INFECTION AND IMMUNITY, Jan. 1989, p. 36–40
0019-9567/89/010036-05$02.00/0

Vol. 57, No. 1

Phospholipase A Activity Associated with the Growth of *Rickettsia prowazekii* in L929 Cells

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

Received 14 July 1988/Accepted 26 September 1988

Cultured L929 cells infected with *Rickettsia prowazekii* had a greatly increased rate of hydrolysis of fatty acid from the oleic acid-radiolabeled phospholipids of the host cell membranes. The incorporation of fatty acid into phospholipid in an infected cell was only moderately inhibited relative to a mock-infected cell. Thus, even if the release of fatty acid from phospholipid represented a steady state between hydrolysis and resynthesis of phospholipids, the increase in release of fatty acid was due principally to increased phospholipase A activity. The increased rate of hydrolysis did not occur only late in the rickettsial infection; this activity began early in infection and continued throughout the course of infection. The addition of tetracycline or chloramphenicol (antibiotics which inhibit rickettsial protein synthesis) to the infected cells caused a rapid and total abatement of this increased rate of phospholipid hydrolysis. In contrast, high concentrations of penicillin affected the morphology of the intracellular rickettsiae, but did not inhibit the phospholipase activity. This phospholipase A activity clearly damages the host cell during the rickettsial infection and may represent the activity by which *R. prowazekii* escapes from the host cell.

Rickettsia prowazekii organisms are obligate intracellular bacterial parasites (1, 13). Ultrastructurally and compositionally, they resemble typical gram-negative bacteria. These organisms can only grow within the cytoplasm of the host cell, in which they are unbounded by either a phagosomal or a phagolysosomal membrane. These bacteria are the etiologic agents of epidemic typhus, a louse-vectored human disease.

Three general challenges to the parasite are presented during its growth in an infected organism. First, the parasite must go from the blood stream or interstitial fluid, where it was deposited by the vector (or by the release of the parasite from previously infected cells), and enter the cytoplasm of the host cell. After first interacting with an unidentified receptor on the host cell surface, the rickettsia, if it is metabolically active and the host cell is competent for the endocytosis of the parasite, can cross the plasma membrane of the host cell by a process termed induced phagocytosis (4, 10, 11). A phospholipase A_2 activity has been observed upon the interaction of extracellular rickettsiae with the surface of eucaryotic cells (17, 18). If the eucaryotic cell is an erythrocyte, a cell that is unable to phagocytize rickettsiae, this phospholipase activity results in hemolysis (16). In contrast, in a competent host cell, it has been proposed that the phospholipase A_2 activity in a concerted reaction both stimulates the host cell to internalize the rickettsiae and provides a means for that rickettsia to escape the phagosome and be released into the cytoplasm (15, 17). It has not been established whether this phospholipase is a rickettsial enzyme or, alternatively, a latent enzyme of the host cell which is activated by the attachment of the rickettsia.

The second challenge for the parasite is to grow in this unusual environmental niche. *R. prowazekii* organisms are able to grow with a generation time of about 8 h and eventually fill the cytoplasm of the host cell (1, 13, 20, 22).

The third challenge to these organisms is to exit from the infected host cell to renew the cycle of infection. During the exponential growth of *R. prowazekii*, very few bacteria exit from the infected cell to set up secondary infections in neighboring cells (20, 22). Instead, *R. prowazekii* grows and fills the cytoplasm until, presumably, the host can no longer support the growth of the parasite. The host cell then bursts and a bolus of hundreds of *R. prowazekii* organisms are released to initiate infections in many new host cells.

The mechanism by which *R. prowazekii* exits from the host cell as a burst, late in infection, has not been elucidated. It seems reasonable to postulate that either the activity responsible for the burst is induced only late in infection or the burst is due to the accumulation of damage to the host cell during the entire course of the infection, an accumulation that eventually overcomes the ability of the host cell to repair such damage. Furthermore, one can suggest a role for phospholipase A in the destruction of the host cell membrane that would be necessary for the exit of rickettsiae from the host cell since *R. prowazekii* has either a phospholipase A or the ability to activate a latent phospholipase A. The existence of a phospholipase A activity associated with the growth of *R. prowazekii* in L929 cells is the subject of this publication. Although at this time proof of a direct and causual relationship between this phospholipase activity and the exit or release of *R. prowazekii* from the host cell is lacking, this study provides the first step in the search for such documentation.

