RICKETTSIAL PHOSPHOLIPASE A2 AS A PATHOGENIC MECHANISM IN A MODEL OF CELL INJURY BY TYPHUS AND SPOTTED FEVER GROUP RICKETTSIAE

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Abstract. Phospholipase A2 activity by typhus group rickettsiae causes hemolysis in vitro. Rickettsial phospholipase A2 has been proposed to mediate entry into the host cell, escape from the phagosome, and cause injury to host cells by both typhus and spotted fever group rickettsiae. In a rickettsial contact-associated cytotoxicity model, the interaction of Rickettsia prowazekii or R. conorii with Vero cells caused temperature-dependent release of 51Cr from the cells. Treatment of rickettsiae, but not the cells, with a phospholipase A2 inhibitor (bromophenacyl bromide) or with antibody to king cobra venom inhibited cell injury. Rickettsial treatment with bromophenacyl bromide inhibited the release of free fatty acids from the host cell. Neither the inhibitor nor antivenom impaired rickettsial active transport of L-lysine. Thus, host cell injury was mediated by a rickettsial phospholipase A2-dependent mechanism.

INTRODUCTION

Rickettsiae of the typhus group have long been known to cause hemolysis of sheep and human erythrocytes by a mechanism that requires rickettsial viability and metabolic activity.1,2 Winkler and Miller1 examined rickettsial hemolysis in vitro in a series of important studies of this model of the rickettsia-host cell membrane interaction culminating in the seminal discovery that rickettsial hemolysis in vitro was associated with the generation of products of phospholipase A2 activity. Whether the phospholipase activity was of host cell or rickettsial origin and whether phospholipase A2 was the cause of the hemolysis remained open questions. Subsequently, Winkler and Miller1 also developed an in vitro model of cell injury by Rickettsia prowazekii: centrifugation of typhus rickettsiae onto a L-929 cell monolayer at a multiplicity of inoculum of 50:1 for 15 min. Similar to the in vitro hemolysis model, death of the L cells is associated with release of free fatty acids and lysophosphatides from the L-cell membrane. The origin and mechanistic role of the phospholipase A2 again were not resolved.

In addition to the presence of phospholipase activity in this immediate cytotoxicity model, its activity is present during the infection of cultured cells with R. prowazekii, where it has been hypothesized to lyse the cell membrane, causing the cell to burst and allowing exit of the rickettsiae.3 Ojcius and others4 used a sensitive fluorometric assay to demonstrate that R. prowazekii and R. typhi secrete extracellular phospholipase A2. The rickettsial hemolytic activity requires divalent cations at a mildly acidic pH, observations that are consistent with the hypothesis that rickettsial phospholipase A2 mediates escape of rickettsiae from the early phagosome into the cytosol.

Spotted fever group rickettsiae may possess a phospholipase A2 enzyme that differs from the typhus group rickettsial hemolytic phospholipase because spotted fever group rickettsiae do not cause in vitro hemolysis with the same rapid kinetics that typhus group rickettsiae do. Phenetermine, a drug that has been reported to inhibit phospholipase A2 activity, reduces cell injury by R. rickettsii in a Vero cell plaque model.5 The drug was not investigated for its potential toxic effect on R. rickettsii but was subsequently shown to be nontoxic for R. prowazekii at the drug concentrations employed.6 In the R. rickettsii plaque model, phospholipase A2 activity was not assayed, thus leaving unresolved whether or not the actual mechanism of reduced cell injury was reduced phospholipase activity.

Silverman and others7 further advanced the understanding of spotted fever group rickettsial phospholipase A2 by demonstrating that treatment of R. rickettsii, but not the host cells, with p-bromophenacyl bromide (BpB) or antibody to king cobra venom phospholipase A2 reduced plaque formation and that the antibody to phospholipase A2 inhibited the spread of rickettsiae to other Vero cells in the monolayer. Again, however, the interpretation relied upon observation of an effect without assays of rickettsial viability, metabolic activity, and the phospholipase A2 activity as measures of the potential toxic and proposed mechanistic effects of the chemical and antibody.

This investigation reports the effects of treatment of rickettsiae only or host cells only with bromophenacyl bromide or antibody to cobra venom phospholipase A2 on injury by R. prowazekii and R. conorii to Vero cells by use of the immediate cytotoxicity model.4 Rickettsial metabolic activity and phospholipase A2 activity were determined, and both typhus group and spotted fever group rickettsial cytotoxic injury were demonstrated to be caused by rickettsial phospholipase A2. Ultrastructural analysis revealed that entry of both R. prowazekii and R. rickettsii into Vero cells appeared to be inhibited by BpB and by antibody to phospholipase A2. Escape of the rickettsiae that did gain entry from the phagosomal vacuoles did not appear to be inhibited by the antibody to phospholipase A2.

