STUDIES ON TYPHUS AND SPOTTED FEVER

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by

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
(1) Two major arms of protective typhus immunity have been investigated: (a) cell mediated immunity which restricts intracellular rickettsial growth local at site of lesions and (b) antibody-macrophage clearance of extracellular rickettsiae. Soluble factors in the supernatant fluids of leukocytes from typhus immune subjects stimulated with R. prowazekii antigen or from non-immune subjects stimulated with phytohemagglutinin had two separable effects on R. prowazekii-infected human somatic (fibroblast, endothelial) or macrophage cells in vitro. (a) Cells pulsed with stimulated supernatant fluids prior to infection
caused the progressive loss of visible and viable rickettsiae without evidence of host cell damage (intracellular antirickettsial action). (b) Cells incubated continuously in the presence of active leukocyte supernatants after infection underwent a progressive cytolysis (cytotoxic action). Treated uninfected host cells were not damaged; treated extracellular rickettsiae remained viable. Human, but not monkey, mouse or chicken cells, were responsive to supernatant fluids from human leukocytes. Strains of R. prowazekii and R. canadensis were susceptible to both intracellular antirickettsial and cytotoxic action; R. mooseri and R. rickettsii, to cytotoxic action only; and strains of R. tsutsugamushi, to neither. The factor(s) in the active supernatants were destroyed by heating to 56°C for 60 min and by exposure to pH 2.

In the non-immune in vitro system, R. prowazekii evades destruction by human peripheral blood monocyte-derived macrophages by rapidly escaping from the phagocytic vacuole into the cytoplasm. Subsequently, in the same macrophage cultures are destroyed by one or another of two mechanisms: (a) late destruction due to unrestricted intracytoplasmic replication of the rickettsiae with eventual breakdown of the rickettsia-laden macrophage as in fibroblasts and other cells; and (b) an early cytotoxic action produced by one or only a few rickettsiae which results in progressive loss of capacity to exclude trypan blue, a marked swelling of the cisternae produced by endoplasmic reticulum, suggestive of loss of capacity to regulate ions and water, and eventual disruption of the plasma membrane. Although chloramphenicol inhibits this cytotoxic action, very little rickettsial replication occurs. Lysosomes remain intact until the plasma membrane breaks down.

By a combination of techniques of intrinsic radiolabeling of R. prowazekii proteins and of surface proteins, immunoprecipitation and SDS-PAGE, it has been shown that some, but not all, surface proteins are precipitated by the antibodies in human typhus convalescent serum. Monoclonal antibodies have been produced which react specifically with a major, high molecular weight surface protein of R. prowazekii but not with R. mooseri. In preliminary studies, monoclonal antibodies against this high molecular weight protein which were derived from different clones of hybridoma vary in their capacity to opsonize R. prowazekii.

Because of the low yields of rickettsiae of the spotted fever group, which precluded the use of the optical method for measuring DNA:DNA hybridization, successfully applied to strains of R. prowazekii, R. mooseri, Rochalimaea quintana and the Baker's vole agent, the nick translation method for producing high specific activity radiolabeled DNA was successfully applied to rickettsial DNA. Optimal conditions for labeling rickettsial DNA and for measuring DNA:DNA hybridization were established and were compared, with good correlation, with the results obtained by the optical method. Conditions were established which could permit the requisite complex pattern of hybridization tests to be performed with the quantities of DNA which could be produced from the suspension of spotted fever group rickettsiae which can be produced in the laboratory with existing levels of support. Purification of the DNA from the 20 spotted fever group rickettsial strains under consideration is well advanced.
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I. IMMUNOLOGICAL STUDIES OF TYPHUS

The long-range objectives of the studies on typhus immunity are as follows:

(i) To elucidate the main mechanisms of immunity to typhus rickettsiae. Such information would define the immunological objectives of a vaccine, i.e., define the subset(s) of immunological responses to be stimulated, would give clues to the method of presenting the vaccine which would optimally elicit the key protective immune responses, and would permit the development of laboratory methods for detecting and measuring protective immunity. (Among other applications, this would permit the development of a reliable potency assay for vaccines.)

(ii) To identify, purify and characterize the rickettsial antigen(s) which elicit the protective immunity.

(iii) To ascertain the way in which the "protective antigen(s)" must be presented as a vaccine to elicit optimally protective immunity - e.g., in soluble form, in some kind of adjuvant, in liposomes, etc., since it has been shown by others that the physical state of the immunogen, the use of adjuvants of various kinds, the dose, route and timing of immunogen presentation may influence substantially and selectively the responses of the different components of the immunological response.

Background. In order to understand the nature of the problem and to evaluate the progress that has been made under this contract, the following overview, selective in nature and restricted to epidemic and murine typhus, is presented to help develop a perspective in this complex, little known, problem. It is restricted to the problems deemed especially pertinent to the defined objectives, despite the existence of a rich, varied and sometimes controversial historical background. It is oriented towards the applied objective of stable, effective and safe typhus vaccine, although this obviously involves some exciting, new types of basic immunological mechanisms and phenomena.

Typhus vaccines of the past, consisting of aqueous suspensions of killed rickettsial bodies, have been only partially effective in preventing disease, although they quite clearly have modified the course of typhus fever and reduced mortality. Over most of this period, the primary focus of vaccine potency was its capacity to elicit antibodies measured by various techniques. Only recently has it been recognized that antibodies play only a limited role in immunity, confined perhaps largely to enhancing clearance of extracellular rickettsiae by phagocytes, and that the control of rickettsial replication in vivo within cells and tissues is largely T-lymphocyte dependent (see below). The capacity of classical killed vaccines given by conventional routes and schedules to elicit cell mediated immunity is undoubtedly limited and variable. Thus, the entire thrust of the classical vaccine was misdirected - the immunological objectives were erroneous, the measure of response (antibody) was inappropriate and the potency test for vaccines did not measure the arm or subset of immunological responses related to protective immunity.

Studies in these laboratories over the years have been directed towards defining the mechanism(s) of protective immunity against typhus fever. These have employed studies with virulent and attenuated strains of Rickettsia prowazekii (agent of epidemic louse-borne typhus) in man and in vitro studies with cells of human origin. Strains of Rickettsia mooseri (R. typhi) (agent of murine typhus) have been employed in animal studies because it tends to produce a better typhus-like disease in guinea pigs. It produces an infection in mice with only 1-2 viable rickettsiae, more akin to the infectivity for Man of R. prowazekii. Mice exhibit a moderately high degree of natural or innate resistance to R. prowazekii, requiring 100-1000 times the inoculum of R. mooseri to pro-
duce an immunizing infection which cannot be maintained in serial mouse passage.

These studies have clearly shown the limitations of antibodies in anti-typhus immunity. Thus, human convalescent serum, even enriched with complement, has no direct rickettsialcidal action and does not prevent \textit{R. prowazekii} from infecting cells in culture. Nevertheless, convalescent serum does have a strong opsonizing action in typhus rickettsiae, markedly enhancing phagocytosis by both human polymorphonuclear leukocytes and monocyte-macrophages \textit{in vivo} (skin window) and \textit{in vitro}. Monocytes/macrophages, a major component of the cellular response in typhus lesions (see below), can phagocytize \textit{R. prowazekii} and \textit{R. mooseri} in the absence of immune serum but the rickettsiae grow in these and destroy them. Convalescent serum–opsonized typhus rickettsiae, however, are rapidly destroyed by human peripheral blood monocyte–derived macrophages in culture. Ultrastructural details were reported in full in last year's annual report. Thus, while neither antibody nor macrophage alone is capable of destroying the rickettsiae, together they are able to destroy effectively accessible, extracellular rickettsiae, but do not have access to intracellular rickettsiae. Nevertheless, although passively transferred antibodies may modify systemic distribution of rickettsiae in guinea pigs, such passively transferred antibodies, even in very high concentration, do not restrict rickettsial proliferation in infected tissues.

On the other hand, passively transferred immune thymus-derived lymphocytes, but not B lymphocytes, confer the capacity to restrict rickettsial growth within tissues (last year's annual report). In the course of typhus infections, human beings develop some types of cell-mediated immunity as evidenced by a delayed type hypersensitivity demonstrable by skin tests and lymphocyte blast transformation. Studies with \textit{R. mooseri} in mice, however, which were reported in detail in last year's annual report, clearly showed that, while the rickettsial infection activated macrophages in spleen for enhanced microbicidal action against \textit{Listeria monocytogenes}, similar activation of the macrophages in non-immune mice with BCG or \textit{Corynebacterium parvum} did not result in enhanced capacity to restrict rickettsial multiplication. Nor did ablation of fixed macrophages with silica permit enhanced rickettsial replication. Thus, the role of activated macrophages in immunity \textit{in vivo} appears to be limited. Guinea pigs infected with \textit{R. mooseri} develop a specific "anergy" mediated through a serum blocking factor which inhibits the expression of delayed type hypersensitivity as measured by skin test or the \textit{in vitro} correlate of MIF production. Yet, capacity to restrict rickettsial growth in tissues is strong and is cell-mediated.

Thus, the evidence from several sources suggests that, although typhus rickettsial infection elicits the classical DTH type of CMI, which depends upon enhanced non-specific microbicidal action of activated macrophages, this well-studied mechanism does not appear to be the basis for that cell-mediated immunity which restricts rickettsial replication in the tissues of an infected animal. Some other kind of T-lymphocyte mediated immunity must be involved.

To understand the immunological problem posed by typhus infections, it is highly instructive to analyze the site of typhus infection and its relationship to immunological mechanisms. The basic lesion in typhus is a focal, but widely distributed endovasculitis. The "target cells" are the endothelial cells of the small blood vessels in which the rickettsiae grow. On the vascular side, infected endothelial cells are bathed in antibody-containing blood after the first third or half of the normal course of human disease. However, we have shown that antibodies in the medium of infected cells have no influence on the rickettsiae within the infected endothelial cells. Nor do monocytes or PMN have access to the intracellular rickettsiae. However, because of the tendency to form fibrin–platelet-leukocyte microthrombi at the site of endothelial infection and damage, it
is theoretically possible that lymphocytes could be trapped in or enter into the thrombus and come in close proximity to, or contact with, infected endothelial cells without being swept away. More important, however, is the fact that in the evolution of immunity an intense perivascular cellular reaction, composed largely of monocytes and lymphocytes, develops around the site of focal rickettsial infection of endothelial cells. An immunologically oriented observer today would immediately suspect the operation of CMI. But the exact mechanisms would be elusive. A basement membrane and adventitia, depending on the size and type of vessel, separate the intravascular endothelial cells from this impressive perivascular array of cells of immunological potential. Both lymphocytes and monocytes/macrophages do have the capacity to enter and pass through small blood vessel walls (diapedesis). However, although one of the drawings in the classical work of Wolbach, Todd and Palfrey (1922) on typhus does show some mononuclear cells in transit across a blood vessel wall, this appears to be the exception rather than the rule. One just does not see many mononuclear cells concentrating in the vessel walls or along the vessel wall side of the endothelial cell.

This suggests that wandering monocyte/macrophages, even if "activated", in the lesions have little opportunity to exert significant antirickettsial action. (Fixed macrophages as in the liver and spleen, even though they become "activated", see above, have even less opportunity to express antirickettsial action because the sites of infection are physically separated by great distances.)

Although the direct action of T-lymphocytes on infected endothelial cells, such as antibody dependent T-lymphocyte mediated cytotoxicity and various other antibody independent cell-cell actions involving different T-cell subsets, as killer cells, natural killer cells, etc., cannot be excluded at this time, the apparent physical separation between the infected target cell (endothelial cell) and the putative effector cells of CMI does suggest the possible participation of diffusible, soluble mediators of immunity.

In last year's annual report, we presented preliminary evidence that soluble factors derived from human blood leukocytes, either from typhus immune subjects specifically stimulated with typhus antigens or from non-immune human subjects non-specifically stimulated with PHA had dramatic actions on R. prowazekii-infected human fibroblasts. This suggested that one or more new phenomena might be operative in immunity to these obligate intracellular bacterial parasites of non-macrophage, i.e. "somatic", cells, which differed substantially from the classical DTH type of anti-tuberculous, anti-Listeria CMI model. The experiments presented in last year's annual report suggested, but did not prove, that two types of action were involved - viz., (i) an intracellular antirickettsial action and (ii) a cytotoxic action against rickettsia-infected host cells. Moreover, there appeared to be host cell species specificity and some variation in the actions on strains of R. prowazekii and on different species of the genus Rickettsia. Such a soluble, diffusible antirickettsial substance(s) would be compatible with the expression of immunity at a short distance by perivascular lymphocytes across a basement membrane on infected endothelial cells.

Although we have not yet advanced to some of the detailed studies of the nature, origin and molecular mode of action of these substances that we had hoped to do this year, we have been able to establish clearly the validity of our earlier observations, to establish firmly the operation of two distinct processes, viz., an intracellular antirickettsial action without gross damage to the host cell and a cytolytic action on infected cells, to develop methods for studying the two types of action, to demonstrate that the action is not directly on the rickettsia but most likely mediated through the host cell, and to complete certain basic characterization studies. Progress on these potentially important, new immunologic phenomena is described in "A" below.
Because *R. prowazekii* (and other rickettsiae causing human disease) must have most successful mechanisms of evading innate host defenses in the non-immune subject (1-10 organisms probably capable of causing disease in Man) and because, although antibody appears to have a definite but restricted role in immunity to typhus, studies have also continued on the definition of the interaction between typhus rickettsiae and human macrophages.

Finally, in pursuit of the long-term objective "2" above, i.e., identification and characterization of important rickettsial antigens, major progress has been made in the polypeptide antigens of *R. prowazekii*, especially those of the outer membrane, as well as on differences between virulent and attenuated strains. The powerful tool of monoclonal antibodies is just now being applied, successful fusions, clonings and amplification having just recently been accomplished.

A. Mechanisms of cell mediated immunity in typhus: characterization of distinct intracellular antirickettsial action and cytotoxic action on infected cells by supernatant fluids from stimulated human leukocytes. (With A. Waddell) Last year's annual report recorded preliminary observations suggesting that immunologically specifically or non-specifically stimulated human leukocytes elaborate soluble factor(s) into the supernatant fluids which appeared to have both intracellular antirickettsial action and a cytotoxic action on *R. prowazekii*-infected human fibroblasts, i.e., "somatic" cells as opposed to macrophages which are effector cells of classical DTH type of cell mediated immunity. These observations suggested that a new mechanism might be operative in antirickettsial CMI. These studies have continued and it is now clear that the two mechanisms exist and can be measured separately. The study is now at a stage at which a major initial publication is in order. The following sections reproduce some key observations reported last year, extend significantly other kinds of observations and present totally new studies that clearly establish and characterize the intracellular antirickettsial action and the cytotoxic (cytolytic) action on rickettsia-infected cells.

