Host Defenses in Experimental Scrub Typhus: Role of Normal and Activated Macrophages

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Resident peritoneal macrophage from BALB/c mice were infected in vitro with Rickettsia tsutsugamushi strain Gilliam, and rickettsial growth was estimated by microscopic examination of Giemsa-stained cells. Both number of infected macrophage per culture and number of intracellular rickettsiae per cell increased with time during culture. Treatment of rickettsiae with immune serum before infection of macrophage cultures reduced the number of infected macrophage by 50%. Macrophage treated in vitro with lymphokines were able to suppress rickettsial growth in the absence of detectable antibody and exhibited a 75% reduction in infection compared with normal macrophage. We also obtained activated macrophage from immune mice and found that they were refractory to in vitro rickettsial infection. Macrophage populations activated in vitro or in vivo contained a small percentage of cells which supported unrestrained growth of rickettsiae. These data suggest that an early immunological event in experimental scrub typhus infection may be the development of activated macrophage capable of suppressing rickettsial proliferation before the appearance of circulating antibody.

Macrophages play an essential role as effector cells in both humoral and cellular immunity (15, 18), and several studies have documented the importance of mononuclear phagocytes in the immune response to obligate intracellular organisms (11, 27). Macrophages are susceptible to infection with several rickettsial species, and in vitro studies have demonstrated the growth of Rickettsia typhi (9) and Coxiella burnetii (6, 10) in the absence of modulating immune factors. When mononuclear phagocytes were exposed to complexes of R. typhi and specific antibody, rickettsial proliferation was suppressed in the majority of cells (9). Conflicting reports concerning C. burnetii suggest either complete suppression (14) or unrestrained growth (10) of these organisms in macrophage cultures after exposure to specific antibody.

Interaction of rickettsiae with macrophage activated by lymphocyte products has been investigated only with C. burnetii, where pretreatment of macrophage cultures with lymphokines resulted in reduced replication of rickettsiae (10). However, the tendency to generalize observations obtained from different groups of rickettsiae may not be justified since rickettsiae are biologically diverse organisms, as determined by both deoxyribonucleic acid base composition and homology studies (20, 25, 28).

Previous investigations from this laboratory documented the onset of humoral and cellular immunity during experimental scrub typhus infection of mice (3, 22). The purpose of this study was to examine the interaction of R. tsutsugamushi with mouse macrophage and to determine the influence of antibody and lymphokines on the fate of intracellular organisms.

MATERIALS AND METHODS

Animals. Female BALB/c mice (18 to 22 g) were obtained from Flow Laboratories, Dublin, Va.

Rickettsiae. R. tsutsugamushi strain Gilliam (163rd egg passage) was plaque-purified, propagated in irradiated L-929 cells, and quantified by plaque assay (17).

Preparation of macrophage cell cultures. Resident peritoneal cells were obtained from mice after intraperitoneal injection of 6 ml of L-15 medium (Microbiological Associates, Walkersville, Md.) supplemented with 2% fetal bovine serum (Microbiological Associates), 50 U of penicillin, and 50 μg of streptomycin per ml. The peritoneal washing was withdrawn through the abdominal wall with a 20-gauge needle. Fluids from 3 to 10 mice were pooled, a sample was taken for total cell counts, and the remainder was centrifuged at 500 × g for 10 min at room temperature in polypropylene tubes (Falcon Plastics, Oxnard, Calif.). The peritoneal cells were adjusted to contain 2 × 10^6 cells/ml in culture medium, which was RPMI 1640 with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (Microbiological Associates) and 20% fetal bovine serum. Portions (2.5
ml each) were added to 35-mm culture wells or petri plates (Costar, Cambridge, Mass.) with glass cover slips and incubated for 4 h at 34°C in 5% CO₂ in moist air. Nonadherent peritoneal cells were removed by aspiration and the remaining adherent cells were washed, received fresh medium, and were incubated for an additional 18 to 20 h at 34°C.

In all experiments using immune serum, where it was necessary to determine the antibody-mediated increase or decrease of rickettsial entry into macrophage, unFractionated peritoneal cells were used. Portions of cells (0.5 ml each) containing 1 x 10⁹ macrophage were dispensed into polypropylene tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.) and incubated for 24 h at 34°C before infection with rickettsiae.

**Estimation of the number of adherent cells.** After 18 h of incubation the culture fluid was aspirated from six samples, and the adherent cells were removed by trypsin and scraping with a rubber policeman. The cells were counted, and an average number of adherent cells per culture was determined. Approximately 50 to 65% of the total peritoneal cells were adherent. Greater than 95% of these cells were macrophage, as identified by morphological criteria.

