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Inhibition of Binding, Entry, or Intracellular Proliferation of *Ehrlichia risticii* in P388D$_1$ Cells by Anti-*E. risticii* Serum, Immunoglobulin G, or Fab Fragment

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The effects of equine antiserum, immunoglobulin G (IgG) specific for *Ehrlichia risticii*, and its Fab fragment on *E. risticii* binding to, internalization into, and proliferation in P388D$_1$ cells were studied by immunofluorescence flow cytometry. Anti-*E. risticii* equine serum or IgG inhibited *E. risticii* at a stage beyond binding and internalization. In contrast, monovalent anti-*E. risticii* equine Fab fragments inhibited *E. risticii* binding and internalization into P388D$_1$ cells. In the presence of control equine serum, IgG, or its Fab fragment, *E. risticii* cells were bound, were internalized and subsequently grew within P388D$_1$ cells, and eventually destroyed the host cells as effectively as was the case without equine serum, IgG, or Fab fragments. Anti-*E. risticii* IgG but not normal horse IgG inhibited L-[^{14}C]glutamine metabolism in Percoll gradient-purified *E. risticii*. These findings suggest that the Fab fragment of intact anti-*E. risticii* IgG blocks the ligands on *E. risticii* responsible for non-IgG-mediated internalization and diverts them to bind via the Fc receptor. Following Fc-mediated entry of *E. risticii*, the antibody interfered with the metabolic activity of *E. risticii* cells, rendering them incapable of proliferation in P388D$_1$ cells and resulting in the eventual destruction of the organisms.

Ehrlichia risticii is a minute obligate intracellular rickettsial organism which infects monocytes/macrophages and intestinal epithelial cells (10). *E. risticii* multiplies in host membrane-lined vacuoles which do not fuse with lysosomes (16). It is the etiologic agent of Potomac horse fever (9). Infected and vaccinated animals (mice and horses) have been shown to produce antibodies against *E. risticii* (6–8, 11–13); however, the role of antibody in protective immunity to *E. risticii* infection is poorly understood.

The involvement of antibody in protective immunity to *E. risticii* was recently indicated by the fact that passive transfer of immune serum or immunoglobulin G (IgG) protects mice, which develop dose-dependent morbidity and mortality, from infection with *E. risticii* (1). The presence of neutralizing activity was demonstrated in infected horse sera in vitro and with a murine model of Potomac horse fever (13, 17).

In a previous paper (4), we reported that *E. risticii* specifically binds at 4°C, internalizes by a monodansylcadaverine-sensitive but cytochalasin D-resistant mechanism, and proliferates in P388D$_1$ murine macrophage cells. The purpose of this study was to elucidate which steps of ehrlichial infection are inhibited by the antiserum. Furthermore, since antibody activity was found predominantly in the IgG subclass of the convalescent sera (5), we prepared an IgG fraction and its Fab fragment to identify their inhibitory activities. Although it is rather easy to distinguish heavily infected cells because ehrlichial organisms tend to cluster when they divide (10, 16), it is very difficult to precisely identify and enumerate every *E. risticii* cell bound to the host cell surface or internalized by conventional light and fluorescence microscopy, since ehrlichial organisms are minute cocci, since a few organisms bind unevenly to the macrophage surface, and since only a few per macrophage internalize (4). We therefore applied our previously devised method using immunofluorescence flow cytometry (4) for this study.

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MATERIALS AND METHODS

***E. risticii*.** *E. risticii* was propagated in a murine macrophage cell line, P388D$_1$ (American Type Culture Collection, Rockville, Md.) in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO) and 2 mM L-glutamine (GIBCO) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, as previously described (4). The degree of *E. risticii* infection in P388D$_1$ cells was assessed by Diff-Quik staining (Baxter, Obetz, Ohio) of cytocentrifuged preparations. Host cell-free *E. risticii* was prepared as described previously (4). Briefly, P388D$_1$ cells which had reached 90% infectivity with *E. risticii* were suspended at a concentration of 10^6 cells per ml in RPMI 1640 medium. Following sonication at 30 kHz at a setting of 3 for 5 s (Ultrasonic Processor W-380; Heat System, Framingdale, N.Y.) and centrifugation at 350 × *g* for 15 min, the supernatant was harvested. *E. risticii* cells were pelleted by centrifugation at 10,000 × *g* for 10 min and resuspended in RPMI medium containing 10% FBS and 2 mM L-glutamine.