1. A complex action of stimulated human leukocyte supernatant fluids on rickettsia-infected cells in vitro: possible host cell specific intracellular antirickettsial and cytotoxic actions. WI-38 diploid human cells and secondary chicken embryo fibroblasts were infected in suspension with *R. prowazekii* (Breinl) and distributed to slideculture chambers in our conventional rickettsial growth system (Wisseman, C.L. Jr., and A.D. Waddell, in preparation). Six hours later, when the cells had attached, the medium was replaced with (1) the supernatant fluid from human typhus immune leukocytes which had been stimulated with killed *R. prowazekii* antigen (ET-S) diluted with an equal quantity of TC medium, (2) the supernatant fluid from unstimulated human typhus immune leukocytes (CS) diluted with an equal quantity of TC medium or (3) TC medium (CM) alone. Once added, the leukocyte products were continuously present during the remainder of the experiment. The slide chamber cultures were incubated at 32°C in a humid atmosphere of 5% CO₂: 95% air. At intervals, usually up to 48-72 h, slides were removed, fixed, stained and counted.

The growth of *R. prowazekii* in WI-38 cells incubated in the presence of supernatant fluid from unstimulated typhus immune human leukocytes (CS) was indistinguishable from that in the presence of TC medium alone which in turn followed the pattern previously described for *R. prowazekii*. In marked contrast, cultures incubated in the presence of supernatant fluids from typhus-immune human leukocytes which had been stimulated with killed *R. prowazekii* antigen (ET-S) showed a dramatic effect on both rickettsiae and host cells. After a lag period of some hours, usually about 12 h, the percent of cells with detectable rickettsiae (p) began a progressive, sharp decline to reach only a fraction of the initial infection rate. A break occurred at the same time in
the growth curve when plotted as $\log_2$ average number of rickettsiae per cell ($\log_2 N$). The effect was much less pronounced when growth curves were plotted as $\log_2$ average number of rickettsiae per infected cell ($\log_2 N_i$), suggesting that some infected cells were not exhibiting the same kind of antirickettsial action. At the same time, a progressive loss of cells from the ET-S treated infected cultures was noted, following a temporal pattern similar to the loss of rickettsiae. On the other hand, when R. prowazekii-infected chicken embryo fibroblasts were cultured in the presence of the same ET-S, there was no demonstrable effect on either rickettsiae or host cells. These observations were reproducible when experiments were repeated with the same ET-S preparation or with ET-S preparations from the stimulated leukocytes from different persons who had experienced previous typhus infections. Figure 1, the results of a single experiment, is representative of these experiments. Results similar to those obtained with irradiated WI-38 cells were obtained with either irradiated or unirradiated F-1000 human foreskin fibroblasts (see below).

Thus, the supernatant fluids from typhus-immune human leukocytes which had been stimulated with killed R. prowazekii antigen had a complex set of effects when added to R. prowazekii-infected human, but not chicken embryo, fibroblasts. These effects appeared often after a lag phase of several hours, were progressive for at least 48 h and appeared to consist of (1) an antirickettsial action on intracellular rickettsiae and (2) a cytotoxic action on infected cultures. In a minority of infected and treated cells, however, rickettsial growth appeared to be relatively uninhibited. Because similar results could have been obtained by selective loss of infected cells as rickettsial growth proceeded over a period of a few hours, it was not possible to prove beyond doubt in this system alone that two separate actions were occurring. New, more specific, assay systems were required (see below). (This basic observations was presented in last year's annual report. The following observations either extend observations reported last year or present totally new sets of experiments.)

Identical results to those described above for supernatants from typhus-immune human leukocytes stimulated with killed R. prowazekii antigen were obtained with supernatants from immune or non-immune human leukocytes stimulated with PHA (PHA-S). Thus, human leukocytes, when stimulated either by an immunologically specific mechanism or by a non-specific T-cell mitogen like PHA, produce soluble factors which display similar antirickettsial-cytotoxic actions on human fibroblasts (host-cell specificity), which are "somatic" cells as opposed to the activated macrophages of classical cell-mediated immunity.

2. Dissociation between intracellular antirickettsial action and cytotoxic action on R. prowazekii (Breinl)-infected human fibroblasts. Since the method described above was incapable of establishing clearly the existence of the two putative kinds of action, i.e., an intracellular antirickettsial action and a cytotoxic action on homologous infected cells, and of measuring these two kinds of action independently, attempts were made to develop methods which would permit measurement of each of these two kinds of action, verify the existence of two separate kinds of action and characterize them in a preliminary way.

In the development of methods for the examination and quantitation of the different components and variables that might enter into the complex interactions between stimulated leukocyte supernatants, rickettsiae and host cells, we drew heavily upon the methods that we have developed or adapted from others and modified over the last several years for the quantitative study of rickettsiae and their interaction with host cells – i.e., rickettsial viability, uptake of rickettsiae by host cells (Wisseman, C. L. Jr., and A. Waddell, in preparation), slide chamber intracellular growth measurements.
the studies to be described below, the use of x-irradiated cells was abandoned because, even with uninfected cells, there was substantial, progressive loss of cells for which it was difficult to compensate in assessing the action of rickettsiae and leukocyte factors. The F-1000 human foreskin fibroblastic cells were chosen for routine tests because of superior growth characteristics in our systems.

In preliminary studies to develop an assay system for the detection and measurement of leukocyte supernatant mediated cytotoxicity, various methods which depended upon the release of radiolabeled amino acids or nucleotides or which depended upon the loss of neutral red binding capacity were tested and were found to be unsatisfactory for one reason or another. Finally, an adaptation of the original method for measuring lymphotoxins, which depends on the direct counting of crystal violet-stained residual cells in microtiter plates, was found to be sensitive, reproducible and reliable. Hence, unless otherwise specified, all studies on cytotoxicity reported here were performed with this test.

Finally, in preliminary experiments, it was found that antirickettsial action could be demonstrated without apparent cytotoxicity by exposure cultures of uninfected F-1000 to stimulated leukocyte supernatant fluids for 18 h, washing, then infecting with rickettsiae and following the fate of the rickettsiae microscopically in slide chamber cultures.

The following series of experiments was performed to characterize the various actions of the leukocyte supernatants on the different components of the rickettsia-host cell system, to establish the existence of both antirickettsial and cytotoxic actions and to validate the methods for demonstrating and quantitating these two kinds of action.

a. Action of stimulated leukocyte supernatant fluids on uninfected host cells. The action of leukocyte supernatant fluids, whether derived from leukocytes of typhus immune subjects stimulated with R. prowazekii antigen (ET-S) or from leukocytes from non-typhus immune subjects non-specifically stimulated with PHA (PHA-S), on uninfected host cells, mostly F-1000 human fibroblasts, was complex and similar.

(1) Clumping of treated cells in suspension. F-1000 human fibroblasts, either pre-treated with ET-S or PHA-S prior to trypsinization from growth flasks or incubated in the presence of the supernatant fluids from stimulated leukocytes, showed a distinct tendency to aggregate or clump in the suspended cell infection system. The observation was made with considerable regularity on numerous occasions and, in this system at least, suggests that exposure to leukocyte supernatants results in some change in host cell surface that enhances clumping. This, therefore, constitutes one observable change induced in rickettsial host cells in which both antirickettsial and cytotoxic actions are expressed. In addition to its theoretical importance as a possible clue to an alteration of host cell which might eventually be correlated with the antirickettsial and cytotoxic mechanisms, this clumping caused some practical technical problems because the quantitative relationships established for the suspended cell system for infection of cells with rickettsiae depends upon monodispersed cells settling freely under the influence of gravity. Hence, in studies in which infection rate was critical (see below), it was necessary either to carry out experiments under conditions which minimized the clumping effect or to employ the stationary host cell-sedimenting rickettsia system (Wisseman and Waddell, in preparation).

(2) Effect of leukocyte supernatants on uninfected cells in microtiter cytotoxicity assay system. Two different kinds of action of active leukocyte supernatants were observed on uninfected cells in the microtiter "cytotoxicity" assay
system, depending upon the kind of exposure.

(a) Cytostasis action in "post-treated" uninfected cells. When the supernatant fluids were present continuously during a cytotoxicity type assay of uninfected F-1000 cells corresponding to the "post-infection treatment" situation, there was a consistent average tendency to cause cytostasis, rarely very slight apparent loss of cells.

(b) Growth stimulatory effect on "pre-treated" uninfected F-1000 cells. In contrast, when the uninfected F-1000 cells were pre-treated for about 18 hrs with stimulated leukocyte supernatant fluids, washed, distributed to the wells of the microtitre plates as for a cytotoxicity assay and subsequently incubated in the presence of normal tissue culture medium without leukocyte supernatant, a distinct growth stimulatory effect was observed. Various growth stimulatory actions have been described by others in the supernatant fluids of stimulated leukocytes.

Thus, supernatant fluids from stimulated leukocytes, whether typhus-immune stimulated by R. prowazekii antigen (ET-S) or non-immune stimulated with PHA (PHA-S), had some effects upon uninfected host cells, depending upon the conditions and kind of assay system employed. None of these, however, were comparable to the effects described in more detail on rickettsia-infected cells and the degree to which they affected the detection of effects in rickettsia-infected cells was minimized by comparison with appropriate controls.

(3) Influence of pre-treatment of host cells with active leukocyte supernatant fluids on rickettsial uptake. On two separate occasions in which great care was taken to assure maximal dispersion of host cells, no effect of pre-treatment of F-1000 cells with leukocyte supernatants was seen on the rate of uptake of untreated R. prowazekii in the suspended cell system (Figure 2). However, in many routine experiments in which pre-treated host cells were infected with R. prowazekii along with appropriate controls (control medium and unstimulated leukocyte supernatant fluids), the per cent cells infected (pi) was often lower in the treated cells. The most likely explanation lies in the distortion of uptake kinetics by the clumping tendency induced by active leukocyte supernatants. However, without a more exhaustive, quantitative study in which all factors are adequately controlled and uptake efficiency is calculated on the basis of successful penetrations per collision (Wisseman and Waddell, in preparation), we cannot categorically state the pre-treatment of host cells has no discernible effect on uptake rate. Nevertheless, it is clear that F-1000 human fibroblasts pre-treated with ET-S or PHA-S can be infected with a relatively high degree of efficiency in comparison with untreated cells and that the major effects described in this communication are not the result of a drastic reduction in infection rate.

b. Direct effect of leukocyte supernatant fluids on viability of extracellular R. prowazekii. Two types of experiment were performed to determine if stimulated leukocyte supernatant fluids exert a direct rickettsicidal action, as on extracellular rickettsiae. In the first type of experiment, dilute yolk sac suspensions of R. prowazekii (Breinl), containing 400-600 PFU/0.2 ml, were mixed with equal volumes of control medium (CM) or supernatant fluids from unstimulated, immune-R. prowazekii stimulated (ET-S) or non-immune-PHA stimulated (PHA-S) leukocytes. The mixtures were incubated at 32 C. After 0, 60, 120 and 180 minutes of incubation, samples were removed for determination of number of plaques in CE cell monolayers, which had been shown to be insusceptible to either the antirickettsial or cytotoxic actions of stimulated leukocyte supernatant fluids (vide supra). Hence, in this type of experiment, residual leukocyte factor would not be expected to influence results through an indirect action on
the host cell. The results, recorded in Table 1, clearly show that, over the exposure period of 180 minutes, neither type of stimulated leukocyte supernatant fluid had any direct effect upon extracellular R. prowazekii (Breinl) which would prevent the rickettsiae from infecting, growing in and forming plaques in CE cells.

A second type of experiment was performed which incorporated the following features. (1) A purified suspension of R. prowazekii was used to minimize the possibility that contaminating host cell material, of a non-responder host (chicken embryo), might inactivate, or otherwise interfere with the action of, leukocyte supernatant factors. (2) A host cell (F-1000) known to be permissive to the antirickettsial action stimulated leukocyte supernatant fluids was used, in the unlikely event that a direct antirickettsial action could be expressed only in susceptible host cells. (3) The assay for antirickettsial action was a direct microscopic measurement of rickettsial growth in the early stages of the first infection cycle, in the event that a putative direct antirickettsial action would only be transient when the antirickettsial factor(s) was continuously present. The results obtained in an experiment of this kind with an active supernatant fluid from ET-stimulated immune leukocytes, shown in Table 2, yield no evidence for direct action of stimulated leukocyte supernatant fluids on extracellular rickettsiae, regardless of whether or not the supernatant was present or absent at the time of infection of the host cells by the treated rickettsiae.

These experiments clearly demonstrate that active leukocyte supernatants, when incubated with extracellular R. prowazekii (Breinl) for 1-3 h, do not exert any direct antirickettsial action which can be detected in tests which measure uptake (infection of host cells), intracellular growth or infection cycle. These findings, along with the apparent host cell specificity, suggest that the action of leukocyte supernatants on rickettsiae may be mediated through the host cell, rather than through a direct action on the rickettsiae.

A comprehensive series of coordinated experiments was performed to define the course of R. prowazekii (Breinl) infection in untreated F-1000 cells and in F-1000 cells which had been pretreated or post-treated with stimulated leukocyte supernatant fluids. Both intracellular antirickettsial action and cytotoxic action of stimulated leukocyte supernatant fluids on infected cells were measured by several different methods. These included, in various combinations, the following general, usually time-course, methods:

(i) Standard slide chamber cultures (vide supra) for the microscopic measurement of intracellular rickettsiae accompanied each set of experiments.

(ii) The microtiter method (vide supra) for counting the host cells remaining attached to the plastic surface of the well bottom was included in each set of experiments.

(iii) A macroplate (35 mm plastic petri dish) method was introduced which provided sufficient cells for the following kinds of determination: total number of cells and percent viable by trypan blue exclusion of cells floating in the medium and cells recoverable by trypsinization from the dish bottom; viability by trypan blue exclusion of attached cells in situ; and host cell damage by measurement of the amount of lactic dehydrogenase (LDH) released into the culture medium, present in floating (detached) cells and remaining in attached cells.