**Infection of macrophage monolayers.** The culture medium was removed by aspiration, and the cells were incubated at room temperature with approximately 15 plaque-forming units (PFU) of rickettsiae per cell in 0.1 ml of brain heart infusion (Difco Laboratories, Detroit, Mich.). The plates were rotated every 10 min for 1 h, cells were washed with Earle balanced salt solution (Microbiological Associates), and culture medium was added to each plate.

**Estimation of rickettsial growth.** Macrophage monolayers on cover slips or cytospin centrifuge preparations of peritoneal cells (Cytospin; Shandon Elliot, Sewickley, Pa.) were Giemsa-stained and examined microscopically to determine percentage of cells infected and number of rickettsiae per infected cell. A total of 200 cells was counted in each sample.

**Preparation of macrophage cell cultures from immune animals.** Peritoneal exudate cells from mice receiving a nonlethal intraperitoneal injection of Gilliam strain R. tsutsugamushi (1,000 PFU) 9 days previously were harvested and cultured in the manner described above for resident peritoneal cells. Activation of macrophage was assessed by their ability to kill syngeneic tumor cells in vitro in the macrophage tumor cytotoxicity assay (4).

**Production of antisera.** Gilliam antiserum was obtained 28 days after inoculation of mice with a single intraperitoneal injection of 1,000 PFU of Gilliam; hyperimmune serum was produced by an initial subcutaneous inoculation of 1,000 PFU of Gilliam rickettsiae followed by an intraperitoneal boost of 1,000 PFU of Gilliam 2 weeks later. All animals were exsanguinated by incision of the right axillary artery. Titers of antisera were determined by the indirect fluorescent-antibody test (2). The immune serum titer was 1:640, and the hyperimmune serum titer was 1:2,560. All sera was inactivated at 56°C for 30 min before use.

**Incubation of rickettsiae with serum.** Gilliam rickettsiae were incubated with dilutions of immune or normal mouse serum for 10 min at 37°C before infection of macrophage. Dilutions were made in brain heart infusion, and rickettsiae were added to the macrophage monolayers at a final concentration of 15 PFU/cell.

**Inactivation of rickettsiae for antigen stimulation of spleen cells.** Gilliam rickettsiae were inactivated by incubation at 60°C for 1 h, then added to the spleen cell suspension at approximately 10⁶ rickettsiae per ml.

**Production of spleen cell supernatants containing lymphokine.** Mice inoculated intraperitoneally with Gilliam rickettsiae were used as a source of spleen cells for the production of lymphokines. The spleens were removed, minced, and pressed through 80-mesh stainless-steel sieves 21 days after infection. The cell suspension was washed twice with RPMI 1640, suspended in RPMI 1640 with 25 mM HEPES to a concentration of 10⁵ cells/ml, and placed in culture with heat-killed rickettsiae. Spleen cells from uninfected mice were cultured in the same manner to produce control supernatants. Spleen cell cultures were incubated for approximately 30 h at 34°C in 5% CO₂ in moist air and centrifuged at 500 x g for 10 min at room temperature, and the culture supernatants were filter sterilized (0.2-μm Nalgene filter, Nalge Sybron Corporation, Rochester, N.Y.). The supernatants were assayed for macrophage-activating lymphokines by the macrophage tumor cytotoxicity assay (19). The supernatants were stored at 4°C. Gilliam-specific antibody could not be detected by the indirect fluorescent-antibody test in lymphokine preparations.

**Activation of macrophage by spleen cell supernatants.** Macrophage monolayers were incubated for 20 h, and then the culture medium was removed and replaced by 1 ml of the appropriate active or control spleen cell supernatant. These cultures were incubated for 4 h at 34°C and then infected with rickettsiae.

**RESULTS**

**Growth of scrub typhus rickettsiae in peritoneal macrophage.** Resident peritoneal macrophage from BALB/c mice cultured in vitro supported the growth of R. tsutsugamushi strain Gilliam. One day after inoculation of macrophage cultures with 15 PFU of Gilliam rickettsiae per cell, 30% of the macrophage contained intracellular rickettsiae (Fig. 1). There was a slight reduction in the number of infected cells on day 2, followed by a constant increase throughout the remainder of the incubation period, suggesting that rickettsiae were able to initiate secondary infections. Approximately 65% of the attached cells were infected with rickettsiae after 4 days of in vitro cultivation. The average number of rickettsiae in each infected cell also increased with time (Fig. 1 and 2). One day after exposure of macrophage to Gilliam rickettsiae, an average of three organisms were localized in the perinuclear region of each infected cell. After 5 days of incubation,
cell; immune serum slightly enhanced both the number of macrophage containing rickettsiae and the number of organisms per cell (Table 1). After 1 day of incubation, cultures exposed to rickettsiae treated with immune serum showed

the entire cytoplasm of macrophage was often filled with scrub typhus rickettsiae. This capacity of *R. tsutsugamushi* to propagate in mouse peritoneal macrophage allowed us to examine several immunological factors which could influence the course of rickettsiae-macrophage interactions.

