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ENTRY OF *COXIELLA BURNETII* INTO HOST CELLS

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Summary. - The attachment to and entry into L mouse fibroblast cells of viable phase I and phase II Nine Mile *Coxiella burnetii* was investigated. The use of ^{32}P-labelled rickettsiae showed that phase II *C. burnetii* attached more readily to L cells than phase I organisms; this probably accounts for the more rapid establishment of infection of host cells by the phase II agents. Two lines of evidence indicated that *C. burnetii* plays a passive role in both attachment and entry into host cells: (1) inactivation of rickettsiae by either heat or glutaraldehyde did not affect either process, and (2) metabolic inhibitors of L cell phagocytic function - NaF and cytochalasin B and D - abolished rickettsiae uptake. These results indicate that it is an endocytotic event. While the presence of purified phase I lipopolysaccharide (LPS) did not interfere with attachment of rickettsiae to the surface of host cells, it markedly impaired entry of *C. burnetii* in both phases. This suggests that LPS is not an adhesin and that it is toxic to the host cell. Treatment of L cells with either pronase, subtilisin or subtilopeptidase A significantly reduced the number of *C. burnetii* that adhered to the host cell surface; this result suggests that proteins are either proximate to or components of the *C. burnetii* attachment site.

Key words: Coxiella burnetii; L929 cells; attachment; entry

Introduction

C. burnetii, the agent of Q fever in humans, is an obligate intracellular prokaryote capable of persistently infecting a variety of cultured cells, including L fibroblasts (Roman *et al.*, 1986) and macrophage cell lines (Baca *et al.*, 1981). This rickettsial agent resides and proliferates within phagolysosomes (Akporiaye *et al.*, 1983; Burton *et al.*, 1978); its entry into host cells has subsequently been presumed to occur by phagocytosis. Entry into host cells of two other obligate intracellular rickettsiae - *Rickettsia tsutsugamushi* and *Rickettsia prowazekii* - apparently requires the active participation of both the parasites and host cells; inactivation of either parasite or host markedly reduces penetration. Heat

inactivation of *R. tsutsugamushi* or deprivation of its primary energy source, L-glutamic acid, reduced penetration into mouse cells (Cohn *et al.*, 1959). Inactivation of either the host cell or *R. prowazekii* sharply reduced parasite internalization (Walker and Winkler, 1978).

In this report evidence is presented that shows that *C. burnetii* passively attaches to the surface of host cells and subsequently is endocytosed. We also show that the relatively avirulent laboratory-derived variant, phase II *C. burnetii*, attaches more readily to the L cell surface than phase I organisms; this apparently accounts for the capacity of the phase II rickettsiae to more readily infect cultured host cells. Purified phase I *C. burnetii* LPS did not significantly affect attachment of the parasite to host cells; however, it impaired entry. Finally, we demonstrate that protease-sensitive components on the L cell surface may be at or near the site of *C. burnetii* adherence.

Materials and Methods

Cultivation, radiolabelling and purification of C. burnetii. Cloned phase I and phase II *C. burnetii*, Nine Mile isolate, were obtained from Drs. R. Ormsbee and M. Peacock of the Rocky Mountain Laboratory, Hamilton, MT. Both phases were cloned by the plaque technique, utilizing chick embryo cells (Ormsbee and Peacock, 1976).

The rickettsiae were propagated and labelled with ^{32}P-phosphate while growing in L cells as previously described (Baca *et al.*, 1981). The L cells were infected with cloned rickettsiae from yolk sac homogenates (phase I - single passage in eggs; phase II - multiple passages) for no more than 30 days. For radiolabelling, infected cells maintained in suspension culture were exposed to [^{32}P]-orthophosphoric acid (1.0 mCi per 50 ml culture) for 3 to 4 days. The host cells were disrupted in a Ten Broeck homogenizer and the released labelled rickettsiae purified from host cell debris by differential centrifugation: 3 cycles of low speed-high speed centrifugation (800 x g and 10 000 x g, 5 min and 30 min, respectively). If required, additional purification was achieved by centrifugation in 30 % to 60 % (w/v) linear sucrose gradients (Thompson *et al.*, 1971). Purified rickettsiae were suspended in Dulbecco's balanced salt solution (DBSS) and their concentration determined by the direct counting procedure of Silberman and Fiset (1968); the specific radioactivity of the purified rickettsiae was determined. Some of the radiolabelled rickettsiae were extracted with trichloroacetic acid; more than 95 % of the radioactivity remained with the TCA-insoluble fraction. Radiolabelled *C. burnetii* were stored at -80 °C.