The results of this series of experiments are presented in Figures 3 through 8 and are analyzed according to type of action below.
c. Characterization of *R. prowazekii* (Breinl) infection cycle in untreated F-1000 cells. The basic characteristics of the infection cycle of *R. prowazekii* (Breinl) in irradiated chicken embryo (CE) cells in culture have been described in some detail in previous publications from these laboratories and constitute essential background information for the study of antirickettsial activity. Those early studies, however, concentrated primarily on the quantitative aspects of growth cycle of the rickettsiae and on the ultrastructural changes seen by transmission and scanning electronmicroscopy in infected cells, the latter somewhat limited in its quantitative and functional aspects. In contrast, the present study requires quantitative knowledge, not only of the growth cycle of the rickettsia in an unirradiated host cell of different origin, i.e., the F-1000 human fibroblast, but also of the fate of the host cell, as a basis for establishing the validity of the two putative types of action of stimulated leukocyte supernatant fluids on *R. prowazekii*-infected cells. For example, preliminary experiments had suggested that the microtiter method for measuring cytotoxic action of leukocyte supernatant fluids on infected cells did not accurately reflect the cell loss expected in infected cultures that were not treated with leukocyte supernatant fluids and in which cell destruction was due solely to the infection. Accordingly, in the series of experiments which were designed to describe and validate the two separate types of action on infected cells, special attention was given to the untreated infected cells undergoing an inhibited infection cycle. Special attention was directed to various measures of host cell damage (e.g., trypan blue exclusion, LDH leakage and total cell counts in situ on the microtiter method and after trypsinization in the macroplate method). The results of this series of observations are summarized below and are recorded in various combinations in Figures 3 through 8.

The basic growth cycle of *R. prowazekii* (Breinl) in the unirradiated F-1000 cells was similar to that previously described for the infection in irradiated CE cells, including the range of generation times in the logarithmic growth phase. Significant also for this study was the fact that, in the F-1000 cells as in the CE cells, the first evidence for spread of the infection, as indicated by an increase in percent cells infected (pi), from infected cells to previously uninfected cells in the culture began after about 48 h of incubation at 32°C. In previous studies with CE cells, it had been shown that *R. prowazekii*, in contrast to *R. rickettsii* and *R. mooseri* (Wisseman, C.L. Jr., and others, to be published), did not have the capacity to escape from the infected cell until there was evidence of host cell breakdown. Thus, in this respect, the infection cycle in the F-1000 human fibroblast cells was similar to that which had been observed previously in the CE cells.

However, in the microtiter assay, which was designed to measure host cell loss or gain, the total cell count showed the expected increase in cell numbers in uninfected cells and either remained relatively constant or showed a slight increase in the first 48 h of incubation of infected cells and did not show a reliable decrease until late, i.e., after 96 to 120 h of incubation, and even then the apparent cell loss was not great. Thus, if the spread of infection in the culture beginning at about 48 h reflected breakdown of infected cells, the microtiter method did not reflect this cell damage for at least another 48 h. The microtiter system depends upon counts under low power of structures which stain with crystal violet and have the general form of cells. It is not capable of detecting finer cytologic signs of cell damage. As will be shown below, remnants of damaged cells remaining attached to the plastic wells and staining with crystal violet accounted for this discrepancy.

When other methods for detecting cell damage or loss were employed in the macroplate system, a different pattern emerged which closely correlated with the results expected from the patterns of rickettsial spread within the culture based on the concept
that, with *R. prowazekii* (but not with *R. rickettsii* and *R. mooseri*), organisms are released in significant numbers only from damaged and deteriorating host cells. Thus, there was no evidence that infected cells detached from the plastic substrate and accumulated in the medium as floating cells in significant numbers prior to eventual breakdown. However, evidence that host cell damage was indeed occurring at about the time of rickettsial spread in the culture was found in the decrease in proportion of attached cells (in situ) capable of excluding trypan blue and in the initiation of progressive loss of LDH from the attached cells into the culture medium. Moreover, cell counts performed in the macroplate system yielded two significant confirming observations—viz. (a) there is no accumulation in the culture medium of detached, damaged cells, (b) there is a progressive decline in the number of trypan blue negative cells recoverable by trypsinization, (c) there is a progressive increase in the number of trypan blue positive cells in situ and (d) there is a progressive increase in the amount of LDH released into the medium, all beginning at about the time cells would be predicted to begin to break down from the effects of *R. prowazekii* infection, i.e., at about 48 h after infection.

These results are consistent with the hypothesis (a) that host cells infected with *R. prowazekii* do not show evidence of marked cell damage, either by previous ultrastructural studies, by various measures of host cell plasma membrane integrity or by total cell count until the infection is far advanced and (b) that the major mechanism of escape of *R. prowazekii* from its host cell is a breakdown in the integrity of the plasma membrane when the host cell is loaded with rickettsiae, due to as yet unknown mechanisms. The failure of the microtiter method to reflect accurately the cell damage is likely due to the fact that host cells damaged by the infection alone are not released from the plastic substrate but rather some parts of the damaged and degraded host cell, which still stain with crystal violet, remain attached and are counted as cells in the assay method. These damaged host cell ghosts, however, are susceptible to the action of trypsin and are lost when counts of surviving cells are made on trypsinized suspensions.

As will be shown below, infected host cells undergoing cytotoxic action on post-exposure to the supernatant fluids of stimulated leukocytes behave differently and appear to be lysed, leaving no residue stainable by crystal violet. Moreover, it must be emphasized that the results described above may apply fully only to cells infected with *R. prowazekii*. There is reason to believe that cells infected with *R. rickettsii* or *R. mooseri* might yield somewhat different results. A comparative study would seem warranted and likely to be informative about the likely diversity of rickettsia-host cell interactions.

d. Characterization of the cytotoxic effect of post-treatment of *R. prowazekii* (Breinl)-infected F-1000 cells with stimulated leukocyte supernatant fluids. When similar methods of study, as described above, were applied to cultures of *R. prowazekii* (Breinl) infected F-1000 cells treated with stimulated leukocyte supernatant fluids 6 h after infection (i.e., post-treated), a very different pattern was observed. The results are also portrayed in Figures 3 to 8.

After a variable lag period, the microtiter method showed a sharp and progressive decline in number of cells. The decrease in number of cells in the microtiter method was closely paralleled by a comparable decrease in the number of cells recoverable by trypsinization in the macroplate method and the shift in LDH from the attached cell population to the cell-free culture fluid. There was no significant increase in detached cell population. In situ the proportion of cells excluding trypan blue showed only a late minor decline.
These results strongly suggest that ET-S or PHA-S treated \textit{R. prowazekii} (Breinl)-infected F-1000 cells undergo, after a variable lag phase, complete lysis. Cells do not detach and accumulate in the culture fluid. Skeletons or ghost of degenerating cells, stainable by crystal violet, do not remain attached to the plastic substrate to confuse the microtiter method. There is no evidence of gradual accumulation of cells with damaged plasma membranes permeable to the entry of trypan blue or the escape of LDH. The pattern differs significantly from that of untreated infected cells. The rickettsial content, measured by both per cent cells infected and average number of rickettsiae per cell, shows a marked decline, but (not shown) the occasional cell appears to permit reasonably uninhibited growth of rickettsiae. However, in this system, it is not possible to discriminate between a specific antirickettsial action and simple selective destruction of infected cells. No such cytolytic effect was demonstrable on uninfected F-1000 cells.

The results of these studies clearly show that the supernatants of stimulated leukocyte exert a dramatic, specific and selective cytolytic effect on \textit{R. prowazekii} (Breinl)-infected F-1000 cells which differs markedly from the pattern of cell destruction seen in untreated infected cells (vide supra) and that the microtiter method reasonably reflects this unique action. Uninfected F-1000 cells similarly treated usually showed only a cytostatic action and rarely showed any detectable cytolytic action. Thus, this cytotoxic (cytolytic) action appears to be a unique, specific effect of stimulated leukocyte supernatant fluids on \textit{R. prowazekii} (Breinl)-infected F-1000 cells.

e. Characterization of the specific intracellular antirickettsial action of stimulated leukocyte supernatant fluids on \textit{R. prowazekii} (Breinl)-infected F-1000 cells without attendant cytolysis. Preliminary experiments suggested that treatment of F-1000 fibroblasts for 18 h with supernatant fluids from either specifically stimulated (ET-S) immune or non-specifically stimulated (PHA-S) non-immune human leukocytes, followed by washing, subsequent infection with \textit{R. prowazekii} (Breinl), plating into slide chamber cultures and following the growth or disappearance of the rickettsiae microscopically as in our conventional growth studies (pre-infection treatment or simply pre-treatment) was followed by rapid disappearance of rickettsiae from the infected cells, whereas the rickettsiae grew in an uninhibited, normal fashion in untreated F-1000 cells held continuously in control medium or pretreated with the supernatant fluids from unstimulated leukocytes. This effect was reproducible and was expressed without the lag period of 12-24 h often seen in cultures exposed to stimulated leukocyte supernatant fluids after infection. Typical responses of \textit{R. prowazekii} (Breinl) in F-1000 cells treated with a stimulated leukocyte supernatant before (pre-treatment) and after (post-treatment) infection are presented in Figures 3 through 8. Microscopic examination of pretreated cultures exhibiting apparent intracellular antirickettsial action failed to reveal the same gross loss of cells observed in the preliminary experiments in which infected cells were treated with stimulated leukocyte supernatant fluids after infection (vide supra) without gross evidence of host cell loss. This phenomenon was subjected to critical study in the same series of experiments described for untreated infected cells and for the post-treatment cytotoxic phenomenon. The results appear along with the others in Figures 3 through 8.

As seen in the preliminary studies, there was progressive loss of rickettsiae from infected cells without a lag period, but without evidence of cell damage or lysis by the several measures employed - viz., microtiter cell counts, count of cells recoverable by trypsinization in the macroplate method, trypan blue exclusion in situ and in suspension and LDH release. Thus, F-1000 cells pretreated with a stimulated leukocyte supernatant fluid exert a prompt and progressive antirickettsial action without undergoing lysis or other evidence of cell membrane damage.
Finally, experiments were performed to ascertain if the loss of stainable, microscopically visible rickettsiae from cells pretreated with stimulated leukocyte supernatant fluids actually reflected a loss of viable organisms. Parallel slide chamber cultures for microscopic examination and flask (75 cm²) cultures for plaque counts were prepared with F-1000 cells which had been pretreated for 18 h with CM, CS, ET-S or PHA-S and which had then been infected with R. prowazekii (Breinl) just prior to plating in the cultures. Following attachment for 6 h, the cultures were washed and supplied with fresh medium containing no leukocyte supernatant fluids. In the two separate experiments of this kind that were performed, slide chamber cultures were harvested for microscopic examination at this time ("0" h) and after 48 h incubation at 32°C. In the first experiment, flasks were harvested for plaque assay only after 48 h incubation, whereas in the second experiment flasks were harvested at both 0 and 48 h. Cells were released from the flasks by trypsinization, sedimented by centrifugation, resuspended in CM, disrupted in a high speed, microblender and assayed for PFU in CE cell monolayers which are insusceptible to the leukocyte factors. The results of the two experiments are recorded in Table 3.

In both experiments, the ET-S preparations showed a marked antirickettsial effect detectable both microscopically and by plaque count. The PHA-S preparations, which were not as potent as the ET-S preparations in these instances, showed a somewhat lesser, though still striking, antirickettsial effect. In retrospect, it would likely have been even more striking to have done the assays at 24-30 h, before the rickettsiae in the apparent resistant cell fraction had multiplied to such a large extent, thus tending to mask somewhat in terms of total culture plaque count the substantial reduction in percent cells infected (p) so readily apparent on microscopic examination.

The intracellular antirickettsial action in pretreated cells, then, appears to be a distinct phenomenon clearly separable from the cytotoxic action on post-treated infected cells. The evidence for the existence of two separate phenomena is thereby greatly strengthened.

3. Some characteristics and properties of stimulated leukocyte supernatant fluids and their actions.

a. Thermal and pH stability of antirickettsial and cytotoxic factors in specifically and nonspecifically stimulated leukocyte supernatant fluids. Both antirickettsial and cytotoxic actions measured in the F-1000 cell-R. prowazekii (Breinl) systems of the supernatant fluids from immunologically stimulated immune leukocytes (ET-S) or non-specifically stimulated non-immune leukocytes (PHA-S) were destroyed either by heating for 1 h at 56°C or by exposure to pH 2 for 24 h (Table 4). Though the stability of the leukocyte factor(s) at other temperatures has not yet been studied systematically, both types of activity have been maintained in supernatant fluids stored in the frozen state at -70°C for weeks to several months. Moreover, in the experiments on exposure to pH 2, the controls which were dialyzed at 4°C for 48 h against glycine buffer at pH 2 in a dialysis tubing with a nominal retention of molecular weight 3000 daltons retained vigorous activity in both systems.

b. Effect of incubation temperature on antirickettsial and cytotoxic actions of stimulated leukocyte supernatant fluids. The antirickettsial and cytotoxic actions of ET-S and PHA-S on F-1000 cells infected with R. prowazekii (Breinl) at 32°C was compared in a series of experiments with these actions at 35°C, the optimal growth temperature for R. prowazekii (Wiseman, C.L. Jr., et al, unpublished observations), and at 37°C, a more physiologic temperature for F-1000 cells (data not shown) and for the action of lymphotoxins. Although there were minor apparent differences in rates, the
patterns of expression of antirickettsial and cytotoxic actions were essentially the same at all 3 temperatures. Significantly, there was no cytotoxic (? lymphotoxins) action on uninfected F-1000 cells at 37°C.

c. Host cell specificity and range of antirickettsial and cytotoxic actions against R. prowazekii (Breinl) infections by specifically and nonspecifically stimulated leukocyte supernatant fluids. Because the preliminary experiments with WI-38 cells and CE cells had suggested a degree of host cell specificity, additional experiments with cells of different types and species of origin were performed, as recorded in Table 5.

Only infected cells of human origin showed either antirickettsial or cytotoxic actions treated with either ET-S or PHA-S. None of the cells of monkey, mouse or chicken origin was susceptible to either type of action. Thus, within the limits of the range of cells tested, both ET-S and PHA-S show a high degree of specificity for cells of human origin.