MATERIALS AND METHODS

Rickettsiae. Rickettsia prowazekii (Breinl strain), provided by G. A. Dasch, Naval Medical Research Institute, Bethesda, Maryland, was passaged twice in yolk sacs of embryonated chicken eggs in our laboratory. The stock of R. conorii (Malish 7 strain) employed was originally obtained from American Type Culture Collection (ATCC; Manassas, VA) and has the passage history as previously reported.8 Both strains of rickettsiae were cultivated in the yolk sacs of 5- to 6-day-old embryonated specific-pathogen-free chicken eggs (SPAFAS, Roanoke, IL). The rickettsiae were harvested, purified by Renografin density gradient centrifugation,9 and used for experiments as freshly prepared materials on the same day. For 51Cr release cytotoxicity assay, the rick-
Rickettsiae were purified by Renografin density gradient centrifugation. In order to decrease the possible influence of cell debris in the ultrastructural investigations, in some experiments, 2 consecutive Renografin purifications were performed, and only the light band was used.

**Target cell and cytotoxicity assay.** Vero cell monolayers (ATCC, CRL1587) were cultivated in 24-well plates (2 × 10^5 cells per well) in Eagle’s minimum essential medium (MEM, Gibco, Grand Island, NY) containing 5% fetal bovine serum (FBS, Hyclone, Logan, UT). After overnight cultivation, the monolayer was labeled with ^31^Cr (Amersham, Arlington Heights, IL; 1 μCi per well) in 0.3 mL MEM containing 2% FBS by incubation for 1 hr at 37°C in an atmosphere containing 5% CO\textsubscript{2}. The labeled plates were washed 3 times with warm MEM containing 5% FBS. In some experiments, before labeling with ^31^Cr, the Vero cell monolayers were treated with a 0.3-mL volume per well containing 10 μM 4-bromophenacyl bromide (BpB; Sigma Chemical, St. Louis, MO) or a 1:10 final concentration of equine antibody to king cobra (Ophiophagus hannah) venom (Sigma) or normal horse serum for 15 min at 37°C in 5% CO\textsubscript{2}, then washed once with warm MEM containing 1% FBS before labeling with ^31^Cr. The complement activity in the sera was inactivated by heating at 56°C for 30 min.

Cytotoxicity assay was performed as described by Winkler and Miller\textsuperscript{4} except that Vero cells were used as the target. ^31^Cr rather than ^86^Rb was used to label the cells, and *R. conorii* was evaluated as well as *R. prowazekii*. Fresh Renografin density gradient-purified rickettsiae in a volume of 0.3 mL was treated as described in Experimental Design, added to duplicate wells of ^31^Cr-labeled Vero cells, and centrifuged onto the monolayers at 500 g for 15 min at 4°C to increase rickettsial contact with the Vero cells. The monolayer was then incubated at 37°C in a 5% CO\textsubscript{2} atmosphere for 30 min except in temperature-dependent experiments, in which for comparison the cytotoxicity assay was performed at 4°C. The cell culture supernatant fluids were harvested and centrifuged at 16,000 × g for 2 min. Each assay was performed in 6 wells of a 24-well plate. The supernatant fluids from 3 wells were pooled, and 150 μL was used for assay in a γ counter. The other 3 wells were also pooled and counted, and the means and standard deviations were calculated from these 2 pooled supernatants. All experiments were performed 3 times to assure reproducibility of the data.

**Electron microscopy.** For ultrastructural investigation monolayers on Thermanox coverslips were fixed at time 0 (immediately after centrifugation at 4°C) and after 15, 30, and 60 min of incubation at 37°C. Rickettsial inoculum or medium was removed, and fixative was added to the wells. The wells were sealed with Parafilm to prevent evaporation of the fixative and its possible action on cells in adjacent wells. A mixture of 1.25% formaldehyde, 2.5% glutaraldehyde, 0.03% trinitrophenol, and 0.03% CaCl\textsubscript{2} in 0.05 M cacodylate buffer (pH 7.3) was used as a primary fixative.\textsuperscript{13} Monolayers were then postfixed in 1% OsO\textsubscript{4} in 0.1 M cacodylate buffer, stained *en bloc* with 1% uranyl acetate in 0.1 M maleate buffer (pH 5.2), dehydrated through a series of ethanol dilutions and a mixture of ethanol and PolyBed, and embedded *in situ* in Poly/Bed 812 resin (PolySciences, War- rington, PA) by use of the caps of Better Equipment for Electron Microscopy (BEEM) capsules. After polymerization, the coverslips were removed, and small blocks of the monolayers were cut out with a razor blade and glued to blank plastic blocks to be cut either flat or perpendicular to the monolayer. Ultrathin sections were cut on a Sorvall MT-6000 ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips 201 electron microscope at 60 kV.