Infection. Confluent monolayers of noninfected P388D$_1$ cells (10^7 cells in a 25-cm^2 tissue culture flask [Corning Glass Works, Corning, N.Y.]) were overlaid with *E. risticii* isolated from 10^6 infected P388D$_1$ cells in 1 ml of RPMI medium containing 10% FBS and 2 mM L-glutamine. Control cultures were similarly treated with the supernatant from uninfected P388D$_1$ cells which had been sonicated. Cell cultures were incubated at 4°C for 3 h to assess binding of ehrlichiae and harvested following incubation at 37°C for 3, 48, or 96 h in humidified 5% CO_2–95% air to permit internalization and growth of ehrlichiae. *E. risticii* infection of P388D$_1$ cells was determined by immunofluorescence flow cytometry and direct microscopy of Diff-Quik-stained cytocentrifuged specimens.

Anti-*E. risticii* serum. The equine antiserum used for the neutralization portion of the study and for immunolabeling was obtained from pony 19, a 2.5-year-old male. Following intravenous challenge with 6×10^7 *E. risticii*-infected U-937 cells, this pony did not develop any clinical signs of disease and was hyperimmunized by three intravenous injections of 6×10^7 *E. risticii*-infected U-937 cells at 2- to 3-month intervals. The indirect fluorescent-antibody titer against *E. risticii* in the antiserum used was 1:2,560. The antiserum was preabsorbed at 4°C for 1 h with 10^6 uninfected P388D_1 cells per ml of serum. Normal equine serum with a negative indirect fluorescent-antibody titer for *E. risticii* was treated similarly. The sera were heat inactivated at 56°C for 30 min.

Preparation of IgG and Fab fragment. IgGs were purified from serum with a MAbTrap G column (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Purified IgG was dialyzed against a 20 mM NaH_2PO_4–10 mM EDTA buffer at pH 7.0 and concentrated to approximately 10 mg/ml, and 1 ml was added to 0.5 ml of prewashed, 50% immobilized papain slurry (Pierce Chemical Co., Rockford, Ill.) in 20 mM NaH_2PO_4–20 mM cysteine HCl–10 mM EDTA, pH 7.2. The digestion mixture was incubated with continuous mixing for 18 h at 37°C, and 1.5 ml of 10 mM Tris HCl (pH 7.5) was added to the digest. Following centrifugation at $1,500 \times g$ for 10 min, the supernatant containing fragments was decanted. The supernatant containing IgG fragments was applied to the MAbTrap G column. Fc fragment and any undigested intact IgG were removed by binding to the MAbTrap G column, and Fab fragment was collected as it flowed through the column. The purity of the Fab fragment fraction was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the fraction (50 μl of a 10-mg/ml sample per lane) in 12.5% polyacrylamide gels under reducing and nonreducing conditions.

Neutralization assay. Anti-*E. risticii* serum or nonimmune equine serum (0.5 ml) or anti-*E. risticii* IgG, nonimmune equine IgG, or monovalent Fab antibodies (0.5 ml at a concentration of 1 mg/ml) were mixed with an equal volume of *E. risticii* and incubated at 37°C for 1 h. As described above, *E. risticii* was isolated from 10^6 infected P388D_1 cells and resuspended in 0.5 ml of RPMI 1640 medium containing 10% FBS and 2 mM L-glutamine. The mixture was then inoculated onto monolayers of P388D_1 cells grown in 25-cm^2 flasks. *E. risticii* binding was determined after incubation for 3 h at 4°C (4). Internalization was determined following 3 h of incubation at 37°C and subsequent treatment with pronase to remove bound *E. risticii* (4). For determination of proliferation, the inoculum was removed after 3 h of incubation, and monolayers were washed twice with RPMI 1640 medium. Then cells were fed with 3 ml of RPMI 1640 medium containing 10% FBS and 2 mM L-glutamine and incubated for 72 or 96 h in 5% CO_2–air.

Immunofluorescence staining for intracellular *E. risticii*. *E. risticii*-infected and control P388D_1 cells were detached by gentle shaking, treated with pronase (Calbiochem, San Diego, Calif.) (1 mg/ml in phosphate-buffered saline [PBS] [0.14 M NaCl, 0.008 M Na_2HPO_4, 0.0015 M KH_2PO_4, 0.0027 M KCl, pH 7.5]) for 10 min at 37°C, washed twice in PBS, fixed for 20 min in 1% paraformaldehyde in PBS, and washed twice with PBS. Staining procedures were done at room temperature in the presence of saponin (Quil A; Accurate Chemicals, Westbury, N.Y.) (0.2% in PBS) to promote internalization of antibody for labeling intracellular *E. risticii*. P388D_1 cells (2×10^6/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 of equine polyclonal antiserum to *E. risticii* or normal equine serum). Cells were washed twice with PBS and incubated for 30 min at room temperature with 100 μl of a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-equine IgG (United States Biochemical, Cleveland, Ohio) diluted in PBS containing 0.2% saponin.

