Characterization of *Ehrlichia risticii* Binding, Internalization, and Proliferation in Host Cells by Flow Cytometry

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The binding, internalization, and proliferation of *Ehrlichia risticii* in P388D₁ cells and equine polymorphonuclear (PMN) leukocytes were studied by immunofluorescent staining and flow cytometric analysis. The binding of ehrlichiae to P388D₁ cells at 4°C was dose dependent, and the antigens of bound organisms were susceptible to pronase treatment. Additionally, the binding of ehrlichiae to P388D₁ cells was diminished when either P388D₁ cells or ehrlichiae were treated with 1% paraformaldehyde for 30 min or 0.25% trypsin for 15 min. These results indicate that the ehrlichial ligand and host cell receptor are likely surface proteins. Following incubation at 37°C, bound *E. risticii* and/or its antigens were removed with pronase and indirect immunofluorescent staining in the presence of saponin was used to examine intracellular ehrlichiae. Our results indicate that *E. risticii* was internalized into P388D₁ cells within 3 h and proliferated by 48 h of incubation. The microfilament-disrupting agent cytochalasin D and the transglutaminase inhibitor monodansylcadaverine were used to differentiate between phagocytosis (sensitive to cytochalasin) and receptor-mediated endocytosis (sensitive to monodansylcadaverine) of *E. risticii* by P388D₁ cells. In concentrations that produced distinctive morphological changes and inhibited phagocytosis of polystyrene latex beads, cytochalasin D did not suppress the infectivity of *E. risticii*. Binding, internalization, or proliferation of *E. risticii* was not affected by cytochalasin D. However, monodansylcadaverine inhibited infection of *E. risticii* in a dose-dependent manner. The agent did not affect the attachment of ehrlichiae to host cells, but it did suppress internalization and proliferation. These results suggest that *E. risticii* is internalized by receptor-mediated endocytosis and that productive infection by *E. risticii* does not depend on phagocytosis by the P388D₁ cells. Although *E. risticii* did not bind to the surface of equine PMN leukocytes at 4°C, organs were taken up by this cell at 37°C. *E. risticii*, however, failed to survive in equine PMN leukocytes.

Members of the tribe *Ehrlichiae* contain obligate intracellular rickettsial parasites with a tropism for either monocytes/macrophages or granulocytes, but not both cell types (13). *Ehrlichia risticii*, the etiologic agent of Potomac horse fever, parasitizes the macrophage and has been shown to proliferate in vitro in the phagosome of a macrophage cell line, P388D₁ (19). Although pivotal to intracellular survival, the component(s) or mechanism(s) involved in adherence to and internalization of *E. risticii* by the macrophage is unknown.

In this study we examined the binding, internalization, and proliferation of *E. risticii* in host cells using three lines of investigation. First, we developed a flow cytometric method to evaluate and characterize ehrlichial attachment to the surface of P388D₁ cells and ehrlichial internalization and proliferation within the host cell. Second, the mechanism of ehrlichial entry into host cells was examined with microfilament and transglutaminase inhibitors. Third, since *E. risticii* has not been found in polymorphonuclear (PMN) leukocytes of infected horses (14), we examined whether *E. risticii* binds, internalizes, or survives in PMN leukocytes.

**MATERIALS AND METHODS**

**Cell line.** P388D₁ cells are a macrophage cell line derived from a DBA/2 mouse (American Type Culture Collection, Rockville, Md.). The cells were maintained in RPMI 1640 (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO) and 2 mM L-glutamine (GIBCO).

**E. risticii.** *E. risticii* was propagated in the P388D₁ cells at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, as previously described (11). The degree of *E. risticii* infection in P388D₁ cells was assessed by Diff-Quik staining (Baxter, Ohio) of cytocentrifuged preparations.

