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Fate of Phase I and Phase II *Coxiella burnetii* in Several Macrophage-Like Tumor Cell Lines

O. G. BACA,[1]* E. T. AKPORIAYE,[1] A. S. ARAGON,[1] I. L. MARTINEZ,[1] M. V. ROBLES,[1] AND N. L. WARNER[2]

Department of Biology[1] and the School of Medicine Departments of Pathology and Medicine,[2] The University of New Mexico, Albuquerque, New Mexico 87131

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Several macrophage-like tumor cell lines of murine origin were exposed to phase I and phase II *Coxiella burnetii*, and the subsequent fate of the parasites was determined by electron and bright-field microscopy. Phase I *C. burnetii* proliferated within and established a persistent infection of P388D1, J774, and PU-5-IR cell lines but not of WEHI-3 and WEHI-274 cell lines. Phase II *C. burnetii*, however, entered into and persistently infected all five cell lines. The parasites proliferated within vacuoles. Macrophage cell lines persistently infected with phase I and phase II *C. burnetii* were maintained for over 200 and 100 days, respectively. Within P388D1 cells, the phase I *C. burnetii* converted, in part, to phase II; phase II organisms remained in the phase II state. The differential fate of the two rickettsial phases after exposure to the WEHI-3 and WEHI-274 cells may be attributable to surface differences such as lipopolysaccharide content.

The mononuclear phagocytic system plays a key role in the control of infectious agents. However, there are several facultative and obligate intracellular parasites that are not destroyed by phagocytes but, instead, poliferate within them. The properties of these microorganisms and the mechanisms involved in avoiding intracellular killing, are, for the most part, unknown. In the case of the diverse group of obligate intracellular procaryotic parasites classified as the rickettsiae, virtually nothing is known about how they avoid destruction within "professional" phagocytes (i.e., polymorphonuclear leukocytes and cells of the monocytic series). In vitro studies have shown that several rickettsial species, including *Coxiella burnetii*, multiply within normal macrophages (4, 6, 8, 11, 12, 17). In the case of *C. burnetii*, both the naturally occurring phase I organism and the phase II variant, that appears after serial passage in embryonated eggs (26), grow within phagocytes. Previous work has also indicated that killed phase II *C. burnetii* are phagocytized by human polymorphonuclear leukocytes to a greater extent than killed phase I *C. burnetii* (33).

One of the long-range goals of our research is to determine the mechanism(s) that accounts for the intracellular survival of *C. burnetii*; this includes the identification of its properties (i.e., surface components) that allow proliferation. We have therefore chosen to examine the interaction, including the entry and fate, of *C. burnetii* with a series of macrophage-like tumor cell lines: PU-5-IR, J774, P388D1, WEHI-3, and WEHI-274. These macrophage-like tumor lines have been chosen as model host cells for the following reasons: (i) with one exception (P388D1), all of the lines are derived from the same inbred mouse strain; (ii) population homogeneity; (iii) they exhibit varying capacities to interiorize particles; (iv) unlike macrophages derived from the host, they divide indefinitely in vitro; and (v) they have been extensively characterized with respect to phagocytic capabilities and other properties characteristic of macrophages (16, 30). A distinct problem with macrophages derived from various animals is the functional heterogeneity exhibited by such phagocytes whether obtained from the same tissues or sites (9, 29). Subsequently, results obtained with such cells must be interpreted with caution.

Recently, we examined the kinetics of association-attachment of ^{32}P-labeled phase I *C. burnetii* with the five cell lines (O. G. Baca, A. S. Aragon, E. T. Akporiaye, I. L. Martinez, and N. L. Warner *in* W. Burgdorfer and R. Anacker (ed.), *RML Conference on Rickettsiae and Rickettsial Diseases*, in press). The order of association-attachment was: J774 > P388D1 > PU-5-IR > WEHI-3 > WEHI-274. In this report we show that phase I *C. burnetii* can proliferate and establish a persistent infection in some of the macrophage-like cell lines but not in others. Phase II *Coxiella* sp., however, proliferates in all the lines.