MATERIALS AND METHODS

Cell culture methods. L-929 mouse fibroblast cells were X-irradiated with 5,000 rads to prevent cell division and suspended in Eagle minimum essential medium supplemented with 10% newborn calf serum without antibiotics. The cell suspension was adjusted to a density of 5×10^5 cells per ml, 0.25 μCi of [9, 10-^{3}H]oleic acid (Du Pont Co., Wilmington, Del.) per ml was added, and 1-ml samples were plated into 35-mm dishes containing a sterile 13-mm glass cover slip. The cells were incubated for 8 h at 34°C. The labeling medium was removed from each dish and replaced for overnight incubation with 1 ml of Iscove modified Dul-

* Corresponding author.

becco medium supplemented with 400 μg of linoleic acid-bovine serum albumin complex per ml, 2 mM L-glutamine, 1.1 mg of sodium pyruvate per liter, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid), 4 g of glucose per liter, 10 μg of insulin per liter, 10 μg of human transferrin per liter, 10 μM 2-aminoethanol, 50 μM 2-mercaptoethanol, and 0.01 μM sodium selenite (IMDM medium). The remainder of the experiment was conducted in this medium.

The next day cells were mock infected (the same manipulation were performed as in the infected cultures but no rickettsiae were added) or infected with *R. prowazekii* at a ratio of 150 viable organisms per L cell (12) in Hanks balanced salts solution plus 0.1% gelatin and 5 mM glutamic acid. The same phospholipase A activity was observed in both the avirulent Madrid E and virulent Breinl strains of *R. prowazekii* as was expected since the growth of these two strains in fibroblast cultures is identical. Prudently, most of the work was done with the Madrid E strain, and only data from this strain are shown. The source of *R. prowazekii* was the final 80% suspension of infected egg yolk sacs purified by a modification (14) of previously described methods (3, 19). After 1 h of infection, the monolayers were washed once with Hanks balanced salt solution plus 0.1% gelatin and 5 mM glutamic acid and once with IMDM, and 1.5 ml of IMDM was added to the cells. At a ratio of 150 rickettsiae per cell during the 1 h of infection, an average of 5 intracellular rickettsiae were found per cell. The rickettsial burden increased throughout the experiment at a rate consistent with an 8-h generation time for the rickettsiae. The rickettsiae were allowed to grow for 24 h before lipid extraction or treatment. After 24 h of rickettsial growth (24 h postinfection [p.i.]), chloramphenicol (50, 5.0, or 0.5 μg/ml), tetracycline (20 μg/ml), penicillin G (200 μg/ml), or nothing was added to the medium. The growth of the rickettsiae within the cells during the investigated intervals of fatty acid release or phospholipid incorporation was monitored by removing the cover slip from the dish just prior to extraction and staining by the method of Gimenez (5).

At the indicated times (usually 24, 28, 32, 48, and 56 h p.i.), either the combined complete culture system of monolayer and medium or just the medium was extracted for lipid content. In the experiments in which the medium and monolayer were extracted separately, the medium was removed for extraction and then the monolayer was washed twice with 1% fat-free bovine serum albumin in phosphate-buffered saline and fresh IMDM was placed on the cells for the next time interval. This procedure was repeated, continuing throughout the length of the experiment, so that medium for several time points was obtained from the same monolayer.

To assess the ability of the cells to reincorporate released fatty acid, cells were infected and grown for 24 or 48 h without label. At this time, labeled oleic acid was added to the medium, the cells were incubated for 8 h, and the monolayer was then extracted to measure incorporation of labeled fatty acid into phospholipids and neutral lipids.