Within the range of cells of human origin infected with R. prowazekii (Breinl) which were tested, however, there was some variation in degree of susceptibility to antirickettsial and cytotoxic actions. For example, among cells of fibroblastic origin, the diploid WI-38 cell of embryonic lung origin and the F-1000 cell line of foreskin origin showed both cytotoxic and antirickettsial actions, whereas an SV-40 transformed WI-38 cell line and a cell line derived from a human fibrosarcoma clearly showed the antirickettsial action but the cytotoxic action was not clear cut. It is unknown if the failure to detect the cytotoxic action in the transformed or malignant cell lines represents a basic change in susceptibility to the action of the leukocyte supernatant fluids or is an artifact of the assay system, as for example cell loss by cytotoxicity offset by an enhanced capacity to grow in the microtiter assay system.

Among epithelial-like cells, the malignant HeLa cell, derived from a cervical carcinoma, failed to exhibit either antirickettsial or cytotoxic effects. Nor did the FL cell of human amnion origin show an antirickettsial action (the cytotoxicity assays were unsatisfactory). Yet the Chang's liver cell exhibited both antirickettsial and cytotoxic actions. It is of interest that the FL (Hu amnion) and Chang's liver cell lines, both listed as showing HeLa markers, showed divergent results.

d. Microbial specificity of antirickettsial and cytotoxic actions of stimulated human leukocyte supernatant fluids. A systematic study of the capacity of ET-S and PHA-S to exert both antirickettsial and cytotoxic action on F-1000 human fibroblasts infected with different members of the typhus, spotted fever and scrub typhus groups of rickettsiae was also performed. These studies are summarized in Table 6. All tests were performed with a single ET-S and a single PHA-S preparation. Three distinct patterns were detected for both ET-S and PHA-S systems, according to the rickettsia tested.

(1) Both antirickettsial and cytotoxic actions expressed. F-1000 cells infected with R. canadensis or, to varying degrees, strains currently classified as R. prowazekii showed both antirickettsial and cytotoxic actions induced by both ET-S and PHA-S.

It is of interest to note that, while all strains of R. prowazekii were associated with a strong cytotoxic action, not all strains were equally sensitive to the antirickettsial action. Thus, antirickettsial action was pronounced with the Breinl strain, an old laboratory strain with a long passage history following its isolation from a patient with epi-
demic typhus in Europe, and the Burundi strain, a recent isolate with short passage history from a typhus fever patient in central Africa. The effect was more variable with the E strain, an attenuated variant with long passage history of a strain isolated from a typhus fever patient in Madrid. Only slight to negligible antirickettsial action was observed with a strain recently isolated in this country from flying squirrels by Bozeman et al. The significance of this variation is unknown. However, minor differences in protein profile obtained by polyacrylamide gel electrophoresis and iso-electric focusing have been detected between the E and flying squirrel strains on the one hand and the classical Breinl strain on the other, despite their high degree of DNA-DNA hybridization.

R. canada, which also was associated with both antirickettsial and cytotoxic action, though currently included in the typhus group on the basis of serology, differs substantially from R. prowazekii, R. mooseri and R. rickettsii in degree of DNA-DNA hybridization, genome size and PAGE protein profiles and may represent a new subgroup under the genus Rickettsia (vide infra).

(2) Cytotoxic action but not antirickettsial action expressed. A distinct cytotoxic action by ET-S and PHA-S was noted with cells infected with R. mooseri or R. rickettsii, but with neither organism was antirickettsial action expressed in a detectable or measurable manner. One important difference between these two species and R. canada and R. prowazekii lies in the fact that they produce rapidly spreading infections in cell cultures, with organisms escaping from host cells from early hours after initial infection. Whether this property inhibits expression or measurement of the antirickettsial action is unknown, but requires additional studies.

(3) Neither antirickettsial nor cytotoxic actions expressed. Neither of two serotypes of R. tsutsugamushi, the Gilliam strain or a Karp-like isolate from the Sialkot region of Pakistan, was susceptible to either antirickettsial or cytotoxic actions of ET-S or PHA-S in the F-1000 fibroblasts. This is especially interesting because Nacy and Osterman and Nacy and Meltzer have shown that mouse lymphokines cause mouse peritoneal macrophages to express some kind of antirickettsial action on the Gilliam strain. In contrast, the factor(s) that we are studying in a human system, which exert both antirickettsial and cytotoxic actions with other rickettsiae in "somatic", non-phagocytic cells, have no effect on R. tsutsugamushi.

These range-finding experiments have revealed an interesting pattern of expression of antirickettsial and cytotoxic actions of stimulated leukocyte supernatants with representatives of different groups of organisms within the genus Rickettsia. With the exception of R. tsutsugamushi strains, the cytotoxic action was detected with all other groups of the genus. Antirickettsial action was more restricted, varying even with strains of the same species. These results suggest, but do not prove, independent mechanisms and entities for the two types of action. In no instance tested to date has antirickettsial action been detected without demonstrable cytotoxic action, but the converse is not true.

e. Preliminary observations on the action of stimulated leukocyte supernatant fluids on rickettsial infection in human umbilical cord endothelial cells and in human peripheral monocyte-derived macrophages. None of the host cells tested above is either a primary "target cell" in which rickettsiae preferentially grow in vivo or a component of the host antimicrobial defense system. However, despite certain technical problems, it was possible to confirm, in preliminary experiments, the antirickettsial action of stimulated leukocyte supernatant fluids in infected cultures of human peripheral monocyte-derived macrophages and of human umbilical vein endothelial cells. Because of the nature of these cells, both pretreatment with supernatant fluids
and infection were carried out on cells attached to glass in slide chamber cultures. Satisfactory methods for quantitating the cytotoxic action on these unique cells have not yet been developed.

In addition to *R. prowazekii* (Breinl) and *R. mooseri*, which have been shown to grow to a modest degree within 6 day cultures of human peripheral monocyte-derived macrophages in the absence of immune serum, *R. rickettsii* (SS) and *R. tsutsugamushi* (Gilliam) were also found to grow in such macrophages (data not shown). Accordingly, the capacity of macrophages pretreated with stimulated leukocyte supernatant fluids to restrict replication of each of the four rickettsial species listed above was tested. As in the case of F-1000 cells pretreated with either ET-S or PHA-S, there was no restriction of growth of *R. tsutsugamushi* (Gilliam) or *R. mooseri* (Wilmington). In a single experiment with *R. rickettsii* (SS), although there was no definite rickettsiastatic or rickettsiacidal action, there was detected in both ET-S and PHA-S pretreated macrophages an inhibition of the spread of infection within the cultures and a slight inhibition of growth rate.

In contrast, the same kind of dramatic antirickettsial action previously demonstrated in *R. prowazekii* (Breinl) infected F-1000 and WI-38 cells was also observed in macrophage cultures (see Figure 9), thus establishing the action of the soluble cell products on a cell type which might play a role in some facets of the immune mechanisms in typhus fever. Although the results are clear-cut, there are, however, some problems with the *R. prowazekii*-human macrophage system. Previous studies had shown that human macrophages, in the absence of immune serum, phagocytizes *R. prowazekii* and permits growth of the organism which eventually destroys the macrophage. Examination of the growth curves in the macrophages revealed that the growth was, on the population average, less than in CE cells. More recently, it has been shown that in this non-immune system, *R. prowazekii* is phagocytized but that the organisms quickly escape from the phagocytic vacuole into the macrophage cytoplasm, where they undergo replication (Meyer, W.A., and C.L. Wisseman, Jr., in preparation, last year's annual report). Although some macrophages supported unrestricted growth, by microscopic examination, it has recently been shown that many macrophages, very soon after ingestion of one or a few *R. prowazekii*, show evidence of severe cell damage (Meyer, W.A., and C.L. Wisseman, Jr., in preparation vide infra). Such damaged macrophages do not appear to support the growth of *R. prowazekii*. On a cell population basis, as in the methods employed here, the net effect would be a reduced apparent growth rate. Despite this potential complicating phenomenon, the results of this preliminary study clearly show that macrophages pretreated with stimulated leukocyte supernatant fluids display the same capacity to restrict *R. prowazekii* replication relative to untreated macrophages as was demonstrated above with fibroblasts.

Endothelial cells of small blood vessels are the best described site of rickettsial replication in hosts infected by "natural", i.e., peripheral routes, as opposed to intraperitoneal and intranasal routes. The classical focal histopathological response to infections with members of the genus *Rickettsia* develops at the sites of endothelial cell infection and contains cells that are now known to be important in immunological and immunopathological processes - i.e., cells of the monocyte-macrophage-histiocyte series, lymphocytes, some polymorphonuclear leukocytes depending upon the stage of development, and occasional plasma cells. Previous studies have shown that the major immunological component in restricting typhus rickettsial growth locally in tissues of infected guinea pigs or mice is T-cell mediated but that the mechanisms probably differ from the classical DTII-tuberculosis-listeria model (last year's annual report). Moreover, the preferred intravascular, endothelial site of rickettsial replication is physically separated from the perivascular accumulation of the monocyte-macrophage-histiocyte...
series of phagocytic cells. Although both lymphocytes and macrophages can migrate through blood vessel walls, they are usually found in a perivascular location in typhus lesions and do not appear to be in physical contact with infected endothelial cells. The case for the operation of a soluble, diffusible mediator of immunity is greatly enhanced. Hence, it becomes imperative to demonstrate the capacity of the soluble factors in stimulated leukocyte supernatant fluids to restrict rickettsial replication in endothelial cells.

The evidence that endothelial cells from different sources may differ in important properties is growing. However, at this time endothelial cells from umbilical veins is the most convenient source of cells of this general type of human origin. Although there have been major advances in recent years of the methods of cultivating umbilical vein endothelial cells for a variety of purposes, the practical requirements for demonstrating antirickettsial activity of stimulated leukocyte supernatant fluids in endothelial cells are far more stringent than those required of many other studies.

Like others, we have found that the growth of endothelial cells in culture varies enormously with cells from one cord to another and that the capacity of endothelial cells derived from different cords to support the growth of rickettsiae varies widely, from no growth after high initial apparent infections to relatively vigorous growth. It is possible that most cultures contain varying mixtures of cells in various stages of cell damage or nutritional deficiency not consistent with good rickettsial growth and of relative healthy cells. We attribute this variation to uncontrolled factors in the system and the general as yet unsophisticated state of the art and science of cultivating endothelial cells.

Nevertheless, by examining endothelial cells from many cords, we have found some that seem to be satisfactory to a limited degree for tests of interest to us. Controls assume an exaggerated importance. Thus, in many cultures which appeared to be in a reasonably healthy state by microscopic examination, it was possible to show vigorous rickettsial uptake but the organisms failed to grow in subsequent incubation. In some preparations, however, it was possible to show that both the Breinl and Burundi strains of *R. prowazekii* and *R. rickettsii* (SS) would grow in reasonably typical fashion. And after several attempts, in which the controls were not satisfactory, it was possible to demonstrate, in one preparation, that cells pretreated with ET-S markedly restricted the growth of *R. prowazekii* (Breinl) (Figure 10). Even this experiment is not ideal. The first 16 hours after pretreatment with ET-S and infection was accompanied by a marked loss of cells in both control and treated populations, which may reflect a starting population of endothelial cells in various states of competence. Nevertheless, after the initial loss of incompetent cells, it is clearly evident that *R. prowazekii* (Breinl) multiplied in untreated endothelial cells and that there was a progressive marked decline in rickettsiae in endothelial cells which had been pretreated with ET-S.

Although the results of these studies warrant further intensive confirmation and exploration, it is apparent that soluble factors derived from antigenically specifically stimulated immune leukocytes or from non-specifically stimulated non-immune leukocytes have the capacity to restrict *R. prowazekii* replication in vitro in infected cells which in the natural peripherally induced infection play diverse roles in the infectious process: (1) "by-stander" cells of fibroblastic origin; (2) "target" cells of endothelial origin; and (3) natural phagocytic cells, i.e., macrophages, whose role in systemic infection of the intact animal remains to be clarified.

4. Summary of main findings.

a. Human peripheral blood leukocytes from typhus immune subjects,
when stimulated specifically in vitro with killed R. prowazekii particulate antigen, elaborate within 24 hrs soluble factor(s) into the supernatant fluid (ET-S) which exert (a) an intracellular antirickettsial and (b) a cytotoxic action on R. prowazekii-infected human fibroblasts in cell culture. When leukocytes from either normal or typhus-immune subjects are stimulated non-specifically with the mitogen, phytohemagglutinin, factor(s) (PHA-S) with identical action are also elaborated.

b. Neither ET-S nor PHA-S have any observable direct effect on extracellular R. prowazekii, as measured by capacity to infect and grow within host cells. Both ET-S and PHA-S may exert a range of effects on uninfected host cells from growth stimulatory to cytostatic, depending upon the manner of presentation, but no cytotoxic or cytolytic action comparable to that observed on infected cells or described for lymphotoxins is observed.

c. The specific cytotoxic or cytolytic action of ET-S and PHA-S is best observed when R. prowazekii infected host cells are grown in medium containing ET-S or PHA-S over a period of 24-48 hours. Treated infected cells progressively lose the capacity to exclude trypan blue, release cytoplasmic lactic dehydrogenase into the medium and lyse completely. The pattern of infected cell destruction by ET-S or PHA-S differs substantially from that eventually produced by the R. prowazekii infection in untreated cells.

d. An intracellular antirickettsial action of ET-S or PHA-S, which does not depend on host cell destruction, is readily distinguished from the cytolytic action by pretreating the host cells with the stimulated leukocyte supernatant fluids before infection with R. prowazekii and subsequently incubating in the absence of ET-S or PHA-S. In such cells, rickettsiae progressively disappear both by microscopic examination and by loss of viability as measured by plaque formation in susceptible CE cell monolayers. The host cells maintain their integrity during the period of rickettsial elimination - there is no increase in permeability to trypan blue, no increased loss of lactic dehydrogenase into the medium and no loss in cell numbers.

e. Both intracellular antirickettsial and cytolytic actions of human origin ET-S and PHA-S are species specific - i.e., they are demonstrable with infected cells (fibroblasts) of human origin but not with infected cells of monkey, mouse or chicken origin. The intracellular antirickettsial action is demonstrable in cultures of R. prowazekii-infected human umbilical vein endothelial cells (the "target" cell type for rickettsial growth in vivo) and human peripheral blood monocyte-derived macrophages.

f. Human fibroblasts infected with strains of R. prowazekii show both antirickettsial and cytolytic actions to varying degrees; those infected with R. mooseri or R. rickettsii show only cytolytic action; and those infected with strains of R. tsutsugamushi show neither.

g. Both intracellular antirickettsial and cytotoxic or cytolytic actions of ET-S and PHA-S are destroyed by heating at 56°C for 60 min and by exposure to pH 2.