MATERIALS AND METHODS

C. burnetii propagation and purification. Phase

I and II *C. burnetii*, Nine Mile strain, were used in these studies. Both phases were obtained from R. A. Ormsbee, Rocky Mountain Laboratory, U.S. Public Health Service, Hamilton, Mont. The organisms were cloned by Ormsbee and Peacock (18) in primary chicken embryo cells. For these studies, the rickettsiae were propagated in mouse fibroblast L cells (L-929) maintained in suspension culture (3). Infected and uninfected L-929 cells were grown in antibiotic-free Eagle minimum essential medium, spinner modified, with L-glutamine, $NaHCO_3$ (0.22%), and 5% heat-inactivated calf serum. Infected cells were disrupted in a Ten Broeck tissue grinder, and the released rickettsiae were purified by differential centrifugation. Three cycles of low speed-high speed centrifugation (800 × *g* and 10,000 × *g*) resulted in rickettsiae essentially free of host material. The purified organisms were suspended in Dulbecco balanced salt solution and stored at −70°C until required. Rickettsiae concentrations were determined by the methods of Silberman and Fiset (24).

Cell cultures were routinely tested for bacterial contamination by inoculation into thioglycolate broth. Mycoplasma testing was periodically performed by standard procedures.

Macrophage tumor cell lines. All tumor cells used in these studies were from established tissue culture cell lines maintained in Dulbecco modified Eagle media with 5 to 10% fetal calf serum with nonessential amino acids included for the PU-5 line. The cultures were passaged every 3 to 4 days as their cell densities approached 10^6/ml. The monocyte-macrophage cell lines (including original reference) used were: J774 (21), P388D1 (14), PU-5-IR (20), WEHI-3 (31), and WEHI-274 (E. Walker, J. B. Richey, and N. L. Warner, Fed. Proc. **38**:1418, 1979). All of these lines were originally derived from BALB/c mice, except P388D1, which originated from the DBA/2 mouse strain. The cells were maintained in suspension culture in plastic petri dishes at 37°C in a 4% CO_2 atmosphere. Cell viabilities were determined by the dye (erythrosin B or trypan blue) exclusion technique (19). Although some of these lines had been originally cloned, this has not been repeated for any of the lines recently. Although these cell lines show distinctly different functional properties, all may be definitively characterized as belonging to the monocyte-macrophage lineage on the basis of specific cell surface markers, e.g., MAC-1 (25; E. Walker, N. L Warner, L. Hammer, and T. Springer, manuscript in preparation) and functional properties (N. L. Warner, R. K. Cheney, L. L. Lanier, M. Daley, and E. Walker *in* M. A. S. Moore (ed.), *Maturation Factors in Cancer*, in press). A summary of these properties is listed in Table 1, with detailed references to these properties being listed by Warner et al., in press.

Experimental procedure. All the macrophage cell lines were exposed to phase I and phase II *C. burnetii* and examined periodically for the presence or absence of cell-associated rickettsiae. Bright-field microscopy of Gimenez-stained cells (7) and transmission electron microscopy were employed. Before exposure to the parasites, all the cell lines were washed and suspended to appropriate concentrations in complete antibiotic-free growth medium and exposed to multiplicities of infection (MOI) of 5, 50, and 500 rickettsiae. The macrophage cell concentrations at the time of exposure were 2×10^4 cells per ml, except P388D1 which was 4×10^4 cells per ml. The cells were kept at 37°C in a 4% CO_2 atmosphere and passaged every 3 days, at which time samples were removed and examined for the presence of cell-associated rickettsiae. At least 200 Gimenez-stained cells from each sample were examined for the presence of cell-associated rickettsiae.

Serological procedure. The complement fixation procedure of Fiset (5) was used.

Electron microscopy. Samples were prepared for electron microscopy by standard techniques. Sedimented material was fixed in 2.5% glutaraldehyde prepared in 0.1 M cacodylate buffer, pH 7.4. The material was washed in buffer and postfixed in 1% OsO_4. After the cells were washed, they were dehydrated through an alcohol series into propylene oxide. The cells were embedded in Spurr medium and sectioned with a Porter-Blum microtome. The sections were placed on specimen grids, double-stained with uranyl acetate and lead citrate, and examined with either an AEI Corinth 275 or 6B transmission electron microscope.