Lipid methodology. To determine the distribution of labeled fatty acid into the various lipids in the cells and the release of fatty acid from the phospholipids, lipids were extracted by a modification of the method of Bligh and Dyer (2). The culture medium (1.5 ml) was removed to an extraction tube, and the cells were scraped into 3.8 ml of methanol, which was pooled with the medium. Chloroform (1.9 ml) was added, and the resulting monophase system was gassed with nitrogen and refrigerated overnight. When a monolayer without medium was extracted, then 1.5 ml of water was added to the extract system. The lipids were separated from the water-soluble material by adding 1.9 ml each of water and chloroform and centrifuging the mixture to separate the phases. The chloroform layer was evaporated to dryness and stored under nitrogen at −20°C. This material was then redissolved in 200 μl of chloroform, and the lipids were separated by elution from a 3-ml column of aminopropylsilane-bonded silica gel (J. T. Baker Chemical Co., Phillipsberg, N.J.), using a manifold system (6). After the column was activated with 4 ml of hexanes, neutral lipids were eluted with 4 ml of chloroform–2-propanol (2:1), fatty acid was eluted with 4 ml of 2% acetic acid in diethyl ether, and phospholipids were eluted with 4 ml of methanol. The eluents were collected in 20-ml glass scintillation vials, evaporated to dryness, and counted by liquid scintillation techniques.

Plasma membrane isolation. The amount of labeled phospholipids associated with the L-cell plasma membrane was determined. Dishes of infected or mock-infected labeled cells were grown for 48 h p.i., during which time the medium had been removed, the cells had been washed with 1% fat-free bovine serum albumin, and IMDM had been replaced every 12 h. At 48 h p.i., the medium was extracted, and the cells were rinsed and scraped into 750 μl of 10 mM Tris–0.25 M sucrose, pH 7.4. The plasma membranes were separated by the method of Morand and Kent (7). Briefly, the cells were broken by Dounce homogenization and incubated on ice with DNase I to reduce clumping of cellular debris, and a portion of this suspension was mixed with Percoll (Pharmacia, Inc., Piscataway, N.J.) at a final concentration of 15% Percoll in a 3-ml volume. Gradient forma-

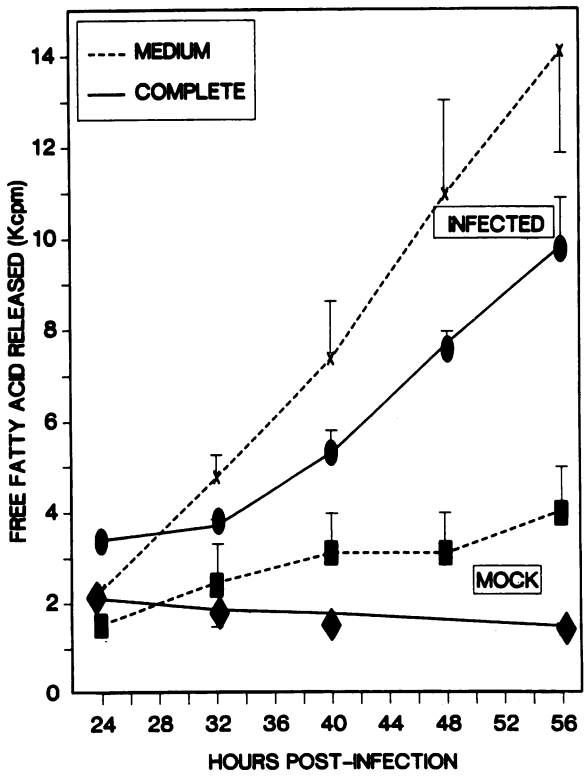

FIG. 1. Release of fatty acid from labeled host cell phospholipids from infected and mock-infected L-929 cells. Means and standard deviations of five experiments are shown.