Infected and uninfected L (L929) cells were cultivated in antibiotic-free Eagle's minimum essential medium (MEM), Spinner modified, with 5 % heat-inactivated calf serum and L-glutamine. Cell counts were performed with a hemocytometer and cell viability determined by dye (trypan blue or erythrosin B) exclusion (Phillips, 1973). Cell cultures were routinely tested for bacterial contamination by inoculation into thioglycollate broth. Mycoplasma testing was periodically performed using standard techniques.

Uptake of rickettsiae by L cells. The basic assay procedure for determining *C. burnetii* attachment-internalization was as follows: ^{32}P-*C. burnetii* were mixed with L cells (11 ml, 10^6 cells/ml) in complete growth medium in 50 ml silicone-stoppered Erlenmeyer polycarbonate flasks. The flasks were incubated at 37 °C and 2 ml aliquots were periodically taken; rickettsiae that were not associated (i. e., attached or internalized) with L cells were removed by pelleting L cells (and attached/internalized rickettsiae) at 160 x g at 4 °C for 15 min, resuspending the pellet in 2 ml of DBSS followed by four additional cycles of centrifugation and resuspension. The four washes were determined to be optimal for removing "free" rickettsiae. The washed L cells-rickettsiae were suspended in detergent-containing (Beckman Bio-Solv BBS-3) liquid scintillation fluid and the radioactivity measured in a Searle Delta 300 liquid scintillation spectrometer. From the specific

radioactivity, the number of rickettsiae associated per cell was ascertained. Only exponentially growing L cells were used in these studies.

Inactivation of C. burnetii. C. burnetii in both phases, suspended in DBSS, were inactivated either by heat (60 °C, 1 hr) according to Downs (1968) or by exposure to 0.5 % or 1.0 % formalin for 24 hr at room temperature (Ormsbee, 1962). Formalin-treated rickettsiae were washed several times with DBSS. The efficiency of the treatments was determined by exposing 6 day-old embryonated eggs and L cells to the treated organisms. After two weeks, no evidence of rickettsial proliferation was detected.

Probing the biochemical nature of the C. burnetii attachment site on the L cell surface. Experiments were performed to begin to identify the biochemical nature of the putative *C. burnetii* attachment site on the L cell surface. Viable host cells were treated with a number of enzymes prior to exposing them to radiolabelled rickettsiae. After treatment, *C. burnetii* were added and the number of attached rickettsiae was immediately determined after washing as described above. Normal uninfected L cells were treated at 37 °C for 30 min (usually in DBSS) with: trypsin (15 μg/ml, bovine pancreas, type III), alpha-chymotrypsin (15 μg/ml, bovine pancreas, type I-s), subtilopeptidase A (5 μg/ml, subtilisin Carlsberg, type III), subtilisin BPN (200 μg/ml, type VII), neuraminidase (1.0 U/ml, type VI from *Clostridium perfringens),* and beta-galactosidase (grade VI from *Escherichia coli*) (all from Sigma) and pronase (15 μg/ml, grade B, nuclease-free, Calbiochem). After treatment, the cells were washed twice with growth medium by centrifugation at 400 x g and resuspended in the appropriate medium. Only those treated cell populations exhibiting > 90 % viability were used in subsequent *C. burnetii* attachment experiments. For each enzyme, a range of concentrations was tested; however, the concentrations indicated are those that had an effect on attachment of *C. burnetii* – if any – and that did not adversely affect host cell viability.

LPS treatment of L cells followed by exposure to C. burnetii. LPS was prepared from purified phase I *C. burnetii* (Baca and Paretsky, 1974). 30 min prior to exposure to rickettsiae, L cells were exposed to 100 μg of purified LPS followed by radiolabelled *C. burnetii.* Both phase I and phase II rickettsiae were used, independently, in these experiments to determine if the presence of LPS affected attachment and entry of the rickettsiae.