RESULTS

Fate of phase I *C. burnetii*. The fate of both phases of *C. burnetii* in all five cell lines was followed over a period of weeks to months. Figure 1 shows that initially all of the cell lines associated with one or more phase I rickettsiae; some of the lines contained a higher percentage of infected cells than others. By day 3 postinfection, the J774 and P388D1 populations contained the highest percentage of cells that were associated with phase I rickettsiae. By day 10, the majority (75 to 85%) of the J774 and P388D1 cells were infected; within 13 to 25 days, 100% of

TABLE 1. *Summary of functional properties of murine macrophage-like cell lines*[a]

Tumor cell line	Phagocytosis	Fc receptor	C3 receptor	Immune suppression	Chemiluminescence	IL-1 production	MAC-1 surface antigen	Lysozyme production
WEHI-274	−	+	±	−	+++		±	−
WEHI-3	+ (subpopulation)	+	++	−	−	++	+	++
PU-5-IR	++	++	+++	±	++	++	++	±
P388D1	+++	+++	+++	++	−	+++	+	±
J774	+++	+++	+++	++	+		+	+

[a] For detailed references see Warner et al., in press, and Morahan (16).

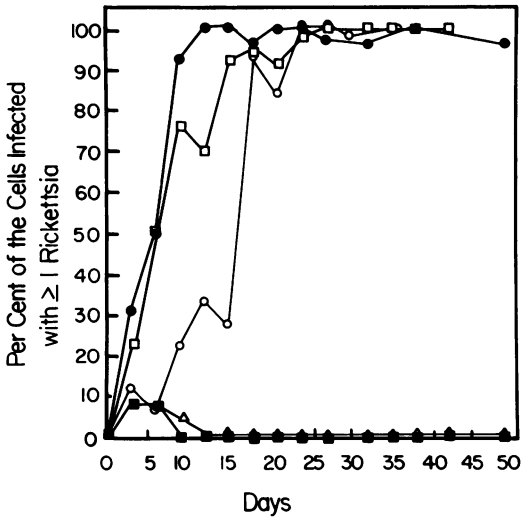

FIG. 1. *Fate of phase I C. burnetii in macrophage-like tumor cell lines J774* (□), *P388D1* (●), *PU-5-IR* (○), *WEHI-3* (△), *and WEHI-274* (■). *MOI was 500. Infectivities were determined as described in the text.*

the P388D1, PU-5-IR, and J774 cells contained phase I *C. burnetii*. Electron microscopy of thin sections of phase I-infected J774 (Fig. 2a), P388D1 (Fig. 2b), and PU-5-IR (Fig. 2c) cells revealed the presence of numerous rickettsiae within vacuoles. Many of the cells possessed over a thousand parasites. Of interest is that all three cell lines became and remained persistently infected for the duration of this experiment. This, of course, implies the existence of a dividing, viable population. Whether or not the highly infected vacuolated cells divide is unknown at this time. In other experiments, persistently infected populations of P388D1 and J774 have been maintained for over 230 and 110 days, respectively.

The viabilities of the infected J774, P388D1, and PU-5-IR populations, as determined by dye exclusion, were relatively high (Fig. 3). At the termination of this experiment, the P388D1 and J774 cells were approximately 90% viable; the PU-5-IR population contained approximately 65% viable cells.

The above results were obtained with macrophages exposed to 500 phase I rickettsiae per cell. At other MOIs (5 and 50) the results were the same, although there was a proportional delay in the attainment of 100% infection.

By day 6 postexposure, the other cell lines WEHI-3 and WEHI-274 peaked in their association with phase I rickettsiae, and thereafter, the number of associated parasites declined and eventually disappeared (Fig. 1). By day 13, none of the WEHI-3 or WEHI-274 cells contained detectable rickettsiae. At other MOIs (5 and 50) similar results were obtained. The disappearance of phase I *C. burnetii* from the WEHI-3 and WEHI-274 populations indicated that either the parasites were interiorized and destroyed or, although they may have attached to the outer surface, they never gained entry and were eventually diluted out upon subsequent cell passage. Because the data for Fig. 1 were obtained using Gimenez-stained cells, it was impossible to distinguish between rickettsiae that were attached to the outer surface and those parasites that were interiorized; electron microscopy was therefore utilized. Extensive examination of thin sections of WEHI-3 and WEHI-274 cells exposed to phase I *C. burnetii* failed to reveal any interiorized rickettsiae. Such examinations were made of cells that were prepared for electron microscopy 6 h, 24 h, and 6 days postexposure.