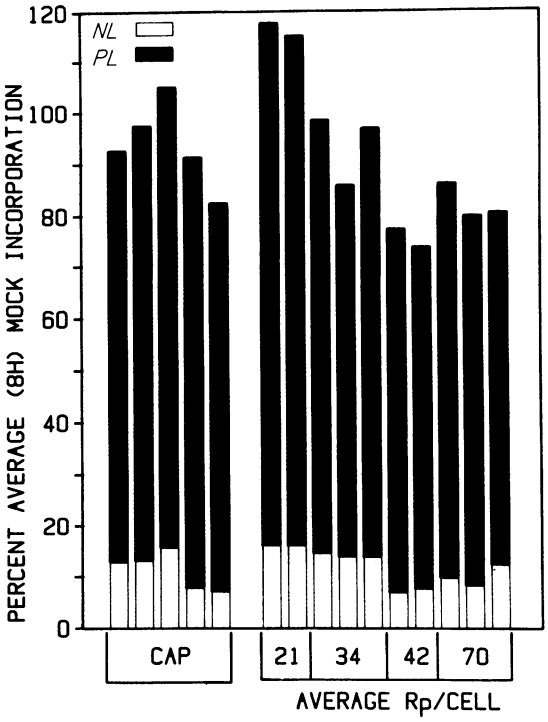

FIG. 2. Effect of rickettsial infection on fatty acid incorporation into host cell neutral lipid (NL) and phospholipid (PL). Total incorporation is shown as percentage of mock-infected cell incorporation. Distribution into neutral lipid and phospholipid is shown by the shading of the bars. Chloramphenicol (CAP; 50 μg/ml) was added where indicated. Rp, *R. prowazekii*.

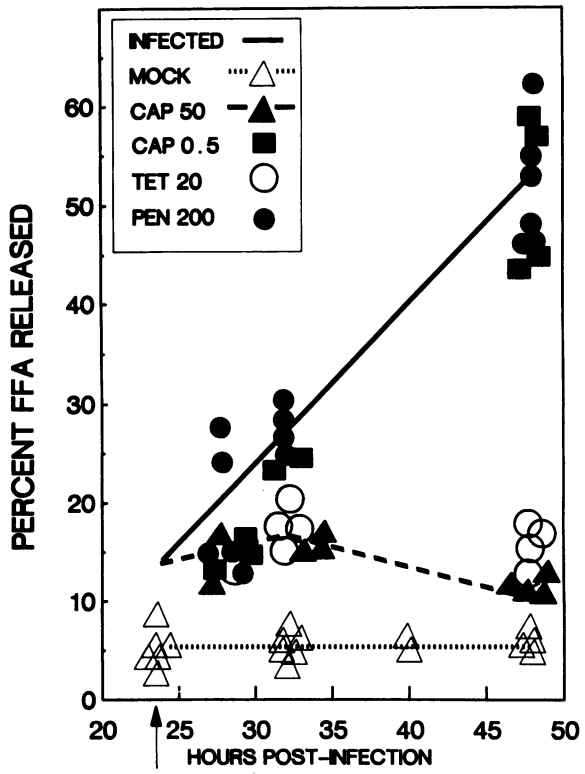

FIG. 3. Effect of antibiotics on phospholipase activity. Shown is a composite of several experiments with the indicated drugs and concentrations (in micrograms per milliliter). For clarity, the data from infected cells without antibiotic are indicated by the linear regression line from 23 points. The complete system extraction protocol was used throughout. CAP, Chloramphenicol; TET, tetracycline; PEN, penicillin G.

tion and membrane separation were accomplished by centrifugation at 28,000 × *g* for 25 min at 4°C in a TL 100.3 fixed-angle rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The gradient was fractionated, and the distribution of radioactivity throughout the gradient was determined by liquid scintillation techniques. The rickettsiae within an infected cell made a negligible contribution to the total protein of the extract. Fractions within the plasma membrane peak were pooled, and the ratios of protein content of these pools in the infected and mock-infected cells were very similar to the protein ratios in the total extracts from these cells. Therefore, the protein content of the extract was used as a basis of normalization so that the lowering of radiolabel in the membranes would not be a result of less membranes.

RESULTS

Release of fatty acid. There was little release of fatty acid from the oleic acid-labeled phospholipids of the mock infected cells whether the combined medium and monolayer were assayed at the indicated times or the medium from the same monolayer was removed and assayed (Fig. 1). In contrast, cultures that were initially infected with about five rickettsiae per cell demonstrated a marked and continual release of fatty acid throughout the course of the experiment (Fig. 1). In both the mock-infected and the infected cultures, the periodic removal and replacement of the medium from the same monolayer of cells was associated with a greater fatty acid release into the medium than when separate cultures were extracted at the various time points. This indicated that essentially all fatty acid released was in the medium (this was confirmed by extraction of both the monolayers and medium separately, but at the same time point).