5. Significance and perspective.

As indicated in the introduction, there is a growing body of information which strongly suggests that the striking immunity that develops during typhus infections to control the rickettsiae and that persists as a solid immunity long after convalescence from infection cannot be explained adequately on the basis of either classical antibody mediated mechanisms or the DTII type of cell mediated immunity which depends on acti-
vation of macrophages to heightened non-specific antimicrobial activities by products of stimulated thymus-derived lymphocytes (1), although components of these well-established mechanisms no doubt do operate to some extent in typhus infections. These considerations, along with the possible unique requirements for the expression of cell mediated immunity in the context of physical constraints suggested by the structure of the basic lesion in typhus, i.e., vasculitis with infection largely confined to endothelial cells and perivascular cellular response not readily demonstrable in intimate contact with the infected "target" cell, suggested the possibility of the operation of a diffusible T-cell derived mediator capable of restricting rickettsial growth in "somatic" cells (e.g., endothelial cells) which are not "professional" phagocytes.

The finding, reported here, that leukocytes from typhus immune human subjects, upon stimulation with R. prowazekii antigen, elaborate soluble factor(s) into the culture supernatant fluid which have the capacity to destroy typhus rickettsiae within "somatic" cells, including fibroblasts and endothelial cells, as well as in macrophages, and to cause the lysis of infected "somatic" cells appears to represent a new kind of cellular immune effector mechanism against these obligate intracellular parasites which selectively infect "somatic" cells instead of macrophages, as do certain other intracellular parasites. Such a mediator can be visualized as potentially effective in the context of the basic typhus lesion. The fact that non-specific (PHA) stimulation of leukocytes from either immune or non-immune subjects leads to the elaboration of soluble factor(s) indistinguishable so far from those produced under immunologically specific stimulation and that the actions are demonstrable to varying degrees on cells infected with R. mooseri and R. rickettsii suggests that we are dealing with a new general class of final effector mechanisms on certain classes of intracellular parasites whose action is indirectly mediated through effects on non-phagocytic as well as phagocytic host cells. Moreover, this factor(s) discriminates between uninfected and infected host cells. It is possible, though as yet unexplored, that these factors may also act upon cells infected with other types of intracellular parasites.

It is clear that the phenomenon described here differs enormously from the better studied DTH type of CMI in which T-lymphocyte products non-specifically activate macrophages to broad enhanced antimicrobial activity (1). There are also some superficial similarities to the properties of interferons (2) and lymphotoxins (3). Not surprisingly, in some preliminary studies (data not shown) we have found that supernatant fluids of these stimulated leukocyte suspensions do also contain varying amounts of interferon, LIF and lymphotoxin activity but no clear association between any of these and activity on typhus rickettsia infected cells has yet emerged.

What relation our factor(s) have to the action of mouse lymphocyte supernatant fluids on R. tsutsugamushi in mouse peritoneal macrophages described by Nacy and coworkers (4, 5, 6) or to the guinea pig factors against Coxiella burnetii infection of macrophages described by Hinrichs and associates (7) is unknown. However, there are certain similarities between our factor(s) and those described by Chinchilla and Frenkel (8) which were active against Toxoplasma and Besnoitia infection in somatic cells. It is possible that a whole family of mechanisms of cell mediated immunity against various obligate intracellular parasites which differ from those operative with facultative intracellular parasites (e.g., Mycobacteria, Listeria, etc.) exist.

Much remains to be done with our antirickettsial and cytolytic factors. If, indeed, they prove to be really important factors in antirickettsial immunity, for both practical and scientific purposes it will become necessary to determine which cells elaborate these factors which rickettsial antigens (see below) elicit this type of immune response and how the antigen(s) can be presented to the human subject so as to stimulate optimally the
desired immune response.

6. References


B. Cytotoxic action of R. prowazekii, but not R. mooseri, on human peripheral monocyte derived macrophages in culture. (With W.A. Meyer) Last year’s annual report presented the ultrastructural details of the escape of R. prowazekii incubated with normal human serum from the phagosomes of human peripheral blood monocyte derived macrophages in culture and the subsequent intracytoplasmic growth and eventual destruction of the macrophage by overwhelming rickettsial infection. In contrast, R. prowazekii incubated with typhus immune human serum was retained in the phagosome and underwent progressive destruction, accompanied by lysosomal fusion with the phagosome and discharge of lysosomal enzymes into the phagosome.

In the ultrastructural study presented last year, as in the earlier light microscope studies of Gambrill and Wisseman (1), there was the distinct impression that in fact that interaction between R. prowazekii and human macrophages in the absence of immune serum was complex and consisted of at least 2 types of patterns exhibited by different macrophages in the same culture: (1) the pattern described in detail in last year’s annual report in which there was relatively unrestricted growth of R. prowazekii in the cytoplasm of the macrophage, similar to that which we have previously described in detail in chicken embryo fibroblasts and in F-1000 human fibroblasts in the preceding section and (2) a pattern in which rickettsial replication is restricted and the macrophage undergoes early degenerative changes. The latter putative phenomenon has now been studied in some detail and has been shown to be a regular component of the interaction of R. prowazekii, but not R. mooseri, with human macrophages in culture in the absence of
Table 1. Failure of pre-treatment of cell-free *R. prowazekii* with leukocyte supernatant fluids to reduce capacity to form plaques in chicken embryo cell monolayers

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>Exp. No.</th>
<th>% Control Plaques after Treatment with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CS³</td>
</tr>
<tr>
<td>0</td>
<td>Exp. 1</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Exp. 2</td>
<td>97</td>
</tr>
<tr>
<td>60</td>
<td>Exp. 1</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Exp. 2</td>
<td>97</td>
</tr>
<tr>
<td>120</td>
<td>Exp. 1</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Exp. 2</td>
<td>100</td>
</tr>
<tr>
<td>180</td>
<td>Exp. 1</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Exp. 2</td>
<td>100</td>
</tr>
</tbody>
</table>

1  Temp. = 32°C
2  Number of plaques in tissue culture medium
3  CS³ = Control supernatant from unstimulated leukocytes
   Exp. 1 donor (PV) non-immune
   Exp. 2 donor (MBD) *R. prowazekii* convalescent
4  ET-S⁴ = Supernatant from leukocytes of *R. prowazekii* convalescent donor (MBD)
   stimulated with *R. prowazekii* antigen
5  PHA-S⁵ = Supernatant from leukocytes of typhus non-immune donor (PV) stimulated
   with phytohemagglutinin
Table 2. Uptake and Growth in F-1000 Human Fibroblasts of *R. prowazekii* (Breinl) Pre-treated in Cell-free State with Immune Leukocyte Supernatant Fluid

<table>
<thead>
<tr>
<th>Incubation Time (h)</th>
<th>Prelim. Wash of Rickettsiae</th>
<th>Rickettsiae in Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p1² CM</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>58</td>
</tr>
<tr>
<td>48</td>
<td>0</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>69</td>
</tr>
</tbody>
</table>

1 Uptake of rickettsiae and subsequent intracellular growth were measured by slide chamber method (15, 18). Purified *R. prowazekii* (Breinl) were first incubated at room temperature for 60 min with control medium (CM) or leukocyte supernatant from typhus convalescent donor (CLW) (ET-S) stimulated with *R. prowazekii* antigen. Half of each mixture was centrifuged and the pellet was resuspended in control medium. All 4 mixtures were used to infect F-1000 cells in slide chambers for 60 min at 32°C. After replacing fluids with control medium, slides were incubated at 32°C in 5% CO₂-air. Replicates were fixed and stained at 0 and 48 h after infection.

2 p1 = % cells infected

3 N = average number of rickettsiae per cell
Table 3. Antirickettsial action on *R. prowazekii* (Breinl) in Human F-1000 fibroblastic cells of supernatant fluids from specifically stimulated immune and non-specifically stimulated non-immune human leukocytes: comparison of microscopic slide chamber method with recovery of plaque forming units from parallel cultures.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Slide Chamber Cultures</th>
<th>Total PFU/Flask (x10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U H</td>
</tr>
<tr>
<td>CM</td>
<td>53 52 1.6 25.7 16.1 ---</td>
<td>1120</td>
</tr>
<tr>
<td>CS</td>
<td>56 51 1.5 24.5 16.3 ---</td>
<td>1180</td>
</tr>
<tr>
<td>ET-S</td>
<td>42 3.7 0.89 0.21 0.24 ---</td>
<td>17.4</td>
</tr>
<tr>
<td>PHA-S (immune)</td>
<td>43 10.7 0.84 3.9 1.6 ---</td>
<td>318</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Slide Chamber Cultures</th>
<th>Total PFU/Flask (x10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U H</td>
</tr>
<tr>
<td>CM</td>
<td>64 64 2.2 41.8 19 4.4</td>
<td>145</td>
</tr>
<tr>
<td>CS (ET-S)</td>
<td>51 45 1.6 22.7 14 4.1</td>
<td>110</td>
</tr>
<tr>
<td>ET-S</td>
<td>49 3.3 1.6 0.48 0.31 4.1</td>
<td>0.8</td>
</tr>
<tr>
<td>CS (PHA-S)</td>
<td>52 52 1.7 28.8 17 4.0</td>
<td>118</td>
</tr>
<tr>
<td>PHA-S</td>
<td>62 26 2.2 12.2 5.6 4.4</td>
<td>26</td>
</tr>
</tbody>
</table>

1 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, with its corresponding CS, were obtained from the leukocytes of a typhus-immune individual whereas the PHA-S, with its corresponding CS, were obtained from a subject not immune to typhus.
## Table 4. Susceptibility of anti-rickettsial and cytotoxic actions of ET-S and PHA-S to inactivation by heat or acid pH.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Effect on Indicated Action*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-Rickettsial</td>
</tr>
<tr>
<td></td>
<td>ET-S</td>
</tr>
<tr>
<td>Acid (pH for 24 h)</td>
<td>marked reduction</td>
</tr>
<tr>
<td>Heat (56°C for 1 h)</td>
<td>marked reduction</td>
</tr>
</tbody>
</table>

* Tested in F-1000 cells infected with *R. prowazekii* (Breinl)
Table 5. Host cell specificity of antirickettsial and cytotoxic actions of "specific" and "non-specific" human leukocyte supernatants on *R. prowazekii* (Breinl) infection.

<table>
<thead>
<tr>
<th>Origin/Designation</th>
<th>Host Cells</th>
<th>State</th>
<th>Type of Action</th>
<th>Anti-Rickettsial</th>
<th>Cytotoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell Type</td>
<td></td>
<td></td>
<td>ET-S&lt;sup&gt;2&lt;/sup&gt;</td>
<td>PHA-S&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Homo Sapiens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WI-38</td>
<td>Embryonic Lung</td>
<td>D</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Fibroblast</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W-38&lt;sup&gt;5&lt;/sup&gt;</td>
<td>SV-40</td>
<td>T</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>(VA 13)</td>
<td>Transformed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT-1080&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Hu Fibrosarcoma</td>
<td>T</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>F-1000</td>
<td>Foreskin Fibroblast</td>
<td>D</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervical Carcinoma Epithelial-like</td>
<td>T</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FL&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Epithelial-like&lt;sup&gt;5&lt;/sup&gt;</td>
<td>T</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Hu Amnion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chang's Liver</td>
<td>Epithelial-like&lt;sup&gt;4&lt;/sup&gt;</td>
<td>T</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Sub-human Primate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBS-FCL-Z</td>
<td>Fetal lung Fibroblast-like</td>
<td>D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VERO&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Normal Af. Green Monkey Kidney Fibroblast-like</td>
<td>T</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LLC-MK&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Normal Rhesus Monkey Kidney Epithelial-like</td>
<td>T</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rodent (Mus)</td>
<td>Mouse embryo</td>
<td>F</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>1</sup> State: D = Dormant, T = Treated
<sup>2</sup> ET-S: Endotoxin-activated T cells
<sup>3</sup> PHA-S: Phytohemagglutinin-activated T cells
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>T</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian</td>
<td>Mouse connective tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>fibroblast-like</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-929</td>
<td></td>
<td></td>
<td>T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken embryo</td>
<td>Fibroblast</td>
<td></td>
<td>P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. D = diploid; T = transformed, malignant or polyploid; P = primary or secondary from indicated tissue.

2. ET-S: Supernatant from R. prowazekii-stimulated leukocytes from human subject convalescent from R. prowazekii infection.

3. PHA-S: Supernatant from PHA-stimulated leukocytes from non-immune subject.

4. Contain HeLa cell markers.

5. Obtained from American Type Culture Collection.
Table 6. Differential antirickettsial and cytotoxic actions of "specific" and "non-specific" human leukocyte supernatants on various rickettsial species and strains in F-1000 human fibroblast cell cultures

<table>
<thead>
<tr>
<th>Species/Strain</th>
<th>Type of Action</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-Rickettsial</td>
<td>Cytotoxic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ET-S¹</td>
<td>PHA-SZ</td>
<td>ET-S¹</td>
<td>PHA-SZ</td>
<td></td>
</tr>
</tbody>
</table>

**R. prowazekii**

<table>
<thead>
<tr>
<th>Strain</th>
<th>ET-S</th>
<th>PHA-SZ</th>
<th>ET-S</th>
<th>PHA-SZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breinl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Burundi</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>+ to ±</td>
<td>+ to ±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fl. sq.</td>
<td>± to -</td>
<td>± to -</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**R. mooseri**

<table>
<thead>
<tr>
<th>Strain</th>
<th>ET-S</th>
<th>PHA-SZ</th>
<th>ET-S</th>
<th>PHA-SZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilmington</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**R. canada**

<table>
<thead>
<tr>
<th>Strain</th>
<th>ET-S</th>
<th>PHA-SZ</th>
<th>ET-S</th>
<th>PHA-SZ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**R. rickettsii**

<table>
<thead>
<tr>
<th>Strain</th>
<th>ET-S</th>
<th>PHA-SZ</th>
<th>ET-S</th>
<th>PHA-SZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheila Smith</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**R. tsutsugamushi**

<table>
<thead>
<tr>
<th>Strain</th>
<th>ET-S</th>
<th>PHA-SZ</th>
<th>ET-S</th>
<th>PHA-SZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gillian</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JC-472 (Karp-like)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1 ET-S: Supernatant from R. prowazekii-stimulated leukocytes from human subject convalescent from R. prowazekii infection.