**Effect of antibody on Gilliam rickettsiae proliferation in normal macrophage.** Treatment of macrophage monolayers with low dilutions of serum resulted in cell detachment during subsequent incubation. Therefore, studies using normal or immune mouse serum employed unfractionated peritoneal cells in suspension. Macrophage were quantified at each time of sampling by estimating the total number of cells by hemacytometer and performing differential counts on Wright’s-stained cell smears. The number of macrophage remained stable at 10⁶/0.5 ml over the 4-day observation period.

Equal volumes of rickettsiae and normal or immune serum (final dilution of serum = 1:2) were incubated for 10 min, then added to peritoneal cell suspensions for 1 h. Examination of cell cultures immediately after exposure to rickettsiae treated with normal serum indicated that 20% of the macrophage were initially infected and contained approximately one organism per

![Fig. 1. Growth of *R. tsutsugamushi*, strain Gilliam, in resident peritoneal macrophage. Symbols: ○, percentage of macrophage infected; □, average number of rickettsiae per infected cell.](http://iai.asm.org/)

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![Fig. 2. Rickettsial growth in macrophage cultures. (a) Uninfected BALB/c peritoneal macrophage. Peritoneal macrophage 3 days (b) and 5 days (c) after in vitro infection with *R. tsutsugamushi* strain Gilliam.](http://iai.asm.org/)

> **Fig. 2.** Rickettsial growth in macrophage cultures. (a) Uninfected BALB/c peritoneal macrophage. Peritoneal macrophage 3 days (b) and 5 days (c) after in vitro infection with *R. tsutsugamushi* strain Gilliam.
a decrease from 29 to 8% of macrophage containing recognizable intracellular rickettsiae. In contrast, cultures infected with rickettsiae treated with normal serum maintained a nearly constant percentage of macrophage containing scrub typhus organisms during the first 3 days of culture. Rickettsiae which persisted in macrophage increased in number in both cultures, and growth did not reflect any differences due to pretreatment with normal or immune serum. By day 4 of incubation, cells in both cultures contained rickettsiae too numerous to count, and there was a concomitant increase in the percentage of macrophage infected, presumably a result of secondary macrophage infections. Comparison of the percentage of infected cells in culture 1 to 3 days after exposure to rickettsiae treated with normal or immune serum indicated that antibody did augment the intracellular killing of scrub typhus organisms. There was a constant twofold reduction in the percentage of cells infected after exposure to rickettsiae treated with immune serum. Use of hyperimmune serum in these experiments did not reduce the percentage of infected macrophage, and rickettsial persistence was noted in these cultures as well. The observation of a small percentage of cells which supported the uninhibited growth of *R. tsutsugamushi* after treatment with immune or hyperimmune serum correlated with the results of other investigators (9) studying the fate of *R. typhi* in human macrophage and suggested to us that additional immune factors may influence in vivo interactions between rickettsiae and mononuclear phagocytes. We examined this possibility by treating peritoneal macrophage before infection with spleen cell supernatants containing lymphokines.

**Effect of lymphokine activation of macrophage on intracellular growth of Gilliam rickettsiae.** A spleen cell supernatant containing lymphokines, or a control preparation without lymphokine activity, was applied to normal macrophage monolayers for 4 h at 34°C. The cultures were then washed and infected with viable Gilliam rickettsiae. By 1 day after infection, 19% of the macrophages exposed to control supernatants contained an average of five rickettsiae per cell (Fig. 3). In contrast, less than 5% of the macrophage exposed to lymphokines contained a similar number of organisms. This 75% reduction in the number of infected macrophage after treatment with lymphokines persisted for at least 48 h; however rickettsiae did proliferate in a small percentage of the cells after lymphokine activation. These data indicated that more than 90% of macrophage activated in vitro by lymphokines were capable of suppressing rickettsial growth in the absence of specific antibody and suggested that macrophage activation was important in the resistance of the intact animal to rickettsial infection.