Other treatments. L cells in complete growth medium were also exposed to NaF (20 mmol/l), cytochalasin B or D (15–25 μg/ml, Aldrich and Sigma), and sodium periodate (5–10 mmol/l). The chemicals were added 30 min prior to exposure to radiolabelled *C. burnetii.*

Electron microscopy. L cells exposed to *C. burnetii* were prepared for electron microscopy by standard techniques. Pelleted material was fixed in 2.5% glutaraldehyde prepared in 0.1 mol/l cacodylate buffer, pH 7.4. The material was washed in buffer and post-fixed in 1 % OsO_4. After washing, the cells were stained with 2 % uranyl acetate and dehydrated through an alcohol series into propylene oxide. The stained cells were embedded in Araldite and sectioned. Thin sections placed on specimen grids were double-stained with uranyl acetate and lead citrate and examined with an AEI Corinth transmission electron microscope.

Results

In most of the experiments, each L cell was exposed to 500 live ^{32}P-labelled rickettsiae. At this concentration and specific radioactivity, it was possible to detect as few as 0.5 *Coxiella* associated per L cell.

Comparative uptake of phase I and phase II C. burnetii by L cells

The association of phase I and phase II *C. burnetii* with L cells in complete growth medium during a 3 hr incubation period was compared. Immediately after the rickettsiae and L cells were mixed at 37 °C, a sample was removed to determine the number of rickettsiae associated per cell; this sample was

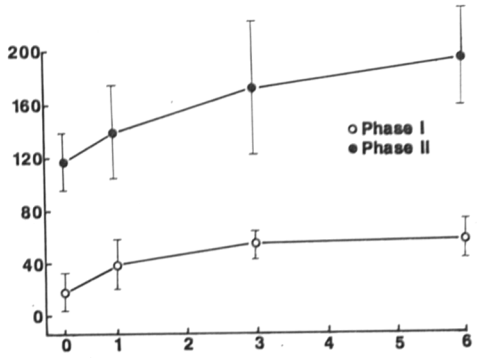

Fig. 1
Uptake of ^{32}P-labelled phase I and phase II *C. burnetii* by L cells at 37 °C
Abscissa: incubation time in hrs; ordinate: number of *C. burnetii* associated per one cell. Mean ±SE of 7 independent experiments performed in duplicate.

designated as the "time zero" sample. At time zero, five times more phase II than phase I *C. burnetii* were associated with the L cells (Fig. 1). Since the elapsed time between mixing of rickettsiae with the host cells and sampling was less than 30 seconds – presumably insufficient time for endocytosis of the rickettsiae to have occurred – it is assumed that the associated rickettsiae were attached to the cell surface and not internalized. This assumption was confirmed by holding *C. burnetii* and L cells at ice-bath temperature throughout a 6 hr period (Fig. 2). It is assumed that endocytosis does not occur at 0 °C. Again, the differential attachment of the phase I and phase II organisms was noted: five times more phase II rickettsiae became associated with the L cells. These results clearly demonstrated that phase II *C. burnetii* has a higher affinity for the host cell surface than phase I.

Electron microscopy of thin sections of L cells exposed to either phase I or phase II *C. burnetii* at time zero and at the end of a 6 hr incubation period was performed. At time zero *C. burnetii* were found attached to the L cell surface and microvilli (Fig. 3). With continued incubation rickettsiae were observed on the surface and within vacuoles (Fig. 4) where they remained and established

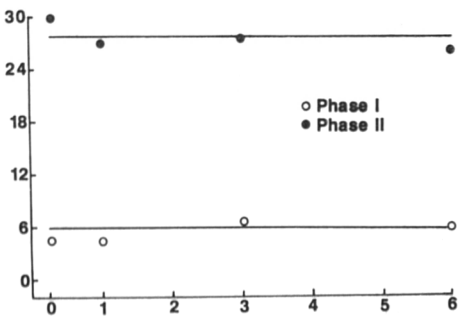

Fig. 2
Association of ^{32}P-labelled phase I and phase II *C. burnetii* by L cells at 0 °C
Abscissa: incubation time in hrs; ordinate: number of *C. burnetii* associated per one cell. This experiment is representative of 3 independent experiments.

a persistent infection.