2 PHA-S: Supernatant from PHA-stimulated leukocytes from non-immune subject.

3 Anti-Rickettsial Action: rickettsiastatic or rickettsiacidal action in cells.

4 Cytotoxic Action: loss of infected cells caused by leukocyte supernatants added 6 h after infection and left in contact with infected cells.
immune serum. Details are presented in the following paragraphs.

Figure 11 contains the quantitative data that we have recently obtained regarding the interaction of typhus rickettsiae with human macrophages. The upper portion of figure 11 illustrates how rapidly rickettsial infected macrophages die, based on loss of their ability to exclude trypan blue, under different experimental conditions. In figure 11, directly below the cytotoxicity data, are the rickettsial growth curves for each experimental condition. The data points from each of the 4 experimental conditions represent mean values obtained from at least 3 replicate experiments.

Each of the 4 experimental conditions demonstrates a unique pattern of rickettsial replication when both major parameters of the growth cycle are examined. The initial uptake of rickettsiae at time 0 hours on the average ranged from 2 to 4 rickettsiae per macrophage for the 4 experimental conditions. In order to compare the growth rates among rickettsiae from different experimental conditions the value of log N (the log 2 of the average number of rickettsia per macrophage) was normalized to a value of 1 at time 0 hours. It can be seen from data in figure 11 that nonimmune human serum treated R. prowazekii and R. mooseri infected cells differ in their growth cycles in the parameter of percent cells infected. This confirms previous, as yet unpublished observations regarding the ability of R. mooseri to exit from infected cells and enter uninfected cells throughout its growth cycle whereas R. prowazekii is restricted to the initially infected cell until it bursts. The average generation time of both organisms, calculated from the slope of the exponential portion of the growth (0—0), is about 18 hours and this indicates that these rickettsiae replicate more slowly in macrophages than other cell systems, but do not significantly differ from each other. In contrast, immune human serum (IHS)-treated R. prowazekii is destroyed by human macrophages, confirming our earlier observation. There is a dramatic rapid decrease in both parameters of the growth cycle. If 20 μg/ml of chloramphenicol is added to macrophages just after their acquisition of NHS-treated R. prowazekii, the organisms do not replicate, although a significant portion of the organism are not destroyed and remain within the macrophage.

The cytotoxicity associated with infection by NHS-treated R. prowazekii is quite evident by the trypan blue assay (Figure 11). The toxic effect was not detectable upon the introduction of the organism into the macrophages, (on the average of 2-3 rickettsiae per cell), but rather was first significantly different from uninfected controls by 18 hours. The percentage of macrophages which were nonviable increased dramatically over the rest of the observation period. In contrast, uninfected cells (data not shown), and IHS-treated R. prowazekii infected macrophages retained > 95% viability through the 72 hour culture period. The viability of NHS-treated R. mooseri infected cells did not differ from that of controls until between 48 and 60 hours post infection when the intracellular rickettsial concentration reached levels which caused cell lysis.

Several experiments were performed to better characterize the requirements for and mechanism of this rickettsial induced cytotoxicity. Figure 11 demonstrates that the macrophage cytotoxicity associated with infection by NHS-treated R. prowazekii can be significantly reduced, but not eliminated, by the incorporation of 20 μg/ml of chloramphenicol in the culture system just after the organisms enter the cytoplasm. This suggests that macrophage cytotoxicity is maximal if the rickettsiae are replicating.

In order to determine if macrophage death during R. prowazekii infection was due to a product released from these cells we transferred 24 hour or 48 hour supernatants collected from NHS-treated R. prowazekii infected macrophages to uninfected macrophages and monitored the viability of those treated cells over the next 48 hours. Despite the fact that those recipient cells received supernatants collected from infected cells
during the peak periods of cytotoxicity (Fig. 11), they did not exhibit an increase in the percentage of cells which were trypan blue positive. This suggests that macrophage cytotoxicity is not the result of products being released from the infected cells themselves.

By comparing the distribution of *R. prowazekii* among macrophages at 0 and 48 h of incubation with similar data from F-1000 fibroblasts (Figure 12), one can demonstrate that *R. prowazekii* indeed undergoes relatively unrestricted replication in some macrophages whereas in others replication is very restricted. The distribution of *R. prowazekii* in macrophages and F-1000 cells at 0 time was comparable. During incubation there was no evidence of significant intercellular spread in cell type since the proportion of cells which had no rickettsiae remained essentially the same between 0 and 48 h. However, *R. prowazekii* underwent the expected division in essentially all F-1000 cells which were infected at 0 time, as evidenced by the fact that almost all infected cells contained more than 20 RLB per cell. In sharp contrast, infected macrophages indeed fell into two major populations at 48 h — (a) a population in which there was relatively unrestricted replication of *R. prowazekii* and (b) an equivalent or larger population in which little or no replication occurred, with an intermediate population showing very limited replication. On the other hand, replication of *R. mooseri* produced the same kind of spreading infection in both macrophages and F-1000 fibroblasts, with a sharp drop in uninfected cells at 48 h and a broad distribution of rickettsiae among cells.

In recent ultrastructural experiments we obtained some insight into the mechanism which might be occurring with *R. prowazekii* induced cytotoxicity. Premature macrophage death appears not to be the result of internal release of lysosomal hydrolases in response to the presence of the rickettsiae. The supporting data for this contention was obtained by labeling macrophage secondary lysosomes with electron dense thorium dioxide, infecting with NHS-treated *R. prowazekii*, and performing transmission electron microscopic observations to determine if the lysosomal marker was released internally. In all cells examined the electron dense marker remained within membrane bound lysosomes until such time as the cells died, as evidenced by breaks in the external plasma membrane.

Ultrastructural experiments with unlabeled macrophages which were infected with NHS-treated *R. prowazekii* did, however, yield information regarding the possible sequence of events which might lead to macrophage cytolysis (Figure 13). Figure 13a is a NHS-treated *R. prowazekii* infected macrophage at 24 hours which is undergoing a cytolytic process. The cell exhibits a swollen endoplasmic reticulum (arrows) and an apparent decrease in the density of the cytoplasmic sap in comparison with the cells in Figure 13b and 13c. The cell in figure 13b is another NHS-treated *R. prowazekii* infected macrophage at 24 hours post infection which is not undergoing a cytolytic process but rather contains rickettsiae (r) which are replicating. The macrophage in figure 13c is a typical NHS-treated *R. mooseri* infected macrophage at 24 hours. Cells infected with *R. mooseri* were found not to exhibit cytopathology, as seen in Figure 13a, but rather retained their ultrastructural integrity and allowed for the replication of the rickettsiae (r). In general the types of cytopathologic changes observed in the *R. prowazekii* infected macrophages (Figure 13a) have been associated by others with a loss in the cell's ability to regulate intracellular ion and water levels (2, 3). We might speculate that *R. prowazekii* has a more deleterious effect on some macrophage's ion and water transport regulatory systems than does *R. mooseri*.

References


C. Polypeptides and polypeptide antigens of R. prowazekii (with Oaks and Smith).

The identification and characterization of rickettsial proteins which are important as virulence factors and protective antigens will lead to a better understanding of the organism's pathogenicity and may identify potential candidates for a sub-unit vaccine. Surface components of the rickettsiae may be extremely important in the disease process especially since the obligatory intracellular parasitism of rickettsiae requires several interactions between the membranous components of the host cell and the surface of the pathogen. In addition, the complex nature of rickettsial pathogenesis probably involves several rickettsial components. A mutation, or lack of any one component necessary to achieve the overall effect of disease, may result in attenuation of the rickettsiae. With the concept of rickettsial pathogenesis in mind we have developed three criteria which will aid in the identification of proteins that are important as virulence factors. The criteria are: (1) those polypeptides which distinguish a virulent strain from an avirulent strain; (2) those polypeptides that are located on the surface of the rickettsiae; and (3) those polypeptides that are precipitated by convalescent or immune serum. Data used to identify R. prowazekii polypeptides important to the virulence of this organism are summarized below.

1. Polypeptide analysis of R. prowazekii strains. Figure 14 is an SDS-polyacrylamide gel of the polypeptides from 5 R. prowazekii strains labeled with \(^3\)H-leucine in emetine-treated L cells. This gel clearly shows that emetine reduces L cell protein synthesis to negligible levels (compare lane B to lane C) and thereby allows specific labeling of rickettsial polypeptides (Lanes D-H) in infected cells. The polypeptide profiles of rickettsiae that have not been purified (lanes D-H) are identical to \(^3\)H-labeled polypeptides of Renografin-purified rickettsiae (lane I). The 5 strains of R. prowazekii in this gel are the flying squirrel strain GVF-IZ (lane D), two recent isolates from Burundi (lanes E and F), the attenuated E strain (lane G) and finally the classic Breinl strain (lane H). The majority of the polypeptides from these five R. prowazekii strains are identical. However, an outstanding difference is present in the attenuated E strain. The 31,000 dalton polypeptide migrates slightly faster in the E strain when compared to the virulent strains. The same results are achieved with \(^3\)H-labeled, Renografin purified organisms (I). This reproducible distinction between the virulent and avirulent strains of R. prowazekii when analyzed by PAGE is in agreement with strain differences determined by isoelectric focusing previously reported by Dasch et al. (4).

2. Analysis of Polypeptides Located on the Surface of R. prowazekii and R. mooseri. Previous studies have used surface labeling techniques to label the surface polypeptides of R. prowazekii (Breinl strain) (2) and the E strain (3). A direct comparison of the surface polypeptides of these two R. prowazekii strains has not been reported, nor has surface labeling of R. mooseri been reported. Therefore, to confirm that the 31,000
polypeptide is indeed the major surface protein of both the E and Breinl strains, and also to analyze R. mooseri for similar surface polypeptides, a surface-specific iodination of rickettsial polypeptides catalyzed by Iodogen (Pierce Chemical Company) was performed (5).

Figure 15 compares $^3$H-leucine labeled polypeptide profiles of R. prowazekii (Breinl and E) and R. mooseri (Wilmington) with surface-labeled polypeptides of the same three organisms. The tritiated profiles clearly show that the murine typhus organism is closely related to epidemic typhus. However, the 31,000 dalton polypeptide, not only distinguishes the avirulent E strain from the Breinl strain of R. prowazekii but the analogous polypeptide in R. mooseri has a lower molecular weight (29,000). Surface iodinations confirm that the 31K polypeptide of R. prowazekii is on the surface of the Breinl and E strain. As expected, the 29K polypeptide of R. mooseri is also labeled by surface specific iodinations. In addition, a low molecular weight polypeptide (16K) is also labeled in R. mooseri.

3. A previous report (annual report 1979) stated that a high molecular weight (138,000) polypeptide and a low molecular weight (32,000) polypeptide of R. prowazekii (Breinl and E strains) were precipitated human immune serum (convalescent). Recent studies show that the same polypeptides are immune precipitated from the Burundi strain (X-16 and V59) and the flying squirrel strain (GVF-12) (see Figure 16). The procedures used to solubilize rickettsial polypeptides for these immune precipitations did not efficiently solubilize the 31K polypeptide. Additional investigations designed to solubilize the 31K polypeptide for immune precipitation indicate that this polypeptide is not solubilized from rickettsial membranes unless high concentrations of guanidine HCl, urea or 2-mercaptoethanol are present. Figure 17 shows immune precipitates, analyzed by SDS-PAGE, of guanidine-HCl solubilized R. prowazekii polypeptides surface labeled with $^{125}$I. The 31K polypeptide is present in the immune serum track (lane C) and absent in the normal serum treated sample (lane D).

Some characteristics of the major R. prowazekii polypeptides are summarized in Table 7. The information in this table indicates that the 138K, 32K and the 31K polypeptides all generate an immune response in the infected host.

TABLE 7

<table>
<thead>
<tr>
<th>Molecular Weight</th>
<th>Immune Serum Precipitated</th>
<th>Surface Oriented</th>
<th>Distinguishes Between Virulent and Avirulent Strains</th>
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<tr>
<td>138,000</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>64,500</td>
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<td>-</td>
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<tr>
<td>32,000</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>31,000</td>
<td>+</td>
<td>+</td>
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</table>

The location of the 138K and the 31K polypeptides on the rickettsiae's surface suggest that these two polypeptides may have a specific role requiring surface orientation. In addition, the different molecular weight forms of the 31K surface polypeptide in the
avirulent E strain, the virulent strains of R. prowazekii and also in R. mooseri, may indicate that this polypeptide is important to the virulence of these organisms in a manner which involves interactions with host-cell membranes.

References


D. Production and Characterization of Monoclonal Antibodies to R. prowazekii (with Oaks and Smith).

The detailed characterization and analysis of specific virulence factors of R. prowazekii can be approached in several ways. The most direct analysis is achieved by using purified preparations of a specific component. Indirect analysis is accomplished by using a highly specific antibody to neutralize specific functions of the rickettsiae. Since protocols for purifying individual rickettsial components are not well developed, we used the recently developed technique for the production of specific monoclonal antibody by lymphocyte hybridomas (1) to characterize specific rickettsial products and their functions. Monoclonal antibody will also aid in purification schemes (immune-affinity chromatography) of specific antigens that will allow more direct analysis.

Initial studies in our laboratories have produced monoclonal antibodies to R. prowazekii. This organism was chosen because knowledge of its polypeptide antigens (see previous section) and protocols for solubilizing these antigens are available.

The immunization of BALB/c mice with live R. prowazekii (1.25 x 10⁸ plaque forming units) and a boost of 6.25 x 10⁸ p.f.u. seven weeks later, produced a pronounced secondary antibody response. Anti-rickettsial antibody measured by indirect fluorescent antibody (IFA) activity increased from an average titer of 1:120 before boosting to an average titer of 1:400 four days after the secondary inoculation. Splenectomy was performed 4 days after the boost because the rapid increase in IFA titer indicated that immune B-cell proliferation and expansion were occurring.