For quantitation of rickettsiae in different locations, pictures were taken of random sections of nucleated cells, and at least 100 rickettsiae were examined.

**Experimental design.** In order to determine whether or not a phospholipase A\textsubscript{2} of *R. prowazekii* and *R. conorii* or of Vero cells was the pathogenic mechanism of cytotoxicity in the model of centrifugation-enhanced immediate cell injury, rickettsiae or Vero cells were incubated with 10 μM BpB, 1:10 dilution of an equine antibody to king cobra venom or normal horse serum, or medium only for 15 min. The rickettsiae were treated in an ice bath, and the Vero cells were incubated with the different treatments at 37°C. Other controls included rickettsiae killed by boiling (100°C) for 10 min and rickettsiae incubated with ethanol (the original diluent of BpB) at the final concentration contained in the BpB-treated wells. To confirm that the model performed as described previously for *R. prowazekii*\textsuperscript{4} and that it was appropriate for investigating *R. conorii*, cytotoxicity was compared for monolayers raised from 4°C to 37°C for 30-min incubation and those held at 4°C for the entire period.

To evaluate whether or not treatment of rickettsiae with BpB, its original diluent (ethanol), equine antibody to king cobra venom, or normal horse serum exerted toxic injury on the rickettsiae, treated and untreated rickettsiae were compared for their ability to actively transport ^14^C-lysine from the extracellular location to within the rickettsiae.\textsuperscript{13} To determine whether or not the putative phospholipase inhibitor, BpB, in fact had an antiphospholipase A\textsubscript{2} effect in this model, free fatty acids as a product of the breakdown of the ^3^H-oleic acid-labeled Vero cell membrane phospholipid were assayed according to the method employed by Winkler and Miller\textsuperscript{4} in their studies that demonstrated the existence of phospholipase A\textsubscript{2} activity in this model. To determine the location of rickettsiae under the different conditions of this experimental cytotoxicity model, electron microscopic examination was performed on rickettsiae-exposed monolayers that were treated as described above, except that the target cells were cultivated on plastic Thermanox coverslips (Nalge Nunc International, Naperville, IL) in 24-well plates, the target cells were not labeled with ^31^Cr, and after centrifugation and incubation at 37°C for 15, 30, or 60 min, the cells were fixed immediately.

**Assay of ^14^C-L-lysine uptake by rickettsiae.** Three microtiter plates containing 0.3 μCi of ^14^C-L-lysine (Amersham) was added to the untreated, Renografin-purified rickettsiae or rickettsiae treated with BpB, antivenom antibody, normal horse serum, or the appropriate dilution of ethanol in a total volume of 0.2 mL and incubated at 37°C for 10 min.\textsuperscript{17} The contents of each tube were diluted by adding 1 mL of sucrose-phosphate-glutamate buffer (SPC; 0.128 M sucrose, 0.0038 M KH\textsubscript{2}PO\textsubscript{4}, 0.0072 M K\textsubscript{2}HPO\textsubscript{4}, 0.0049 M monosodium L-glutamic acid)\textsuperscript{14} and then passed through a 0.45 μm filter membrane. The unincorporated ^14^C-L-lysine was removed by flushing 10 mL of SPG through the filter. The
selenite; sodium pyruvate 1.1 mg/L; and 2 mM L-glutamine.

RESULTS

The immediate cytotoxic effect of both R. prowazekii and R. conorii on Vero cells was temperature dependent and required rickettsial viability (Table 1). Cell injury measured as release of $^5$Cr from Vero cells at 4°C was only 2.5–13.5% of that at 37°C in 3 replicate sets of experiments performed on different days.