The optimum temperature for *C. burnetii* uptake by host cells was approximately 37 °C. Incubation of rickettsiae and L cells at other temperatures (0, 28 and 40 °C) during a 3 hr period resulted in the following decrease of L cell – associated rickettsiae when compared with incubation at 37 °C: 58 %, 24 % and 33 %, respectively (means of 3 independent experiments).

Attempts to saturate "attachment sites" with either phase I or phase II *C. burnetii* did not succeed (Fig. 5). The number of radiolabelled rickettsiae added per cell varied from 100 to 2 500. Attachment of both phases was linear with increasing number of rickettsiae. This experiment also demonstrated the differential attachment of phase I and phase II rickettsiae to the L cells.

Uptake of inactivated rickettsiae by host cells

To determine whether or not active participation of *C. burnetii* was required for attachment and entry, uptake experiments were performed with rickettsiae

Fig. 3

Electron micrograph of phase I *C. burnetii* attached to the surface and being engulfed by a microvillus of an L cell

This sample was prepared for electron microscopy immediately after the rickettsiae were mixed with the host cells (time zero). The rickettsiae are approximately 1 μm in length.

inactivated with either heat or formaldehyde. These treatments had no significant effect on either attachment or entry of both phase I and phase II *C. burnetii;* the same number of rickettsiae, whether viable or dead, associated with the L cells (data not shown).

Uptake of C. burnetii by phagocytically-inhibited L cells

The results obtained up to this point indicated that entry of the parasite was exclusively a phagocytosis related event. Evidence that this is the case was obtained with the use of the glycolysis inhibitor NaF and cytochalasin B and D. The latter fungal metabolites are selective inhibitors of phagocytosis (Casella *et al.*, 1981; Howard *et al.*, 1981; Painter *et al.*, 1981). Pretreatment of L cells with either cytochalasin B or D (15–25 μg/ml, 37 °C, 30 min) prior to exposure to rickettsiae resulted in nearly complete abolition of *C. burnetii* internalization – both phases; with either cytochalasin B or D, attachment of the rickettsiae was

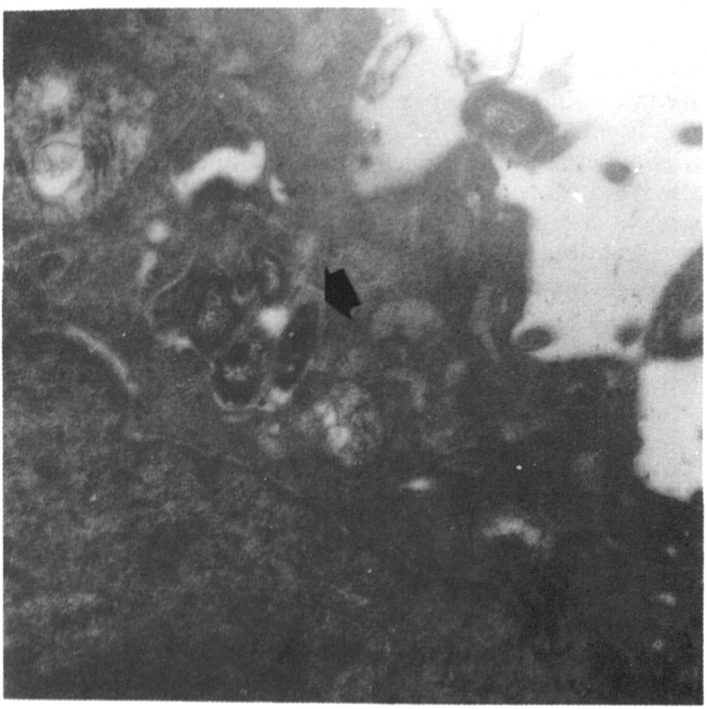

Fig. 4

Electron micrograph of phase I *C. burnetii* attached to the surface and within a vacuole of an L cell The sample was prepared 6 hr after initial contact between rickettsiae and host cells (the same experiment as that depicted in Fig. 3). The arrow points to a vacuole.