**Infection of P388D₁ cells.** P388D₁ cells which had reached 90% infectivity with *E. risticii* were detached by gentle scraping from the bottom of the flask and suspended at a concentration of 10⁶ cells per ml in RPMI 1640. Following sonication at 30 kHz at a setting of 3 for 5 s (Ultrasonic Processor W-380; Heat System, Farmingdale, N.Y.) and centrifugation at 350 × g for 15 min, the supernatant was harvested. The supernatant was centrifuged a second time at 600 × g for 15 min to remove remaining P388D₁ cellular debris. *E. risticii* was pelleted at 10,000 × g for 10 min and resuspended in RPMI 1640 containing 10% FBS and 2 mM L-glutamine. Disruption of the P388D₁ cells and the presence of freed *E. risticii* were confirmed by cytocentrifugation of the sample followed by Diff-Quik staining and light microscopic examination. This procedure is routinely utilized in our laboratory to isolate ehrlichiae from host cells (11).

Confluent monolayers of noninfected P388D₁ cells, 10⁶ cells in a 25-cm² tissue culture flask (Corning Glass Works, Corning, N.Y.), were overlaid with *E. risticii* isolated from 10⁶ infected P388D₁ cells in 1 ml of RPMI 1640 containing 10% FBS and 2 mM L-glutamine. Control cultures were
similarly treated with the supernatant from uninfected P388D1 cells which had been sonicated. Cell cultures were incubated at 4°C for 3 h to assess binding of ehrlichiae and harvested at 3, 48, or 96 h following incubation at 37°C in a humidified 5% CO2-95% air atmosphere to permit internalization and proliferation of ehrlichiae. *E. risticii* infection of P388D1 cells was determined by direct microscopy of Diff-Quik-stained cytocentrifuged specimens.

In certain experiments, a microfilament inhibitor, cytochalasin D (Sigma Chemical Co., St. Louis, Mo.); the stock solution was 500 μg/ml in dimethyl sulfoxide), or a transglutaminase inhibitor, monodansylcadaverine (Sigma; stock solution was 25 mM in methanol, adjusted to pH 7.3 with HCl), was present in the medium starting 3 h prior to inoculation with *E. risticii*.

To verify the activity of cytochalasin D, polystyrene latex particles (Sigma; average diameter, 1.1 μm) were added to P388D1 cells at a final concentration of 0.005% in the presence or absence of cytochalasin D or monodansylcadaverine at 37°C for 1 h. The numbers of P388D1 cells with internalized latex particles were counted in 200 cells under a light microscope after Diff-Quik staining. The experiment was done in duplicate and repeated twice.

**Infection of equine PMN leukocytes.** Peripheral blood PMN leukocytes were isolated from 100 ml of heparinized equine blood (seronegative for *E. risticii*) on a Ficoll-sodium diatrizoate gradient (Histopaque-1119; Sigma). The interface fraction contained 92% PMN leukocytes with cell viability of 94% as assessed by trypan blue exclusion. The concentration of PMN leukocytes was adjusted to 106 cells per ml, and 1 ml was added to a 25-cm2 tissue culture flask. As described above, PMN leukocytes were overlaid with *E. risticii* isolated from 106 infected P388D1 cells in 1 ml of RPMI 1640 containing 10% FBS and 2 mM L-glutamine. Binding, internalization, and proliferation of *E. risticii* in PMN leukocytes were assessed by flow cytometry. *E. risticii* infection was also quantitated by enumerating organisms in 200 cells on Diff-Quik-stained cytocentrifuged preparations.

**Immunofluorescent staining for intracellular *E. risticii*.** *E. risticii*-infected and control P388D1 cells were detached by gentle shaking, treated with 1 mg of pronase (Calbiochem, San Diego, Calif.) per ml in phosphate-buffered saline (PBS; pH 7.5; 0.14 M NaCl, 8 mM Na2HPO4 · 1.4H2O, 1.5 mM KH2PO4, and 2.7 mM KCl) for 10 min at 37°C to remove bound organisms, washed twice in PBS, fixed for 20 min in 1% paraformaldehyde (Sigma) in PBS, and washed twice with PBS. Staining procedures were done at room temperature in the presence of saponin (Accurate Chemicals, Westbury, N.Y., 0.2% in PBS) to promote internalization of antibody for labeling intracellular ehrlichiae. P388D1 cells, 2
× 10⁶/ml, were incubated for 30 min at room temperature in 100 μl of 0.2% saponin in PBS and 500 μl of the primary antibody (1:100 dilution in PBS containing 0.2% saponin of equine antisera to E. risticii or normal equine serum). Cells were washed twice with PBS containing 0.2% saponin and incubated for 30 min at room temperature with 100 μl of a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-equine immunoglobulin G (United States Biochemicals, Cleveland, Ohio) diluted in PBS containing 0.2% saponin.

