In Vitro Killing of Ehrlichia risticii by Activated and Immune Mouse Peritoneal Macrophages†

NEIL M. WILLIAMS‡* AND PETER J. TIMONEY

Department of Veterinary Science, University of Kentucky, Lexington, Kentucky 40546-0099

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Normal resident murine peritoneal macrophages inoculated in vitro with Ehrlichia risticii readily phagocytized the organism but were unable to suppress ehrlichial replication as determined by indirect fluorescent-antibody staining of the inoculated cells. In contrast, macrophages from Corynebacterium parvum-inoculated and E. risticii-recovered mice rapidly eliminated the ehrlichiae. Macrophages from E. risticii-recovered mice were as effective as the C. parvum-activated cells in phagocytizing and eliminating the organism. Opossonization of E. risticii with homologous antiserum prior to inoculation of macrophage cultures resulted in enhancement of phagocytosis and greater suppression of E. risticii replication in all macrophage groups. These findings indicate that the pathogenesis of E. risticii infection centers on the ability of the organism to enter and replicate within the macrophage with avoidance of macrophage antimicrobial effects. An immune response results in macrophage activation with enhancement of the macrophage's ability to eliminate E. risticii. Opossonization of E. risticii with anti-E. risticii serum renders E. risticii more susceptible to macrophage destruction.

Ehrlichia risticii is a rickettsial organism in the family Rickettsiaceae, tribe Ehrlichiae. Members of the genus Ehrlichia cause a variety of diseases in humans and animals (30). E. risticii causes a disease of horses known as equine monocytic ehrlichiosis which is characterized by fever, depression, profuse diarrhea, and death in 30% of affected animals (16). E. risticii is an obligate intracellular organism which preferentially replicates within cells of the monocyte-macrophage lineage. Inside the cell the organism resides within host cell-derived vacuoles and, similar to chlamydiae, exhibits variations in morphology and electron density (17).

E. risticii possesses mechanisms which allow for its survival in the otherwise hostile intracellular environment of the macrophage (36). Precisely what these are and how they function await complete elucidation. As with toxoplasmas (20), chlamydiae (39), mycobacteria (2), and legionellae (18), E. risticii may inhibit phagolysosome formation. This is supported by findings in P388D1 cells, in which lysosomes generally failed to fuse with phagosomes containing E. risticii (36). The absence of any detectable respiratory burst activity in mouse resident peritoneal macrophages following phagocytosis of E. risticii is suggestive of the possibility of multiple mechanisms for preventing intramacrophage destruction (37).

The ability of E. risticii to enter and replicate within the macrophage constitutes something of a paradox. Cell-mediated immunity is the principal mechanism by which intracellular pathogens are destroyed, the macrophage being the effector cell (1). The pathogenesis of E. risticii infection appears to be dependent upon the ability of the organism to circumvent macrophage antimicrobial action. Acquisition of antihrlichial activity by the macrophage is a likely prerequisite to the individual's ability to overcome infection. The nature of this antihrlichial activity and the mechanism by which it is elicited are still unknown. Thioglycolate broth-elicited mouse peritoneal macrophages developed the ability to eliminate intracellular E. risticii following treatment with several immunopotentiating agents (29); however, it is not known whether these agents per se also have direct antihrlichial effects. Concerning the humoral immune response, passive transfer of immune serum protected E. risticii-challenged mice from developing signs of disease (22). The effect of a specific anti-E. risticii immune response on the macrophage's ability to suppress E. risticii replication remains to be determined.

The overall goals of this study were to attempt to more clearly understand the pathogenesis of E. risticii infection through investigation of the interaction of E. risticii with the macrophage and to further establish the mouse as a useful model for the study of E. risticii infection. Specific objectives of the study were (i) to evaluate the ability of peritoneal macrophages obtained from E. risticii-sensitized individuals to engulf and eliminate E. risticii in the presence or absence of ehrlichia-immune serum and (ii) to compare the phagocytic activities of murine peritoneal macrophages from normal individuals and individuals receiving a macrophage-activating agent.