Immunofluorescence labeling of extracellular attached *E. risticii*. Cells were washed once in cold PBS and then fixed at room temperature for 20 min in 1% paraformaldehyde. Immunostaining was performed at 4°C in the absence of saponin as previously described (3, 4).

Flow cytometry. Fluorescence was measured by flow microfluorometry analysis on an EPICS V 753 cell sorter. For each of the fluorescence profiles, experiments were performed in duplicate and 5,000 intact cells were analyzed. Data are presented as fluorescence profiles, with cell frequency plotted as a function of fluorescence intensity. Mean channel fluorescences of histograms were obtained and data were analyzed by using Easy 2 Flow Cytometry software (Coulter Corp., Hialeah, Fla.). Experiments were repeated at least two times.

$^{14}CO_2$ production from L-[^{14}C]glutamine by Percoll-purified *E. risticii*. *E. risticii*-infected P388D_1 cells were disrupted by homogenization as described previously (14, 15) and centrifuged at $600 \times g$ for 5 min. The supernatant was incubated with 0.5 mg of trypsin (GIBCO) per ml at room temperature for 3 min, and then 0.125 mg of trypsin inhibitor (GIBCO) per ml was added. The suspension was centrifuged at $11,670 \times g$ for 10 min, and the pellet was resuspended in SPK buffer (0.2 M sucrose and 0.05 M potassium phosphate buffer, pH 7.4). The suspension was mixed with Percoll (Sigma) and centrifuged at $61,900 \times g$ for 30 min as described by Weiss et al. (15). The lower layer was harvested, mixed with SPK buffer, and centrifuged at $11,670 \times g$ for 10 min to remove Percoll. The pellet was resuspended in SPK buffer containing 1 mM L-glutamine. The *E. risticii* suspension (0.25 ml) was incubated with 50 μl of a 1-μCi/ml (231.6-mCi/mmol) solution of L-[^{14}C]glutamine (New England Nuclear Corp., Boston, Mass.) without or with 100 μg of anti-*E. risticii* or normal horse IgG per ml. The tubes were sealed with a rubber stopper top (Kontes Scientific Glassware/Instruments, Vineland, N.J.) from which a plastic center well (Kontes) was suspended. The tubes were incubated in a 37°C water bath with shaking for 2 h, and the reaction was stopped with injection of 250 μl of 25% trichloroacetic acid per tube. Methylbenzethonium hydroxide (1.0 M) (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was injected into the center well, the tubes were further incubated at 37°C in the water bath, and then methylbenzethonium hydroxide in the well was transferred to a scintillation vial and Safety-Solve High Flash Point Cocktail (Research Products International Corp., Mt. Prospect, Ill.) was added to measure $^{14}CO_2$ generated in the tube as described previously (14). Experiments were repeated twice.

The protein concentration of the purified *E. risticii* suspension was determined by the Coomassie brilliant blue dye-binding assay (2). To remove residual Percoll, a mixture of 100 μl of purified *E. risticii* suspension and 100 μl of 0.025% (wt/vol) Triton X-100 (Sigma) in 0.25 N NaOH was centrifuged at $12,000 \times g$ for 15 min. A volume of 50 μl of the supernatant was mixed with 1 ml of Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Richmond, Calif.) which had been diluted to 1:5 with distilled water. To make a protein standard, bovine serum albumin was prepared in an identical manner. The A_{595} was measured in a DU-70 spectrophotometer (Beckman).

Statistical analysis. The Kolmogorov-Smirnov (K-S) test with $n_1 = n_2 = 5,000$ cells and $\alpha = 0.001$ was applied for the analysis of histograms generated by fluorescence flow cytometry (18). The D critical value (D_{crit}) was 0.04. When $D > D_{crit}$, we rejected the null hypothesis that these two populations, as