A brief treatment of either R. prowazekii or R. conorii with BpB before centrifugation onto the Vero cell monolayer strongly inhibited the cytotoxic effect, but pretreatment of the Vero cells with BpB did not prevent the cytotoxic effect on the cells by either the typhus or spotted fever group rickettsiae (Figures 1 and 2). A similar brief treatment of either R. prowazekii or R. conorii with equine antibody to cobra venom also prevented both species of rickettsiae from exerting a cytotoxic effect on the monolayer (Figure 3). Treatment of the rickettsiae with normal horse serum as a negative control resulted in a moderate decrease in cytotoxicity of the untreated rickettsiae at 37°C was considered to be 100%. The actual levels of radioactivity released by labeled Vero cells were 5,715 cpm for these exposed to untreated rickettsiae; 50 ± 10 cpm for those exposed to inhibitor-treated rickettsiae, and 5,372 ± 500 cpm for inhibitor-treated Vero cells exposed to untreated rickettsiae.

Assay of phospholipase A$_2$ activity as free fatty acid released from radiolabeled Vero cells. As described previously, $^5$Cr-labeled Vero cells/mL were irradiated with 5,000 rads and labeled with $^3$H-oleic acid (0.25 μCi/5 x 10$^5$ Vero cells) (DuPont NEN Research Products, Boston, MA). The cells were then stirred at 37°C for 8 hr, the medium was changed to serum-free Iscove modified Dulbecco medium, supplemented to contain linoleic acid–bovine serum albumin complex 400 mg/mL; 15 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid); glucose 4 g/L; insulin 10 μg/L; human transferrin 10 μg/L; 10 μM 2-amino-nomethanol; 50 μM 2-mercaptoethanol; 0.01 μM sodium selenite; sodium pyruvate 1.1 mg/L; and 2 mM L-glutamine.

After overnight incubation at 37°C in a 5% CO$_2$ atmosphere, the BpB-treated or untreated R. prowazekii or R. conorii was added to the Vero cell monolayer and centrifuged at 500 × g for 15 min. After incubation at 37°C for 24 hr, the culture supernatant was collected, and the cell monolayer was scraped into 7.6 mL of methanol per plate, then mixed with the supernatant. The total lipid was extracted by adding 3.8 mL of chloroform. This mixture was gassed with nitrogen and kept at 4°C overnight. The next day, this mixture was centrifuged at 2,000 × g for 30 min at 4°C. The chloroform in the bottom layer was collected, evaporated to dryness with nitrogen, dissolved in 0.3 mL of chloroform, and added to a hexane-activated, aminopropylsilane-bonded silica gel column (J. T. Baker, Phillipsburg, NJ). Neutral lipids were eluted with 4 mL of a 2:1 mixture of chloroform:2-propanol, the free fatty acids were eluted with 4 mL of 2% acetic acid in diethyl ether, and the phospholipids were eluted with 4 mL of methanol. Each fraction was evaporated to dryness, 3 mL of scintillation fluid (Betafluor National Diagnostics, Atlanta, GA) was added, and the radioactivity of the vials was measured in a β counter.

The abrogation of cytotoxicity caused by the rickettsiae was not due to a toxic effect of the BpB, ethanol, or the antibody to cobra venom on the rickettsiae as judged by the preservation of the ability of the rickettsiae to actively transport lysozyme into their cells. This rickettsial metabolic activity was lost upon rickettsial death caused by heat (100°C), but not after the experimental treatments with the phospholipase inhibitors or the diluent and serum controls (Table 2). These
The actual levels of radioactivity released by labeled Vero cells for each set of conditions were (1) 5,847 cpm, (2) 116 ± 10 cpm, (3) 3,391 ± 292 cpm, (4) 2,896 cpm, (5) 98 ± 12 cpm, and (6) 2,027 ± 114 cpm.

Data indicate that the cytotoxic effect is caused by a rickettsial function that is inhibited in rickettsiae that have maintained their viable, metabolically active state.

Analysis of the release of 3H-oleic acid label incorporated into the Vero cells in the free fatty acid fraction demonstrated that BpB functioned in this cytotoxicity model as an inhibitor of phospholipase A2. The percent free fatty acid released from labeled Vero cells interacting with BpB-treated R. conorii was decreased to the baseline level of normal cells.

The uptake of 14C-lysine by untreated rickettsiae was considered to represent 100% (25,279 cpm for R. prowazekii; 22,025 cpm for R. conorii).