MATERIALS AND METHODS

Mice. Four- to six-week-old female BALB/c and C3H/HeN mice (Harland Sprague-Dawley, Indianapolis, Ind.) were housed in microisolator cages and provided commercial rodent feed and autoclaved tap water.

E. risticii. The strain of E. risticii selected for use was a field isolate obtained from a case of equine monocytic ehrlichiosis in Kentucky in 1987. The isolate, designated PHF-K6, had been subcultured eight times in the continuous mouse macrophage-like cell line P388D1 (American Type Culture Collection, Rockville, Md.) in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal calf serum (GIBCO). E. risticii-infected cells were aliquotted into cryoampules (Cryule; Wheaton, Millville, N.J.) in 1-ml amounts at a concentration of 2.0 × 10⁶ cells per ml of cryopreservation medium and frozen in liquid nitrogen (31).
“Cell-free” ehrlhiae were produced by combining and pelleting the contents of several flasks containing E. risticii-infected P388D1 cells and Doune homogenizing the cells (9). Briefly, the pellets were suspended in 5.0 ml of RPMI 1640 medium and Doune homogenized (300 strokes) with a tissue grinder (2.0-cm² straight-wall grinder; Radnoti Glass Technology, Inc., Monrovia, Calif.). The ground-cell suspension was centrifuged at 500 x g for 10 min at 4°C, and the supernatant containing the cell-free ehrlhiae was diluted in RPMI 1640 medium containing 20% fetal bovine serum and 7.5% dimethyl sulfoxide and frozen in liquid nitrogen. Ampules of cell-free E. risticii were quick thawed and tested for viability by mouse and cell culture inoculation and were determined to contain approximately 1.0 x 10⁸ organisms per ml by indirect fluorescent-antibody (IFA) staining and counting in a hemacytometer by fluorescence microscopy.

**Inoculation of mice.** The study comprised three separate but identical trials. Within each trial, two mice of each strain received an intraperitoneal injection of either 0.3 mg of Corynebacterium parvum (Burroughs Wellcome, Research Triangle Park, N.C.) (24) in 0.5 ml of 0.85% saline or 1.0 x 10⁸ E. risticii-infected P388D1 cells (infectivity, -90%) in 0.5 ml of RPMI 1640 medium. Sufficient numbers of untreated mice served as a source of normal resident peritoneal macrophages. Mice receiving E. risticii-infected cells were reinoculated intraperitoneally 20 days later with 1.0 mg of E. risticii antigen in 0.5 ml of 0.85% saline.

**E. risticii antigen.** Two ampules of cell-free ehrlhiae held frozen in liquid nitrogen were rapidly thawed and combined, and the ehrlhiae were pelleted by centrifugation at 15,000 x g for 10 min at room temperature. The supernatant was decanted, and the pellet was rinsed twice with 1 ml of 0.85% saline. The ehrlhiae pellet was resuspended in 2 ml of 0.85% saline and sonicated (sonicator from Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) at the point of cavitation in an ice bath. The protein content of the sonicated ehrlhiae was determined by the Bio-Rad Coomassie blue technique (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, Calif.). The ehrlhiae antigen was diluted with RPMI 1640 medium and sonicated into polypropylene tubes in 1-ml volumes, and frozen at -20°C until used.

**Macrophages.** Peritoneal cells were harvested from the C. parvum-inoculated mice on day 7 postinoculation (p.i.) (C. parvum-activated macrophages) and on days 2 to 3 following the injection of ehrlhiae antigen in the mice previously inoculated with E. risticii (E. risticii-sensitized macrophages). Following euthanasia by cervical dislocation, the skin was reflected from the abdominal wall and peritoneal cells were harvested by abdominal lavage with 8 ml of cold erythrocyte lysis buffer (Sigma Chemical Co., St. Louis, Mo.) (7). The peritoneal wash was transferred to a centrifuge tube on ice and immediately centrifuged at 500 x g for 10 min at 4°C. Following centrifugation, the cells were resuspended in RPMI 1640 medium at a concentration of 1.0 x 10⁶ cells per ml.

