AD _____

BIOCHEMICAL BASIS OF VIRULENCE IN EPIDEMIC TYPHUS

Annual Progress Report

Herbert H. Winkler, Ph.D.

August 1980

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-79-C-9018

University of South Alabama
College of Medicine
Mobile, Alabama 36688

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. A134 277	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) BIOCHEMICAL BASIS OF VIRULENCE IN EPIDEMIC TYPHUS		5. TYPE OF REPORT & PERIOD COVERED Annual Report for contract awarded 1-1-80 to 12-31-80.
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Herbert H. Winkler		8. CONTRACT OR GRANT NUMBER(s) DAMD17-79-C-9018
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Microbiology and Immunology University of South Alabama, College of Medicine Mobile, Alabama 36688		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A.3M161102BS10.AC.052
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701		12. REPORT DATE August, 1980
		13. NUMBER OF PAGES 45 pages
14. MONITORING AGENCY NAME & ADDRESS(If different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)
Approved for Public Release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
Rickettsia, Macrophage, Virulence, Typhus, Plasmid, Fatty-acid-composition;

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The avirulent, Madrid E, and virulent, Breinl, strains of Rickettsia prowazeki were compared with respect to their interaction with (a) mouse macrophage-like cell lines, (b) a human macrophage-like cell line, (c) mouse peritoneal macrophages and (d) guinea pig peritoneal macrophages. Furthermore, the fatty acid composition and plasmid DNA content of these strains were compared.

The mouse macrophage-like cell lines and the guinea pig peritoneal

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

(con't, no. 20)

macrophages could differentiate between the strains. The avirulent strain was eliminated and the virulent strain grew within the cytoplasm of these cells. The human macrophage-like cell line and the mouse peritoneal macrophages, on the other hand, could not distinguish between the two strains. Mouse macrophages destroyed both strain and both strains grew in the human macrophage-like cell line.

The fatty acid composition of the two strains were compared. The hypothesis was that the high level of unsaturated fatty acid known to be in the avirulent strain might not occur in the virulent strain and that this might be a target for selective killing via lipid peroxidation. Our investigation established that the fatty acid composition was not different in the two strains.

Plasmids are often a source of virulence factors. The two strains were compared and neither was found to have detectable plasmid DNA.

AD _____

BIOCHEMICAL BASIS OF VIRULENCE IN EPIDEMIC TYPHUS

Annual Progress Report

Herbert H. Winkler, Ph.D.

August 1980

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-79-C-9018

University of South Alabama
College of Medicine
Mobile, Alabama 36688

SUMMARY

The purpose of the work is to elucidate the biochemical basis of virulence in epidemic typhus. The basic method at this stage of the project is a comparison of the E strain and Breinl strain (avirulent and virulent, respectively) of Rickettsia prowazeki and their interaction with the host defense system.

The avirulent, Madrid E, and virulent, Breinl, strains of Rickettsia prowazeki were compared with respect to their interaction with a) mouse macrophage-like cell lines, b) a human macrophage-like cell line, c) mouse peritoneal macrophages and d) guinea pig peritoneal macrophages. Furthermore, the fatty acid composition and plasmid DNA content of these strains were compared.

The mouse macrophage-like cell lines and the guinea pig peritoneal macrophages could differentiate between the strains. The avirulent strain was eliminated and the virulent strain grew within the cytoplasm of these cells. The human macrophage-like cell line and the mouse peritoneal macrophages, on the other hand, could not distinguish between the two strains. Mouse macrophages destroyed both strain and both strains grew in the human macrophage-like cell line.

The fatty acid composition of the two strains were compared. The hypothesis was that the hihg level of unsaturated fatty acid known to be in the avirulent strain might not occur in the virulent strain and that this might be a target for selective killing via lipid peroxidation. Our investigation established that the fatty acid composition was not different in the two strains.

Plasmids are often a source of virulence factors. The two strains were compared and neither was found to have detectable plasmid DNA.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences-National Research Council.

ANNUAL REPORT TABLE OF CONTENTS

List of Table and Figures

Differentiation of Virulent and Avirulent Strains of *Rickettsia prowazeki* by Macrophage-like Cell Lines

Jenifer Turco and Herbert H. Winkler

Department of Microbiology and Immunology, University of South Alabama, College of Medicine, Mobile, Alabama 36688.

Abstract

Avirulent (Madrid E) and virulent (Breinl) strains of Rickettsia prowazeki were tested for their ability to grow in four mouse macrophage-like cell lines (RAW264.7, J774.1, P388D1, and PU5), one human macrophage-like cell line (U937-1) and the mouse fibroblast line L-929. All cells were X-irradiated prior to infection with rickettsiae.

The E and Breinl strains grew equally well in L-929 cells. However, all of the mouse macrophage-like cell lines clearly restricted the growth of the E strain relative to that of the Breinl strain. Most of these cell lines exhibited a non-uniform response to infection with the E strain such that E strain rickettsiae were cleared from the majority of the infected cells, but multiplied in some of the remaining infected cells. The human line U937-1 was much less effective at restricting the growth of the E strain than were the mouse macrophage-like cell lines.

Treatment of the Breinl or E strains of R. prowazeki with rabbit antirickettsial antiserum prior to infection of L-929 cells caused a marked decrease in the initial infection but had no effect on the subsequent growth of the rickettsiae. In contrast, such treatment of Breinl or E strain rickettsiae prior to infection of macrophage-like cell lines caused no change or an increase in the initial infection. In mouse macrophage-like cell lines infected with antiserum-treated Breinl strain rickettsiae, the percentage of cells infected and the average number of rickettsiae per cell were markedly depressed in comparison with the controls at 24 and 48 hours after infection. The human macrophage-like cell line U937-1 was less effective than the mouse macrophage-like cell lines at clearing antiserum-treated rickettsiae.

These data indicate that mouse macrophage-like cell lines should be useful for studying rickettsia-macrophage interactions and for defining the biochemical basis of virulence in R. prowazeki.

INTRODUCTION

Ability to resist destruction in the phagocytic cells of the host is a definite advantage for an obligate intracellular parasite. Yet relatively few studies have dealt with the fate of Rickettsia species in macrophages.

Nacy and Osterman (11) recently reported that Rickettsia tsutsugamushi multiplied within resident mouse peritoneal macrophages, and that treatment of the rickettsiae with immune serum prior to infection of the macrophges led to destruction of many (though not all) of the rickettsiae within the macrophages. In addition, they along with Nacy and Meltzer (10), provided evidence that activated macrophages are involved in host defense against R. tsutsugamushi .

Gambrill and Wisseman (6) showed that both Rickettsia typhi and the virulent Breinl strain of Rickettsia prowazeki could multiply in human monocyte-derived macrophages. Incubation of either organism with immune serum prior to infection of human macrophages resulted in destruction of the rickettsiae within the cells (1,5). Unlike the virulent Breinl strain, the avirulent E strain of R. prowazeki failed to multiply in most human macrophages (6). This observation suggested that detailed study of the interaction of R. prowazeki and macrophages might allow definition of the basis for the difference in virulence between the E and Breinl strains.

In recent years several continuous macrophage-like cell lines have been developed (4,7,12). Each cell line exhibits some, though not all, of the properties characteristic of macrophages. Because these cells can easily be grown in culture and would readily lend themselves to a variety of studies, we have examined the interaction of avirulent and virulent strains of R. prowazeki with several macrophage-like cell lines.

MATERIALS AND METHODS

Cell cultures . RAW264.7 and J774.1 cells were obtained from the Cell Distribution Center at the Salk Institute. P388D1 and PU5 cells were obtained from Dr. Janet Oliver. U937-1 cells were provided by Dr. Hillel S. Koren. L-929 cells were purchased from Flow Laboratories.

Mouse macrophage-like cell lines were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum (P388D1 and PU5), 10% calf serum (RAW264.7), or 7.5% fetal calf serum and 7.5% horse serum (J774.1). The human macrophage-like cell line U937-1 was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. The mouse fibroblast line L-929 was grown in Eagle's Minimum Essential Medium supplemented with 10% calf serum. All cell cultures were maintained in tissue culture dishes or flasks and were kept in a CO_2 incubator at 34°C. The cell lines J774.1 and RAW264.7 grew attached to the substratum; the cell lines PU5 and P388D1 grew both attached to the substratum and suspended in the medium; the cell line U937-1 grew suspended in the medium.

Rickettsiae. Rickettsia prowazeki Madrid E and Breinl strains were grown in 6-day embryonated, antibiotic-free chicken eggs. Eggs were inoculated from seed pools prepared from the Madrid E strain or the Breinl strain. Rickettsiae were harvested and purified from infected yolk sacs 8 days after inoculation. Methods of rickettsial purification were modified from those of Bovarnick and Snyder (3) and Wisseman et al. (18) as described previously (16).

Purified rickettsiae were suspended in the sucrose phosphate glutamate solution of Bovarnick et al. (2) and were stored at -70°C in 0.2 ml aliquots. Viable rickettsiae were enumerated by the antibody hemolysis method of Walker and Winkler (15), and total rickettsia-like bodies were enumerated by a modification of the method of Silverman, Fiset, and Wisseman (14).

Infection of cells with rickettsiae. All cells were x-irradiated in a G.E. Maximar 100 prior to infection with rickettsiae at a dose adequate to prevent cell division without causing loss of metabolic integrity. RAW264.7 and J774.1 cells were irradiated with 3000 rads; L-929 cells were given a dose of 5000 rads. For U937-1, PU5, and P388 cells, doses ranging from 700 to 1500 rads were used. After irradiation, RAW264.7 or J774.1 cells were adjusted to a density of 2.5×10^5 viable (trypan blue excluding) cells/ml and were planted in 8-chambered Lab-Tek slides (0.3 ml per chamber). L-929 cells were adjusted to a density of 1.0×10^5 viable cells/ml and were similarly planted in chamber slides. The cells were then incubated at 34°C for 36 to 48 hours prior to infection. PU5, P388D1, and U937-1 cells were infected in suspenion in siliconized glass tubes at a density of 1.0×10^6 cells/ml immediately after irradiation.