These results also suggested that some of the hydrolyzed fatty acid in the medium was reincorporated into the phospholipids of the cells so that periodic removal of the medium would lead to an observed higher level of release. The level of oleic acid incorporation into phospholipid and neutral lipid by infected cells with a rickettsial burden of 21 to 70 rickettsiae per infected cell was only slightly less than that observed in mock-infected cells (Fig. 2). Thus, even if the release of fatty acid from phospholipid represented a steady state between hydrolysis and resynthesis of phospholipids, the increase in release of fatty acid was due principally to increased phospholipase A activity. The addition of chloramphenicol to infected cells at 24 h p.i. inhibited rickettsial growth but had no effect on the incorporation of oleic acid over the next 8 h.

Effect of inhibition of rickettsial growth. Tetracycline and chloramphenicol inhibit rickettsial protein synthesis and growth. The addition of either of these antibiotics to the infected cultures 24 h p.i. inhibited the subsequent release of oleic acid from the phospholipids of the host cell (Fig. 3). The *P* values determined from Student's *t* test at 28, 32, and 48 h p.i. were <0.2, <0.02, and <0.001 for chloramphenicol at 50 μg/ml and <0.3, <0.03, and <0.001 for tetracycline. The inhibition of the phospholipase A activity associated with the rickettsial infection could be observed as rapidly as 4 h after antibiotic addition and was absolute at the high concentrations. Chloramphenicol at 0.5 μg/ml had no effect,

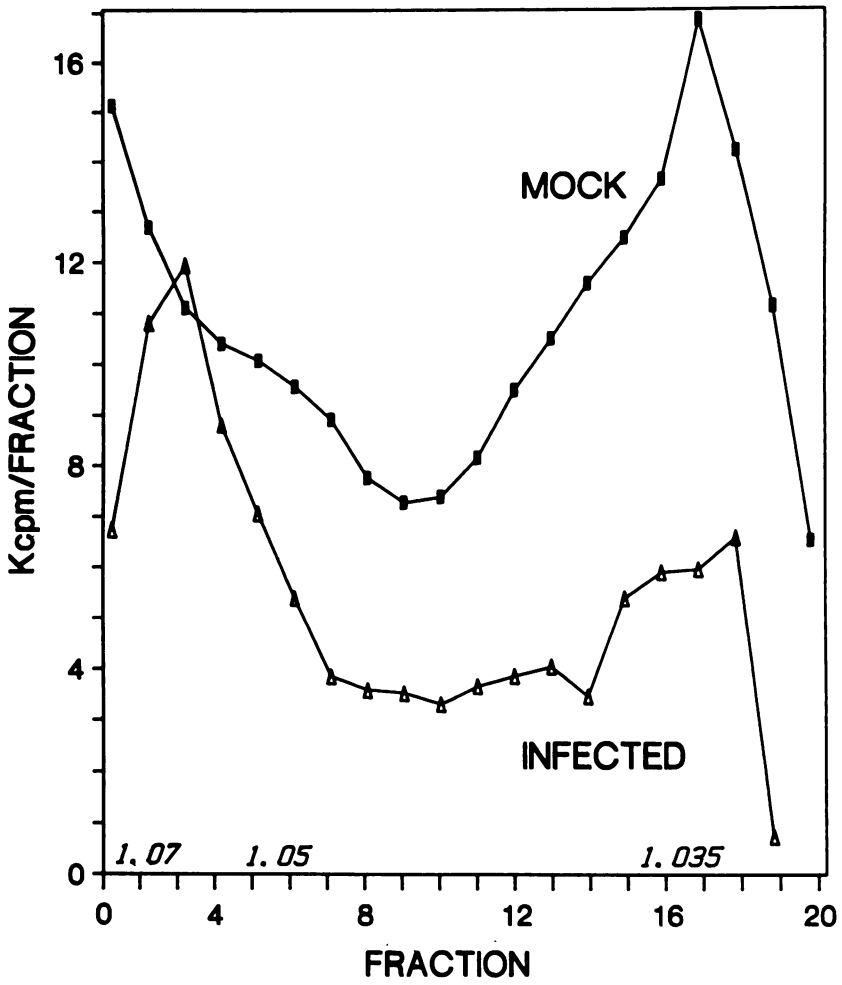

FIG. 4. Fractionation of membranes from infected and mock-infected cells: a representative experiment in which the medium had been changed on the cells daily and the cells were fractionated at 48 h p.i. The protein content of the infected cell homogenate was 74% of the mock-infected homogenate, and the infected cell curve was normalized so that it would represent the same protein concentration as the mock-infected cell curve.