Fate of phase II *C. burnetii*. At all three MOIs (5, 50, and 500) phase II rickettsiae entered and multiplied within all five cell lines, including WEHI-3 and WEHI-274. Phase II rickettsiae established a persistent infection of all five cell lines (Fig. 4). All infected populations continued to divide after infection (Fig. 4) and with a relatively high degree of viability (Fig. 5). Electron microscopy revealed the presence of many rickettsiae within the J774, P388D1, and PU-5-IR cells (Fig. 6); and, in contrast to phase I, phase II parasites were detected within WEHI-3 and WEHI-274 cells (Fig. 7). In all five cell lines, the rickettsiae were localized within vacuoles.

Chronically infected phase II cultures were maintained for over 100 days. Both phase I and phase II *C. burnetii* proliferated within vacuoles. Eventually, heavily infected cells contained one large vacuole that occupied most of the volume of the cell, with the nucleus pushed to one side (Fig. 2 and 6–8). It is possible that the large vacuole resulted from the fusion of several rickettsiae-containing vacuoles (note the multi-vacuolated P388D1 cell in Fig. 2b) or the enlargement of an infected vacuole or both.

As the infection with either rickettsial phase progressed, the macrophages became progressively larger. An example of this enlargement is shown in Fig. 8, which shows lightly infected J774 cells (which are near normal in size) alongside heavily infected and enlarged cells.

Phase analysis of rickettsiae isolated from persistently infected cells. Rickettsiae were isolated from P388D1 cells that had been infected chronically for 191 days and 98 days with phase I and phase II *C. burnetii*, respectively. Complement fixation block titration (5)