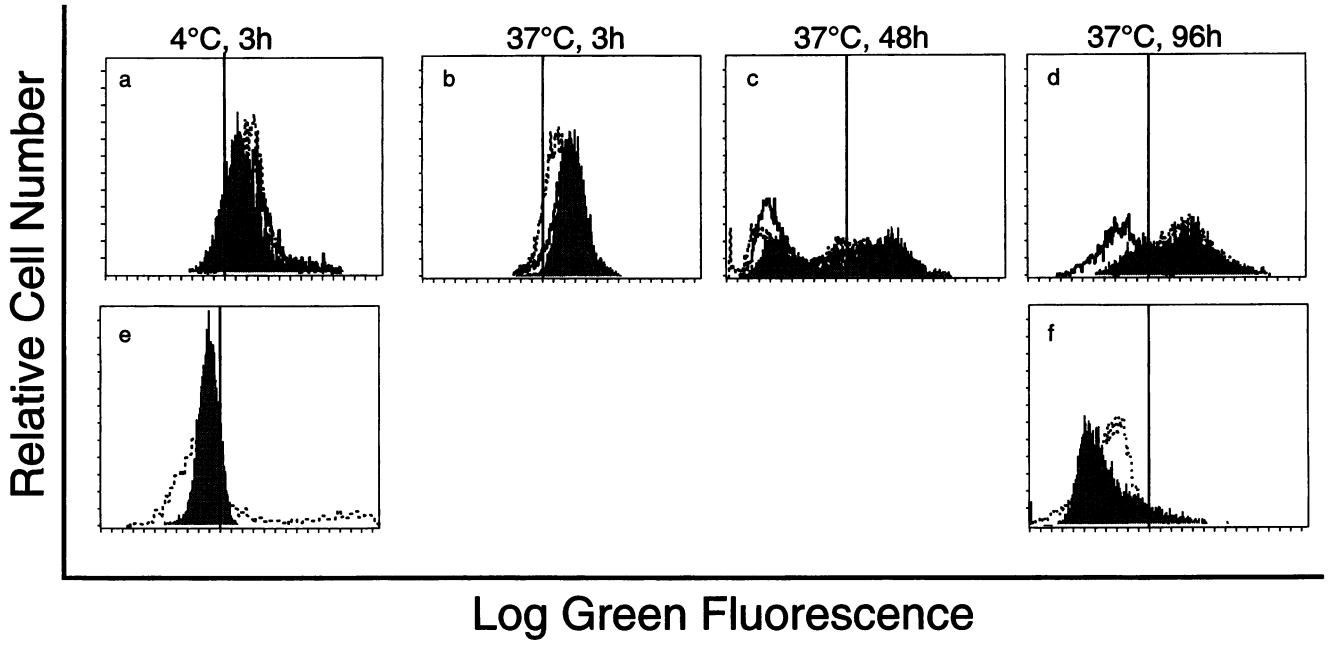

FIG. 1. Influence of anti-*E. risticii* serum on binding (4°C, 3 h), internalization (37°C, 3 h), and proliferation (37°C, 48 and 96 h) of *E. risticii* in P388D_1 cells. (a) *E. risticii* cells were incubated with P388D_1 cells for 3 h at 4°C to evaluate the binding of *E. risticii* to P388D_1 cells following no treatment (shaded histogram), treatment with normal serum (dotted line), and treatment with anti-*E. risticii* serum (solid line). Cells were then washed twice in PBS to remove any unbound *E. risticii* and fixed in 1% paraformaldehyde. Cells were stained with horse anti-*E. risticii* serum followed by fluorescein-conjugated goat anti-horse IgG in the absence of saponin and analyzed by flow cytometry. (b, c, and d) Internalization (37°C, 3 h) and growth (37°C, 48 and 96 h) of *E. risticii* in P388D_1 cells were examined following no treatment (shaded histogram), treatment with normal serum (dotted line), and treatment with anti-*E. risticii* serum (solid line). Cells were treated with pronase to remove bound *E. risticii*, immunostained in the presence of 0.1% saponin, and analyzed by flow cytometry. (e) Uninfected P388D_1 cells were labeled with anti-*E. risticii* serum in the absence of saponin (shaded histogram), or P388D_1 cells were incubated with *E. risticii* at 4°C for 3 h and then immunostained with normal horse serum in the absence of saponin (broken line). (f) Uninfected P388D_1 cells were immunostained with anti-*E. risticii* serum (shaded histogram), or infected P388D_1 cells were immunostained with normal horse serum (dotted histogram) in the presence of saponin. Vertical lines, cutoff for uninfected P388D_1 cells immunostained with anti-*E. risticii* serum or P388D_1 cells infected with or attached to *E. risticii* and immunostained with normal serum.

characterized by their histograms, were the same at the 99.9% confidence level. The statistical significance of the results for L-glutamine metabolism was determined by analysis of variance and Tukey's studentized range test; $P < 0.05$ was considered significant.