Rickettsiae were diluted in Hank's Balanced Salts Solution supplemented with 0.1% gelatin and 4.9 mM L-glutamic acid, monopotassium salt. Rickettsiae were added to cells which had been washed once in this solution at multiplicities ranging from 30 to 80 viable rickettsiae per cell for RAW264.7 or J774.1 cells, and from 75 to 200 viable rickettsiae per cell for L-929 cells. For cells infected in suspension (PU5, P388D1, and U937-1) multiplicities ranging from 10 to 50 viable rickettsiae per cell were used. Cells and rickettsiae were incubated together for one hour at 34°C. The cells were then washed twice, given fresh medium, and incubated at 34°C for 48 hours. At 0 hours, 24 hours, and 48 hours after infection, chamber slides were

washed gently in 0.85% NaCl and were dried with a warm air stream. Cell smears were prepared from suspension cultures by cytocentrifugation. All slides were fixed in 1% formalin in 0.1M sodium phosphate buffer, pH 6.8 . The slides were washed in three changes of buffer and were then stained by a modification of the Gimenez method, as described by Wisseman et al . (19).

All treatments were done in duplicate at each time point. Slides were examined microscopically under oil immersion and the number of rickettsiae present in each of 100 cells was counted for each duplicate of each treatment. When rickettsiae were present in clumps, the number present was estimated. When a cell contained over 100 rickettsiae, it was assigned a value of 100. From the data collected the percentage of cells infected with rickettsiae (%R), the number of rickettsiae per infected cell (IR), and the average number of rickettsiae per cell (NR) were determined. Much of the data was normalized to the zero time infection, e.g., NR at a given time divided by NR at time zero times 100 gives the average number of rickettsiae per cell as a percentage of that present at zero time.

ANTISERUM . Antiserum was prepared in rabbits immunized with Madrid E strain rickettsiae in adjuvant as previously described (17). Antiserum was heat-inactivated at 56°C for 1 hour.

RESULTS

Growth of the Madrid E and Breinl strains in L-929 cells or in macrophage-like cell lines.

The experiments described here were performed with several different preparations of the E and Breinl strains of Rickettsia prowazeki . The range of multiplicities of infection used in these experiments resulted in infection of from 25-93% of the cells with rickettsiae, and an average of 0.3 to 7.1 rickettsiae per cell immediately after infection.

The Madrid E and Breinl strains grew equally well in L-929 cells (Fig. 1). During a 48 hour period, the percentage of L-929 cells infected remained constant or increased slightly and the average number of rickettsiae per cell increased about 30-40 fold. The growth of the Breinl strain in RAW264.7 cells was similar to the growth of the E or Breinl strains in L-929 cells. In contrast, the E strain grew poorly in RAW 264.7 cells. Although no change in the percentage of E strain-infected RAW264.7 cells was apparent at 5 hours or 12 hours after infection, by 24 hours after infection, the percentage of E strain-infected RAW264.7 cells had dropped to about 47% of its initial value. At 48 hours after infection, the percentage of RAW264.7 cells infected with the E strain was only about one-third of its initial value. Some of the remaining infected cells, however, supported the growth of the E strain. The number of E rickettsiae per infected RAW264.7 cell increased about 7 fold in this small population, whereas the number of Breinl rickettsiae per infected cell increased about 20 fold during a 48 hour period.

RAW264.7 cells phagocytized E and Breinl strain rickettsiae which had been killed by heating at 45°C for 2 1/2 - 3 hours. Clearance of such rickettsiae was not complete by 24 hours after infection, but was almost complete by 48 hours after infection. We were concerned that in some cases our suspensions of E and Breinl rickettsiae contained different percentages of dead rickettsiae. We therefore performed some experiments with RAW264.7 cells using E and Breinl rickettsial suspensions which fortuitously contained equivalent percentages of dead rickettsiae. In other experiments killed rickettsiae were added to one of the 2 rickettsial suspensions to make the percentages of dead rickettsiae equivalent. The results in these experiments were similar to those in which no adjustment was made.

The growth of the E and Breinl strains in J774.1 cells was very similar to that observed in RAW264.7 cells (Fig. 1). Once again, the Breinl strain grew well and the E strain grew very poorly.

The remaining two mouse macrophage-like cell lines tested also restricted the growth of the Madrid E strain relative to that of the Breinl strain. The E strain failed to grow in P388D1 cells (Fig. 1); all parameters used to assess rickettsial growth decreased during a 48 hour period. However, during the same period in Breinl-infected P388D1 cells, all rickettsial growth parameters increased; there was about a 13 fold increase in the average number of rickettsiae per cell. The Breinl strain multiplied to a similar extent during a 48 hour period in PU5 cells (Fig. 1). Once again, the growth of the E strain in these cells was poor . The percentage of PU5 cells infected with the E strain decreased and the average number of rickettsiae per cell increased slightly.

The human macrophage-like cell line, U937-1, was much less effective than the mouse macrophage-like cell lines at restricting the growth of the E strain

(Fig. 1). Although the percentage of U937-1 cells infected with the E strain decreased slightly during a 48 hour period, the average number of rickettsiae per cell increased about 6 fold. In U937-1 cells infected with the Breinl strain, the percentage of cells infected increased slightly and the average number of rickettsiae per cell increased about 16 fold during a 48 hour period.

Effect of antiserum treatment of rickettsiae on their ability to infect and grow in L-929 cells or macrophage-like cell lines. The addition of 10% normal rabbit serum to suspensions of the Breinl strain of R. prowazeki just prior to infection of L-929 or RAW264.7 cells had no effect on the initial infection (Table 1) or on the ability of the rickettsiae to grow in either of these cell lines (Fig. 1.). In contrast, the addition of 10% rabbit antirickettsial antiserum to rickettsial suspensions (Madrid E or Breinl strains) just prior to infection of L-929 cells caused a marked decrease in the initial infection (Table 1). Although the percentages of L-929 cells infected with antiserum-treated rickettsiae were much reduced in comparison with the values for cells infected with untreated rickettsiae, these percentages remained constant during a 48 hour period (Fig. 1). The average number of rickettsiae per cell in L-929 cultures infected with antiserum-treated rickettsiae increased similarly to that observed in L-929 cultures infected with untreated rickettsiae.

The interaction of antiserum-treated rickettsiae and mouse macrophage-like cell lines differed significantly from that of antiserum-treated rickettsiae and L-929 cells in at least two respects. Firstly, antiserum treatment of the rickettsiae prior to infection of the mouse macrophage-like cell lines caused an increase in or had no effect on the initial infection (Table 1). Secondly, most of the antiserum-treated rickettsiae (Breinl, as well as E strain) disappeared from the cells during a 48 hour period (Fig.1). There were occasional cells in these experiments with antiserum-treated rickettsiae which manifested rickettsial growth and which account for the slight increases in the number of rickettsiae per cell after 48 hours.

In the case of U937-1 cells infected with antiserum-treated Breinl strain R. prowazeki , both the percentage of cells infected and the average number of rickettsiae per cell decreased during the first 24 hours incubation, although the number of rickettsiae per infected cell doubled (Fig. 1). From 24 to 48 hours, however, all rickettsial growth parameters increased. Hence antiserum-treated rickettsiae were less effectively destroyed in U937-1 cells than in the mouse macrophage cell lines.

DISCUSSION

Although many properties of mouse macrophage-like cell lines have been described (9) very little is known about the bactericidal potential of these cells. All of the mouse macrophage-like cell lines tested in this study clearly differentiated the avirulent E and virulent Breinl strains of R. prowazeki by restricting the growth of the former and supporting that of the latter. In contrast, resident or elicited mouse peritoneal macrophages have been shown to clear both the E and Breinl strains of R. prowazeki (Winkler and Daugherty, this report). The mechanism(s) by which R. prowazeki is killed in mouse macrophage-like cell lines and in mouse macrophages are not known. It is

tempting, however, to speculate that (1) killing of R. prowazeki occurs by different mechanisms in mouse macrophages and mouse macrophage-like cell lines or that (2) killing of R. prowazeki occurs by the same mechanism in both types of cells, but that some step in the killing process occurs more slowly or operates less efficiently in mouse macrophage-like cell lines than in mouse macrophages. Our results with mouse macrophage-like cell lines are similar to those of Gambrill and Wisseman (6), who infected human macrophages with the E and Breinl strains. Interestingly, the human macrophage-like cell line U937-1 was less effective than the mouse macrophage cell lines at restricting the growth of the E strain. Both strains of rickettsiae grew equally well in the mouse fibroblast line L-929.

The responses of four mouse macrophage-like cell lines to infection with the E and Breinl strains were not identical. In all cases the percentage of cells infected with Breinl strain rickettsiae remained constant or increased slightly during a 48 hour period, and the average number of rickettsiae per cell increased about 13 to 30 fold, depending on the cell line. However, with the cell lines RAW264.7, J774.1, and P388D1, the percentage of cells infected with E strain rickettsiae decreased very dramatically during a 48 hour period, whereas with the PU5 cell line, the percentage of cells infected with E strain rickettsiae decreased by a smaller amount. The average number of E strain rickettsiae per cell decreased dramatically during a 48 hour period in P388D1 cells, and less dramatically in J774.1 cells. This same parameter increased slightly during the same period of time in PU5 or RAW264.7 cells infected with E strain rickettsiae. The differences among the mouse macrophage cell lines in their response to infection with E strain rickettsiae may reflect actual differences in the expression of macrophage-like characteristics by these cell lines. Ralph et al. (12) have emphasized the heterogeneity among the various mouse macrophage-like cell lines. It is important to remember also that (1) variants of macrophage-like cell lines can arise on passage, (9), and (2) it is possible that variants of a particular macrophage-like cell line might also differ in their ability to restrict the growth of the E strain.

Although RAW264.7 was not the most effective mouse macrophage-like cell line at clearing E strain rickettsiae, it was undoubtedly the easiest mouse macrophage-like cell line to culture and use in our studies. This cell line grew well in medium supplemented with 10% calf serum and adhered firmly to the chamber slides we used. Although the cell line J774.1 adhered firmly to the substratum, these cells tended to become very vacuolated. Adherence of the cell lines PU5 and P388D1 to the substratum was influenced by the density of cell planting; hence we did our experiments with cells maintained in suspension. The cell line P388D1 was in our experiments the most effective line at killing the E strain of R. prowazeki .

Although the mouse macrophage-like cell lines RAW264.7 and J774.1 had been cloned, the cells of these lines were not uniform in their response to infection with the E strain. The rickettsiae disappeared from most of the cells, but usually multiplied in a small population of cells. We recloned RAW264.7 and infected 15 clones with the E strain. Each clone was nonuniform in its response to infection with the E strain. Whether variability in the cells or in the rickettsiae (or both) accounts for this heterogeneous response is not known. Gambrill and Wisseman (6) reported similar findings with human macrophages. It is difficult to speculate about how fast E strain rickettsiae were killed in RAW264.7 cells because dead rickettsiae were cleared so slowly

from these cells. One could obtain information about the rate of killing of E strain rickettsiae by lysing infected cells and assaying the lysate for viable rickettsiae. It is also not clear in our study whether the surviving E strain rickettsiae multiplied at the same rate as the Breinl strain rickettsiae, since it was impossible to distinguish microscopically how many of the E strain rickettsiae present were dead at the time of slide preparation.