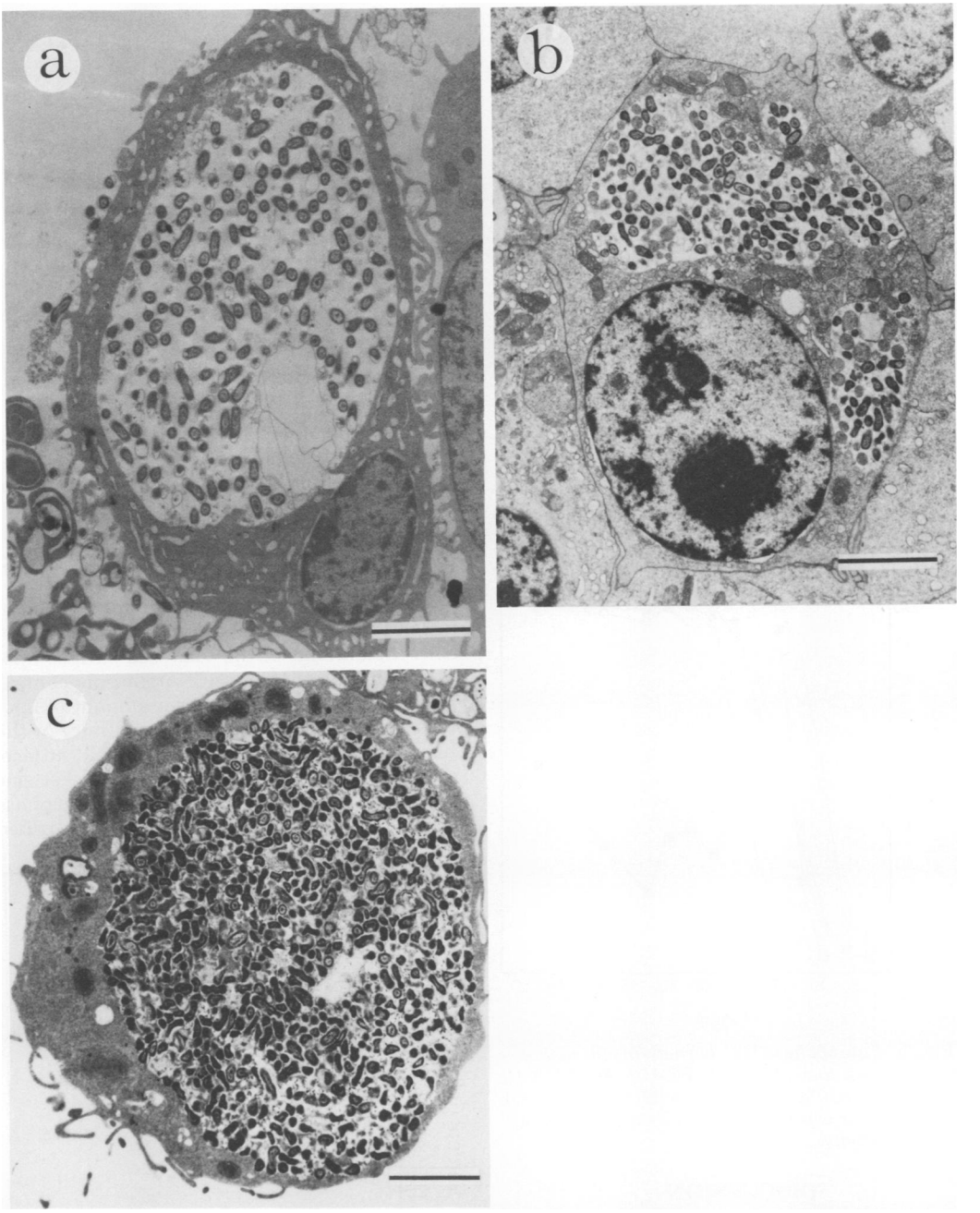

FIG. 2. *Phase I C. burnetii-infected (a) J774, (b) P388D1, and (c) PU-5-IR cells. J774 and PU-5-IR cells were infected for 27 days at the time of preparation for transmission electron microscopy; the P388D1 cells were infected for 15 days. MOI was 500. Markers of Fig. 2a and 2b represent 2 μm, and that of Fig. 2c represents 3 μm.*

showed that the rickettsiae isolated from cells that were initially infected with cloned phase I were now 50% phase II. One hundred percent of the rickettsiae isolated from the phase II-infected P388D1 cells were antigenically phase II. Similar phase analysis was not performed on rickettsiae derived from the other persistently infected cell lines.

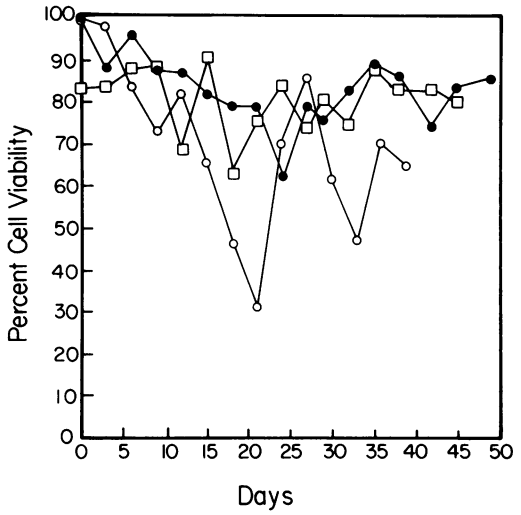

FIG. 3. *Percent cell viability of phase I C. burnetii-infected J774 (□), P388D1 (●), and PU-5-IR (○) cells. Viabilities were determined as described in the text.*

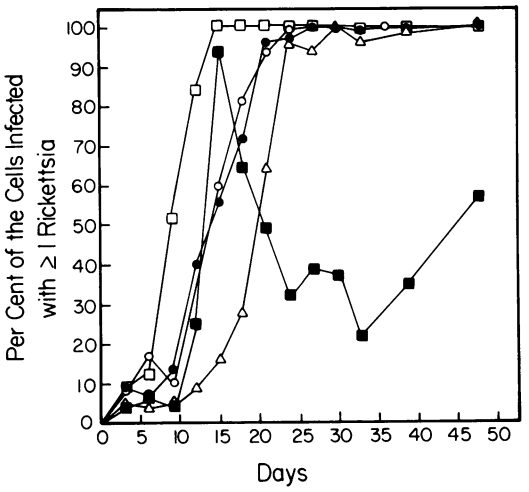

FIG. 4. *Fate of phase II C. burnetii in macrophage-like tumor cell lines J774 (□), P388D1 (●), PU-5-IR (○), WEHI-3 (△), and WEHI-274 (■). MOI was 500. See the text for experimental details and determination of infectivity.*