**Attachment assay.** Isolated chelirchiae were added to cultures of P388D₁ cells as described above and incubated at 4°C for 3 h or at 37°C for 1 h. Unbound chelirchiae were separated from chelirchiae attached to P388D₁ cells by centrifuging cells through 10 ml of FBS at 200 × g for 5 min at 4°C. Cells were washed once in cold PBS, either treated with 1 mg of pronase per ml in PBS for 10 min at 37°C or left untreated at 4°C, and then fixed at room temperature for 20 min in 1% paraformaldehyde. Immunostaining was performed at room temperature in the absence of saponin.

**Trypsin, paraformaldehyde, and heat treatments of E. risticii or host cells.** E. risticii isolated from 10⁶ P388D₁ cells were pelleted by centrifugation at 10,000 × g for 10 min and either resuspended in 1 ml of RPMI 1640 containing 10% FBS and 2 mM L-glutamine, digested with 1 ml of 0.25% porcine trypsin (GIBCO) for 15 min at room temperature, or reacted with 1 ml of 1% paraformaldehyde for 30 min at room temperature. Suspensions of E. risticii in RPMI 1640 were incubated at 60°C for 5 or 15 min. Treated E. risticii was washed once with RPMI 1640 containing 10% FBS and 2 mM L-glutamine, pelleted at 10,000 × g for 10 min, and resuspended in 1 ml of RPMI 1640 containing 10% FBS and 2 mM L-glutamine. The samples were overlaid onto confluent monolayers of P388D₁ cells in a 25-cm² tissue culture flask, incubated at 4°C for 3 h. Confluent monolayers of noninfected P388D₁ cells in a 25-cm² tissue culture flask were treated similarly with 0.25% trypsin for 15 min or 1% paraformaldehyde for 30 min, gently detached from the bottom of the flask, and washed twice by centrifugation with RPMI 1640 containing 10% FBS and 2 mM L-glutamine before the cells were incubated with E. risticii at 4°C for 3 h. The binding of untreated chelirchiae to untreated P388D₁ cells was also assessed. All samples were then fixed in 1% paraformaldehyde for 20 min, washed twice with PBS, and immunofluorescently labeled.

**Flow cytometry.** Fluorescence was measured by flow microfluorometry analysis on an EPICS V 753 flow cytometer (Coulter Corp., Hialeah, Fla.). For each of the fluorescence profiles, experiments were performed in duplicate and 5,000 cells were analyzed. Data were presented as fluorescence profiles, plotting cell frequency as a function of log fluorescence intensity. Mean channel fluorescence of histograms was obtained, and data were analyzed by using Easy 2 Flow Cytometry software (Coulter Corp.). Various controls, including uninfected P388D₁ cells and immunostaining with normal horse serum, were run in each experiment. Each experiment was repeated more than three times. Representative histograms of one set of experiments run concurrently are shown in Results.

**Statistical analysis.** The Kolmogorov-Smirnov test with n₁ = 5,000 and n₂ = 5,000 cells and at α₀ = 0.001 was applied for the analysis of histograms generated by fluorescence flow cytometry (20). The D critical value was equal to 0.04. When D was greater than the D critical value, these histograms were considered significantly different at the 99.9% confidence level.