The cellular site of killing of the E strain of R. prowazeki in macrophage-like cell lines is also unknown and is of great interest. One may hypothesize that both E and Breinl strain rickettsiae are phagocytized by the macrophage and that most of the E strain rickettsiae are killed in the phagosome whereas the Breinl strain rickettsiae escape to the cytoplasm and multiply. Using electron microscopic techniques, Meyer and Wisseman have demonstrated the escape of the Breinl strain of R. prowazeki from the phagosome in human macrophages, (W.A. Meyer III and C.L. Wisseman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D10, p.39). In addition, they demonstrated the destruction in the phagosome of dead rickettsiae and rickettsiae which had been treated with immune human serum.

On the other hand, both strains of rickettsiae may escape to the cytoplasm and killing of the E strain and survival of the Breinl strain may be determined there. Nacy and Meltzer (10) postulated that killing of R. tsutsugamushi in mouse macrophages treated with lymphokines after infection occurred in the cytoplasm.

Several macrophage-like cell lines are capable of responding to treatment with lymphokines by acquiring certain characteristics of activated macrophages (7,8,9,13, J.E.R. Potter and P. Ralph, Fed. Proc. 38:1095, 1979.). Experiments are in progress to determine the effect of lymphokine treatment of macrophage-like cell lines on the fate of the E and Breinl strains of R. prowazeki in these cells.

The results obtained in this study with macrophage-like cell lines differ from those reported by Gambrill and Wisseman (6) in two respects. Firstly, in the present study, the percentage of cells infected with Breinl strain rickettsiae remained constant or increased slightly during a 48 hour period. Gambrill and Wisseman found that the percentage of macrophages infected with Breinl strain rickettsiae decreased somewhat, but not as dramatically as did the percentage of macrophages infected with E strain rickettsiae. Secondly, in the present study, the average number of Breinl strain rickettsiae per cell increased to a greater extent during a 48 hour period than did the same parameter for human macrophages infected with Breinl strain rickettsiae (6). These data suggest that mouse macrophage-like cell lines may lack a limited ability possessed by human macrophages for inhibiting the growth of the Breinl strain of Rickettsia prowazeki.

Treatment of rickettsiae with antiserum differentially affected their ability to infect L-929 cells or macrophage-like cell lines. Rickettsial infection of L-929 cells was suppressed after antiserum treatment, whereas infection of macrophage-like cell lines (which bear receptors for the Fc portion of immunoglobulin) was enhanced or unaffected. The results with L-929 cells were unexpected, since Wisseman et al. (20) found that treatment of R.

prowazeki with immune human serum did not decrease the infection of chicken embryo cells by the rickettsiae. The reason for the difference in our results is not known, but it may reflect a difference in the antibody compositions of the antisera used or in the host cell lines.

The ability of rickettsiae to grow in L-929 cells or in macrophage-like cell lines was also differentially affected by treatment of the rickettsiae with antiserum. On one hand, the growth of antiserum-treated rickettsiae was unrestricted in L-929 cells. Wisseman et. al (20) likewise showed that rickettsiae treated with immune human serum grew normally in chicken embryo cells . On the other hand, the majority of the antiserum treated rickettsiae disappeared from the mouse macrophage-like cell lines.

The human macrophage-like line U937-1 was less effective than the mouse macrophage-like cell lines at clearing antiserum-treated rickettsiae. This observation and the imperfect clearance of antiserum-treated rickettsiae by some of the other macrophage-like cell lines may be related to the percentages of the cells which bear Fc receptors. The variability among the macrophage-like cell lines with regard to their ability to be infected with antiserum- treated rickettsiae is probably also related to Fc receptor expression. Larrick et al. (8) reported that 35-40% of U937 cells expressed Fc receptors whereas Ralph et al. (12) indicated that the percentages of PU5-1.8, P388D1, J774.1, and RAW264.10 cells which expressed Fc receptors were 62, 96, 100, and 68% respectively. Alternatively, imperfect clearance of rickettsiae by macrophage-like cell lines may be a reflection of insufficient coating of some of the rickettsiae with antibody.

This study has characterized the interaction of R. prowazeki and several macrophage-like cell lines, and has identified four cell lines which are capable of clearly differentiating the avirulent E and virulent Breinl strains of R. prowazeki. These cell lines, especially RAW264.7 and P388D1, should be useful models for studying rickettsia-macrophage interactions and for defining the basis for the difference in virulence between the E and Breinl strains of R. prowazeki. In addition, this study has provided information about the interaction of bacteria and macrophage-like cell lines. Comparison of our data with that of Winkler and Daugherty (this report), who demonstrated that neither the E nor the Breinl strains of R. prowazeki could grow in mouse peritoneal macrophages, indicates that some significant difference(s) exists between the continuous mouse macrophage-like cell lines and mouse macrophages.

REFERENCES

1. Beaman, L. and C.L. Wisseman, Jr. 1976. Mechanisms of immunity in typhus infections VI. Differential opsonizing and neutralizing action of human typhus rickettsia-specific cytophilic antibodies in cultures of human macrophages. Infect. Immun. 14:1071-1076.

2. Bovarnick, M.R., J.C. Miller, and J.C. Snyder. 1950. The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae. J. Bacteriol. 59:509-522.

3. Bovarnick, M.R., and J.C. Snyder. 1949. Respiration of typhus rickettsiae. J. Exp. Med. 89:561-565.

4. Defendi, V. 1976. Macrophage cell lines and their uses in immunobiology, p.

275-290. In D.S. Nelson (ed.), Immunobiology of the Macrophage. Academic Press, New York.

5. Gambrill, M.R. and C.L. Wisseman, Jr. 1973. Mechanisms of immunity in typhus infections III. Influence of human immune serum and complement on the fate of Rickettsia mooseri within human macrophages. Infect. Immun. 8:631-640.

6. Gambrill, M.R. and C.L. Wisseman, Jr. 1973. Mechanisms of immunity in typhus infections. II. Multiplication of typhus rickettsiae in human macrophage cell cultures in the nonimmune system: influence of virulence of rickettsial strains and of chloramphenicol. Infect. Immun. 8:519-527.

7. Koren, H.S., S.J. Anderson, and J.W. Larrick. 1979. In vitro activation of a human macrophage-like cell line. Nature 279:328-330.

8. Larrick, J.W., D.G. Fischer, S.J. Anderson, and H.S. Koren. 1980. Characterization of a human macrophage-like cell line stimulated in vitro: A model of macrophage functions. J. Immunol. 125:6-12.

9. Morahan, P.S. 1980. Macrophage nomenclature: Where are we going? J. Reticuloendothel. Soc. 27:223-245.

10. Nacy, C.A. and M.S. Meltzer. 1979. Macrophages in resistance to rickettsial infection: Macrophage activation in vitro for killing of Rickettsia tsutsugamushi . J. Immunol. 123:2544-2549.

11. Nacy, C.A. and J.V. Osterman. 1979. Host defenses in experimental scrub typhus: Role of normal and activated macrophages. Infect. Immun. 26:744-750.

12. Ralph, P., I. Nakoinz, H.E. Broxmeyer, and S. Schrader. 1978. Immunologic functions and in vitro activation of cultured macrophage tumor lines. Natl. Cancer Inst. Monogr. 48:303-310.

13. Russell, S.W., G.Y. Gillespie, and J.L. Pace. 1980. Comparison of responses to activating agents by mouse peritoneal macrophages and cells of the macrophage line RAW264. J. Reticuloendothel. Soc. 27:607-619.

14. Silverman, D.J., P. Fiset, and C.L. Wisseman, Jr. 1979. Simple, differential staining technique for enumerating rickettsiae in yolk sac, tissue culture extracts, or purified suspensions. J. Clin. Microbiol. 9:437-440.

15. Walker, T.S. and H.H. Winkler. 1979. Rickettsial hemolysis: rapid method for enumeration of metabolically active typhus rickettsiae. J. Clin. Microbiol. 9:645-647.

16. Winkler, H.H. 1974. Inhibitory and restorative effects of adenine nucleotides on rickettsial adsorption and hemolysis. Infect. Immun. 9:119-126.

17. Winkler, H.H. 1977. Rickettsial hemolysis:adsorption, desorption, readsorption, and hemagglutination. Infect. Immun. 17:607-612.

18. Wisseman, C.L., Jr., E.B. Jackson, F.E., Hahn, A.C. Ley, and J.E. Smadel. 1951. Metabolic studies of rickettsiae. I. The effects of antimicrobial substances and enzyme inhibitors on the oxidation of glutamate by purified rickettsiae. J. Immunol. 67:123-136.

19. Wisseman, C.L., Jr., A.D. Waddell, and W.T. Walsh. 1974. In vitro studies of the action of antibiotics on Rickettsia prowazeki by two basic methods of cell culture. J. Infect. Dis. 130:564-574.

20. Wisseman, C.L., Jr., A.D. Waddell, and W.T. Walsh. 1974. Mechanisms of immunity in typhus infections. IV. Failure of chicken embryo cells in culture to restrict growth of antibody-sensitized Rickettsia prowazeki . Infect. Immun. 9:571-575.

Table 1. Effect of rabbit antiserum on the ability of Rickettsia prowazeki to infect L-929 cells or macrophage-like cell lines.

Rickettsial Strain	Rickettsial Treatment	L-929		RAW264.7		J774.1		P815		P817.1	
		Percent Infected[b]	Number Rickettsiae[c]	Percent Infected	Number Rickettsiae	Percent Infected	Number Rickettsiae	Percent Infected	Number Rickettsiae	Percent Infected	Number Rickettsiae
Breinl	none	76±7	1.8±0.4(4)[a]	74±12	2.1±0.9(5)	83±2	2.7±0.4(2)	55±1	1.2±0.2(2)	57±4	1.0±0.1(3)
Breinl	normal serum[d]	76±7	2.0±0.4(2)	75±7	2.0±0.9(2)	ND	ND[e]	ND	ND	ND	ND
Breinl	antiserum[d]	15±5	0.3±0.1(4)	67±18	3.2±2.0(5)	99±1	8.2±0.4(2)	88±7	4.1±1.9(2)	47±7	1.1±0.5(3)
E	none	84±1	2.7±0.3(2)	81±10	2.6±0.8(3)	78±1	2.3±0.1(2)	ND	ND	ND	ND
E	antiserum	11±1	0.2±0.0(2)	78±3	5.5±2.0(3)	97±2	8.6±0.8(2)	ND	ND	ND	ND

[a]Each value represents the mean ± the standard deviation. The number of experiments is in parentheses.