**Effect of in vivo activation of macrophage on intracellular growth of Gilliam rickettsiae.** Previous studies from this laboratory have established the temporal development of cellular immunity in experimental scrub typhus (22). Based on this work, we harvested macrophage from mice infected 9 days previ-

| Table 1. Infection of macrophage by Gilliam rickettsiae pretreated with either normal or immune mouse serum |
|---|---|---|---|
| Time (days) | % Cells infected | Avg no. of rickettsiae/infected cell |
| | Normal serum | Immune serum | Normal serum | Immune serum |
| 0 | 20 | 29 | 1.2 | 2.1 |
| 1 | 18 | 8 | 3.1 | 3.5 |
| 2 | 17 | 7 | 8 | 7.4 |
| 3 | 22 | 9 | 14 | 13 |
| 4 | 50 | 32 | >25 | >25 |

![Fig. 3. Rickettsial growth in normal and activated macrophages. Open bars indicate the percentage of infected macrophage in cultures pretreated with spleen cell supernatants without lymphokine activity; solid bars indicate the percentage of infected macrophage in cultures pretreated with lymphokine-containing culture supernatants. Symbols: ●, rickettsial replication in normal macrophage cultures; ○, rickettsial replication in lymphokine-activated macrophage cultures.](http://iai.asm.org)
ously with a nonlethal dose of Gilliam rickettsiae. Macrophage from these mice did not contain identifiable rickettsiae after Giemsa staining and were capable of killing tumor cells in the macrophage tumor cytotoxicity assay (data not shown), suggesting that these cells were activated. When the cells were placed in culture and infected with approximately 15 PFU of Gilliam rickettsiae per cell only 8% of the macrophage contained intracellular rickettsiae 1 day after infection (Fig. 4). The percentage of infected macrophage did not increase during 4 days of incubation. Comparison of these data with those of normal macrophage cultures (Fig. 1), in which the percentage of infected macrophage increased from 30 to 65 during 4 days of incubation, suggested that macrophage activated in vivo during the course of scrub typhus infection suppressed rickettsial proliferation in vitro and also prevented secondary infection of cells. However, it is important to note that rickettsiae did proliferate in a small number of cells and that replication was similar to that observed in normal peritoneal macrophage.

Effect of antibody on Gilliam rickettsiae persistence in activated macrophage. Neither antibody nor lymphokines alone enabled macrophage cultures to completely suppress rickettsial infection. The effect of both modulating factors was examined by exposing in vivo-activated macrophage to rickettsiae treated with antibody. Peritoneal exudate cells from mice inoculated 9 days previously with 1,000 PFU of rickettsiae were exposed to Gilliam rickettsiae pretreated for 10 min at 37°C with either normal or immune mouse serum. As in normal peritoneal cell cultures, immune serum enhanced the initial uptake of rickettsiae by activated macrophage (Table 2). By 1 day after infection, however, rickettsiae were seen in only 4 to 5% of macrophage exposed to either normal or immune serum-treated Gilliam. The percentage of infected cells continued to decline in both macrophage cultures, although there was still an occasional cell in which rickettsiae grew in an unrestrained manner.

**DISCUSSION**

This study has clearly shown that both humoral and cellular responses play a role in facilitating the destruction of *R. tsutsugamushi* by mouse macrophage during scrub typhus infection. Resident peritoneal macrophage from BALB/c mice supported the growth of *R. tsutsugamushi* strain Gilliam, and within 4 to 5 days they contained rickettsiae in numbers too large to quantify by microscopic observation. This growth was substantially modified by the interaction of antibody with rickettsiae before internalization by these phagocytic cells. The initial uptake of the rickettsiae-antibody complex was slightly enhanced, but the ensuing intracellular events resulted in a marked decrease in the percentage of cells which remained infected with rickettsiae. The mechanism of rickettsial elimination in these cells is unknown, but phagosome-lysosome fusion may result in digestion of phagocytized organisms (16). The interaction of macrophage with rickettsiae-antibody complexes was not uniform, however, and a minority of the cell population sustained rickettsial growth, leading to secondary infection of macrophages and involvement of a substantial proportion of the cell population after 4 days of incubation. Those cells showing unrestricted rickettsial

![Figure 4](http://iai.asm.org)

**FIG. 4. Growth of *R. tsutsugamushi*, strain Gilliam, in macrophage activated in vivo. Macrophage were harvested from mice infected 9 days previously with Gilliam rickettsiae. Symbols: %, percentage of macrophage infected; O, average number of rickettsiae per infected cell.**

**Table 2. Infection of in vivo-activated macrophage by Gilliam rickettsiae pretreated with either normal or immune mouse serum**