**RESULTS**

Attachment of E. risticii to P388D₁ cells. As shown in Fig. 1a, when E. risticii was stained with anti-E. risticii or normal horse serum as the control for immunostaining after fixation with paraformaldehyde in the absence of saponin, significant binding of E. risticii to P388D₁ cells was evident after both 4°C incubation for 3 h and 37°C incubation for 1 h. With these cells were treated with pronase before fixation and immunolabeling, there was significant reduction in positively labeled cells to the level or less than the level of the uninfected cells labeled with anti-E. risticii serum or the infected cells labeled with normal horse serum (Fig. 1 and 2). Thus, bound chelirchiae were removed and/or their surface antigens were stripped off by pronase treatment. Most likely bound chelirchiae were removed, since when pronase-treated cells were further cultured, there was no infection of P388D₁ cells (data not shown). Slightly less E. risticii and/or their antigen bound at 37°C was removed by pronase treatment than those bound at 4°C (Fig. 1b). When pronase-treated cells were immunostained in the presence of saponin to observe intracellular chelirchiae, no significant labeling at 4°C was seen but at 37°C positive labeling of intracellular E. risticii was seen (data not shown). Because (i) at 37°C for 1 h, binding and internalization could not be clearly distinguished because they concurrently occurred, (ii) pronase treatment could not completely remove extracellular E. risticii bound at 37°C, likely because of partial internalization, and (iii) the amount of E. risticii bound at 4°C was not less than that at 37°C, in the following studies E. risticii binding to P388D₁ cells was determined at 4°C.

In Fig. 2, uninfected P388D₁ cells and P388D₁ cells incubated with E. risticii at 4°C for 3 h were compared by immunofluorescence. Pronase treatment not only removed E. risticii bound on P388D₁ cells but also slightly reduced weak nonspecific immunofluorescent staining of uninfected P388D₁ cells (Fig. 2), as well as nonspecific staining of
infected cells with normal serum (data not shown). These nonspecific labelings were so weak that the labelings were not noticeable by immunofluorescence microscopy. Binding of *E. risticii* to P388D1 cells was dose dependent (Fig. 3).

**Trypsin or paraformaldehyde treatment of *E. risticii* or host cells or heat treatment of *E. risticii*.** As shown in Fig. 4, the attachment of *E. risticii* to P388D1 cells was partially abrogated by pretreatment of either ehrlichiae or host cells with trypsin or paraformaldehyde. Heat-treated ehrlichiae lost their ability to attach to the host cells (Fig. 5). There was no significant reduction in immunostaining of paraformaldehyde-fixed *E. risticii* in the absence of saponin by prior trypsin, paraformaldehyde, or heat treatment, as determined by flow cytometry of host cell-free *E. risticii* (data not shown).

**Internalization and proliferation of *E. risticii* in P388D1 cells.** As seen in Fig. 6, following 3 h of incubation at 37°C and immunolabeling after pronase treatment in the presence of saponin, a distinct shift in the fluorescence intensity of the histogram was observed. This shift was not observed by

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**Fig. 3.** Flow cytometric histogram for dose response for binding of *E. risticii*. *E. risticii* dilutions of 1:16 (a), 1:4 (b), and 1:1 (c) were incubated at 4°C with P388D1 cells. Shaded histogram, uninfected P388D1 cells. All immunostained with anti-*E. risticii* serum.

**Fig. 4.** Flow cytometric histograms for attachment of *E. risticii* to P388D1 cells after treatment of ehrlichiae or P388D1 cells with trypsin or paraformaldehyde. Solid line, *E. risticii* or P388D1 cells treated with paraformaldehyde or trypsin and then incubated at 4°C with untreated P388D1 cells or untreated *E. risticii*, respectively, and immunofluorescently stained with anti-*E. risticii* serum; shaded histogram, uninfected P388D1 cells; dotted line, untreated *E. risticii* bound to untreated P388D1 cells.

**Fig. 5.** Flow cytometric comparisons of the effects of heating *E. risticii* on its attachment to P388D1 cells. *E. risticii* was heated at 60°C for 5 or 15 min. Shaded histogram, uninfected P388D1 cells; dotted line, P388D1 cells plus untreated *E. risticii*; solid line, P388D1 cells plus heat-treated *E. risticii*. All immunofluorescently stained with anti-*E. risticii* serum.
labeling with normal serum (data not shown) or immunolabeling after pronase treatment in the absence of saponin (result similar to that in Fig. 1b). This shift in fluorescence intensity correlated with positive immunolabeling of 94% of the P388D₁ cells as observed by fluorescence microscopy. However, as previously reported (11), few to no E. risticii cells per P388D₁ cell were seen under light microscopy after Diff-Quik staining at 3 h. At 48 h a distinct population of negatively and positively stained cells (37% infectivity by Diff-Quik staining) was apparent by both flow cytometry and fluorescence microscopy. Saponin treatment permeabilized not only host membranes but also ehrlichial membranes, resulting in immunolabeling of the interior of ehrlichiae as well. In contrast, in paraformaldehyde fixation given prior to immunostaining for the binding study, the antibody could not penetrate the ehrlichial membrane, resulting in ringlike immunolabeling (8). By 96 h the progression of the intracellular proliferation of E. risticii was demonstrable (Fig. 6). As shown in Fig. 6, staining infected cells with anti-E. risticii serum and normal serum demonstrates distinct peaks.