Active transport of 14C-lysine by rickettsiae treated with bromophenacyl bromide or anti-phospholipase A2 antibody

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rickettsia prowazekii</th>
<th>Rickettsia conorii</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100%*</td>
<td>100%*</td>
</tr>
<tr>
<td>BpB†</td>
<td>73.7 ± 34.7%</td>
<td>141.9 ± 51.6%</td>
</tr>
<tr>
<td>Ethanol</td>
<td>89.3 ± 1.9%</td>
<td>99.4 ± 9.4%</td>
</tr>
<tr>
<td>Anti-phospholipase A2 antibody</td>
<td>59.0 ± 20.3%</td>
<td>109.7 ± 64.6%</td>
</tr>
<tr>
<td>Normal horse serum</td>
<td>97.5 ± 15.9%</td>
<td>122.8 ± 52.4%</td>
</tr>
<tr>
<td>Heated (100°C)</td>
<td>3.2 ± 2.2%</td>
<td>1 ± 0.3%</td>
</tr>
</tbody>
</table>

* The uptake of 14C-lysine by untreated rickettsiae was considered to represent 100%
† BpB = p-bromophenacyl bromide.

Ultrastructural examination of numerous sections from many blocks under each set of conditions of these experiments revealed that heat-killed rickettsiae did not enter the Vero cells, BpB- and antivenom-treated rickettsiae appeared to have a reduction of entry into the cells, and native R. conorii and R. prowazekii invaded the host cell by a frequently hypothesized route that had not been previously visualized and documented completely for R. prowazekii. Both R. prowazekii and R. conorii were observed in apposition to the Vero cell plasma membrane that often manifested nearby projections suggestive of ruffles (Figure 6A). The successive stages of engulfment by phagocytosis were identified, including numerous rickettsiae completely surrounded by a phagosomal membrane (Figure 6A, D). The morphologic appearance of rickettsial escape from the phagosomal vacuole consisted of loss of segments of the vacuolar membrane. Parts of the rickettsial cell wall were observed adjacent to the host cell cytosol and other parts of the cell wall of the same rickettsia were still adjacent to a normal-appearing vacuolar membrane (Figure 6B, C).

Because entry of BpB- or antivenom-treated rickettsiae was only decreased and not completely prevented, the addition of larger quantities of rickettsiae resulted in the presence of similarly proportionally larger quantities of treated rickettsiae in the host cell, even though rickettsial entry was without any rickettsial contact (Figure 4). The percentage of free fatty acid released by BpB-treated R. conorii was also significantly reduced, although not to the baseline level (Figure 5).
inhibited. These results are consistent with those of Silverman and others, who showed a reduction of rickettsial entry to approximately half of that of the untreated rickettsiae. Comparison of the relative proportions of treated and control rickettsiae inside of intact vacuoles, inside of vacuoles with partially lysed vacuolar membrane, and free in cytosol did not demonstrate significant reduction in phagosomal escape by intracellular rickettsiae that had been exposed to inhibitors of phospholipase A₂ activity. Similar quantities of these rickettsiae had escaped into the cytosol (Table 3).

**DISCUSSION**

This study extended the characterization of the model of immediate cytotoxicity associated with *R. prowazekii* contact with a host cell monolayer and phospholipase A₂ activity. The pathogenic mechanism of the cytotoxic effect was shown to be rickettsial phospholipase A₂ activity that was blocked by the phospholipase A₂ inhibitor, BpB, and by an antibody directed against king cobra venom phospholipase A₂. This cytotoxic rickettsial phospholipase A₂ was present not only in the prototype typhus group rickettsia, *R. prowazekii*, but also in the typical spotted fever group rickettsia, *R. conorii*.

This investigation confirmed all the features of the previously described rickettsia-induced cytotoxicity model, including its temperature dependence. It is noteworthy that Vero cells in these experiments exhibited the same cytotoxic phenomenon as the L-929 cells did in the previous studies, indicating, not unsurprisingly, that the cytotoxicity was not limited to a single cell type.

This rickettsia-induced, phospholipase A₂-dependent cytotoxicity offers a model for rickettsial pathogenesis research into rickettsial phospholipase A₂ as a potential mechanism of injury to eukaryotic cells. It had previously been hypothesized that rickettsiae enter and injure cells by a phospholipase A₂-dependent mechanism. The interpretation by Silverman and others that fewer BpB-treated rickettsiae were present inside of the Vero cells than in untreated rickettsiae was corroborated by this study, in which additional observations were documented, namely viability of the rickettsiae, inhibition of phospholipase A₂ activity, and the ultrastructural intracellular location. Moreover, the phospholipase A₂ dependence of rickettsial entry into the cell was extended to a rickettsia of the typhus group.