RESULTS

Effect of anti-*E. risticii* serum on binding, internalization, and proliferation of *E. risticii* in P388D_1 cells. Binding of *E. risticii* was determined by incubating *E. risticii* and P388D_1 cells at 4°C for 3 h and then immunostaining in the absence of saponin after paraformaldehyde fixation. Intracellular ehrlichiae were determined by immunostaining ehrlichiae in the presence of saponin after pronase treatment to remove extracellular ehrlichial antigens. Immunofluorescence micrographs and flow cytometric data with various controls to validate these procedures were previously reported (3, 4). As shown in Fig. 1a, the levels of shift from controls (Fig. 1e) in fluorescence histograms caused by the binding of *E. risticii* preincubated with normal or anti-*E. risticii* serum or without equine serum were similar. Thus, the presence of normal or immune equine serum in the incubation medium had very little influence on either *E. risticii* binding or immunofluorescence labeling. After 3 h of incubation at 37°C, there was a significant shift in fluorescence histograms caused by the internalization of *E. risticii* preincubated with normal or anti-*E. risticii* serum or without equine serum (Fig. 1b) compared with controls (Fig. 1f). Visual observation supported the flow cytometry data. After 3 h of incubation at 37°C, 95% of cells took up a few *E. risticii* cells with or without antiserum treatment as observed visually by immunofluorescence microscopy. After 3 h at 37°C, the distributions of fluorescence intensity among P388D_1 cells were homogeneous (sharp peaks in Fig. 1b). By 48 h, there was a broader distribution of fluorescence intensity among P388D_1 cells, and two populations of cells became apparent. These two populations appeared as two peaks in flow cytometry (Fig. 1c). One peak occurred below the control fluorescence intensity, and another peak occurred above the control fluorescence intensity (Fig. 1c). By 48 h, infected-cell populations could be easily identified by Diff-Quik staining, since *E. risticii* multiplied in a quantity sufficient to make clusters for easy visual recognition. By Diff-Quik staining, 11% of P388D_1 cells were infected with *E. risticii* which had been preincubated with anti-*E. risticii* serum, and 30% of P388D_1 cells were found to be infected with *E. risticii* which had been preincubated with control equine serum. In the absence of equine serum, 37% of P388D_1 cells were found by Diff-Quik staining to be infected. The flow cytometry finding was not distinct at 48 h. The peaks of the uninfected cell populations of the three groups corresponded to the Diff-Quik results. With anti-*E. risticii* serum, the peak of the uninfected cell population (left side of the vertical lines in Fig. 1) was greater than those of P388D_1 cells incubated with *E. risticii* preincubated with and without normal

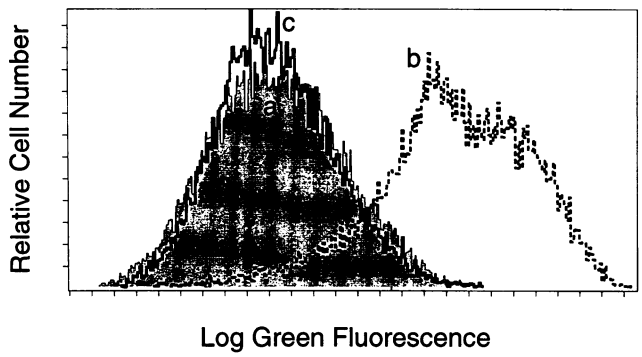

FIG. 2. Flow cytometric analysis of fluorescein-conjugated protein G staining of isolated *E. risticii*. Shaded histogram (a), *E. risticii* reacted with normal equine IgG; dotted line (b), *E. risticii* reacted with equine anti-*E. risticii* IgG; solid line (c), *E. risticii* reacted with equine anti-*E. risticii* Fab fragment.

horse serum (Fig. 1c). However, the peaks of infected cells (right side of the vertical line) with normal horse serum were shifted toward the left, indicating a higher proportion of infected cells with fewer organisms compared with the control. At 96 h, the number of cells with a higher fluorescence intensity increased in normal serum-treated and untreated groups. Thus, normal equine serum did not influence overall *E. risticii* proliferation or immunofluorescence labeling (Fig. 1d). With the group treated with anti-*E. risticii* serum, the number of cells with a lower fluorescence intensity increased at 96 h (Fig. 1d). The percentage of infected cells as determined by Diff-Quik staining was 58% with normal serum and 6% with anti-*E. risticii* serum, which coincided with the histogram data (Fig. 1d). Without equine serum, the percent infected cells at 96 h was 64% by Diff-Quik staining. There was no difference in immunofluorescence labeling histograms of *E. risticii* whether *E. risticii* had been preincubated with normal or anti-*E. risticii* equine serum, because the primary antibody used for immunofluorescence labeling was also the same anti-*E. risticii* equine serum. The secondary antibody used for immunolabeling was fluorescein isothiocyanate-conjugated anti-equine IgG, which binds both opsonized equine antibody and primary labeling equine antibody (4).

IgG and Fab fragments from equine antibodies specific for *E. risticii*. Equine anti-*E. risticii* IgG, equine normal IgG, and their respective purified papain-digested Fab fragments were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions. Examination of Fab preparations derived from equine normal IgG and equine anti-*E. risticii* IgG under reducing conditions showed the absence of full-length heavy chain (50 kDa) and the appearance of the expected Fab fragments migrating at approximately 31 kDa. Under nonreducing conditions, the Fab fragments migrated as 50-kDa polypeptides as expected, and the absence of intact IgG migrating at approximately 150 kDa was confirmed (data not shown).