Effects of cytochalasin D and monodansylcadaverine on ehrlichial infectivity. There was no difference in the proliferation of ehrlichiae in P388D₁ cells treated with 0.5, 1.0, or 4.0 μg of cytochalasin D per ml (Table 1). Compared with those of untreated ehrlichiae, the binding, internalization, and intracellular development of E. risticii assessed by Kolmogorov-Smirnov testing of histograms generated by flow cytometry were not inhibited by cytochalasin D treatment (Fig. 7). Without treatment, 94% of P388D₁ cells ingested latex particles. With 1 and 4 μg of cytochalasin D per ml, 33 and 11% of P388D₁ cells, respectively, phagocytized latex particles. P388D₁ cells became round and multinucleated because of inhibition of cytokinesis but not nuclear division by cytochalasin D. Monodansylcadaverine at 250 μM had no effect on latex particle uptake by P388D₁ cells.

Monodansylcadaverine exhibited a dose-dependent effect on the intracellular proliferation of E. risticii in P388D₁ cells (Table 1). The percentage of P388D₁ cells infected with E. risticii increased in the absence of this inhibitor, but a 250 μM concentration of monodansylcadaverine inhibited the intracellular proliferation of ehrlichiae at 48 and 96 h by 75 and 88%, respectively. By Kolmogorov-Smirnov testing of histograms generated by flow cytometry, monodansylcadaverine (250 μM) had minimal effects on ehrlichial binding to P388D₁ cells compared with that in the untreated control culture (Fig. 8). However, the agent significantly reduced internalization and subsequent growth of ehrlichiae in the host cell, which was seen as a significant decrease in cells stained at a higher intensity at 48 h than at 3 h of incubation at 37°C (Fig. 8).

Binding, internalization, and proliferation of E. risticii in equine PMN neutrophils. E. risticii infection was quantified by enumerating organisms in 200 cells on Diff-Quik-stained cytospin centrifuged preparations. As seen in Fig. 9, binding of E. risticii to PMN leukocytes at 4°C was insignificant. However, following 3 h of incubation at 37°C, a significant shift in the fluorescence intensity of the histogram for PMN leukocytes was observed. This shift in fluorescence intensity correlated with the internalization of E. risticii by 86% of the PMN leukocytes as observed by light microscopy (Fig. 10). By 24 h, fluorescence intensity declined significantly compared with that at 3 h. Ehrlichial organisms were observed by light microscopy in only 36% of the PMN leukocytes. At 48 h E. risticii infectivity of the PMN leukocytes had declined to 19%.

**DISCUSSION**

Although heavily infected cells are easily recognized, since (i) ehrlichial organisms are relatively small cocci to visually enumerate, (ii) very few organisms are bound or ingested per macrophage, and (iii) the number of organisms bound or internalized per cell varies (11), objective and quantitative analysis of bound or internalized ehrlichiae in macrophages by conventional microscopy is difficult to impossible. We developed the flow cytometric method to study attachment and internalization of E. risticii. Furthermore, with saponin to permeabilize antibodies, proliferation...
of *E. risticii* was objectively observed in 5,000 cells by flow cytometry, rather than in a few hundred cells by light microscopy. Logarithmic expression of fluorescence intensity in flow cytometry is superior to fluorescence microscopy in discriminating positive and negative cells. Especially at a weaker fluorescence intensity range, the difference is rather clear by flow cytometry. However, flow cytometry is inferior to light microscopic observation of Diff-Quik-stained cells in differentiating among more heavily infected cells.