[b]Percentage of cells infected.

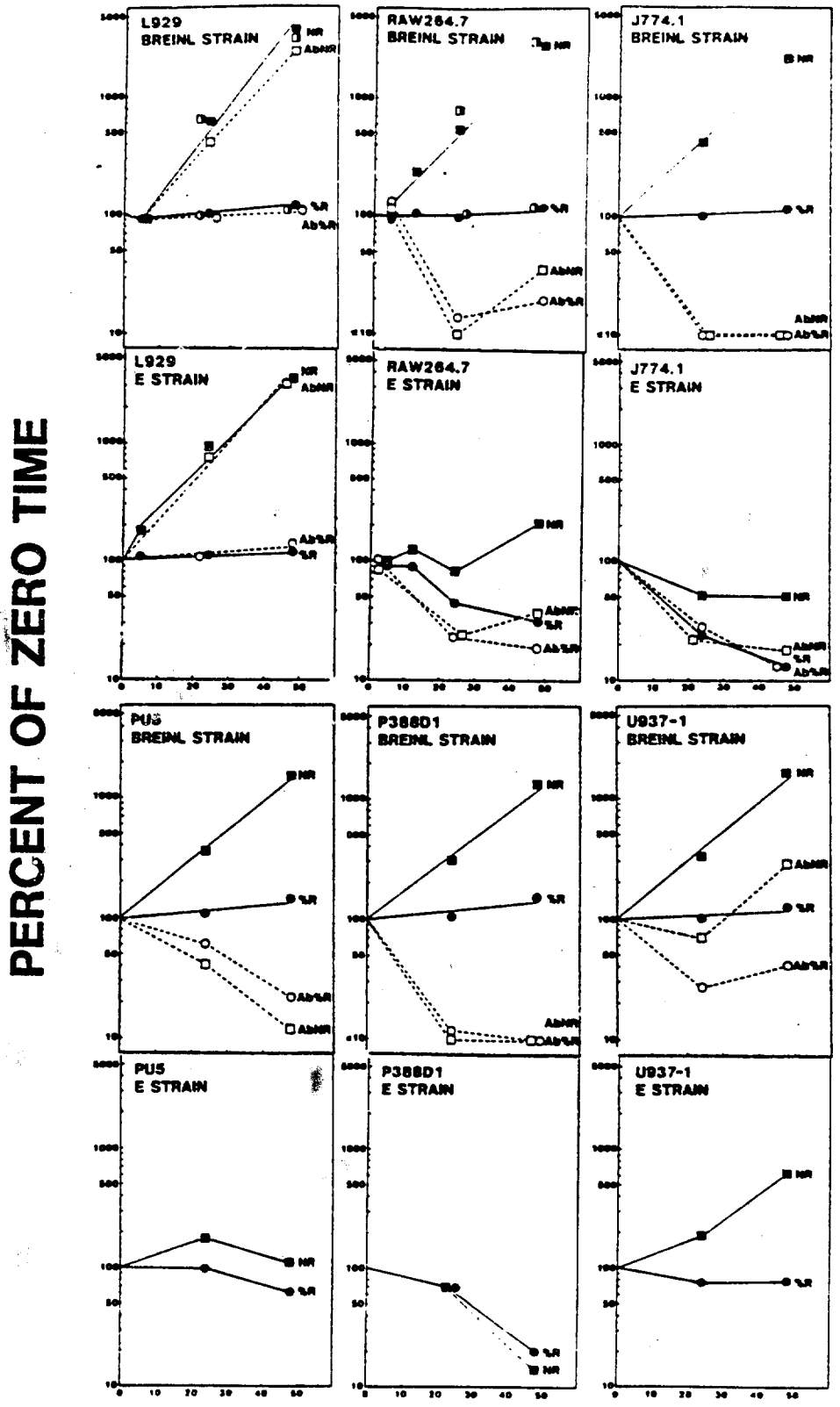
[c]Average number of rickettsiae per cell.

[d]Normal rabbit serum or rabbit antiserum was added to rickettsial suspensions at a concentration of ten percent just prior to infection of the cells.

[e]Not done.

Figure 1. Growth of the Madrid E or Breinl strains of Rickettsia prowazeki in L-929 cells or macrophage-like cell lines. The percentage of cells infected (% R, circles) and the average number of rickettsiae per cell (NR, squares) are expressed as percentages of those observed at zero time and are plotted against the time after infection in hours.

Open symbols with dashed lines depict antiserum-treated rickettsiae (Ab% R, AbNR), closed symbols with solid lines depict untreated rickettsiae, and half-closed symbols with solid lines represent normal rabbit serum-treated rickettsise. The values shown are the averages of the following numbers of experiments: L929, n=7 except 3 at 5 hrs, 3 with antiserum, and 2 with normal serum; RAW264.7, n=17 for E, 13 for Breinl strains except 2 at 12 hrs, 5 at 5 hrs, and 3 with antiserum; J774.1, n=2; PU-5, n=7 except 2 with antiserum; P388D1, n=2 except 1 with antiserum; U937-1, n=3. Normal rabbit serum or rabbit antiserum was added to rickettsial suspensions at a concentration of ten percent just prior to infection of the cells.


L929
BREINL STRAIN
RAW264.7
BREINL STRAIN
J774.1
BREINL STRAIN
L929
E STRAIN
RAW264.7
E STRAIN
J774.1
E STRAIN
PU5
BREINL STRAIN
P388D1
BREINL STRAIN
U937-1
BREINL STRAIN
PU5
E STRAIN
P388D1
E STRAIN
U937-1
E STRAIN
NR
AbNR
%R
Ab%R
PERCENT OF ZERO TIME
HOURS

Clearance of *Rickettsia prowazeki* by Mouse Peritoneal Macrophages

Herbert H. Winkler* and Robin M. Daugherty

Department of Microbiology and Immunology, University of South Alabama, College of Medicine, Mobile, Alabama 36688.

INTRODUCTION

A role for the macrophage in the pathogenesis of rickettsial diseases is strongly suggested. Nacy et al. (9,10) showed that activated mouse macrophages in vitro could eliminate R. tsutsugamushi but that resident macrophages could not. Gambrill and Wisseman (8) showed that cultured human monocytes could kill the avirulent strain of R. prowazeki. However, the virulent strains of R. prowazeki and R. typhi grew within these monocytes unless these virulent strains had been reacted with antiserum in which case they were destroyed by the monocyte (2,7,8).

The advantages of the mouse peritoneal macrophage as a model for the pathogenesis of epidemic typhus infections and as a means of exploring the differences in the virulent and avirulent strains of R. prowazeki are numerous. Little has been described on the interaction of mouse macrophages and R. prowazeki.
In this study we describe the interactions of the E strain and Breinl strain of R. prowazeki with resident, elicited and activated peritoneal macrophages from three strains of mice including the "beige" strain which has impaired lysosomal degranulation (4).

METHODS AND MATERIALS

Basic preparation and characterization of cells . Unstimulated or treated (5% oyster glycogen, Corynebacterium parvum , or E strain-immunized mice were killed by cervical dislocation and the peritoneum was injected with 8-10 ml of Dulbecco's Modified Eagle Medium (DMEM) containing 2 mM glutamine, 10% fetal calf serum, and 5U Heparin per mililiter. After gentle massaging of the abdominal wall, the fluid containing peritoneal exudate cells (PEC) was removed. Routinely 1-4 x 10^5 PEC per glycogen-elicited mouse were obtained by this procedure. An aliquot of the PEC suspension was stained with 0.1% crystal violet in 10% acetic acid and then counted in a Fuchs-Rosenthal Ultraplane counting chamber. Only cells having a small round nucleus and a relatively small amount of cytoplasm were enumerated. The PECs were then adjusted to a concentration of 1 x 10^6 cells/ml in DMEM + 10% FCS and 0.3 ml aliquots were delivered to each well of a chamber slide (Lab-Tek no. 4808, 8 chambers/slide), allowing one slide for each time point to be observed. Initial enrichment and separation of non-adherent cells (mostly lymphocytes) from the adherent peritoneal cell population was accomplished by allowing the cells to adhere to glass for 2 hr at 34°C in 5% CO_2. Non-adherent cells were then removed with three vigorous washings with Hank's Balanced Salt Solution. Approximately 50% of the cells adhere and these are assumed to be macrophages.

Rickettsiae. Rickettsia prowazeki Madrid E and Breinl strains were grown in 6-day embryonated, antibiotic-free chicken eggs. Eggs were inoculated from seed pools prepared from the Madrid E strain or the Breinl strain. Rickettsiae were harvested and purified from infected yolk sacs 8 days after inoculation. Methods of rickettsial purification were modified from those of Bovarnick and Snyder (6), and Wisseman et al. (15) as described previously (14).

Purified rickettsiae were suspended in the sucrose phosphate glutamate buffer of Bovarnick et al. (5) and were then stored at -70°C in 0.2 aliquots.

Viable rickettsiae were enumerated by the antibody hemolysis method of Walker and Winkler (13), and total rickettsiae-like bodies were enumerated by a modification of the method of Silverman, Fiset, and Wisseman (12).

Infection of macrophages with rickettsiae. Rickettsiae were diluted in Hank's Balanced Salt Solution supplemented with 0.1% gelatin and 4.9 mM L-glutamic acid, monpotassium salt (HBSSGG). Rickettsiae were then added to the washed macrophages in 0.2 ml aliquots, giving multiplicities ranging from 50 to 200 viable rickettsiae per adherent cell. E strain-immune rabbit serum (which had been heat-imactivated at 56°C for 1 hour) was used to achieve opsonization when desired. Macrophages and rickettsiae were then incubated together for 60 minutes at 37°C, room air. The infections were then removed and the macrophages washed three times with DMEM + 10% FCS. The cells were then given fresh medium and allowed to incubate up to 48 hours. At 0 hours, 4 hours, 24 hours, and 48 hours post infection chamber slides were then washed gently in 0.85% NaCl and dried with a warm air stream. The slides were then fixed in 1% formalin in 0.1 M sodium phosphate buffer (pH 6.8) and stained by a modification of the Gimenez method as described by Wisseman et al. (16). A sample of glycogen-elicited macrophages from C57/6Bl or C57/J6 bg mice was stained in a 1 x 10^6 dilution of acridine orange in phosphage-buffered saline and examined by fluorescence microscopy for giant lysosome formation.