To confirm that the Fab fragments derived from polyclonal equine anti-*E. risticii* IgG were free of intact IgG, host cell-free *E. risticii* was reacted with either polyclonal equine anti-*E. risticii* IgG, normal equine IgG, or purified papain-digested Fab fragments of polyclonal equine anti-*E. risticii* IgG, washed, and subsequently reacted with fluorescein-labeled protein G (Fig. 2), which binds specifically to the Fc fraction of IgG. Binding of intact polyclonal equine anti-*E. risticii* IgG to the organism was apparent by a significant shift in fluorescence intensity compared with that of *E. risticii* reacted with nonimmune equine IgG (negative control). By K-S testing to compare the histograms generated by *E. risticii* reacted with the Fab fragments of equine anti-*E. risticii* IgG and *E. risticii* reacted with control equine IgG (negative control), no significant difference was found ($D = 0.04$). Histograms of *E. risticii* reacted with Fab fragments of anti-*E. risticii* IgG were significantly different ($D = 0.74$) from the histogram for intact equine anti-*E. risticii* IgG. These data confirmed the absence of intact anti-*E. risticii* IgG in the purified papain-derived Fab fragments. Fab fragments of anti-*E. risticii* bound to host cell-free *E. risticii* as evidenced by positive immunofluorescence staining with fluorescein isothiocyanate-conjugated anti-horse IgG (data not shown).

Effect of equine anti-*E. risticii* IgG on binding, internalization, and proliferation of *E. risticii* in P388D$_1$ cells. To determine whether the inhibitory activity of the antiserum resides in the IgG fraction, the effect of anti-*E. risticii* IgG was examined. As shown in Fig. 3a, in the presence of control equine IgG and equine anti-*E. risticii* IgG, *E. risticii* bound to P388D$_1$ cells after 3 h of incubation at 4°C. By the K-S test to compare the histograms generated following treatment with control or anti-*E. risticii* IgG with that of the uninfected P388D$_1$ cells immunostained with anti-*E. risticii* serum (Fig. 3g), significant binding ($D = 0.62$ and 0.77, respectively) to the host cells was apparent. *E. risticii* coated with anti-*E. risticii* IgG showed slightly enhanced binding ($D = 0.33$) to P388D$_1$ cells compared with control equine IgG (Fig. 3a). Additionally, as seen in Fig. 3b, anti-*E. risticii* IgG had no inhibitory effect on the internalization of *E. risticii* at 37°C for 3 h compared with control IgG. By the K-S test to compare the histograms generated for internalization of ehrlichial organisms following treatment with control or anti-*E. risticii* IgG (Fig. 3b) with that of uninfected P388D$_1$ cells immunofluorescence labeled with anti-*E. risticii* serum (Fig. 3h), significant ($D = 0.91$ and 0.92, respectively) internalization to the host cells was apparent. As seen in Fig. 3c, however, anti-*E. risticii* IgG had a marked inhibitory effect on the intracellular proliferation of ehrlichiae. The flow cytometry data are in agreement with light microscopic observation of Diff-Quik-stained cells. Anti-*E. risticii* IgG-treated *E. risticii* cells had lower infectivity (6%) than control IgG-treated organisms (55%) after 96 h at 37°C. These results showed that although *E. risticii* was able to bind to and be internalized by P388D$_1$ cells, anti-*E. risticii* IgG had an inhibitory effect on the intracellular proliferation of *E. risticii*.

Effect of equine Fab fragment on binding, internalization, and proliferation of *E. risticii* in P388D$_1$ cells. To examine whether any direct inhibitory activity resides in the Fab fragment, the effect of Fab fragment on ehrlichial infection was examined. Since Fab fragment is capable of only monovalent attachment, aggregation of ehrlichiae does not occur and the absence of the Fc region precludes internalization by Fc receptors located on P388D$_1$ cells. As shown in Fig. 3d, in the presence of control equine Fab fragments, *E. risticii* binds to P388D$_1$ cells; however, *E. risticii* coated with equine anti-*E. risticii* Fab fragment showed reduced binding to the host cell. By the K-S test to compare the histograms generated following treatment with control equine Fab fragment with that of the uninfected P388D$_1$ cells immunofluorescence labeled with anti-*E. risticii* serum (Fig. 3g), significant ($D = 0.62$) binding to the host cells was apparent. However, *E. risticii* coated with anti-*E. risticii* Fab fragment did not bind to P388D$_1$ cells compared with *E. risticii* incubated with control equine Fab fragment (Fig. 3d) or compared with uninfected P388D$_1$ cells (Fig. 3g). As seen in Fig. 3e, equine anti-*E. risticii* Fab fragment had a significant ($D = 0.92$) inhibitory effect on the internal-