Peritoneal cells were plated onto 12-mm-diameter round glass coverslips (Fisher Scientific Co., Cincinnati, Ohio) in individual wells of 24-well plates (Costar, Cambridge, Mass.) at a concentration of 1.0 x 10⁶ cells per well in a total volume of 1.0 ml of RPMI 1640 medium. Macrophages were allowed to adhere for 45 min at 37°C, after which time the nonadherent cells were removed by washing with RPMI 1640 medium and the macrophages were reincubated in 1 ml of fresh medium. In preliminary experiments, IFA staining of macrophages from mice previously infected with E. risticii failed to demonstrate ehrlhiae at the postinoculation interval described in the experimental protocol.

**Macrophage inoculation.** Each plate well containing macrophages cultured on coverslips in 1.0 ml of medium was inoculated with 100 µl of RPMI 1640 medium containing approximately 5.0 x 10⁵ cell-free ehrlhiae (-50 ehrlhiae per cell). The ehrlhiae were treated in one of two ways just prior to inoculation of the macrophages. One treatment consisted of opsonization of ehrlhiae with horse serum negative for antibodies to E. risticii by the IFA test (normal serum). Two milliliters of a cell-free suspension of E. risticii was incubated with 2 ml of normal serum and 2 ml of RPMI 1640 medium for 30 min at 37°C. In the case of the second treatment, cell-free E. risticii was opsonized with convalescent-phase horse serum containing anti-E. risticii antibody (IFA titer, 1:2,560) (immune serum). Two milliliters of immune serum and 2 ml of RPMI 1640 were incubated with 2 ml of ehrlhiae suspension for 30 min at 37°C.

Following inoculation with either normal serum- or immune serum-opsonized E. risticii, normal resident, E. risticii-sensitized, and C. parvum-activated macrophages were evaluated for phagocytosis and killing of E. risticii. Macrophage-containing coverslips were harvested at 2, 24, 48, 72, 96, 120, and 144 h p.i., stained by the IFA technique (31), and examined by fluorescence microscopy. Briefly, coverslips were immersed in 0.85% saline and then in 100% ethanol for 5 min. The coverslips were then dipped in 0.85% saline, 1 drop of aqueous ant-E. risticii serum (titer, 1:2,560) diluted 1:200 in 0.85% saline containing 0.1% sodium azide (Sigma) was placed on the coverslips, and the coverslips were incubated at 37°C for 20 min in a humid atmosphere. The coverslips were then washed in 0.85% saline for 10 min, 1 drop of fluorescein-conjugated goat anti-equine immunoglobulin (National Veterinary Services Laboratory, Ames, Iowa) was placed on the coverslips, and the coverslips were incubated for another 20 min. The coverslips were then washed and placed facedown on a glass slide with a drop of a 50-50 mixture of glycerine and 0.85% saline for the mountant.

**Enumeration of E. risticii within infected macrophages.** Fifty randomly selected cells having macrophage morphology were examined per coverslip, and the percentage of cells infected with E. risticii and the number of ehrlhiae per cell (up to a maximum count of 25 organisms) were determined. Three replicate coverslips per treatment variable were examined for each mouse strain, and the average percent infectivity and average number of ehrlhiae per infected cell were determined for each time interval. Group means were compared by the Mann-Whitney rank sum test for two independent samples (35). P values of <0.01 were taken as significant.

**RESULTS**

**Phagocytosis of E. risticii.** Results of the three replicate experiments were consistent, with the macrophage populations from both the BALB/c and C3H/HeN strains responding in similar fashions in each experiment. Two hours following inoculation of the cell cultures with E. risticii, the organism was observed within the cytoplasm of the majority of macrophages (Fig. 1 and 2). With respect to the normal resident, C. parvum-activated, and E. risticii-sensitized macrophages, the infectivity among these groups ranged from 59 to 91% (Fig. 1 and 2). There were no differences among the three groups of macrophages with respect to their ability to phagocytize E. risticii, as determined by the presence of
detectable organisms within cells and analyzed by the Mann-Whitney test.