Like the attachment of *Rickettsia conorii* to L-929 cells (7), *E. risticii* bound to P388D₁ cells at 4°C and the binding was dose dependent. Furthermore, the sensitivity of *E. risticii* binding to parafomaldehyde or trypsin treatment of *E. risticii* or P388D₁ cells or heat treatment of *E. risticii* was similar to that of *R. conorii* (7). These results indicate that the ehrlichial ligand and host cell receptor are likely surface proteins.

Although quantitative flow cytometric assay was used to examine only binding of rickettsial organisms to host cells (7), we performed cytometric analysis of intracellular *E.
risticii by the use of saponin to permeabilize cell membranes. Saponin acts mainly by solubilizing cholesterol and leaves most antigens unaltered (5, 17). It should be cautioned, however, that since saponin treatment allowed ehrlichial cytoplasmic antigen to be also immunolabeled, flow cytometric data of ehrlichial binding and internalization are not directly comparable. Our flow cytometry results indicate that after 3 h incubation at 37°C nearly 100% of the macrophages become positive for cytoplasmic ehrlichial antigens. Ehrlichial antigens disappeared in 50 to 70% of P388D1 cells by 48 h, and then nearly 100% of cells again became positive in 4 to 6 days postinfection. A likely explanation for this observation is that following in vitro culture and/or sonication a mixture of viable, nonviable, or debris of ehrlichiae is produced, all of which are endocytosed and positively immunolabeled, although only viable and intact ehrlichiae prevent phagosome-lysosome fusion and go on to multiply. The rest of the ehrlichial antigens were digested by lysosomal enzymes and disappeared. Immunolabeling cannot discriminate viable from nonviable ehrlichiae. Alternatively, ehrlichiae, like chlamydiae (10), may have infectious and replicative forms. Both forms may be endocytosed, but only one form may be able to survive and replicate in macrophages. A further possibility is that P388D1 cells initially are able to kill viable E. risticii but eventually are overwhelmed with infection. Observation of very few intracellular ehrlichiae by Diff-Quik staining after 3 h of incubation at 37°C favors the first explanation, since the basis for its positive staining is the presence of nucleic acids of ehrlichiae.

Internalization of E. risticii by PMN leukocytes has not previously been reported. Unlike P388D1 cells, E. risticii does not bind at 4°C but is internalized by PMN leukocytes at 37°C, and then the organisms fail to survive or proliferate in this cell. This may be the reason why we did not see infected PMN leukocytes in the horse (14). Apparently equine PMN leukocytes lack receptors to which E. risticii can bind at 4°C.

The data reported here indicate that the entry of E. risticii into P388D1 cells occurs by a mechanism resistant to cytochalasin D and sensitive to monodansylcadaverine. Cytochalasins are inhibitors of actin filament polymerization and have been used to block phagocytosis of bacterial pathogens by host cells (1, 9). Monodansylcadaverine is a potent inhibitor of transglutaminase and interferes with receptor-mediated endocytosis by blocking receptor clustering and recycling (2, 6). These inhibitors have been used to characterize endocytosis and phagocytic mechanisms of entry utilized by rickettsiae and chlamydiae (3, 12, 16, 18). Like a number of viruses (4) and chlamydiae (15, 16), E. risticii appears to exploit the process of receptor-mediated endocytosis to provide for its entry into eucaryotic cells and does not involve the microfilament-dependent process of phagocytosis. Whether the uptake of E. risticii by P388D1 cells occurs in coated or uncoated membranes is unknown. The nature of the signal for receptor-mediated endocytosis of E. risticii by the P388D1 cell is not known. However, work in our laboratory (11) suggests that the signal for entry of E. risticii into the macrophage may involve formation of a calcium gradient, since thioglycolate-induced peritoneal macrophages became refractory to E. risticii infection following treatment with the Ca²⁺ ionophore A23187.

These findings are important in providing the technique for future studies toward identifying the ehrlichial ligand and target cell receptor involved in this attachment, internalization, and survival of ehrlichiae.

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REFERENCES


