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STUDIES ON TYPHUS AND SPOTTED FEVER
ANNUAL PROGRESS REPORT

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by

Charles L. Wisseman, Jr., M. D.

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University of Maryland School of Medicine
Department of Microbiology
Baltimore, Maryland 21201

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The studies reported here suggest that one of the mechanisms by which cell mediated immunity may control and kill intracellular rickettsiae is through a sequence which begins with an immunologically specific step in which immune T lymphocytes are stimulated by rickettsial antigens to produce a soluble factor which induces in rickettsia-infected cells in a non-immunologically specific reaction (1) an antirickettsial action which is dependent upon new host cell protein synthesis and (2) a cytolytic action specific for infected cells which is not dependent upon the synthesis of proteins by either host cell or rickettsia.		

20. ABSTRACT (continued)

Within the limits of specificity and purity of the reagents which were available at the time of these studies, the major contributor to both of these actions has been identified as gamma or immune interferon. It is currently unknown if other components of the complex leukocyte supernatant fluids contribute to reactions of this kind in a minor or enhancing manner. *Keywords: Reaction kinetics;*

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SUMMARY

The studies reported here suggest that one of the mechanisms by which cell mediated immunity may control and kill intracellular rickettsiae is through a sequence which begins with an immunologically specific step in which immune T lymphocytes are stimulated by rickettsial antigens to produce a soluble factor which induces in rickettsia-infected cells in a non-immunologically specific reaction (1) an **antirickettsial action** which is dependent upon new host cell protein synthesis and (2) a **cytolytic action** specific for infected cells which is not dependent upon the synthesis of proteins by either host cell or rickettsia. Within the limits of specificity and purity of the reagents which were available at the time of these studies, the major contributor to both of these actions has been identified as **gamma or immune interferon**. It is currently unknown if other components of the complex leukocyte supernatant fluids contribute to reactions of this kind in a minor or enhancing manner.

IMMUNE MECHANISMS IN TYPHUS

Background. A significant component of this contract over the years has been the elucidation of the basis for immunity against typhus infections. Studies in guinea pigs and mice had shown that immune T lymphocytes, but not immune serum, controlled Rickettsia mooseri replication at the site of intracellular growth in infected tissues. Only guinea pigs that showed the cell mediated immunity (CMI) correlate of typhus-specific MIF production were immune to challenge with viable organisms. The CMI correlates of typhus specific delayed type hypersensitivity and of lymphocyte blast transformation were demonstrated in human beings had experienced infection with virulent R. prowazekii or R. mooseri or with the attenuated living E strain R. prowazekii typhus vaccine and who were immune to these diseases. These findings suggested that CMI is a major contributor to immunity in typhus worthy of further investigation.

Activation of macrophages by antigen-specific mechanisms for non-specific enhanced microbicidal action is one accepted effector mechanism of CMI which had received much early attention as an effector mechanism of CMI in facultative intracellular infections such as tuberculosis. However, our studies with R. mooseri in mice (see previous annual reports) suggested that this mechanism was probably not responsible for most of the T lymphocyte mediated control of rickettsial replication in tissues. Some other effector mechanism of CMI must be operational in typhus infection.

In the course of our continuing studies on immune mechanisms, a new phenomenon was discovered (see last year's annual report) - namely, that the supernatant fluids from cultures of typhus antigen-stimulated human typhus immune blood leukocytes or from phytohemagglutinin-stimulated leukocytes from nonimmune subjects have two distinct, separable effects on R. prowazekii-infected cultures of human fibroblasts, endothelial cells and macrophages: (1) an apparent antirickettsial effect on intracellular rickettsiae and (2) cytolysis of rickettsia-infected cells. These observations suggested that one or more soluble mediators of CMI are produced by the immunological specific stimulation of immune leukocytes and that these factor(s) then act upon infected cells in such a way as to cause the destruction of intracellular rickettsiae and the specific lysis of infected cells. The results of the studies suggested the possibility that two separate factors might be involved.

Progress in Past Year. The studies of the past year have been directed largely at characterizing the phenomenon described above and in attempting to identify the factor(s) involved.

Test Systems. The studies described herein used Rickettsia prowazekii (Breinl strain) as test organism and F-1000 diploid human fibroblasts as host cell. The source of active factor(s) was the supernatant fluid from R. prowazekii-antigen stimulated cultures of human typhus immune blood leukocytes (ET-S) and from phytohemagglutinin-stimulated human nonimmune blood leukocytes (PHA-S). In later studies, either international standard human IFN preparations or commercial highly purified natural human IFN preparations were tested for activity and commercial rabbit polyclonal anti-IFN sera were used in neutralization tests.

Routine tests for antirickettsial action were performed in slide chamber cultures of F-1000 cells which had been pretreated for ca. 18 h with the desired factor, washed, infected with rickettsiae and the intracellular rickettsiae counted microscopically after Gimenez staining at predetermined intervals to determine the fate of the rickettsiae. In this system there was no detectable lysis of infected cells.