DISCUSSION

Several in vitro studies have shown that phase I and phase II *C. burnetii* enter and proliferate within normal monocytes and macrophages and, eventually, destroy the phagocytes (4, 8, 10–12). Upon activation with lympokines, the macrophages can, to a degree, control the interiorized rickettsiae (8). Various continuous cell lines have been infected, some persistently, with the Q fever agent (2, 3, 13, 22, 23). Collectively, these in vitro observations may reflect the in vivo state in humans and experimental animals in which Q fever occasionally results in a chronic, persistent infection (15, 27, 28, 32). In humans and animals, the types of cells that may sequester the rickettsiae during latency have not been identified. Although there is no evidence, phagocytic cells such as macrophages may be the cells in which the parasite persists. Because the cells of the monocytic series play a key role in the control of infectious agents, it is important to attain an understanding of the mechanisms involved in the control of infectious agents. In the case of the Q fever agent, nothing is known about its interactions with macrophages or other host cells that accounts for its ability to avoid destruction. The in vitro system that we have chosen to study may provide clues to such mechanisms, especially since phase I *C. burnetii* enters and multiplies within some cell lines and phase II proliferates in all. This differential ability to infect cells, depending on the phase, is extremely interesting because it strongly suggests that surface differences may account for their differential fates. Recently, we demonstrated that phase II *C. burnetii* contains one-tenth the amount of the toxic cell surface lipopolysaccharide that phase I organisms contain (1). It is possible that the lower amount of lipopolysaccharide may enhance the affinity of the phase II rickettsiae for the host cell surface. Indeed, we have found that phase II rickettsiae attach more readily to L-929 cells than do phase I *C. burnetii* and that the presence of purified

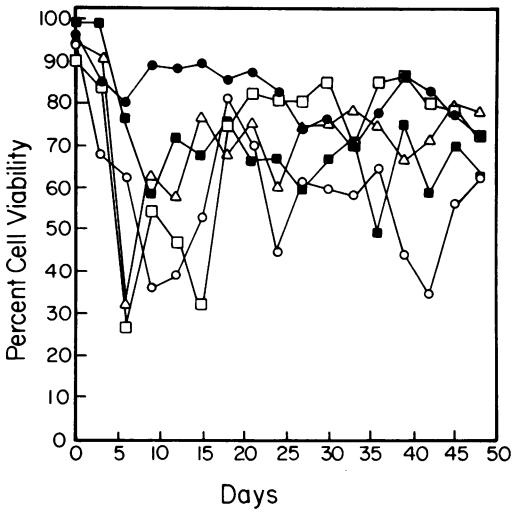

FIG. 5. *Percent cell viability of phase II C. burnetii-infected J774 (□), P388D1 (●), PU-5-IR (○), WEHI-3 (△), and WEHI-274 (■) cells. See the text for description of viability determination.*