When phagocytosis of ehrlichiae by macrophages from the two mouse strains was compared, the normal resident and C. parvum-activated macrophages from the BALB/c strain were significantly less efficient \((P < 0.01)\) in phagocytizing E. risticii, as measured by percent infectivity (Fig. 1 and 2) and the average number of ehrlichiae per cell (Fig. 3 and 4) at 2 h p.i., compared with E. risticii-sensitized macrophages from BALB/c mice and all three types of macrophages from the C3H/HeN mice.

Incubation of E. risticii in immune serum prior to inoculation of the macrophage cultures resulted in increased phagocytosis of E. risticii, as evidenced by a slightly higher percentage of cells being infected at 2 h p.i. compared with the corresponding rate in macrophages inoculated with ehrlichiae previously opsonized with normal serum. Similarly, the average number of ehrlichiae was significantly higher \((P < 0.01)\) in macrophages inoculated with immune serum-opsonized E. risticii (Fig. 1 through 4).

**Macrophage elimination of E. risticii.** By 24 h p.i., the percentage of cells infected with E. risticii and the number of ehrlichiae per cell decreased dramatically in both the C. parvum-activated and E. risticii-sensitized macrophages while the percentage of cells infected and the number of organisms per cell either decreased slightly, remained constant, or increased in the normal resident macrophages (Fig. 1 through 4). A similar pattern was observed throughout the remainder of the study, and by 144 h p.i., the average percentage of C. parvum-activated and E. risticii-sensitized macrophages infected with ehrlichiae ranged from 5 to 29% (Fig. 1 and 2), with averages of 1 ehrlichia per cell in some C.
parvum-activated macrophages to up to 10 organisms per cell in some normal resident macrophages (Fig. 3 and 4). There was a significant difference \( P \leq 0.01 \) in the average infectivity rate in the normal resident macrophages at 144 h p.i. compared with that in the C. parvum-activated and E. risticii-sensitized cells. The percentage of cells that became infected ranged from 40 to 65% in normal resident macrophage treatment groups, with average counts of from 9 to 17 ehrlichiae per cell (Fig. 1 through 4).

The different groups of macrophages from both the BALB/c and C3H/HeN strains of mice exhibited significantly enhanced ability \( P \leq 0.01 \) to suppress the growth and multiplication of E. risticii when there was prior opsonization of the organism with immune serum compared with the corresponding macrophage treatment groups inoculated with normal serum-treated ehrlichiae. At 96 h p.i., 8 to 12% of the C. parvum-activated and E. risticii-sensitized macrophages and 50% of the normal resident macrophages had become infected with an average of two and eight ehrlichiae per cell, respectively, in the groups inoculated with immune serum-opsonized E. risticii. In contrast, the infectivity of normal serum-treated ehrlichiae in C. parvum-activated and E. risticii-sensitized macrophages averaged 21 to 34% with three to nine organisms per cell, respectively. The corresponding rate in the normal resident macrophage groups averaged 66 to 72% with 15 to 16 organisms per cell (Fig. 1 through 4). By the termination of the study (144 h p.i.), infectivity in the C. parvum-activated and E. risticii-sensitized macrophages inoculated with immune serum-opsonized ehrlichiae ranged from 5 to 11% and 0.9 to 2.6 organisms per cell, compared with 10 to 29% and 1.4 to 10.4 organisms per cell in corresponding macrophages inoculated

FIG. 3. Average number of ehrlichiae per cell in peritoneal macrophages following inoculation in vitro with E. risticii treated with normal serum. Solid lines, BALB/c cells; broken lines, C3H/HeN cells; ■, resident cells (normal peritoneal macrophages from mice); □, activated cells (peritoneal macrophages from C. parvum-inoculated mice); ✶, immune cells (peritoneal macrophages from mice previously infected with E. risticii).

FIG. 4. Average number of ehrlichiae per cell in peritoneal macrophages following inoculation in vitro with E. risticii opsonized with homologous immune serum. Solid lines, BALB/c cells; broken lines, C3H/HeN cells; ■, resident cells (normal peritoneal macrophages from mice); □, activated cells (peritoneal macrophages from C. parvum-inoculated mice); ✶, immune cells (peritoneal macrophages from mice previously infected with E. risticii).
with normal serum-opsonized ehrlichiae. The infectivity in normal resident macrophages inoculated with immune serum-opsonized ehrlichiae ranged from 40 to 44% and 9 to 12.5 organisms per cell, compared with 46 to 65% and 16.7 ehrlichiae per cell in corresponding macrophages that had received ehrlichiae treated with normal serum (Fig. 1 through 4).