and only partial inhibition was seen at 5 μg/ml (*P* values were <0.3, <0.2, and <0.01 at 28, 32, and 48 h p.i., respectively). Penicillin G did not inhibit (*P* >0.9) the phospholipase A activity even though the staining characteristics of the rickettsiae were altered by this concentration of the drug, presumably due to the formation of spheroplasts (20). Penicillin had no effect on the phospholipase A activity in mock-infected cultures, eliminating the possibility that penicillin did in fact inhibit the infection-associated phospholipase but masked this inhibition by activating another phospholipase not related to the rickettsiae (data not shown).

Isolation of plasma membranes from the infected cultures. If this phospholipase A activity is related to the exit of the rickettsiae from the host cell, then the plasma membrane would be a likely target. The plasma membranes from infected and mock-infected cells at 48 h p.i. were fractioned on Percoll gradients (Fig. 4). The labeled oleic acid content of the phospholipids of plasma membrane fraction (*d* = 1.035) in the mock-infected cells was prominent after the 48-h incubation. However, the plasma membrane fraction from the infected cells was greatly decreased in radioactivity.

DISCUSSION

There was an accumulation of fatty acid derived from the phospholipids of host cell membranes in the culture medium of cells in which *R. prowazekii* organisms were growing. This accumulation was due to an increased rate of hydrolysis of phospholipid (phospholipase A) in infected cells since the rate of incorporation of fatty acid into phospholipid was little changed. Fatty acid began to accumulate in the infected cultures as early as could be measured (24 h p.i.) and continued to accumulate until the infection destroyed the host cell culture. It seems likely that the graphs do not illustrate an exponential increase in phospholipase activity with rickettsial growth because the substrate concentration was falling at the same time as the rickettsial numbers were increasing.

The dependence of phospholipase activity upon continued rickettsial protein synthesis is most striking. The addition of rickettsiostatic antibiotics that inhibit rickettsial protein synthesis caused a very rapid reversal in the augmentation of the phospholipase activity. When the antibiotic was added at 24 h p.i., no additional fatty acid release was observed 4 h later.

However, the inhibition of the rickettsia-associated phospholipase activity by chloramphenicol and tetracycline cannot establish whether the phospholipase per se is a rickettsial or a host cell enzyme. This inhibition does indicate that a relatively labile protein is produced by the rickettsiae which is necessary for phospholipase A activity. The labile rickettsial protein could be the phospholipase A itself, an accessory protein (for example, a protein necessary to bring the rickettsia in apposition to the host cell membrane), or a protein which activates a latent host cell phospholipase A.