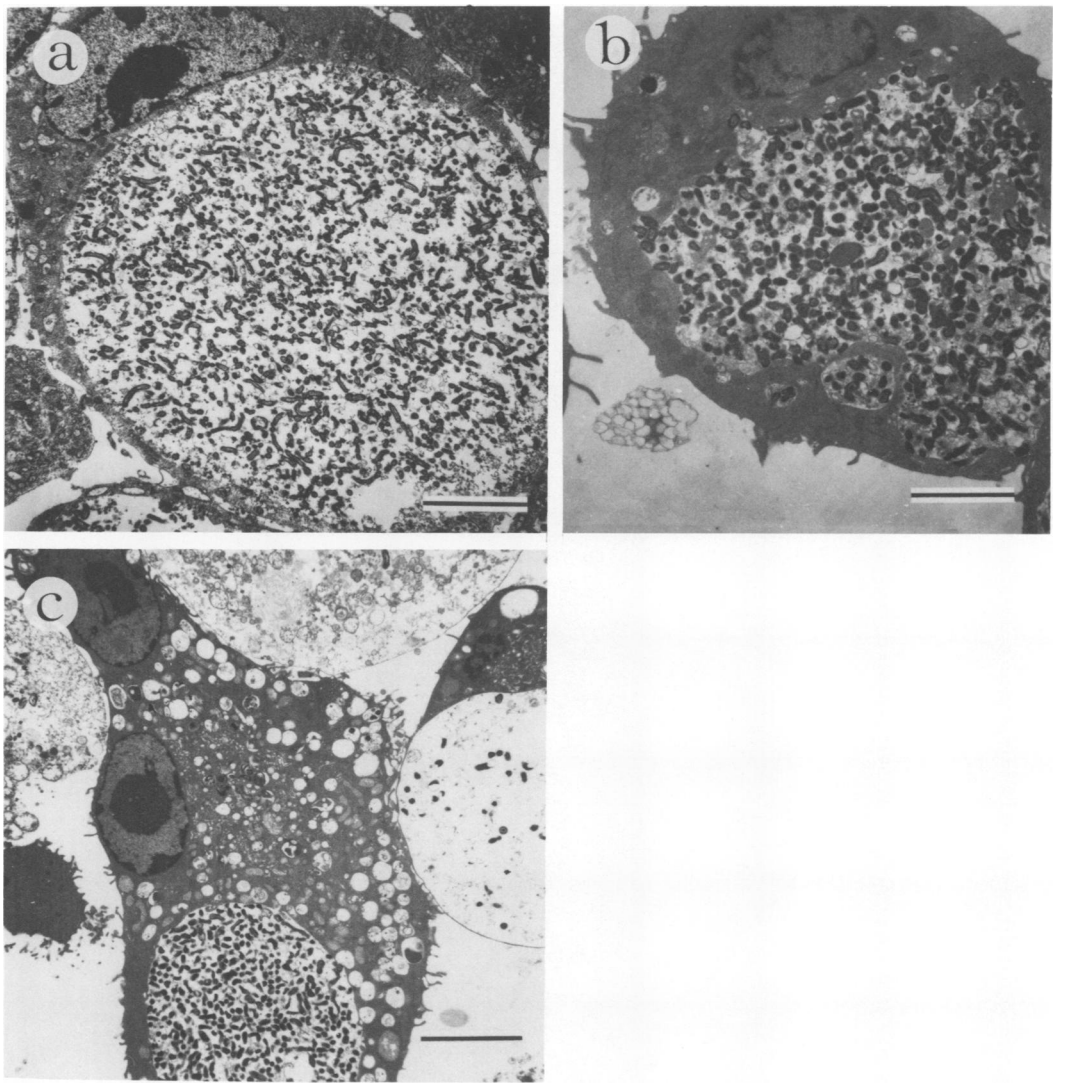

FIG. 6. *Phase II C. burnetii-infected (a) J774, (b) P388D1, and (c) PU-5-IR cells. J774 cells were infected for 63 days at the time of preparation for electron microscopy; P388D1 and PU-5-IR cells were infected for 36 days. MOI was 500. Markers each represent 5 μm.*

lipopolysaccharide in incubation mixtures results in decreased attachment (of either phase) of *C. burnetii* to L-929 cells (unpublished data). Extensive electron microscopy of WEHI-3 and WEHI-274 cells exposed to phase I *C. burnetii* has failed to reveal interiorized parasites; this strongly suggests that phase I parasites are probably not phagocytized. It is possible, however, that they may gain entry but are rapidly destroyed. That phase I rickettsiae do not attach as readily to WEHI-3 and WEHI-274 cells (Baca et al., in press) as they do to the other cell lines might explain, in part, the resistance of WEHI-3 and WEHI-274 to infection.

The use of different macrophage-like cell lines for these studies may provide further information on the mechanisms responsible for preventing or, alternatively, permitting rickettsial growth. Previous studies reported elsewhere (Warner et al., in press) suggest that these different cell lines may represent different macrophage subpopulations, or alternatively, different stages in maturation within monocyte-macrophage differentiation. Of interest in this regard is that the two phase I nonpermissive lines, WEHI-3 and WEHI-274, are by some criteria the "less mature" of these macrophage-like lines, however, they do possess certain active enzy-