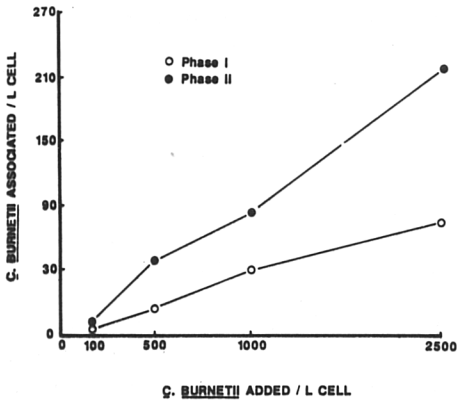

Fig. 5
Attachment of phase I and phase II *C. burnetii* to L cells at different concentrations of rickettsiae
The number of L cells was held constant (10^6/ml). Immediately after the L cells were exposed to the rickettsiae, samples were processed as described in *Methods* to assess the number of attached *C. burnetii*. This experiment is a representative of 3 independent experiments.

consistently slightly depressed. The depicted results (Fig. 6) are with cytochalasin D and phase I *C. burnetii;* similar results were obtained with cytochalasin B and in the case of phase II, the fungal metabolites had similar effects (data not shown). Other investigators have reported that the cytochalasins have little effect on the attachment of relatively large particles such as bacteria (Davies and Allison, 1978; Elliot and Winn, 1986; Klaus, 1973; Malawista *et al.*, 1971; Painter *et al.*, 1981; Spilberg and Mehta, 1981; Van Obberghen *et al.*, 1976). In our experiments, we consistently saw this slight effect on attachment; apparently, disruption of microfilament structure by the cytochalasins affects surface topography. Treatment of L cells with NaF (20 mmol/l, 20 min, 37 °C) prior to exposure to rickettsiae also resulted in diminished ingestion; attachment of the rickettsiae was unaffected (Table 1). After the first hour of incubation, the number of rickettsiae associated with the treated L cells remained the same

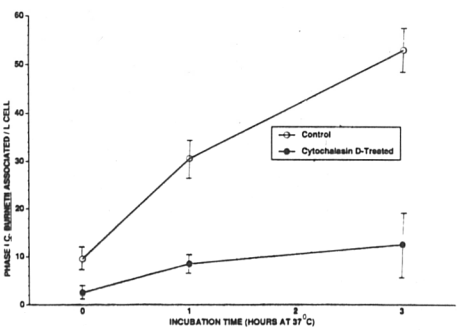

Fig. 6
Effect of cytochalasin D on the uptake of radiolabelled phase I *C. burnetii* by L cells
The L cells were treated with cytochalasin (15 μg/ml) for 30 min prior to 3 hr exposure to the ^{32}P-*C. burnetii*. Mean ±SE of 3 independent experiments performed in duplicate.

Table 1. Association of phase I C. burnetii with L cells pretreated with NaF

L cells	Number of *C. burnetii* associated per L cell		
	Time (hr)		
	0	1	3
Untreated	9	36	59
Treated	9	22	19

This experiment is representative of 2 independent experiments. The results are averages of duplicate samples.

through the 3rd hour. These combined results indicate that entry of *C. burnetii* into host cells is via the energy-requiring process, phagocytosis.

Effect of purified LPS on rickettsial attachment and uptake

We have investigated the possibility that LPS itself may be *C. burnetii*'s adhesin by exposing L cells to purified LPS and observing whether or not attachment of *C. burnetii* (either phase) was impeded. Pretreatment of L cells with LPS from phase I organisms only slightly affected attachment of either phase I or phase II *C. burnetii* to the L cells; however, subsequent entry of rickettsiae was apparently inhibited because very few additional organisms associated with the L cells during the subsequent 4.5 hr incubation period (Fig. 7, 8).