The stained slides were examined microscopically under oil immersion and the number of rickettsiae present in each of 100 macrophages were counted for each multiplicity of infection. From the data collected the percentage of macrophages infected with rickettsiae (%R) and the average number of rickettsiae per cell (NR) were determined. Much of the data was normalized to the zero time infection, e.g. NR at a given time divided by NR at the zero time times 100 gives the average number of rickettsiae per macrophage as a percentage of that present at zero time.

RESULTS

Mouse peritoneal macrophages can clear their cytoplasm of R. prowazeki in 24 to 48 hours. This clearance is rapid in onset: by 4 hrs after infection a significantly lower fraction of the macrophages have any rickettsia within them. Any macrophages, unable to clear the rickettsiae by 24 to 48 hrs, a number in general less than 5%, are no longer even rickettsiastatic and the number of rickettsiae within these few macrophages increases. These rickettsiae will eventually fill and kill these macrophages.

The macrophages of both BALB/c and C57/6Bl mice were able to kill intracellular rickettsiae. Activation of the macrophages was unnecessary; resident and oyster shell glycogen elicited macrophages were as rickettsiacidal as those activated by the administration of C. parvum or immunization with R. prowazeki of either strain. Alterations in activation-state as a result of in vitro cultivation of the macrophages was also not crucial since macrophages which had been cultivated for seven days, 24 hrs or infected with rickettsiae the same day they were removed from the mouse were all able to kill rickettsiae.

Both the virulent Breinl strain and the avirulent E strain were eliminated from the macrophages and no tendency for survival or growth of the

virulent strain relative to the avirulent strain was present in any of the macrophage types. Opsonization of either the E or Breinl strains resulted in more phagocytosis and hence a greater initial infection but no effect on the subsequent clearance of these rickettsiae was evident.

The beige mutant of C57/6 has a defect in degranulation and its neutrophils are unable to kill bacteria as well as those of the wild type mouse. The oyster glycogen elicited peritoneal macrophages of these mice were found to contain giant lysosomes when stained by acridine orange as has been seen in their neutrophils by other groups. In spite of this defect, the clearance of both avirulent and virulent rickettsiae occurred, and in this series of experiments was slightly better than in the macrophages of the wild type mouse.

DISCUSSION

Mice in general are unable to be infected by R. prowazeki although they can be rapidly killed by large inoculations of viable rickettsiae through a toxicity reaction of unknown mechanism (3). The three strains of mice used in the present study were injected intraperitoneally with 10^9 Breinl strain rickettsiae and no morbidity or mortality appeared after the first 24 hrs. The striking ability of the mouse peritoneal macrophage to eliminate R. prowazeki correlates with the resistance of the mouse to infection by this species of rickettsiae. R. tsutsugamushi , on the other hand, infects mice and cannot be eliminated by the macrophage unless the macrophage is activated (9,10). It must be emphasized that the basis of the resistance of mice, and animals in general except man, to R. prowazeki infection has not been established and hence the causal significance of this correlation is unknown. We have investigated the peritoneal macrophages of the hamster and gerbil. These cells, like those of mice, kill both the Breinl and E strain of R. prowazeki. In fact, large doses of the Breinl strain produced no deaths within 31 days observation in our hands. Investigations of the interaction of guinea pig and cotton rat macrophages, macrophages from animals that are susceptible to infection by R. prowazeki, (1,11) may be more rewarding in terms of obtaining macrophages that will show differential killing of the two strains.

It had been hoped that the beige mouse would be unable to clear the Breinl strain but able to kill the E strain. This would have shown the importance of lysosomal fusion in the killing of rickettsiae and have strongly suggested that the virulence of the Breinl strain results, at least in part, from its ability to lyse its way out of the phagosome more effectively than its avirulent counterpart. However, in spite of its abnormal macrophage lysosomes and the reduced bacteriacidal capacity of its neutrophils, the peritoneal macrophages of the beige mouse were able to clear rickettsiae of both strains. It may be that lysosomal granules are unimportant in rickettsial killing and that the oxidative mechanisms within the primary phagosome are sufficient. We have shown that rickettsiae are sensitive to superoxide anion and H_2O_2 in a cell free system. Alternatively, since the beige mouse is "defective" but not lacking lysosomes, the limited degranulation that occurs may be sufficient because of the extended time that the rickettsiae are within the cell. A third possibility is that the rickettsiae are actually eliminated from the cytoplasm of the cell by an undescribed mechanism rather than from the phago(lyso)somal vesicle.

REFERENCES

1. Anderson, C.R., 1944. Experimental typhus infection in the eastern cotton rat (Sigmodon hispidus hispedus). J. Exper. Med. 80: 341-356.

2. Beaman, L. and C.L. Wisseman, Jr. 1976. Mechanisms of immunity in typhus infections VI. Differential opsonizing and neutralizing action of human typhus rickettsia-specific cytophilic antibodies in cultures of human macrophages. Infect. Immun. 14:1071-1076.

3. Bentson, I.A., N.H. Topping and R.G., Henderson. 1945. Epidemic typhus: Demonstration of a substance lethal for mice in the yolk sac of eggs infected with Rickettsia prowazeki . Natl. Inst. Health. Bull. 183:25-29.

4. Bennet, J.M., R.S. Blume and S.M. Wolff. 1969. Characterization and significance of abnormal leukocyte granules in the beige mouse: a possible homologue for Chediak-Higashi Aleutian trait. J. Lab. Clin. Med. 73:235- .

5. Bovarnick, M.R., J.C. Miller, and J.C. Snyder. 1950. The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae. J. Bacteriol. 59:509-522.

6. Bovarnick, M.R., and J.C. Snyder. 1949. Respiration of typhus rickettsiae. J. Exp. Med. 89:561-565.

7. Gambrill, M.R. and C.L. Wisseman, Jr. 1973. Mechanisms of immunity in typhus infections III. Influence of human immune serum and complement on the fate of Rickettsia mooseri within human macrophages. Infect. Immun. 8:631-640.

8. Gambrill, M.R., and C.L. Wisseman, Jr. 1973. Mechanisms of immunity in typhus infections. II. Multiplication of typhus rickettsiae in human macrophage cell cultures in the nonimmune system: influence of virulence of rickettsial strains and of chloramphenicol. Infect. Immun. 8:519-527.

9. Macy, C.A. and M.S. Meltzer. 1979. Macrophages in resistance to rickettsial infection: Macrophage activation in vitro for killing of Rickettsia tsutsugamushi. J. Immunol. 123:2544-2549.

10. Macy, C.A. and J.V. Osterman. 1979. Host defenses in experimental scrub typhus: Role of normal and activated macrophages. Infect. Immun. 26:744-750.

11. Perez Gallardo, F. and J.P. Fox. 1948. Infection and immunization of laboratory animals with Rickettsia prowazekii of reduced pathogenicity, Strain E. Am. J. Hyg. 48:6-21.

12. Silverman, D.J., P. Fiset, and C.L. Wisseman, Jr. 1979. Simple, differential staining technique for enumerating rickettsiae in yolk sac, tissue culure extracts, or purified suspensions. J. Clin. Microbiol. 9:437-440.

13. Walker, T.S. and H.H. Winkler. 1979. Rickettsial hemolysis: rapid method for enumeration of metabolically active typhus rickettsiae. J. Clin. Microbiol.

9:645-647.

14. Winkler, H.H. 1974. Inhibitory and restorative effects of adenine nucleotides on rickettsial adsorption and hemolysis. Infect. Immun. 9:119-126.

15. Wisseman, C.L., Jr., E.B. Jackson, F.E. Hahn, A.C. Ley, and J.E. Smadel. 1951. Metabolic studies of rickettsiae. I. The effects of antimicrobial substances and enzyme inhibitors on the oxidation of glutamate by purified rickettsiae. J. Immunol 67:123-136.

16. Wisseman, C.L., Jr., A.D. Waddell, and W.T. Walsh. 1974. *In vitro* studies of the action of antibiotics on *Rickettsia prowazeki* by two basic methods of cell culture. J. Infect. Dis. 130. 564-574.

Table 1.

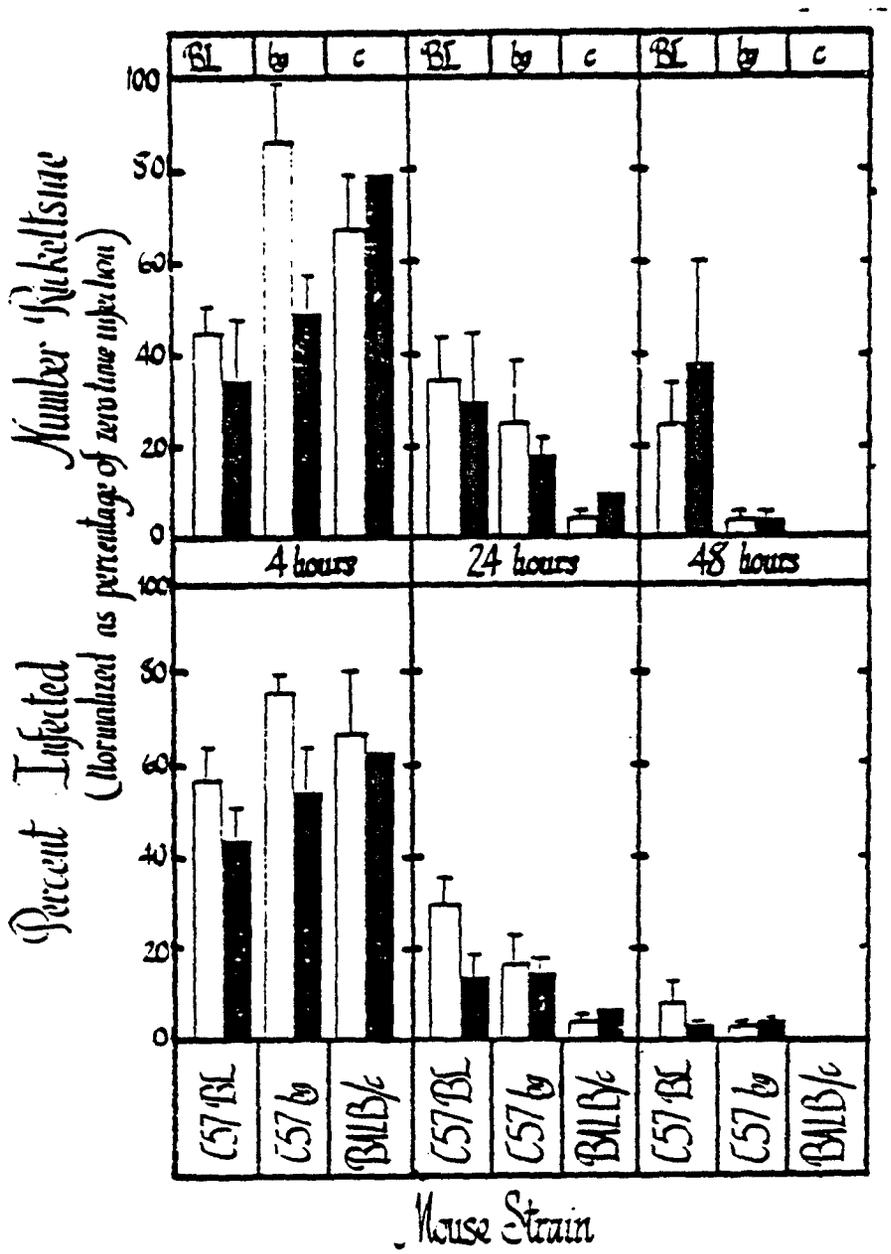
Effect of Macrophage State on Clearance of Rickettsiae