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<tr>
<th>Time (days)</th>
<th>% Cells infected</th>
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<tr>
<td></td>
<td>Normal serum</td>
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TABLE 2. Infection of in vivo-activated macrophage by Gilliam rickettsiae pretreated with either normal or immune mouse serum.
growth could result from a lack of sufficient antibody to complex with each infective rickettsia in the inoculum. We reacted similar rickettsial inocula with both immune and hyperimmune sera with similar results, but we recognize that the interaction of scrub typhus organisms with homologous antiserum may be an inefficient process (1). Alternatively, the rickettsial persistence in these cultures could reflect a macrophage population heterogeneous with respect to surface receptors and the ability to ingest and degrade antibody-antigen complexes. It is known that peritoneal macrophages from rabbits are composed of several different subpopulations which differ quantitatively or qualitatively or both with regard to immunoglobulin receptor sites (26). If an analogous situation exists for mouse macrophage, it is possible that rickettsial proliferation simply reflected the mechanism of internalization, with Fc receptor-deficient cells undergoing an infection with rickettsiae-antibody complexes similar to that observed by others with fibroblast (13, 29) and lymphoblast (5) cells.

The activation of macrophage, which is accompanied by an enhanced listericidal activity, is an inducible phenomenon mediated by lymphokines (7, 24). We investigated the interaction of activated macrophage with rickettsiae under controlled conditions of in vitro lymphokine activation. These macrophage responded much differently after exposure to rickettsiae than did normal macrophage. Only 5% of the lymphokine-activated macrophage contained rickettsiae 1 day after infection, whereas 19% of normal macrophage contained identifiable scrub typhus rickettsiae. This difference in susceptibility to rickettsial infection was sustained through 2 days of incubation. Since the activated state of macrophage exposed to lymphokines in vitro is of short duration (24 to 36 h [19]), the experiments were terminated before the time secondary infection would have occurred in normal macrophage cultures. Similar results were achieved by using activated macrophage obtained from immunized mice. In this instance, cultivation was extended to 4 days, but the pattern was similar to that observed after in vitro activation of phagocytes. Rickettsial infection was suppressed, and proliferation of organisms occurred in less than 10% of the cultured macrophage. It is important to note that regardless of the method of activation, in vivo or in vitro, these macrophage demonstrated rickettsial persistence. In a small percentage of cells, rickettsial proliferation was similar to that observed in normal peritoneal macrophage cultures. This rickettsial persistence was not reduced in activated macrophage cultures by prior treatment of rickettsiae with antibody, but the level of infection in these cultures was so small that the contribution of antibody would be difficult to detect unless the culture was completely free of rickettsiae. Occasional cells in activated macrophage cultures exposed to rickettsiae-antibody complexes clearly supported the unrestricted growth of rickettsiae, and these cells may play a role in the persistence of rickettsiae in the immune host (23).

The lower initial infection rate for activated macrophage compared with normal macrophage (Fig. 3) suggests the possibility that activated macrophage may influence rickettsial infection by elaboration of extracellular factors. Other investigators have described the extracellular killing of *L. monocytogenes* by a monokine elaborated by peritoneal cells of guinea pigs convalescent from infection with *R. typhi* (21) and *C. burnetii* (12). The effect of this monokine on rickettsial virulence is unknown, but if a similar factor is released after lymphokine activation of macrophage it is possible that *R. tsutsugamushi* could be modified or inactivated before internalization by phagocytes.

Our previous in vivo studies on experimental scrub typhus infection suggested that events critical for animal survival were primarily dependent upon cellular responses and occurred before the detection of circulating antibody in the serum (22). The present study supports that concept and provides evidence for the ability of activated macrophage to suppress rickettsial growth in the absence of antibody. As the humoral response develops, we postulate that rickettsiae-antibody complexes are presented to macrophage, and this study has indicated that such modification of infecting organisms augments the rickettsiacidal capacity of nonactivated macrophage, further ensuring host control of the infective process. The principal contribution of the humoral response may occur in the convalescent or latent stage of infection, when the protective capacity of lymphocytes declines (22). Since both animals (8) and humans (23) endure a nonsterile immunity, the ability of the host to control the smoldering infection during this period may be dependent upon the presentation of rickettsiae-antibody complexes to nonactivated macrophage.

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LITERATURE CITED


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Volume 26, no. 2, p. 744: As the result of a misunderstanding of editorial policy, the word "macrophage" was printed throughout this article in both the singular and plural senses. The Publications Office regrets the error and apologizes to the authors.