There were no significant differences between macrophages from the BALB/c and C3H/HeN strains of mice in their respective abilities to suppress growth and replication of E. risticii nor in the ability of C. parvum-activated and E. risticii-sensitized cells to phagocytize or kill ehrlichiae.

**DISCUSSION**

This study confirms the mouse as a useful model for the study of E. risticii infection and that differences in mouse strain response to ehrlichial infection may offer an additional method by which the pathogenesis of infection can be investigated. With the exception of E. risticii-sensitized macrophages from the BALB/c mice, cells from the C3H/HeN mice were superior in their overall ability to phagocytize E. risticii. The C3H/HeN mice represent a mouse strain which is resistant to the development of disease following challenge with E. risticii (37). In contrast, infection of the BALB/c strain of mouse with E. risticii results in clinical disease with significant mortality (37). It is not known whether the enhanced phagocytosis of E. risticii by macrophages from C3H/HeN mice is related to the observed resistance to disease in this strain.

The results of the present study demonstrated that the macrophage responds with enhanced antichrlichial activity following the occurrence of an immune response. E. risticii-sensitized macrophages had equal or even enhanced phagocytic ability compared with that of C. parvum-activated macrophages. Intraperitoneal inoculation of C. parvum into mice stimulates a large influx of macrophages that have been shown to be fully immunologically activated (21). Since E. risticii-sensitized macrophages phagocytized and killed E. risticii as effectively as did C. parvum-activated cells, it can be concluded, at least by the criteria of this study, that E. risticii-sensitized cells are immunologically activated to a similar extent.

Administration of anti-E. risticii antibody has been shown to protect mice against subsequent ehrlichial challenge (22). The present study tends to confirm the role of antibody in the elimination of E. risticii. Immune serum-incubated ehrlichiae were phagocytized in greater numbers than ehrlichiae treated with antibody-negative serum. This suggests the possibility that antibody opsonizes E. risticii with binding of the antibody molecule to Fc receptors on the macrophage, thus triggering internalization of the bound ehrlichiae (33). Since the number of Fc receptors is increased on the surface of activated macrophages (12), these cells consequently would be expected to have a greater phagocytic rate. The findings of the present study were consistent with this. The activated and E. risticii-sensitized macrophages engulfed greater numbers of immune serum-opsonized ehrlichiae than did normal resident macrophages. In contrast, the average number of normal serum-treated ehrlichiae phagocytized was roughly the same for all types of macrophages whether activated or not. Fc receptor-mediated internalization has also been shown to occur with other intracellular organisms, including chlamydiae (39), rickettsiae (3), toxoplasmas (20), and salmonellae (19).

At 24 h p.i. the percentage of infected cells and the number of ehrlichiae per cell were found to decrease sharply in the C. parvum-activated and E. risticii-sensitized macrophages, indicating efficient killing of the organism by these cells. The reduction in the percentage of cells that became infected was more dramatic in the activated and sensitized macrophages inoculated with normal serum-treated ehrlichiae than in the same cells inoculated with immune serum-opsonized ehrlichiae. This possibly is a reflection of the latter group having initially phagocytized a greater number of organisms on a per cell basis. In support of this is the finding that the average number of ehrlichiae per cell in the macrophages inoculated with immune serum-opsonized ehrlichiae approximated the average number in the normal serum-treated ehrlichia-inoculated macrophages even though the macrophages in the former groups initially averaged over twice as many organisms per cell. Similar findings were reported in a study of the effects of immune serum on the survival of Rickettsia prowazekii in monocytes (3).

The role of complement in the destruction of E. risticii was not determined in the present study. Complement was not inactivated in either the antibody-positive or antibody-negative serum prior to incubation of ehrlichiae in the serum. Binding of antibody to a membrane can activate the complement system (23), resulting in the effects of antibody, in terms of increased phagocytosis and killing, being enhanced by complement (38). Since in the present study normal resident macrophages inoculated with immune serum-opsonized ehrlichiae did not eliminate infection nearly as effectively as activated and sensitized cells, it would appear that complement activation was not a major factor in the increased killing of E. risticii under the conditions of these experiments.