Mouse Strain	Macrophage Type	Rickettsial Strain	Percent of zero time at 24 hrs	
			Percent Infected	Number Infected
C57 BL	Resident	E	13±5	23±12 (5)[b]
"	"	Breinl	15±8	17±8 (5)
"	Oyster (1)[a]	E	30±6	35±9 (13)
"	"	Breinl	26±12	30±15 (11)
"	Oyster (2)[a]	E	4±2	3±2 (5)
"	" (2)[a]	Breinl	0±0	0±0 (4)
Balb/c	Oyster (1)[a]	E	4±2	4±1 (7)
"	" (1)[a]	Breinl	7±7	10±10 (2)
"	" (7)[a]	E	3±2	3±2 (2)
"	" (7)[a]	Breinl	15±1	11±2 (2)
"	C. parvum	E	3±3	2±2 (4)

a) number refers to time of aging of macrophages in vitro before rickettsiae added. (1) rickettsiae added on same day as macrophages taken from mouse, (2) rickettsiae added on 2nd day, (7) rickettsiae added on seventh day.

b) mean, standard error and number of determinations.

Fig. 1. Clearance of R.prowazeki by mouse peritoneal macrophages. The effects of rickettsial strains, mouse strains and time are shown. All values are normalized to that at zero time. The standard error is shown. The Balb/c 48 hr data were not determined.


Number Infected
(Normalized as percentage of zero time injection)
Percent Infected
BL
b
c
100
50
40
20
0
4 hours
24 hours
48 hours
C57BL
C57b
BALB/c
Mouse Strain

Selective Elimination of the Avirulent, Madrid E, strain of *Rickettsia prowazeki* by Guinea Pig Peritoneal Macrophages.

Herbert H. Winkler and Robin M. Daugherty

Laboratory of Molecular Biology
Department of Microbiology and Immunology
University of South Alabama College of Medicine
Mobile, Alabama 36688.

ABSTRACT

The oyster shell glycogen elicited peritoneal macrophage of the guinea pig interacts differently with the avirulent E strain and the virulent Breinl strain of Rickettsia prowazeki . 24 h after a wide range of initial infections the Breinl strain survives in about 71% of those macrophages it infected and the number of rickettsia per macrophage increases about four-fold. The E strain, on the other hand, survives in only about 9% of those macrophages it infected and the number of rickettsiae per macrophage falls to about 13% of that present initially.

The guinea pig has been the most widely used animal model for the study of the pathogenesis of epidemic typhus. Only the cotton rat, which is not readily available as a laboratory strain in this country, is an alternative. Mice and albino rats have been shown to be uninfectible by Rickettsia prowazeki and we have extended this list to include the golden hamster and gerbil.

An avirulent strain, the Madrid E strain, of R. prowazeki can be distinguished from the common virulent (Breinl) strain by its ability to immunize guinea pigs and man without causing mortality or significant morbidity. However, the biochemical basis of virulence in R. prowazeki is unknown. To date the only published in vitro data bearing on this are those of Gambrill and Wisseman who showed that cultured human monocytes can destroy the avirulent strain but that the virulent strain grows within the phagocytes. However, we have in preparation studies of rickettsial interaction with mice and macrophage-like cell lines.

We have investigated the peritoneal macrophage of guinea pigs to develop a flexible animal model for the study of pathogenesis and the basis of virulence. The macrophages of the guinea pig are shown to differentiate between the Breinl and E strains: allowing the former to grow and eliminating the latter.

The Methods and Materials are as described in the preceeding manuscript on mouse macrophages and rickettsiae and for brevity are not repeated.

The oyster glycogen elicited peritoneal macrophages of guinea pigs were infected with the E strain or the Breinl strain of R. prowazeki . The initial infections resulted in infection of from 4 to 100% of the macrophages (mean of 31 for E and 37 for Breinl) and the number of rickettsiae per 100 macrophages had a mean of 79 for E and 114 for the Breinl strain (Fig. 1). The ability of the macrophage to deal with this wide range of infection during the next 24 h was dependent on the strain. The E strain was eliminated by the macrophage: only 9% of those cells infected at zero time time had rickettsiae at 24 h and the number of rickettsiae per macrophage fell to 13% of the zero time level (Fig. 1). The Breinl strain, on the other hand, survived and grew in most of the macrophages. At 24 hrs. 71% of those cells infected at zero time with the Breinl strain remained infected and the number of rickettsiae per macrophage increased 397% (Fig. 1).

The mechanisms by which the guinea pig macrophage is able to differentiate between the virulent and avirulent strains of R. prowazeki are

unknown. The mouse macrophage-like cell lines (Turco and Winkler, this report) and the human monocyte are also able to eliminate the E strain while allowing the virulent strain to survive and grow (Gambrill and Wisseman). The mouse peritoneal macrophage, however, is unable to discriminate and both strains are killed (Winkler and Daugherty, this report). The guinea pig macrophage are, as would be expected, more heterogenous than those of the mouse macrophage-like cell lines in that some of the macrophages infected with the Breinl strain eliminate it during the first 24 hrs whereas the percent of the macrophage-like cell lines infected with the Breinl strain remains essentially constant through 48 h.

Future work will investigate the mechanism by which the E strain is killed and the Breinl strain is able to avoid destruction. The role of the activation state and the metabolic state of the macrophage will also be probed. We hope to develop the tools to allow manipulation of the macrophage so that either strain can be made to survive or be killed.

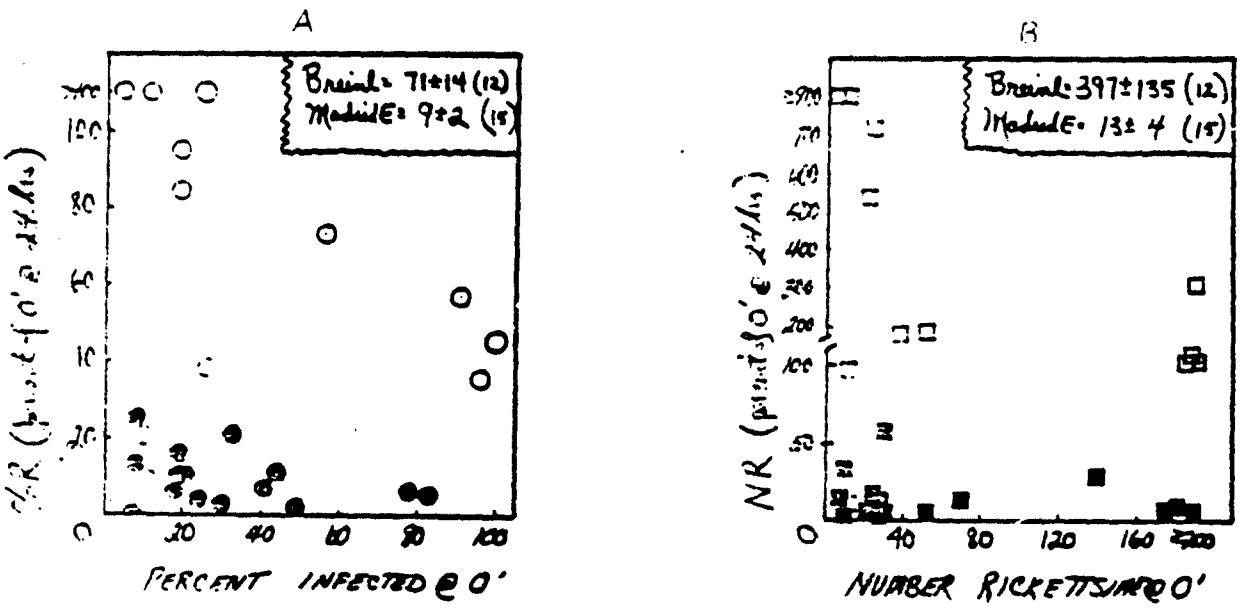


Fig. 1. Elimination or growth of rickettsiae in guinea pig macrophages. Growth parameters of the D strain (●,■), and the Breinl strain (○,□) are plotted against that parameter at initial infection. A) The percentage of macrophages infected at 24 h expressed as a percentage of the macrophages infected at zero time is shown for both strains. B) The number of rickettsiae per 100 macrophages at 24 h post-infection expressed as a percentage of the number of rickettsiae per 100 macrophages at zero time is shown for both strains. Note the break in the y-axis. The numbers in the boxes are the mean percent of zero time, the standard error and the number of determinations.

Comparison of the Fatty Acid Composition of Avirulent and Virulent Strains of *Rickettsia prowazeki*

Herbert H. Winkler and Elizabeth T. Miller

Laboratory of Molecular Biology
Department of Microbiology and Immunology
University of South Alabama
College of Medicine
Mobile, Alabama 36688

The fatty acid composition of the phospholipids of the avirulent Madrid E and virulent Breinl strains of Rickettsia prowazeki were compared. The fatty acids 16:0,16:1 and 18:1 are the major fatty acids of both strains and in sum account for 90% of the fatty acid in these organisms. Large amount of mono-unsaturated fatty acid (>65%) was detected in both strains. Thus, a differential susceptibility to lipid peroxidation is not a likely hypothesis to account for virulence in the Breinl strain.

