protein synthesis from newly transcribed messenger RNA under the influence of the factor. Additional properties are consistent with those of immune interferon (IFN-γ): (*a*) type of stimulus

required for production in leukocyte cultures (antigen, mitogen); (*b*) rapid production of factor(s) (1–3 d) (data not shown); (*c*) sensitivity to heat and pH 2; and (*d*) reversible cytostatic action on uninfected fibroblasts (27).

The cytolytic action of stimulated leukocyte culture supernatant fluids is specific for *R. prowazekii*-infected cells, does not act on uninfected "bystander" cells, results in the complete lysis of infected cells (Wisseman, C. L., Jr., and A. Waddell, manuscript in preparation) and does not require host cell protein synthesis. The change from resistance to susceptibility to lysis can be induced in a cell by infection with 1–3 rickettsiae, even if rickettsial replication and protein synthesis are continuously inhibited with chloramphenicol. Although some properties are superficially similar to those reported for lymphotoxin (21, 28), i.e., independence from host cell protein synthesis and slow action over 24+ h, the cytolytic factor of ET-S and PHA-S exhibited several properties that are substantially different: (*a*) more rapid production in stimulated leukocyte cultures; (*b*) action over a greater temperature range; (*c*) greater sensitivity to heat inactivation; (*d*) far more restricted range of host cell species; and (*e*) action limited to infected cells. On the other hand, growing experience with IFN-γ suggests that, in addition to its reversible cytostatic effect on normal fibroblasts, it may interfere with the growth of tumor cells and cause death of transformed cells (27, 29, 30). Thus, it is conceivable that IFN-γ could have a cytolytic action on host cells that have been altered in some way by the intracellular rickettsial infection. Indeed, a subsequent report (Wisseman, C. L., Jr., and A. Waddell, manuscript in preparation), presents definitive evidence to indicate that IFN-γ is responsible for both antirickettsial and cytolytic actions of ET-S.

Lymphokines have been described that one way or another restrict the replication of, or cause the death of, *R. tsutsugamushi* and *Coxiella burnetii* (11, 31) within macrophages. However, there is a great paucity of information on the capacity of soluble factors from specifically or nonspecifically stimulated lymphocytes to restrict replication of nonviral agents in cells that are not "professional" phagocytes, i.e., "somatic" or parenchymal cells, such as the endothelial target cells of typhus infection. The reports of Kazar et al. (32) on inhibition of *Chlamydia trachomatis* growth in L cells and of Chinchilla and Frenkel (33) on inhibition of toxoplasma and besnoitia in fibroblasts and kidney cells suggest that such phenomena may occur with other types of intracellular parasites. In a recent abstract, Turco and Winkler (34) reported an inhibition of *R. prowazekii* growth in macrophages and fibroblasts by lymphokines that may be similar to the antirickettsial action described here.

A hypothesis was presented in the Introduction that cell-mediated immune effector mechanisms other than that of the classical activated macrophage variety logically must operate to explain the control of *R. prowazekii* infection in its endothelial target cells in tissues. The present study supports this possibility by demonstrating that, in in vitro systems, an immunologically specific stimulus causes immune human peripheral blood leukocytes to produce a soluble factor(s) that can inhibit and kill *R. prowazekii*, not only within the cells in which it normally grows to produce the characteristic pathophysiology of typhus, i.e., endothelial cells, but also in fibroblasts and in macrophages, and can cause selective lysis of infected cells. The possibility that other types of effector mechanisms exist, such as other soluble factors as well as the direct interaction between various subpopulations of T lymphocytes, natural killer

cells, and activated macrophages with infected endothelial cells remains to be explored.

Summary

Unique features of the primary site of rickettsial replication in typhus fevers, i.e., within the endothelial cells of small blood vessels in tissues, suggest that effector mechanisms, other than those dependent on phagocytosis by activated macrophages with enhanced microbicidal properties, most likely are necessary to explain the cell-mediated immune control of intracellular rickettsial replication in these sites. Theoretically, such mechanisms might involve contact between infected endothelial cells and activated T lymphocyte subpopulations or macrophages or immunologically induced soluble factors or lymphokines. Support for the existence of at least one of these alternative effector mechanisms is presented here for *Rickettsia prowazekii*. Cultures of human blood leukocytes, upon immunologically specific stimulation with *R. prowazekii* antigen or nonspecific stimulation with the mitogen phytohemogglutinin, produce soluble factor(s) in the supernatant fluid which, in culture, have (*a*) an intracellular *antirickettsial* action on *R. prowazekii*-infected human endothelial cells, fibroblasts, and macrophages, and (*b*) a specific cytolytic action on *R. prowazekii*-infected, but not uninfected bystander, human fibroblasts. Neither action is demonstrable in *R. prowazekii*-infected chicken embryo fibroblasts. The factor(s) has no direct antimicrobial action on extracellular rickettsiae and is inactivated by heating at 56°C for 1 h or by acid treatment at pH 2. Expression of the antirickettsial action requires new host cell messenger transcription and protein synthesis, whereas the cytolytic action does not. The circumstances of production and action and the properties of the factor(s) responsible for the intracellular antirickettsial, and perhaps also the cytolytic action are consistent with those of immune interferon (IFN-γ).

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