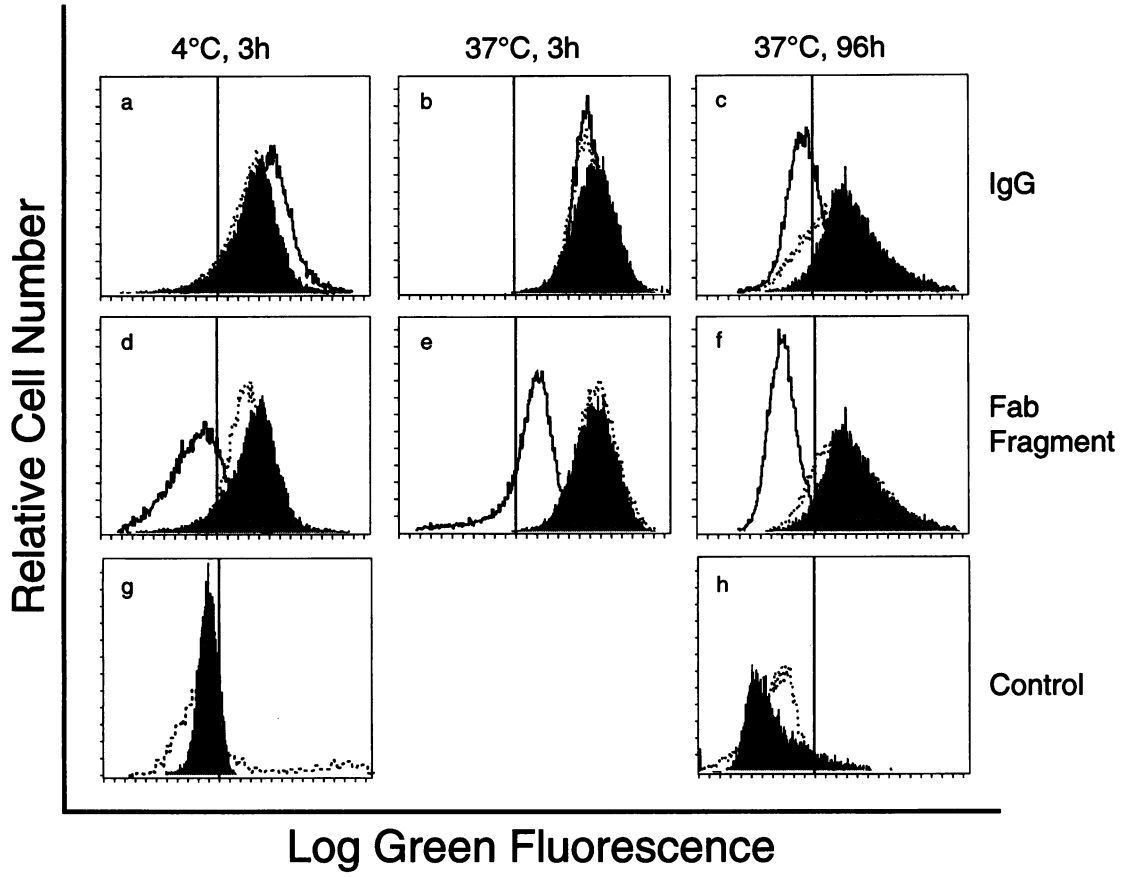

FIG. 3. Binding (4°C, 3 h), internalization (37°C, 3 h), and proliferation (37°C, 96 h) of *E. risticii* to P388D$_1$ cells in the presence of IgG or Fab fragment. *E. risticii* cells were incubated with nonimmune IgG or Fab fragment (a to f, dotted lines) and anti-*E. risticii* IgG or Fab fragment (a to f, solid lines) for 1 h at 37°C, and this mixture was overlaid on a monolayer of P388D$_1$ cells. The mixtures were incubated for 3 h at 4°C to assess binding (a and d). Cells were harvested, fixed, and stained by an indirect immunofluorescence procedure. Internalization and proliferation were examined after incubation at 37°C for 3 h (b and e) and 96 h (c and f), respectively. The results obtained with unopsonized *E. risticii* are in shaded histograms. Sham-infected P388D$_1$ cells were labeled with anti-*E. risticii* serum in the absence (g) or presence (h) of saponin (shaded histograms), and P388D$_1$ cells were incubated with *E. risticii* at 4°C for 3 h (g) or at 37°C for 96 h (h) and immunofluorescence labeled with normal horse serum (broken lines). Vertical lines, cutoff for uninfected P388D$_1$ cells immunostained with anti-*E. risticii* serum or P388D$_1$ cells infected with or attached to *E. risticii* and immunostained with normal equine serum.

ization of *E. risticii* compared with control Fab fragment. The inhibitory effect of anti-*E. risticii* Fab fragment on proliferation of *E. risticii* was evident by the shift of cells toward the lower fluorescence intensity side at 96 h (Fig. 3f). By Diff-Quik staining, the percent infected P388D$_1$ cells with control Fab fragment was 52% and that with anti-*E. risticii* Fab fragment was 2%.