The rickettsiae were harvested from infected chicken egg yolk sac, purified through Renografin, the lipids were extracted with chloroform-methanol, the fatty acids of the phospholipids were cleaved and converted to methyl esters with methanolic HCl and the methyl esters were separated and quantitated by gas liquid chromatography, all as previously described by Winkler and Miller, (J. Bacteriol., 1978).

The phospholipids of the avirulent and virulent strains of R. prowazeki contain the same fatty acids (Table 1). The slightly higher levels of 16:0 and 18:0 in the Breinl strain are probably not significant since variation in the phosphatidyl choline content of the rickettsial preparation (which, in the E strain, we showed was host derived) would be most strongly reflected in these fatty acids. The fatty acid composition of the individual phospholipid classes (PE,PG,PC) was not determined for the Breinl strain. However, published data for the E strain showed little differences in the fatty acids of PE and PG, the major phospholipids of this strain (Winkler and Miller).

R. prowazeki , Madrid E strain, was shown to have a surprisingly high percentage of its fatty acids unsaturated (Winkler and Miller). We hypothesized that the E strain was much higher in percentage of unsaturated fatty acids than the Breinl strain. This could account for a greater susceptibility to destruction through lipid peroxidation in the E strain. The E strain is selectively eliminated relative to the Breinl strain in human monocytes, (Gambrill and Wisseman) guinea pig macrophages (Winkl. and Daugherty) and mouse macrophage-like cell lines (Turco and Winkler). The results do not support this hypothesis.

Table 1. Comparison of the fatty acid composition of the phospholipids of the Madrid E and Breinl strains of R. prowazeki.

FATTY ACID	PERCENT OF TOTAL		
	MADRID E STRAIN		BREINL STRAIN
14:0	5.5,6.2	(4)[a]	4.7,5.5
16:0	20,22	(24)	26,25
16:0	28,30	(22)	25,26
18:0	1.5,1.6	(6)	3.0,3.2
18:1	45,40	(44)	41,39
18:2	0,0	(0)	0,1

a) The results of two determinations are shown, the number in parentheses is the literature value for the E strain.

ABSENCE OF DETECTABLE PLASMID DNA IN MADRID E AND BREINL STRAINS OF *RICKETTSIA PROWAZEKI*

David O. Wood and Herbert H. Winkler

University of South Alabama
College of Medicine
Department of Microbiology
Mobile, Alabama 36688

ABSTRACT

The virulent Breinl and avirulent Madrid E strains of Rickettsia prowazekii were examined for the presence of plasmid DNA by cesium chloride-ethidium bromide density equilibrium centrifugation and by agarose gel electrophoresis of small volume, crude lysates. No plasmid DNA was detected in either strain using these techniques.

Plasmids are circular pieces of DNA that exist extrachromosomally in bacterial cells. Although they are generally considered nonessential to the survival of the host cell, they confer a variety of phenotypic properties that can impart advantages to the bacterium. Such properties include antibiotic resistance, toxin production, fertility, bacteriocin production, and production of virulence factors (8). Numerous examples of plasmids conferring such phenotypes are found both in gram-positive and gram-negative bacteria.

Plasmids exist within the bacterial cell in the form of a covalently closed circular (CCC) molecule of DNA which replicates independently of the chromosome (1). This physical configuration has been exploited for the purpose of isolating plasmid DNA by cesium chloride-ethidium bromide density gradients (7). Plasmid DNA in the form of CCC molecules binds less ethidium bromide than chromosomal DNA and bands at a denser position. The DNA bands can be visualized with ultraviolet light and the plasmid band removed. After extraction of the ethidium bromide, this procedure results in a pure preparation of plasmid DNA. Generally, however, initial detection of plasmids is achieved by subjecting cell lysates to agarose gel electrophoresis (5). Purified plasmid DNA from CsCl-EB gradients can also be characterized by this method. DNA subjected to agarose gel electrophoresis migrates according to size and configuration and can be visualized by staining the gel with ethidium bromide.

Rickettsia prowazekii is an obligate intracellular bacterium which is the causative agent of epidemic typhus in man. This bacterium's unusual intracellular existence, the mechanism by which it enters eucaryotic cells, and its virulence properties have prompted numerous investigations. Since plasmid DNA is associated with surface antigens and virulence factors, we recently turned our attention to an examination of plasmid DNA in two well-characterized strains of R. prowazekii , the avirulent Madrid E strain and the virulent Breinl strain. With the techniques used in this study, which we believe to be the best available, we were unable to detect plasmid DNA in either strain.

MATERIALS AND METHODS

Bacterial strains. The R. prowazekii strains used in this study are the Breinl and Madrid E strains. In addition, Escherichia coli strain V517 (3), E. coli strain MCB79 (D. Wood), and Pseudomonas aeruginosa strain MCB82 (D. Wood) were employed as plasmid-positive controls. The rickettsia were grown and incubated as described

previously (9). The E. coli and P. aeruginosa strains were routinely grown in L-broth which contained tryptone (10 g), yeast extract (5 g), NaCl (5 g), glucose (1 g) and water (1 l).

Materials. Reagents and sources were as follows: Lysozyme (Grade 1), ribonuclease-A, protease (type V) and ethidium bromide (EB), from Sigma; cesium chloride (technical grade) from Kawecki Berylco Industries.

DNA extraction. 10 ml of the purified Madrid E and Breinl strains of R. prowazekii at a protein concentration of about 1 mg/ml or 30 ml of early stationary phase cells of E. coli or P. aeruginosa were pipetted into 50 ml centrifuge bottles and centrifuged at 9,000 RPM for 10 min at 4°C. The supernatants were discarded and the pellet suspended in 1 ml of 25% sucrose in 0.05 M tris (hydroxymethyl)-aminomethane (Tris), pH 8.0. 0.2 ml lysozyme (10 mg/ml in 0.25 M Tris) was added and the mixture incubated at room temperature for 15 min. 400 ul of 0.25 M disodium ethylene-diaminetetraacetic acid (EDTA), 14 ul of 25% sodium dodecylsulfate (SDS) and 100 ul of protease (20 mg/ml in TES buffer [0.05 M Tris, 0.05 M NaCl, and 0.005 M EDTA]; autodigested for 10 min at 65°C) were then added and the mixture incubated at 37°C for 2 h and 60°C for 30 min. The lysate was sheared by pipetting it in and out of a 1 ml pipette twenty times. 1 ml of this sheared lysate was mixed with 2.9 ml of sterile water, 1.6 ml of EB (2 mg/ml in phosphate buffer, pH 7) and 5.22 g of CsCl. This mixture was then placed in a polyallomer centrifuge tube, overlayed with light mineral oil, and centrifuged in a Beckman TY65 rotor using a Beckman L5-65 preparative ultracentrifuge. Centrifugation was for 48 h at 50,000 rpm and 20°C. After centrifugation, the gradient was examined with ultraviolet light (356 nm). Photographs were made with a Polaroid MP-4 camera unit, using Polaroid type 665 film and a Wratten No. 9 filter.

Small volume screening of strains for plasmid DNA was performed as follows: 1.5 ml of overnight growth of E. coli and P. aeruginosa strains and various amounts of the purified Breinl and Madrid E strains of R. prowazekii were transferred to a 1.5 Eppendorf tube and centrifuged 3 min in an Eppendorf centrifuge. The resulting cell pellet was suspended in 250 ul of 25% sucrose in 0.05 M Tris, pH 8.0, 6 ul of ribonuclease-A (5 mg/ml in 0.05 M sodium acetate, pH 5.0), 17 ul of 1% lysozyme in TES buffer, and 13 ul of 0.25 M EDTA, pH 8.0. The suspension was incubated for 15 min at room temperature before 20 ul of SDS in 0.01 M Tris and 50 ul of 5 M NaCl were added. This mixture was placed in ice for at least 3 h before centrifuging the lysates at 16,000 rpm for 30 min at 0°C. The supernatant was mixed with 0.3 ml phenol (saturated with TES buffer) and 0.3 ml chloroform:isoamyl alcohol (24:1) and centrifuged for 8 min in an Eppendorf centrifuge. The upper aqueous layer was mixed with ether to remove phenol and the ether layer discarded. After the remaining ether had evaporated, 13 ul of 5M NaCl was added, the tube was filled with 95% ethanol, and placed at -20°C overnight. The precipitated DNA was collected by centrifugation at 7,000 rpm for 20 min at -20°C, and dissolved in 50 ul of sterile water. Normally 25 ul of this preparation was examined by agarose gel electrophoresis.

Agarose gel electrophoresis. DNA samples were subjected to electrophoresis in a standard vertical slab gel apparatus according to the method described by Meyers, et. al. (5). Electrophoresis was generally carried out at 45 mA (100 V) until the tracking dye reached the bottom of the gel. The gel was stained with ethidium bromide and photographed under ultraviolet light with a Polaroid MP-4 camera unit, using Polaroid type 665 film and a Wratten No. 9 filter.

RESULTS

Analysis of strains by equilibrium centrifugation. Lysates of the Breinl and Madrid E strains of R. prowazekii, E. coli strains V517 and MCB79 and P. aeruginosa strain MCB82 were subjected to CsCl-EB equilibrium centrifugation. When examined by ultraviolet light, the centrifuged gradients of V517, MCB79 and MCB82 lysates exhibited 2 fluorescent bands (Fig. 2, A). The lower band corresponded to CCC plasmid DNA. Similar gradients of the Breinl and Madrid E strains, however, exhibited only one fluorescent band (Fig. 1, B). When this band was removed, the ethidium bromide extracted and the DNA examined by agarose gel electrophoresis, the band gave a pattern consistent with linear, chromosomal DNA (not shown).

Analysis of strains by agarose gel electrophoresis. Examination of small volume lysates of the rickettsial strains by agarose gel electrophoresis also revealed only contaminating chromosomal DNA (Fig. 2, TOP). No plasmid DNA bands analogous to those seen for plasmid-carrying strains of E. coli or P. aeruginosa could be detected in either the Breinl or Madrid E strains. The masking of plasmids in the 6 X 10^6 to 15 X 10^6 dalton size range by chromosomal DNA in crude lysates has been reported (4). However, low voltage electrophoresis (20 V in the system described in Materials and Methods) induces plasmids in this size range to migrate ahead of chromosomal DNA in 15 h electrophoresis runs (4). When lysates of the Breinl and Madrid E strains were subjected to such low voltage agarose gel electrophoresis (Fig 2, BOTTOM), no plasmid DNA was detected.