Immune mouse spleen cells were fused with P3-X63 Ag8 myeloma cells using PEG 1000 as the "fusagent" according to the procedure of Kennett (2). Hybrid cells were selected by a medium containing hypoxanthine, thymidine and aminopterin. After two weeks of growth in 96-well plates the hybridoma medium of each well was tested for anti-rickettsial antibody by IFA. Approximately 60% (156/261) of the initial hybrids
were IFA positive. Growing cells producing anti-rickettsial antibody were passed to 24-well plates, and then transferred to 25 cm² flasks. Fifty hybridoma lines were eventually cloned on soft agar over a feeder layer of human fibroblasts. Ten to fifteen clones from each parent were passed in culture and tested by IFA. Those retaining IFA activity were maintained in culture or stored frozen in liquid nitrogen.

Twenty-eight clones have been selected for more detailed analysis. Results from immune precipitations of ³H-labeled R. prowazekii polypeptides, IFA titrations and R. prowazekii specificity assays are in Table 8. Several (10) of the monoclones are producing antibody that immune precipitates the 138K polypeptide of R. prowazekii (Figure 18). Five of these monoclones (23A6, 23B3, 27B2, 27B6 and 30A4) are also epidemic typhus specific as determined by a negative IFA against R. mooseri. Other clones are producing epidemic typhus specific antibody but not able to immune precipitate the 138K polypeptide. These monoclones may be reacting with an undetermined species-specific antigen.

Other clones that are IFA positive remain uncharacterized with regards to antigen specificity. These clones may be recognizing rickettsial components such as the carbohydrate slime layer or the 31K polypeptide which is not efficiently solubilized in the immune precipitation procedures used.

A few monoclones were also analyzed for opsonizing activity (see Table 9) as determined by increased uptake of treated rickettsiae by human macrophages. Four out of six clones tested had the capacity to opsonize rickettsiae. The same four monoclones also produced antibody which immune precipitated ³H-labeled R. prowazekii polypeptides. One clone (46B2) immune precipitated ³H-labeled polypeptides of R. prowazekii but did not opsonize. The antigen specificities of these clones remain to be determined.

The production of specific antibody by lymphocyte hybridomas has enormous potential in the field of rickettsiology. Early work in our laboratories has generated a monoclonal antibody for an individual rickettsial component (the 138K polypeptide). Detailed characterization of the biological properties of this polypeptide and its purification by immune-affinity chromatography are now possible. Several clones are also producing R. prowazekii specific antibody which could be used as a standardized reagent in diagnostic and epidemiological investigations throughout the world. Experiments analyzing the role of specific antibodies in rickettsiae-host-cell interactions have identified four monoclones producing antibody that have the ability to opsonize R. prowazekii while other clones that are IFA positive do not have this capacity. Further characterization of monoclonal antibody activity in other rickettsial functions are currently in progress.

References
### TABLE 8

**CHARACTERISTICS OF ANTI-R. PROWAZEKII MONOCLONAL ANTIBODIES**

<table>
<thead>
<tr>
<th>Clone&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Polypeptide Immune Precipitated&lt;sup&gt;b&lt;/sup&gt;</th>
<th>IFA Titer&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Epidemic Typhus Specific&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td>19B2</td>
<td>138K</td>
<td>64</td>
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<tr>
<td>19C1</td>
<td>138K</td>
<td>1024</td>
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<td>19D1</td>
<td>138K</td>
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<td>16</td>
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<td>42C2</td>
<td>138K</td>
<td>1024</td>
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<tr>
<td>43B5</td>
<td>138K</td>
<td>256</td>
<td>+</td>
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<sup>a</sup> Cell lines were cloned on soft agar prior to these analyses. Each clone represents a different parent line from the original 96-well plates.

<sup>b</sup> Detergent solubilized <sup>3</sup>H-labeled polypeptides were reacted with hybridoma supernatants, followed by absorption with *Staphylococcus aureus* (Cowen I) protein A. Absorbed complexes were solubilized with electrophoresis sample buffer and subsequently electrophoresed on SDS-PAGE gels. Fluorography was used to detect <sup>3</sup>H-polypeptide profiles.

<sup>c</sup> Reciprocal IFA titers were determined on *R. prowazekii* (Breinl) infected chicken fibroblasts using fluorescein conjugated rabbit anti-mouse IgG. Four-fold dilutions of hybridoma culture supernatants were made in PBS.

<sup>d</sup> Epidemic typhus specificity was determined by IFA assays of undiluted hybridoma supernatants against *R. prowazekii* infected chick fibroblasts and *R. mooseri* infected chicken fibroblasts. Those supernatants not reactive with *R. mooseri* antigen slides but reactive with *R. prowazekii* antigen slides were considered epidemic typhus specific.
### TABLE 9
CHARACTERISTICS OF MONOCLONAL ANTIBODY WITH OPSONIZING CAPABILITIES

<table>
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<tr>
<th>Clone</th>
<th>IFA titer</th>
<th>Capacity to opsonize&lt;sup&gt;a&lt;/sup&gt; (≥5 rick/cell)</th>
<th>5pm of solubilized&lt;sup&gt;b&lt;/sup&gt; &lt;sup&gt;3&lt;/sup&gt;H-labeled polypeptides immune precipitated</th>
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<tr>
<td>46C3</td>
<td>64</td>
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<td>3852</td>
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<td>+</td>
<td>3340</td>
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<tr>
<td>HAT medium</td>
<td>Neg.</td>
<td>-</td>
<td>1427</td>
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</table>

<sup>a</sup> Dialyzed hybridoma supernatants were incubated with live *R. prowazekii* for 30 min at 25°C. The rickettsiae-hybridoma supernatant mixture was added to seven day old human macrophage Leighton tube cultures for 2 hours at 34°C. After washing, the cells were air dried and stained by the method of Gimenez. Two hundred cells were counted for rickettsial content.

<sup>b</sup> Detergent solubilized <sup>3</sup>H-labeled polypeptides were reacted with hybridoma supernatants, followed by absorption with *Staphylococcus aureus* protein A. Absorbed complexes were solubilized with SDS and mercaptoethanol and subsequently counted for <sup>3</sup>H activity.
II. Studies on Rickettsial Nucleic Acids (with Dr. Wm. F. Myers).

A. Studies on the taxonomic relationships among the rickettsiae based on analyses of their deoxyribonucleic acid. Our studies on the genetic relatedness among the various rickettsiae have been continued. We have based our assessment of relatedness on (1) genome size, (2) %G+C, and (3) the degree of DNA-DNA hybridization between any two rickettsial strains. We have continued to apply the optical methods (5, 6) that were described in our last report to determine DNA melting points for %G+C determinations and for reassociation kinetics for determination of genome size and degree of hybridization. These optical methods were employed primarily to complete our study on the relatedness of R. canada to R. prowazekii, R. mooseri, and R. rickettsii. We noted in the last report that these optical approaches do require relatively large amounts of DNA and, thus, their application to the study of relatedness among the spotted fever group is essentially precluded. Therefore, a more sensitive method using the principles of intense radiolabelling of DNA by nick translation methods (7, 12) and the determination of free association kinetics for genome size and degree of hybridization (1, 4, 16) is being adapted.

1. Preliminary studies on the application of the radiolabeled DNA technique to the determination of the degree of DNA hybridization. In the previous report we described in some detail the mechanisms of in vitro labeling by the nick translation method (7, 12). The procedure can be described briefly as follows. The use of pancreatic DNAase I, which produces random nicks, or breaks, in the DNA chain, in conjunction with E. coli DNA polymerase I, which has both exonuclease and polymerase activity, permits the introduction of radioactive nucleotides from a nucleotide pool into a previously unlabeled double-stranded DNA. In this procedure one strand of the DNA acts as a template for that strand being replaced, and thus a faithful copy is made with no loss in chain length (7).

Radiolabeled rickettsial DNA with high specific activity has been prepared from R. canada, R. prowazekii, R. mooseri and R. rickettsii using the nick-translation method. We employed a nick translation kit prepared by New England Nuclear. It was modified, however, by NEN to incorporate dTTP-3H instead of the usual dCTP-32P. Although dCTP can be obtained with a higher specific activity in terms of its radiolabel by employing 32P, it was considered preferable to employ 3H, considering the very short half-life for 32P (14.3 days). The use of the tritium label thus provides a radiolabel stability not otherwise available. Although the commercial kit is normally supplied as the cytidine nucleotide, we have substituted this with the thymidine nucleotide since there is approximately twice as much thymidine as cytidine in rickettsial DNA, and, thus, a higher specific activity is realized.

The kinetics of free-solution reassociation are now well understood and it is possible to adjust the concentrations of the radiolabeled DNA and the unlabeled DNA such that at most only 1-2% of the radiolabeled DNA renamels on itself while greater than 95% of the unlabeled DNA anneals on itself within a given time period. In practice, this involves using a thousand to ten thousand greater quantity of the unlabeled DNA relative to the labeled component. In our current hybridization studies between R. canada, R. rickettsii, R. prowazekii and R. mooseri, we have typically employed 1 ng of labeled DNA (10^4 dpm/ng) which would be allowed to react against 1.5 ug of unlabeled DNA in a total volume of 25 ul. At this concentration of unlabeled DNA (75 ng/ml) essentially complete renaturation could be obtained in approximately 4 hrs. A series of experiments was conducted concerning the kinetics of DNA renaturation, employing both the optical as well as the radiolabel methods. These involved DNA renaturations performed at varying concentrations of labeled and unlabeled DNA as well as variations in the total reac-
tion volume. The results obtained led to the experimental conditions described above. Difficulties were experienced initially when attempting to maintain DNA solutions at elevated temperatures (62°C) and at volumes of less than 50 ul without problems of evaporation and condensation and attendant changes in DNA concentrations. These particular problems have been overcome by using small volume containers (100 ul) which are heated uniformly in aluminum blocks.

After hybridization has occurred by the radiolabel method, there is an additional step which involves the use of the enzyme nuclease $S_1$. Nuclease $S_1$ has the characteristic of being much more active in degrading single-stranded DNA as opposed to the double-stranded form. Previous investigators (1) have studied the conditions for obtaining optimal degradation of single-stranded DNA by nuclease $S_1$, and at the same time minimizing its effect on double-stranded DNA. Optimal results were obtained by these investigators at a pH of 4.8, a NaCl concentration of 0.2M, and a zinc ion concentration of 1 mM. We have adopted these conditions and have applied them in a series of experiments where such other factors as the nuclease $S_1$ concentration, temperature of nuclease reaction, and the time factor were varied. These studies have led to our current protocol. Thus, after the 4 hr hybridization period the hybridization reaction solution is diluted two-fold by the addition of an equal volume (25-50 x1) of nuclease solution (nuclease $S_1$, 2000 units/ml., Zn 50 2 mM; in Na acetate buffer, 0.06 M, pH 4.8). The nuclease solution is pre-heated to the temperature of the hybridization reaction (62°C) prior to its addition. The nuclease reaction is then maintained at 62°C for 40 min. The reaction is stopped in an ice bath, carrier DNA (calf thymus DNA, 50 μg) is added, followed by trichloroacetic acid (10%). After setting in the cold for 10 to 15 min the precipitated DNA is collected on Millipore filters and the radioactivity is determined by liquid scintillation counting.

2. Genetic relatedness between R. canada, R. rickettsii, R. prowazekii, and R. mooseri. In our previous report we reviewed the literature regarding R. canada which suggested that this organism might belong to the typhus group on the basis of certain serological cross-reactions (9). However, other biological properties, including intracellular growth, its pattern of infection in several kinds of ticks, and its transovarial passage (3) suggested a phenotypic kinship to the spotted fever group. Certain studies have suggested that R. canada may be able to cause a severe illness in man clinically similar to Rocky Mountain spotted fever (2).

Our more recent follow-up studies on the taxonomic position of R. canada vis a vis the typhus and spotted fever groups support our previous contention that R. canada is not as closely related to the typhus group as certain earlier serological studies had suggested. This conclusion is based on a continuation of our studies on the genome sizes of R. canada and R. rickettsii as well as additional determinations on the degree of DNA hybridization between R. canada, R. rickettsii, R. prowazekii, and R. mooseri. The degree of DNA hybridization was determined not only by the optical technique but included the more sensitive method of free-solution reassociation of radiolabeled DNA.

a. Genome size of R. canada, R. rickettsii, R. prowazekii, and R. mooseri. We have extended our study of the genome size of R. canada and R. rickettsii. Table 10 summarizes the data obtained from 11 separate evaluations for genome size in R. canada and 12 evaluations in the case of R. rickettsii. The genome size in R. canada was determined to be $1.49 \times 10^9$ daltons, while in R. rickettsii the value was $1.30 \times 10^9$ daltons. A distinct difference is thus observed. Genome size values for other rickettsiae are included for comparison. It should be noted in particular that R. canada DNA ($1.49 \times 10^9$ daltons) is distinctly larger than both R. prowazekii DNA ($1.08-1.14 \times 10^9$ daltons) and R. rickettsii ($1.30 \times 10^9$ daltons). Thus, on the basis of genome size, R. canada is
different from both the typhus and spotted fever groups.

b. **Degree of hybridization** between *R. canada*, *R. rickettsii*, *R. prowazekii*, and *R. mooseri* as measured by the initial rate of renaturation (optical) and radiolabel methods. Table 11 summarizes the results from a series of hybridization experiments involving various hybridization pairs of rickettsial DNA. The data obtained by the optical method had been reported last year and is included here for comparison. In general, there is fairly good agreement between the two methods. Such agreement, thus, strengthens our confidence in the validity of both methods. Since the genomes involved differ in size it is necessary to give two sets of values for each hybridization pair. In the optical procedure the two sets of values are generated by mathematical manipulations of the raw data obtained in a single experiment, while in the radiolabel procedure the two sets of values (genome A and genome B) are generated in two separate experiments, where genomes A and B are separately employed as radiolabels. With the exception of the *R. prowazekii*-*R. mooseri* pair, none of the other hybridization pairings seem to indicate a particularly close genetic relationship, although the values obtained would seem to support the view that they are sufficiently related to each other for continued inclusion within the single genus *Rickettsia*.