$^{14}CO_2$ production from L-[^{14}C]glutamine in Percoll gradient-purified *E. risticii*. Host cell-free *E. risticii* can metabolize L-glutamine and generate CO_2 for a few hours (14, 15). We examined whether antibody against *E. risticii* had a direct influence on ehrlichial metabolism. Anti-*E. risticii* IgG inhibited 50 to 70% of $^{14}CO_2$ production from L-[^{14}C]glutamine by Percoll gradient-purified *E. risticii*: when *E. risticii* was treated with sucrose potassium phosphate (control), normal horse IgG (100 μg/ml), and anti-*E. risticii* IgG (100 μg/ml), 1.67 ± 0.32, 2.48 ± 0.56, and 0.87 ± 0.10 μmol of $^{14}CO_2$ per mg of ehrlichial protein, respectively, were generated in 2 h (means ± standard deviations; $n = 3$). The results for the control and for normal horse IgG are not significantly different by Tukey's studentized range test.

DISCUSSION

This study revealed that anti-*E. risticii* horse serum did not inhibit apparent binding or internalization of *E. risticii* into P388D$_1$ murine macrophages but that it inhibited the proliferation of *E. risticii* in P388D$_1$ cells. The reduction in percent infected cells (6% at 96 h compared with 30% at 48 h) indicates not only that proliferation was inhibited but also that *E. risticii* (antigen) was destroyed. By using purified IgG, it was shown that the inhibitory activity resides in the IgG fraction. The mechanism whereby anti-*E. risticii* IgG blocked the proliferation of *E. risticii* in P388D$_1$ cells is unknown. One possibility is that anti-*E. risticii* IgG-coated *E. risticii* bound to the P388D$_1$ cells via Fc receptor or other binding sites. Since ehrlichial organisms coated with Fab fragment blocked the ligand on *E. risticii* responsible for non-IgG-mediated uptake, binding of immune IgG-coated *E. risticii* most likely took place via the Fc receptor. This Fc receptor binding then subsequently delivered antibody-coated *E. risticii* to the different intracellular compartments, which readily fused with lysosomes. Concurrently, *E. risticii* binding via Fc receptor sites may not trigger

an appropriate transmembrane signal, and as a result, macrophages might have been activated and killed *E. risticii*.

Alternatively or concurrently, simple antibody coating rendered *E. risticii* unable to proliferate in $P388D_1$ cells. The L-[^{14}C]glutamine metabolism assay revealed that anti-*E. risticii* IgG directly inhibited energy metabolism of *E. risticii*. Thus, it is possible that the direct metabolic inhibition of *E. risticii* cells by the antibody made them incapable of proliferating in $P388D_1$ cells regardless of the involvement of Fc receptor. Inhibition of ehrlichial metabolic activity may lead to lysosomal fusion with the ehrlichial inclusion membrane. Wells and Rikihisa (16) previously found that prokaryotic protein synthesis inhibition by oxytetracycline treatment induces lysosomal fusion with ehrlichia-containing vacuoles.

The reason for incomplete inhibition of L-glutamine metabolism with antibody is unknown. Since some purified ehrlichiae appeared to be still partly enveloped by phagosomal membrane by electron microscopy as shown by Weiss et al. (14), it is possible that the limited accessibility of the antibody to ehrlichiae might be the cause for the incomplete inhibition. It remains to be determined whether the metabolic inhibition by anti-*E. risticii* IgG is due to inhibition of either L-glutamine transport or a specific enzyme by the antibody or due to generalized steric hindrance of energy transduction process.

E. risticii infects equine intestinal epithelial cells (10). Our study revealed that IgG not only induced Fc-mediated internalization but also inhibited *E. risticii* from binding to the receptor on $P388D_1$ cells that is responsible for non-Ig-mediated uptake. This suggests that immune IgG can prevent ehrlichial infection of intestinal epithelial cells which lack Fc receptor.

Lack of binding is, however, not a prerequisite for the lack of internalization of *E. risticii*. We demonstrated that *E. risticii* does not bind to equine polymorphonuclear leukocytes at 4°C after incubation for 3 h but that it readily internalizes at 37°C (4). Fab fragment-coated *E. risticii* not only did not bind but also failed to internalize and thus establish intracellular residence. Ehrlichial ligand and receptor molecules are still unidentified. Our previous study suggests that both the ehrlichial ligand and receptor are proteins (4). Our current study indicates that the use of Fab fragment of immune IgG and perhaps monoclonal IgG are useful in studying ehrlichial binding to its receptor on Fc-receptor-bearing macrophages, whereas intact IgG is inappropriate for this purpose.

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