DISCUSSION

The techniques used in this study for isolating plasmid DNA have been found to be applicable for a number of different bacteria. Our failure to detect plasmid DNA with these techniques in the two R. prowazekii strains we examined strongly suggests that plasmid DNA is absent from these strains. Certainly, there was no problem in lysing the cells with SDS. A similar procedure was used successfully in an earlier report to isolate rickettsial chromosomal DNA for genome size determinations (6). Of course, plasmid DNA not present as CCC molecules would not be detected in the dye-buoyant equilibrium gradients. However, all plasmids studied to date have been shown to exist in the CCC form (1). In addition, a uniform molecular weight molecule in the normal plasmid size range (2-50 Mdal) would have been detected by agarose gel electrophoresis whether it was in the CCC form or not.

The procedures used for plasmid extraction in this report were rather vigorous and could result in the fragmentation of plasmid molecules of high molecular weight. Such molecules would not have been detected in either the CsCl-EB gradients or by agarose gel electrophoresis since the linear segments of the plasmid would be indistinguishable from those of the chromosome. We are currently examining the rickettsial strains by a technique designed to isolate large plasmids (2). This technique has been used successfully to isolate plasmids ranging from 60 to 312 megadaltons. Preliminary experiments examining the Madrid E strain by this method have not revealed any detectable plasmid DNA.

Genetic analysis of R. prowazekii has been limited to determinations of G+C content and genome size (6). Obviously, the intracellular existence of this bacterium presents many problems. The examination of R. prowazekii for plasmid DNA has been our

first step in applying techniques of microbial genetics to this genus. Future research will be directed toward techniques designed to isolate large plasmids and examinations of a larger number of R. prowazekii strains.

LITERATURE CITED

1. Clowes, R.C. 1972. Molecular structure of bacterial plasmids. Bacteriol. Rev. 36:361-405.

2. Hanson, Jeffrey B. and Ronald H. Olsen. 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. 135:227-238.

3. Macrina, Francis L., Dennis J. Kopecko, Kevin R. Jones, Deborah J. Ayers, and Sara M. McCowen. 1978. A multiple plasmid-containing Escherichia coli strain; Convenient source of size reference plasmid molecules. Plasmid 1: 417-420.

4. Markowitz, Sheldon M., Francis L Macrina, and Paul V. Phibbs, Jr. 1978. R-factor inheritance and plasmid content in mucoid Pseudomonas aeruginosa. Infect. Immun. 22:530-539.

5. Meyers, J.A., D. Sanchez, L.P. Ellwell, and S. Falkow. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol 127:1529-1537.

6. Myers, William F. and Charles L. Wisseman, Jr. 1980. Genetic relatedness among the typhus group of Rickettsiae. International J. of Systematic Bacteriology. 30:143-150.

7. Radloff, Roger, William Bauer, and Jerome Vinograd. 1967. A dye-buoyant density method for the detection and isolation of closed circular duplex DNA: The closed circular DNA in Hela cells. PNAS. 57:1514-1521.

8. Timmis, F.N., S.N. Cohen, and F.C. Cabello. 1978. DNA cloning and the analysis of plasmid structure and function. Progress in Molecular and Subcellular Biology. Vol. 6. Ed. Fred H. Hahn. Springer-Verlag. Berlin, Heidelberg, New York.

9. Walker, T. Stuart and Herbert H. Winkler. 1978. Penetration of cultured mouse fibroblasts (L Cells) by Rickettsia prowazekii. Infect. Immun. 22:200-208.

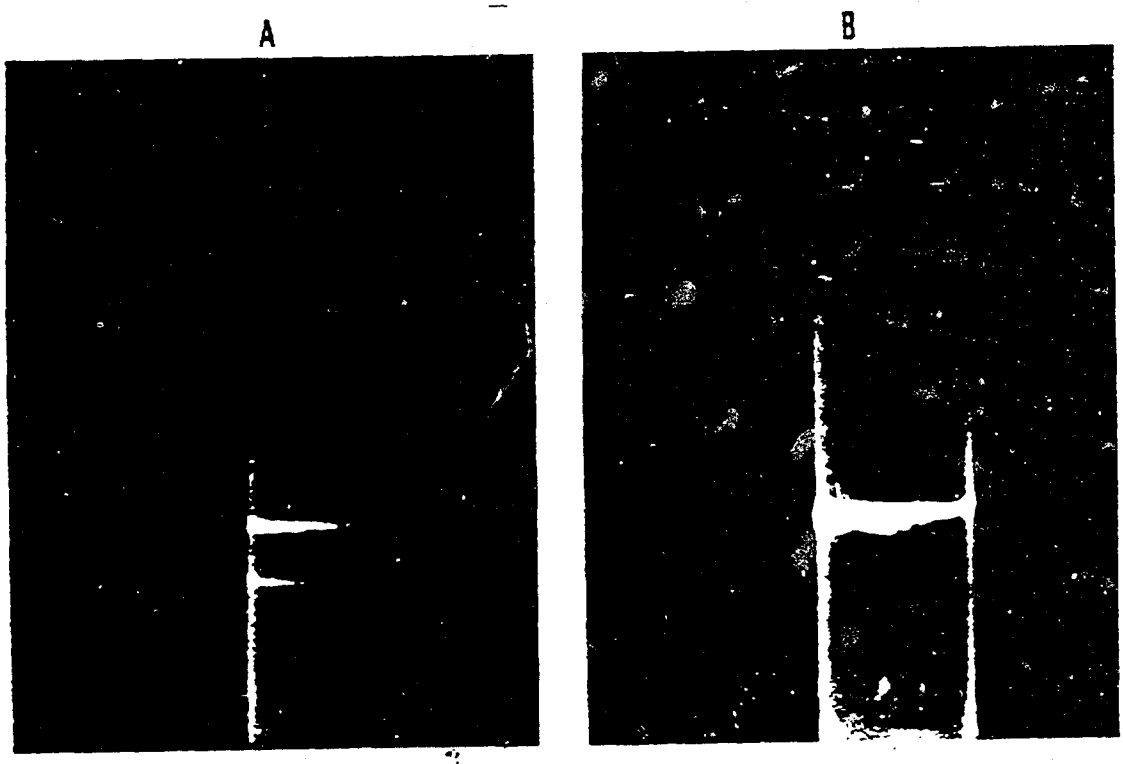


Fig. 1. CsCl-EB dye buoyant equilibrium density gradients of whole cell lysates of (A) P. aeruginosa (MCB82) and (B) R. prowazekii (Breinl). Gradients were centrifuged at 50,000 rpm at 20°C for 48 h. DNA bands were visualized by viewing the gradients with ultraviolet light. Photographs were taken with a polaroid MP-4 system using Type 665 Polaroid film and a Wratten #9 filter.

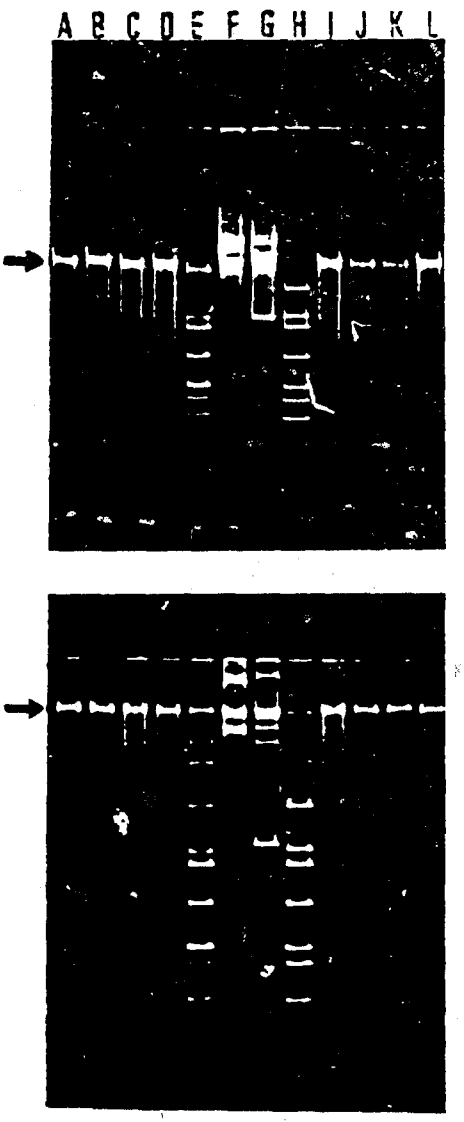


Fig. 2. Agarose gel electrophoresis analysis of small volume cell lysates of TCP: (A-D) Breinl strain of R. prowazekii . 0.05, 0.1, 0.5, and 1.0 mls respectively of a cell suspension at a protein concentration of approximately 10 mg/ml. (E) E. coli V517 (F) E. coli (MCB79) (G) P. aeruginosa (MCB82) (H) purified plasmid DNA from E. coli V517 (3). (I-L) Madrid E strain of R. prowazekii. 0.05, 0.1, 0.5, and 1.0 mls. respectively, of a cell suspension at a protein concentration of approximately 10 mg/ml. Arrows indicate band of contaminating chromosomal DNA. The additional bands in E, F, and G are various physical configurations (CCC, open circular, linear) of plasmid DNA. BOTTOM: Similar samples run under conditions of lower voltage. In both cases, agarose concentration was 0.8%, and migration was from top to bottom. The cell concentrations of the E. coli and P. aeruginosa strains were approximately 10-fold less than those of the R. prowazekii strains.

DISTRIBUTION LIST

12 Copies	Director Walter Reed Army Institute of Research ATTN: SGRD-UWZ-AG Walter Reed Army Medical Center Washington, DC 20012
4 Copies	Commander US Army Medical Research and Development Command ATTN: SGRD-AJ/Mrs. Madigan Fort Detrick, Frederick, MD 21701
12 Copies	Defense Documentation Center (DDC) ATTN: DDC-TCA Cameron Station Alexandria, VA 22314
1 Copy	Superintendent Academy of Health Sciences, US Army ATTN: AHS-COM Fort Sam Houston, TX 78234
1 Copy	Dean, School of Medicine Uniformed Services University of the Health Sciences Office of the Secretary of Defense 4301 Jones Bridge Road Bethesda, MD 20014