3. **Genetic relatedness** among the spotted fever group of rickettsiae. We will shortly complete the preparation of high quality DNA from 20 different spotted fever rickettsiae (established species and isolates from Pakistan, Israel, Thailand, and Czechoslovakia). Approximately 25 to 50 µg of DNA was recovered from each preparation. This is about ten-fold less than realized with the typhus rickettsiae. In our most recent DNA hybridization experiments involving the typhus rickettsiae we have employed hybridization reaction volumes of 25 or 50 µl. At the 25 µl volume we have used a total of 1.5 µg of unlabeled rickettsial DNA per reaction tube. If the volume could be reduced below this figure we could maintain the same DNA concentrations but reduce the total amount of DNA required. There are difficulties, of course, in maintaining such minute volumes at elevated temperatures over a span of several hours. However, with the experience gained from applying these radiolabel procedures to the taxonomic relationships of *R. canada*, we feel reasonably confident that useful data will be forthcoming in regard to spotted fever group rickettsial taxonomy.

**Literature Cited**


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<th>Biogroup/strain</th>
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<td>Experimental^a</td>
<td>Published^b</td>
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<tr>
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^a Our data, see ref. 10 and 11.
^b Data from literature, see ref. 8, 13, 14, 15 and 17.
^c SD, Standard deviation.
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<th>HYBRIDIZATION PAIRS (A + B)</th>
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<th>Genomes B</th>
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Figure 1

Comparison of Cell Viability and Growth in WI-38 Cells (irradiated) and CE Cells under different conditions. The graphs show the change in cell viability and growth over time under various incubation conditions. The plots illustrate the effect of different stimuli on cell loss and the logarithm of cell number (log$_2$N) over time (Incubation Time, h).
Apparent Host-Specific Antirickettsial and Cytotoxic Actions on *R. prowazekii* (Breinl)-Infected Cells (Irradiated) Treated 6 h Post-Infection with Supernatant Fluid from *R. prowazekii*-Stimulated Typhus-Immune Human Leukocyte.

Note that the growth of *R. prowazekii*, in terms of per cent cells infected (p), average number of rickettsiae per cell (N) and average number of rickettsiae per infected cell (N_i), followed expected, established patterns in chicken embryo fibroblasts (CE cells) and human diploid fibroblastic cells (WI-38 cells) in the presence of control medium and the supernatant fluids from unstimulated typhus-immune human leukocytes. However, in WI-38 cells in the presence of *R. prowazekii* stimulated typhus-immune leukocyte supernatant (ET-S), the per cent cells infected (p) began a progressive decline after a lag of a few hours, accompanied by visible evidence of cell loss. After a similar lag, the growth of rickettsiae expressed as N ceased (and in some experiments the curve actually declined). When only infected cells (N_i) were counted, no such break was evident, although in this experiment the slope was less than the control. In other experiments, the slope of \( \lg_2 N_i \) vs time more closely approximated that of the controls. No such effect on growth was apparent in CE cells.
Suspended Cell Uptake:
F-1000 Hu Fibroblasts
Pre-treated with
-○○ Unstim. sup. (CS)
-○○ ET stim. sup. (ET-S)
*R. prowazekii* (Breinl)

$P_I$ (probits)

log t (min)
Figure 2

Effect on Uptake of *R. prowazekii* (Briinl) by F-1000 Human Foreskin Fibroblasts Pre-treated for 18 h with Supernatant Fluids of Unstimulated and *R. prowazekii*-Stimulated Human Typhus-Immune Leukocytes: A Kinetic Study.

Note that pre-treatment of host cells with an active supernatant under conditions which permitted expression of intracellular antirickettsial action (see Figure 2) failed to influence significantly the capacity of *R. prowazekii* to infect them.
Figures 3 and 4

Comparison of total F-1000 cells measured by recovery of attached cells by trypsinization (macroplate method) with those counted in situ in the microtiter method in the case of uninfected cells, *R.* prowazekii-infected cells and infected cells post-treated with ET-S. Note that, in the untreated infected system, the expected progressive loss of cells recoverable by trypsinization (macroplate method) beginning about the end of the first rickettsial growth cycle (ca. 48h) is not detected in the microtiter system which may permit counting of partially degenerated cell ghosts remaining attached to plastic. However, both methods reflect the rapid, early, progressive cell loss in ET-S treated infected cells, which appear to undergo complete lysis, since no great accumulation of detached cells occurs. Cells recoverable by trypsinization appear to be viable by trypan blue exclusion (Fig. 4).

*Figure 4*

Uninfected | Infected | Infected + ET-S
---|---|---
Trypan Blue Exclusion
- Attached Cells*
- Floating Cells

*MACRO-PLATE METHOD*
% Cells viable

*Tested after trypsinization*
RICKETTSIAL GROWTH
(slide chamber method)

MACRO-PLATE METHOD

(% LDH in)

UNINFECTED

Attached Cells

Supernatant

Floating Cells

INFECTED

pi (% "O" time)

log₂ N

Hours

ET-S

INFECTED +
Figure 5

Lactic dehydrogenase loss from F-1000 cells due to cell damage caused by R. prowazekii infection alone in untreated cells and by the action of ET-S on infected cells. These results are consistent with the kinetics of cell damage, shown in Figures 3 and 4, due to infection alone and to cytolitic action of ET-S on infected cells. In the case of untreated infected cells, the pattern of LDH loss from attached cells is similar to that of the loss of trypsin-resistant cells.
Figure 6

MACRO-PLATE METHOD

LDH (% per Dish)

Attached
- - Uninfected
- - Infected

Floating:
- - Uninfected
- - Infected

Supernate:
- - Uninfected
- - Infected

ET-S PRE

CM

ET-S POST

MICROTITER METHOD

Cells (% of inoculum)

0 50 100

0 50 100

0 50 100

RECSIAL GROWTH

t (h)

0 50 100

0 50 100

0 50 100

Incubation Time (h)
Figure 6

Failure of ET-S pre-treated F-1000 cells infected with R. prowazekii and exhibiting intracellular anti-rickettsial action to show significant evidence of cell loss or damage by microtiter method or LDH release as compared with ET-S post-treated infected cells, which reproduce the findings of earlier experiments (Figs. 3-5).
Figure 7

**CONTROL MEDIUM**

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<th>Attached (tryp)</th>
<th>Floating</th>
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<tr>
<td>Inf.</td>
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<td>Uninf.</td>
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**MACRO-PLATE METHOD**

- Cells (% "O" time)
- Time (0-100)

**MICROTITER METHOD**

- Cells (% "O" time)
- Time (0-100)

**IN SITU TRYPAN BLUE**

- % Cells Viable
- Control, Pre, Post

Legend:
- Infected
- Uninfected

Graphs show the comparison of infected and uninfected cells in different media conditions (Control Medium, ET-S PRE, ET-S POST).
Failure of ET-S pretreated F-1000 cells infected with R. prowazekii and exhibiting intracellular antirickettsial action to show cell loss by either microtiter method or trypsinized cell count in macroplate method or for attached cells in situ to show damage detectable by trypan blue exclusion. In contrast, post-treatment of infected cells with ET-S causes rapid, early cell loss by both methods, as well as evidence of increasing cell damage in situ by trypan blue exclusion.

Figure 8

[Graph showing slide chamber growth with incubation time for control, ET-S pre, and ET-S post conditions]
Intracellular antirickettsial action by pre-treatment with ET-S of 6-day cultures of human peripheral blood monocyte-derived macrophages infected with R. prowazekii.
Intracellular antirickettsial action by pre-treatment with ET-S of human umbilical vein endothelial cell cultures infected with *R. prowazekii*.
Figure 11

This figure illustrates the quantitative aspects of human macrophage infection by typhus group rickettsiae. For each of the four experimental conditions there is plotted, (1) a measure of macrophage viability (upper portion of the figure), and (2) directly below that the corresponding rickettsial growth cycle data. The kinetics of the rickettsial growth cycles (lower portion of the figure) are illustrated by two parameters: (1) the percentage of macrophages which were infected (o--o) and (2) the adjusted number of rickettsiae per cell expressed on log_{2} scale (o--o). Data points are an average of at least 3 experiments for each of the four conditions.
Figure 12

No. Rickettsiae per Cell

% Cells with Indicated Rickettsiae

48 hrs

R. mooseri (WIM)

R. prowazekii (Bretl)

Hu F-1000 Fibroblasts

Hu Macrophages

0

1-5

6-10

11-15

16-20

>20
This figure illustrates the distribution of human macrophages and human F-1000 fibroblasts based on their intracellular content of R. prowazekii or R. mooseri at 0 and 48 hours post infection. The data are expressed as the percentage of cells (ordinate) at a particular time interval which contain a specified range of rickettsia (abscissa). The distribution of R. prowazekii infected human fibroblasts is relatively homogeneous at both the 0 and 48 hour time intervals. The distribution of R. prowazekii infected human macrophages at 48 hours is not homogeneous as evidenced by the existence of a subpopulation of cells with 20 rickettsiae/cell and one with 1-5 rickettsiae/cell. R. mooseri, which is known to spread from cell to cell beginning at the early stages of its infection cycle (1), does not exhibit a significantly different distribution pattern of infected human macrophages when compared to infected human fibroblasts.
This figure illustrates the cytopathology associated with the introduction of typhus group rickettsiae into in vitro cultivated human macrophages. These ultrathin sections are from macrophage cultures which were infected 24 hours previously with an average of 2-4 organisms/cell. Figure 11A is a representative NHS-treated R. prowazekii infected macrophage which is undergoing a cytolytic process. The cell contains severely swollen endoplasmic reticulum (arrows), swollen mitochondria (m), clumped nuclear chromatin, and a decrease in the density of its cytoplasmic sap. Figure 11B illustrates a typical NHS-treated R. prowazekii infected macrophage which does not exhibit the cytopathology like the cell in (A). The rickettsiae (r) are replicating in the cell's cytoplasm which is of moderate density and contains morphologically normal mitochondria (m) and endoplasmic reticulum (arrow) which is not swollen. Figure 11C is an ultrathin section of an NHS-treated R. mooseri infected macrophage which exhibits ultrastructural details which are similar to those seen in (B). The ultrastructural details of infected cells in frames (B) and (C) do not differ from those of uninfected controls. (Bar 1 μ).
Figure 14

SDS-PAGE Analysis of *R. prowazekii* Polypeptides. Specific labeling of rickettsiae was accomplished by using emetine (1 μg/ml) to inhibit L-cell protein synthesis. Cultures were harvested (72 hours post infection) after 18-24 hours of isotope (3H-leucine, 50 uCi/ml) incorporation by solubilizing the monolayer with hot electrophoresis sample buffer (4). Lanes B and C are L-cells labeled in the absence and presence of emetine, respectively. Five *R. prowazekii* strains are represented in this gel; flying squirrel strain GVF-12 (lane D); Burundi isolate X-16 (lane E); Burundi isolate V-59 (lane F); avirulent E strain (lane G); Breinl (lane H); and also Renografin purified Breinl (lane I). Lanes A and J are molecular weight markers with their corresponding molecular weights in the right hand column. The slightly lower molecular weight 31K polypeptide of the E strain is indicated by the arrow.
Figure 15

Surface Polypeptides of R. prowazekii and R. mooseri. Renografin purified rickettsiae, surface-labeled with 125-I, are in lanes B (R. mooseri), C (R. prowazekii, E strain) and D (R. prowazekii, Breinl strain). SDS-solubilized R. prowazekii (Breinl) polypeptides, that were iodinated after solubilization, are in lane A. On the right are \(^3\)H-polypeptide profiles of the Breinl (lane E) and E strains (lane F) of R. prowazekii, and R. mooseri (lane G). The gel on the left (lanes A-D) is an autoradiograph while the gel on the right (lanes E-G) is a fluorograph.
Figure 16

SDS-PAGE Analysis of Immune Serum Precipitated Polypeptides of *R. prowazekii* Strains. Detergent (1% Triton X-100, 0.5% deoxycholate in PBS) solubilized $^3$H-labeled polypeptides of 5 *R. prowazekii* strains were incubated with human convalescent serum for 18 hours at 4°C, followed by absorption with *Staphylococcus aureus* protein A. The absorbed complexes were washed and subsequently solubilized with hot electrophoresis sample buffer. Immune precipitates of the following strains are shown: flying squirrel GVF-12 (lane B); Burundi X-16 (lane D); Burundi V-59 (lane F); Madrid E (lane H), and Breinl (lane J). Non-immune serum controls were run with each strain (lanes A, C, E, G, I). Lane K is a $^3$H-labeled polypeptide profile of *R. prowazekii* (Breinl).
Immune Precipitation of the 31K Polypeptide of *R. prowazekii*. Surface-labeled (125-I) *R. prowazekii* were solubilized in 1.0% Triton X-100, 0.5% deoxycholate and 0.1% sodium dodecyl sulphate (TDS) containing 7.2 M guanidine HCl. After centrifugation, the supernatant was dialyzed against TDS. The dialysate was used for immune precipitations. Lane A is surface-iodinated *R. prowazekii* (Breinl), lane B is a dialysate, lane C is immune serum treated dialysate absorbed with Staph protein A, and Lane D is non-immune-serum treated dialysate absorbed with protein A.
SDS-PAGE Analysis of $^3$H-labeled *R. prowazekii* Polypeptides Precipitated by Monoclonal Antibody. Hybridoma culture supernatants were incubated with TDS solubilized *R. prowazekii* polypeptides followed by immune absorption with *Staph aureus* protein A. Absorbed complexes were solubilized with hot electrophoresis sample buffer and subsequently electrophoresed. The clones tested are characterized further in Table 8. IHS is immune human serum and HAT is hypoxanthine, aminopterin, thymidine medium.
III. PUBLICATIONS


The following papers were presented at the Second Conference on Rickettsiae and Rickettsial Diseases which was held from 2-6 Sept 1980 at the Rocky Mountain Laboratory, Hamilton, Montana. They are in press in the Proceedings of the Conference to be published by Academic Press.

5. Wisseman, C.L. Jr. Some biological properties of rickettsiae.


9. William F. Myers, Charles L. Wisseman, Jr. The taxonomic relationship of Rickettsia canada to the typhus and spotted fever groups of the genus rickettsia.

The following papers on rickettsiae, not related to this contract, were also presented at the Conference and are also in press.


12. Robert Traub and William L. Jellison. Evolutionary and biographic history and the phylogeny of vectors and reservoirs as factors in the transmission of diseases from other animals to man.
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Walter Reed Army Medical Center  
ATTN: SGRD-UWZ-AG  
Washington, DC 20012 |
| 4      | HQDA      | U.S. Army Medical Research and Development Command  
Fort Detrick  
Frederick, MD 21701 |
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