The role of this phospholipase A activity is unknown. This activity could be simply the continued vestigial action of the system involved in the entry of *R. prowazekii* and have no value once the rickettsiae have been internalized in the cell. More interestingly, this activity could be responsible for the destruction of the host cell membrane and the release of the rickettsiae. It would be difficult to understand how such extensive hydrolysis of the host cell phospholipid and the resulting cellular pathology could be evolutionarily advantageous for the parasite unless this activity had a role in the exit of the parasite. The activity appears to be constitutive during the intracellular growth of the rickettsiae rather than temporally regulated by the rickettsiae to coincide with an optimal time for the rickettsiae to be released from the host cell. Presumably while the rickettsiae are growing, the host cell can repair the damage to its membranes and thus remain viable and intact, allowing the parasite to have a suitable external milieu. In fact, by electron microscopy there is little intracellular pathology as *R. prowazekii* (in contrast to *R. rickettsii*) grows in the cytoplasm (8, 9). However, rickettsial growth will eventually produce very extensive damage that is beyond the ability of the host cell to repair it. We hypothesize that, when this eventually occurs, the rickettsial burden is such that the parasite population can no longer expand at a reasonable rate within this host cell and would benefit from exit from the current host cell and entry into new cells that can be efficiently parasitized.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant AI-15035 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Austin, F. E., and H. H. Winkler.** 1988. Relationship of rickettsial physiology and composition to the rickettsia-host cell interaction, p. 29–50. *In* D. H. Walker, (ed.), Biology of rickettsial diseases, vol. 2. CRC Press, Inc., Boca Raton, Fla.
2. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
3. **Bovarnick, M. R., and J. C. Snyder.** 1949. Respiration of typhus rickettsiae. J. Exp. Med. **89**:561–565.
4. **Cohn, Z. A., F. M. Bozeman, J. M. Campbell, J. W. Humphries, and T. K. Sawyer.** 1959. Study on growth of rickettsiae. V. Penetration of *Rickettsia tsutsugamushi* into mammalian cells *in vitro*. J. Exp. Med. **109**:271–292.
5. **Gimenez, D. F.** 1964. Staining rickettsiae in yolk sac culture. Stain Technol. **39**:135–140.
6. **Kaluzny, M. A., L. A. Duncan, M. V. Merrett, and D. E. Epps.** 1985. Rapid separation of lipid classes in high yield and purity used bonded phase columns. J. Lipid Res. **26**:135–140.
7. **Morand, J. N., and C. Kent.** 1986. A one-step technique for the subcellular fractionation of total cell homogenates. Anal. Biochem. **159**:157–162.
8. **Silverman, D. J.** 1984. *Rickettsia rickettsii*-induced cellular injury of human vascular endothelium in vitro. Infect. Immun. **44**:548–553.
9. **Silverman, D. J., C. L. Wisseman, Jr., and A. Waddell.** 1980. In vitro studies of rickettsia-host cell interactions: ultrastructural study of *Rickettsia prowazekii*-infected chicken embryo fibroblasts. Infect. Immun. **29**:778–790.
10. **Walker, T. S.** 1984. Rickettsial interactions with human endothelial cells in vitro: adherence and entry. Infect. Immun. **44**: 205–210.
11. **Walker, T. S., and H. H. Winkler.** 1978. Penetration of cultured mouse fibroblasts (L cells) by *Rickettsia prowazekii*. Infect. Immun. **22**:200–208.
12. **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.
13. **Weiss, E., M. E. Dobson, and G. A. Dasch.** 1987. Biochemistry of rickettsiae: recent advances. Acta Virol. **31**:271–286.
14. **Winkler, H. H.** 1976. Rickettsial permeability: an ADP-ATP transport system. J. Biol. Chem. **251**:389–396.
15. **Winkler, H. H.** 1986. Early events in the interaction of the obligate intracytoplasmic parasite, *Rickettsia prowazekii*, with eucaryotic cells: entry and lysis. Ann. Inst. Pasteur (Paris) **137A**:333–336.
16. **Winkler, H. H., and E. T. Miller.** 1980. Phospholipase A activity in the hemolysis of sheep and human erythrocytes by *Rickettsia prowazekii*. Infect. Immun. **29**:316–321.
17. **Winkler, H. H., and E. T. Miller.** 1982. Phospholipase A and the interaction of *Rickettsia prowazekii* and mouse fibroblasts (L-929 cells). Infect. Immun. **38**:109–113.
18. **Winkler, H. H., and E. T. Miller.** 1984. Activated complex of L-cells and *Rickettsia prowazekii* with *N*-ethylmaleimide-insensitive phospholipase A. Infect. Immun. **45**:577–581.
19. **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**: 123–126.
20. **Wisseman, D. L., Jr., D. J. Silverman, A. Waddell, and D. T. Brow.** 1982. Penicillin-induced unstable intracellular formation of spheroplasts by rickettsiae. J. Infect. Dis. **146**:147–158.
21. **Wisseman, C. L., Jr., and A. D. Waddell.** 1975. In vitro studies on rickettsia-host cell interactions: intracellular growth cycle of virulent and attenuated *Rickettsia prowazekii* in chicken embryo cells in slide chamber cultures. Infect. Immun. **11**:1391–1401.
22. **Wisseman, C. L., Jr., A. D. Waddell, and D. J. Silverman.** 1976. In vitro studies on rickettsia-host cell interactions: lag phase in intracellular growth cycle as a function of stage of growth of infecting *Rickettsia prowazekii* with preliminary observations on inhibition of rickettsial uptake by host cell fragments. Infect. Immun. **13**:1749–1760.