Rickettsial entry into the host cell by induced phagocytosis has been demonstrated for *R. prowazekii* by functional studies including inhibition by cytochalasin B, but to our knowledge, this is the first demonstration of the ultrastructural morphology of its entry by phagocytosis into a vacuole from which the organism escaped by observed lysis of the vacuolar membrane. Ultrastructural demonstration of the entry of *R. conorii* and *R. japonica* via phagosomal vacuoles and lysis of the vacuolar membrane has been described previously. The lack of significant evidence that escape of *R. prowazekii* or *R. conorii* from the phagosomal vacuole is reduced by inhibition of a rickettsial phospholipase A₂ suggests that phospholipase A₂ may not be the mechanism of phagosomal escape. However, it is conceivable that the population of rickettsiae that had gained entry into the host cell possessed a larger quantity of uninhibited phospholipase A₂, enabling not only entry but also lysis of the phagosomal membrane. Indeed, *R. prowazekii* appears to possess 2 different extracellularly secreted membranolytic activities, one of which is substantially more effective at the mildly acidic pH of the early phagosomal compartment than the other hemolytic activity. Other mechanisms of lysis of the vacuolar membrane, including rickettsia-secreted pore-forming proteins, have been proposed.

Although sequencing and annotation of the entire genome of the attenuated Madrid E strain of *R. prowazekii* did not identify a gene for phospholipase A₂, this conclusion should be recognized as only tentative and perhaps likely to be revised. Although phospholipase A₂ activity has been detected in many bacteria, DNA sequences for well-characterized bacterial phospholipase A₂ genes are virtually nonexistent. Thus, searches of gene data banks for similarity of DNA sequences are unlikely to identify a bacterial phospholipase A₂. Moreover, there is an abundance of open reading frames in the annotated genome of *R. prowazekii* for which the function is unknown or for which the putative function is based on a low level of one functional activity. Indeed, analysis of the *R. prowazekii* genome revealed a rickettsial protein belonging to the alpha/beta hydrolase fold, which itself contains several lipases, including human phospholipase A₂. The rickettsial protein contains sequences similar to the catalytic domain of phospholipase A₂ and is also similar to the cytotoxic protein, ExoU, of *Pseudomonas aeruginosa*. Sequence analysis of the genome of *R. prowazekii* also revealed 2 genes (lyC and lyB) that encode proteins with similarity to hemolysins of *Treponema hondysenteriae*. A gene of *R. typhi* that is homologous to lyC of *R. prowazekii* conferred hemolytic activity on a nonhemolytic strain of *Proteus mirabilis*. The mechanism of the membranolytic activity of the putative hemolysins is not known, nor has their role in typhus pathogenesis been determined.

Whether or not the phospholipase A₂-dependent cytotoxic mechanism described in this work is important as a pathogenic mechanism in vivo during rickettsial infection remains undefined. The mechanism by which host cells filled with typhus group rickettsiae burst and die has been hypothesized to be associated with phospholipase A₂ activity but...
FIGURE 6. Early events in the interaction of rickettsiae with Vero cells. Bar = 0.5 μm. (A) Rickettsia prowazekii (Breinl strain), native, 30-min incubation. Two rickettsiae are surrounded by host cell surface projections (arrows) suggestive of ruffles. Another rickettsia is membrane-bound within a phagosome (thick arrow). (B) Rickettsia prowazekii (Breinl strain), native, 30-min incubation. Rickettsia exits from the phagosome into the cytosol. Most of its circumference is free of the phagosomal membrane. Part of the phagosomal membrane is still preserved (between arrows). (C) Rickettsia prowazekii (Breinl strain), native, 30-min incubation. Rickettsiae are in the process of exiting from the phagosome into the cytosol. Fragments of the phagosomal membrane are preserved (between arrows). (D) Rickettsia conorii (Malish 7 strain), native, 30-min incubation. Many rickettsiae are still extracellular; some of them adhere to the host cell surface (arrowheads). At 2 sites of rickettsial adherence, the plasmalemma is partially invaginated (thick arrows), apparently the early stages of engulfment of the rickettsiae. Most of the intracellular rickettsiae are inside phagosomes (arrows).
remains undetermined. This model could be used to evaluate a phospholipase A2-deficient rickettsia constructed by gene knockout methodology. Furthermore, tools should be developed to investigate bona fide phospholipase A2 in studies of pathogenicity in animal models.

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