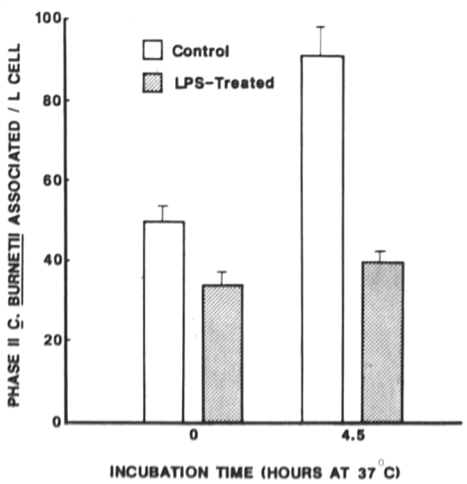

Fig. 7
Effect of purified phase I LPS on the attachment and uptake of radiolabelled phase I *C. burnetii* by L cells
Values at time zero correspond to the attachment. Mean ±SE of 3 independent experiments performed in duplicate.

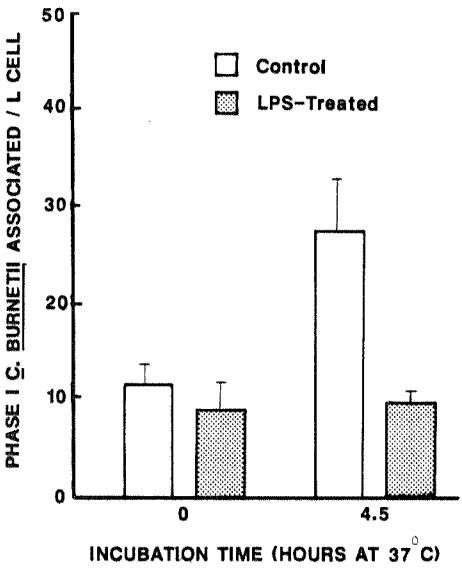

Fig. 8
Effect of purified phase I LPS on the attachment and uptake of radiolabelled phase II *C. burnetii* by L cells
Values at time zero correspond to the attachment. Mean ±SE of 3 independent experiments performed in duplicate.

Biochemical nature of the rickettsial attachment site

To probe the biochemical nature of the components on the L cell surface that may function as rickettsial "attachment sites", cells were treated with periodate, a number of potential competitors of attachment (concavalin A, sugars, including D-galactose, D-mannose, D-xylose), and enzymes, including proteases and glycohydrolases. The only treatments that affected *C. burnetii* (phase I) attachment were exposure to the proteases pronase, subtilisin and subtilopeptidase A.

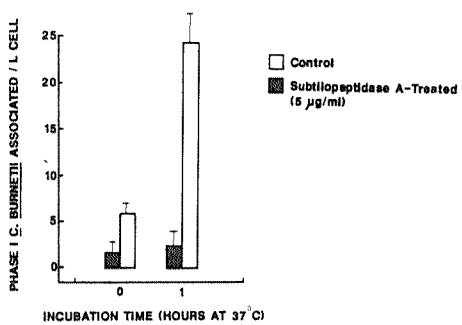

Fig. 9
Effect of subtilopeptidase A treatment of L cells on the attachment of phase I *C. burnetii* by L cells
Mean ±SE of 3 independent experiments performed in duplicate.

Treatment with pronase caused a 50 % reduction in the number of attached *C. burnetii* (data not shown) while treatment with subtilopeptidase A resulted in approximately a 70 % reduction (Fig. 9). Subtilisin treatment reduced the amount of attached *C. burnetii* (phase I) by approximately 50 % and continued incubation at 37 °C for up to three hours revealed continued differential attachment and uptake (data not shown).

Discussion

Other investigators (Burton *et al.*, 1978) have speculated that *C. burnetii* enters into host cells via phagocytosis; our data substantiate such speculation. In an earlier report (Akporiaye *et al.*, 1983) we demonstrated conclusively that this intracellular agent resides within phagolysosomes. The results presented in this report show that the parasite does, indeed, enter host cells via phagocytosis and that it does not actively promote its entry, but rather, enters passively. Inactivation of the rickettsiae with glutaraldehyde or heat did not significantly affect the number of ^{32}P-*C. burnetii* that attached to the cell surface or that subsequently entered. Furthermore, treatment of host cells with the metabolic inhibitor NaF and the specific phagocytosis inhibitors cytochalisin B or D resulted in almost complete abolition of parasite entry.