While infectivity of the C. parvum-activated and E. risticii-sensitized macrophages approached zero toward the end of the study, a few cells in which ehrlichial replication continued to occur were always observed. This observation probably reflects heterogeneity within the macrophage population and the fact that not all macrophages respond to activating stimuli in a similar or synchronous fashion (32). This inability to completely clear infection has also been observed in macrophages infected in vitro with Rickettsia tsutsugamushi (27). It appears that a cellular mechanism enabling persistence of this organism, in that viable rickettsiae can be isolated from lymph nodes for up to a year following recovery from scrub typhus (13).

In a recent study of the infectivity and growth of E. risticii in peritoneal macrophages from CF1 mice (29), it was reported that cells treated in vitro with recombinant murine gamma interferon prior to or during the first 3 days of infection became resistant to infection and were able to kill ingested ehrlichiae. The findings of the present study, which represent the first reported observations on E. risticii interaction with E. risticii-sensitized and in vivo-activated macrophages, are in agreement with these results. T lymphocytes produce gamma interferon following a specific immune response, which in turn leads to macrophage activation. The findings of both studies emphasize the requirement for T-cell-dependent activation of macrophages to have occurred for effective killing of E. risticii to take place.

The macrophage represents the body’s major defense against intracellular pathogens (1). In spite of this, a number of pathogens, including mycobacteria (34, 40), Chlamydia psittaci (39), Legionella pneumophila (18), Toxoplasma gondii (20), Yersinia pestis (25), Salmonella typhimurium (25), Trypanosoma cruzi (28), and Coxiella burnetii (4), like E. risticii, are capable of intramacrophage survival. Diverse
mechanisms which enable such pathogens to avoid destruction by the macrophage, among which are the ability to escape from the phagosome (28), inhibition of fusion of lysosomes with phagosomes (2, 18, 39, 40), resistance to lysosomal enzymes (6, 8, 11, 14, 25), and failure to stimulate respiratory burst activity (5, 10), have been identified. Although these mechanisms for avoiding the bactericidal effects of macrophages favor survival and replication of the invading organism, they may be overcome following full immunologic activation of the macrophage or upon binding of antibody to the bacteria. There is often an increase in phagocytic rate, respiratory burst activity, and phagosome-lysosome fusion with enhanced microbicidal effects when activated macrophages encounter organisms which normally survive in nonactivated macrophages (15, 26). Such mechanisms appear to be operative in E. risticii infection.

The results of this study add to our understanding of the pathogenesis of E. risticii infection and the host responses necessary for erlichial elimination. It is apparent that an immune system response is required for the killing of E. risticii, with the macrophage clearly functioning as the effector cell. Macrophages from C. parvum-inoculated mice and E. risticii-sensitized macrophages were highly effective in eliminating erlichiae. Normal resident macrophages, on the other hand, were ineffective and became heavily infected. The specific mechanisms used by immune activated macrophages to kill E. risticii, however, remain to be defined.

It appears that both cell-mediated and humoral immune responses may function to inhibit E. risticii infection. Phagocytosis and elimination of the organism were enhanced when there was prior incubation of E. risticii in immune serum. Even normal resident macrophages exhibited improved killing. Since it was not determined that the positive effect of immune serum was attributable solely to antibody, other humoral factors possibly present in the convalescent-phase serum may have also had an enhancing effect.

The differing responses in the macrophages from the two strains of mice are intriguing. Normal resident macrophages from the C3H/HeN strain inherently phagocytized more organisms and subsequently exhibited a reduction in percent infectivity and erlichiae per cell at 24 h.p.i. However, after 24 h.p.i., cells from the two strains had comparable levels of infectivity. How these initial differences relate to the observation that mice of the C3H/HeN strain are resistant to E. risticii-induced disease while BALB/c mice are highly susceptible is unknown but warrants further investigation in our attempts to understand more fully the pathogenesis of this infection.

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