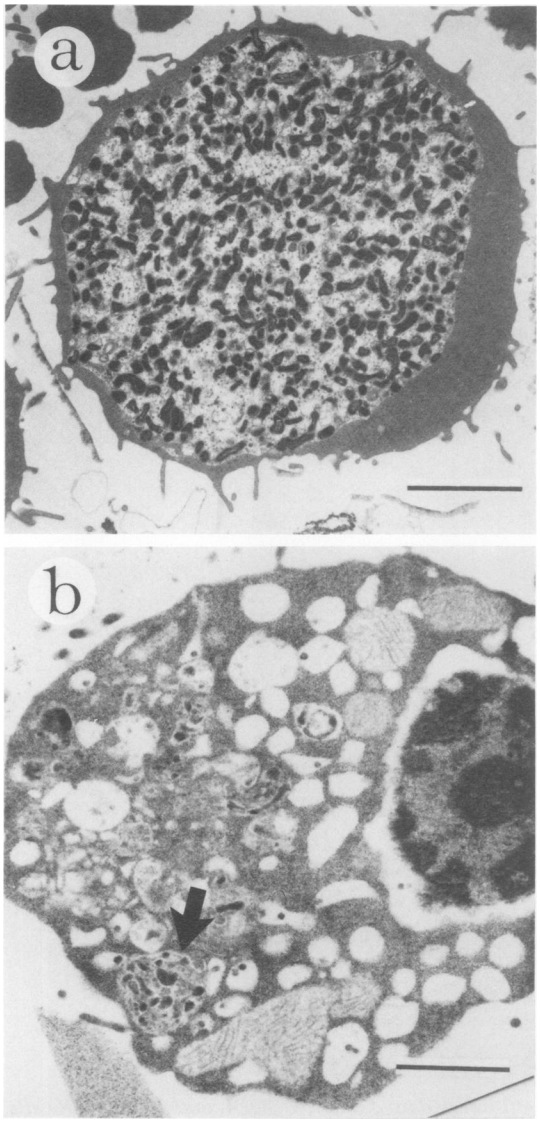

FIG. 7. *Phase II C. burnetii-infected (a) WEHI-3 and (b) WEHI-274 cells. At the time of preparation for electron microscopy, the WEHI-3 and WEHI-274 populations were infected for 36 and 104 days, respectively. MOI was 500. Markers represent 3 μm (a) and 1 μm (b). Arrow (b) points to rickettsiae.*

matic functions such as lysozyme (WEHI-3 [31]) and superoxide anion (WEHI-274 [Walker et al., Fed. Proc.]).

It is interesting to note that the cloned phase I rickettsiae shifted, in part, to phase II during persistent infection of P388D1 cells. Such antigenic shifting of phase I *C. burnetii* to phase II has previously been reported to occur during prolonged (more than 1 year) infection of L-929 cells (3). The mechanism that accounts for phase variation in *C. burnetii* is still unknown. Both the L-929 and the macrophage cell lines may prove valuable in probing the basis of phase variation. That the entire phase II *C. burnetii* population remained as phase II, at least for the first 98 days might indicate that phase II organisms are antigenically stable and do not revert to phase I; however, before such a conclusion is warranted, additional phase analyses must be made of rickettsiae derived from infected cells that have been maintained for longer periods of time.

The conditions for predictably infecting and maintaining the cell lines have been established.

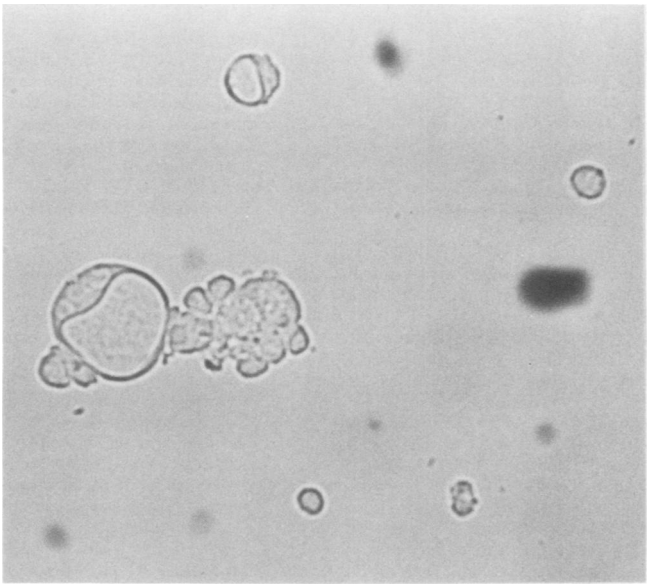

FIG. 8. *Phase I C. burnetii-infected J774 cells photographed 43 days postinfection. MOI was 500, and the cells were maintained as described in the text.*

Experiments are in progress to determine the levels of various lysosomal enzymes and putative killing agents (i.e., superoxide anion) within normal and infected cells. Whether or not lysosomes fuse with *C. burnetii*-containing vacuoles is being examined.

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