Unlike *C. burnetii*, other rickettsial agents actively promote their entry into host cells. The classic work of Cohn *et al.* (1959) demonstrated that *R. tsutsugamushi* actively penetrated host cells. This conclusion was based on the observation that inactivation of the parasite with metabolic inhibitors, heat, formalin or UV irradiation resulted in marked reduction of host cell penetration. Later, similar results were obtained with *R. prowazekii* when it was reported that parasite entry into L cells required the active participation of both the rickettsia and the host cell (Walker and Winkler, 1978). It has been proposed (Walker, 1984; Walker and Winkler, 1978) that internalization of *R. prowazekii* occurs through a process of "induced phagocytosis": attachment of live rickettsiae to an unidentified site signals the cell to phagocytose – inactive rickettsiae adhere to the cell membrane but are slowly internalized. A similar mechanism had been previously proposed by Byrne and Moulder (1978) when they presented evidence that *Chlamydia psittaci* was phagocytosed by nonprofessional phagocytes (L and HeLa cells) through a process they termed "parasite-specified phagocytosis".

The biochemical nature of the *C. burnetii* attachment site on the L cell surface remains unidentified; however, our investigation provides circumstantial evidence that proteins are at or near the attachment site since *C. burnetii* adherence was substantially reduced by treating L cells with proteases.

Kazár *et al.* (1975) previously reported that macrophages were more readily infected by phase II rickettsiae than by phase I. Our findings that phase II *C. burnetii* attach more readily to L cells than do phase I organisms likely accounts

for this differential infection rate which we have also observed with L cells. The reason for such differential attachment is not known. One possible explanation may be that phase I *C. burnetii* surface LPS may impede attachment. LPS is present in substantially greater amount in phase I organisms (Baca *et al.*, 1980), and phase II agents exhibit rough-type LPS while phase I possess smooth-type LPS (Schramek and Mayer, 1982). The presence of purified phase I LPS during uptake of the parasite (either phase) by L cells only caused a slight reduction in attachment; however, unexpectedly, we found that LPS markedly reduced parasite entry. These results indicate that the LPS is toxic to L cells. They also indicate that LPS is not an adhesin as has been reported for some bacteria. For example, Berlanger *et al.* (1990) recently demonstrated that purified LPS from smooth *Actinobacillus pleuropneumoniae* impeded attachment of the bacterium to tracheal rings.

Other workers have reported that qualitative differences in the LPSs of *Salmonella typhimurium* variants could be correlated with differential uptake by phagocytic cells (Standahl and Edebo, 1972). Variants with shorter LPS lengths were more readily phagocytosed. This is consistent with our finding that phase II *C. burnetii* is more readily internalized by L cells, that phase II contains one-tenth the amount of LPS present in phase I *C. burnetii* (Baca *et al.*, 1980), and that the carbohydrate portion of phase II LPS is devoid of many of the sugars present in phase I LPS (Amano and Williams, 1984; Hackstadt *et al.*, 1985; Schramek and Mayer, 1982). The lower amount of LPS possessed by phase II organisms and reduced carbohydrate content of LPSs probably enhance rickettsial surface hydrophobicity which, in turn, results in greater affinity for other hydrophobic structures such as host membranes and phase II rickettsiae. Indeed, suspended, purified phase II *C. burnetii* tend to clump; after pelleting by centrifugation, they are not readily resuspended as are their phase I counterparts.

Relatively recent genomic and biochemical evidence (Hackstadt *et al.*, 1985; Samuel *et al.*, 1985) indicates that different *C. burnetii* isolates may be responsible for different disease manifestations, including acute and chronic disease (i.e., infectious endocarditis). The possibility that "endocarditis strains" contain unique adhesins that promote colonization of target cardiac tissues needs to be explored. The identification and characterization of such putative adhesins and complementary host cell receptors could be important for designing effective vaccines/blockers. At present, efficacious vaccines and drugs for preventing and treating life-threatening chronic Q fever endocarditis are unavailable.

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