Routine tests for cytolytic action were performed in microtiter plates bearing 96 flat-bottomed wells. Rickettsia-infected cells or uninfected controls were distributed to the wells, allowed attach and then covered with medium containing the desired factors or controls. The fate of the cells, i.e., growth, cytostasis or cytolysis, was determined at intervals up to about 48 hours. In early studies, the number of cells per well was determined by directly counting them under a binocular microscope after staining with crystal violet. Later, this was modified by eluting the crystal violet retained by the cells in an alcoholic solution and reading the optical density on an automated plate reader. There was a good correlation between the two methods, as determined by rather extensive testing. This latter optical method greatly facilitated studies of the cytolytic phenomenon.

Antirickettsial Action. Major findings of the studies on the intracellular antirickettsial action of leukocyte culture supernatants are summarized below.

1. In all instances studied, the ET-S and PHA-S preparations behaved in an indistinguishable manner.

2. Loss of rickettsiae from cytokine¹ pretreated cells

¹ Since the active supernatant fluid were generated by stimulating cultures of the mixed leukocytes present in blood, the cellular source of the active principle(s) cannot be specified at this time. Hence, the noncommittal term, cytokine, is used instead of the commonly, but inappropriately anticipatory, used term, lymphokine.

as measured microscopically in Gimenez-stained preparations was paralleled by a loss of rickettsial bodies detectable by staining with fluorescein-labeled typhus immune serum and by a loss of infectious units (PFU) recoverable by the plaque technique. Thus, the observed loss of stainable rickettsiae reflects an intracellular rickettsiacidal action. Since, as documented in the previous report, the active supernatant fluids displayed no direct antirickettsial action on extracellular organisms, the antirickettsial action may be indirect, mediated in some manner by the host cell.

3. Active supernatant fluids did not induce the antirickettsial action in F-1000 cells in the presence of mitomycin C, actinomycin D or cycloheximide. This suggests that the antirickettsial action depends upon the synthesis of protein by the host cell from newly transcribed messenger RNA.

4. The characteristics of the antirickettsial action of the cytokine preparations from blood leukocytes are reminiscent of the antiviral action of an interferon (IFN). The sensitivity to inactivation by acid and heat, described in the previous report, is consistent with the properties of immune or gamma interferon.

Cytolytic Action. Major characteristics uncovered for the cytolytic action are summarized below.

1. Cytokines from both immune and nonimmune sources, ET-5 and PHA-S, behaved in an indistinguishable manner in all cytolytic systems studied.

2. The cytolytic action was confined to, and selective for, rickettsia-infected cells. Uninfected "by-stander" cells were not lysed. This suggests that sensitivity to lysis is a unique property of infected cells and is not the result of the production of a diffusible non-specific secondary cytotoxin which might lyse any cell in the vicinity.

3. Cells infected and maintained in the presence of chloramphenicol at a concentration several times the MIC for R. prowazekii are lysed by active leukocyte supernatant fluids. Thus, the mere entry of non-replicating rickettsiae whose protein synthesis is inhibited induces susceptibility to lysis by active leukocyte supernatants.

4. Infected cells whose protein synthesis is inhibited are lysed by ET-5.

5. Although the cytolytic action of stimulated leukocyte supernatant fluids is destroyed by acid and heat, its insensitivity to inhibition of host cell protein synthesis is not consistent with the usual concept of interferon action. The possibility remains that the two types of action, antirickettsial

and cytolytic, may be induced by different components of the leukocyte supernatants which are known to be complex mixtures of biologically active substances.

Neutralization Tests. Because certain key features of the antirickettsial action were consistent with an interferon-like action whereas the cytolytic action had certain features in common with other cytokines, such as lymphotoxin, attempts were made to neutralize these actions in ET-S and PHA-S with the then available polyclonal rabbit antisera against natural human interferons and lymphotoxin. (Remember that at this time gamma interferon had just been discovered and that monoclonal antibodies against these substances were not yet available, nor were recombinant interferons yet available.)

Both the antirickettsial action and the cytolytic action were strongly neutralized by antiserum against gamma interferon but not by the antisera against alpha interferon, beta interferon or lymphotoxin.

Action of Interferons and Lymphotoxins on R. prowazekii-Infected Cells. Commercial preparations of highly purified and highly active preparations of natural human alpha, beta and gamma interferons, international standard preparations of natural human alpha and beta interferon (a standard preparation of gamma interferon was not yet available) (NIH) and a preparation of natural lymphotoxin (Granger) were tested for antirickettsial and cytolytic action.

Gamma interferon preparations, but not those of alpha interferon, beta interferon or lymphotoxin, exhibited strong antirickettsial and cytolytic actions indistinguishable in kinetics or otherwise from those induced by ET-S and PHA-S.

Unfortunately, this contract was terminated at this exciting stage. (However, it may be noted that the major findings reported here have subsequently been confirmed under other auspices with monoclonal antibodies and recombinant interferons, thus eliminating the possibility that they were due to minor contaminants of other biologically active molecules present in the natural products.)