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Macrophages in Resistance to Rickettsial Infections: Early Host Defense Mechanisms in Experimental Scrub Typhus

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Several early nonspecific host defense mechanisms were examined in resistant (BALB/c) and susceptible (C3H/He) mice after intraperitoneal inoculation with *Rickettsia tsutsugamushi* strain Gilliam. Inflammatory exudates were formed in both mouse strains in response to rickettsial inoculation, but the inflammatory response of C3H animals was delayed several days, and influx of peroxidase-positive macrophages occurred late in infection. Peritoneal cells of C3H mice became progressively infected, with 40% of both macrophages and lymphocytes containing intracellular rickettsiae by day 10. The early flammatory response of BALB/c mice was unexpectedly associated with a low percentage of infected peritoneal cells (1 to 2%). In vitro, no difference was detected in ability of resident macrophages of either strain to support the growth of *R. tsutsugamushi* or to become activated by treatment with lymphokines for rickettsiacidal activity. In vivo, however, macrophages from C3H mice inoculated with Gilliam were not activated on days 6 and 7 after infection, whereas BALB/c macrophages were continuously activated beginning on day 4. The lack of in vivo C3H macrophage activation was not secondary to deficient lymphokine production by infected lymphocytes, as levels of lymphokines produced by peritoneal lymphocytes of both strains were similar and peaked on day 7 after infection. Susceptibility to infection appears to be related to defective regulation of macrophage responses rather than to defects in macrophage function.

Genetic resistance to intraperitoneal (i.p.) inoculation of *Rickettsia tsutsugamushi* strain Gilliam is controlled in inbred mouse strains by a single autosomal dominant gene (4). This gene (*Ric*) has been mapped on chromosome 5 (4a), but the gene product(s) or target cell(s) responsible for resistance has not been identified. Histopathology of lethal and immunizing doses of *R. tsutsugamushi* in mice suggested that resistance to infection is mediated by the macrophage (3) and correlates with the development of cellular immunity (15). Additional studies with mouse peritoneal macrophages activated in vivo by nonlethal rickettsial infection (11) or nonspecific macrophage-activating agents (C. A. Nacy, M. S. Meltzer, P. K. Russell, and J. V. Osterman, Fed. Proc. **38**:1078, 1979), or in vitro by soluble T-lymphocyte products (10, 11), were substantially less susceptible to Gilliam infection than resident peritoneal macrophages. In this study we examined several early nonspecific host defense mechanisms in a resistant (BALB/c) and a susceptible (C3H/He) mouse strain in an effort to further clarify the role of macrophages in host susceptibility.

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MATERIALS AND METHODS

Animals. Adult (6 to 8 weeks old) female BALB/c and C3H/He mice were obtained from Flow Laboratories, Dublin, Va.

Rickettsiae. The Gilliam strain of *R. tsutsugamushi* was grown in irradiated L-929 cell monolayers, harvested, and quantified by methods previously described (11, 12). All animals were inoculated i.p. with 1,000 plaque-forming units (PFU) of rickettsiae.

Peritoneal cells. Peritoneal exudate cells (PC) from infected or control mice were harvested after i.p. injection of 8 to 10 ml of L-15 medium (Microbiological Associates, Bethesda, Md.) supplemented with 10 U of sodium heparin (Abbott Laboratories, King of Prussia, Pa.) per ml, 10% fetal bovine serum, and 2 mM glutamine (Microbiological Associates). Peritoneal fluid was withdrawn through the anterior abdominal wall with a 20-gauge needle and pooled, and samples were removed for differential cell counts. The remainder of the fluid was centrifuged in polypropylene tubes (Falcon Plastics, Oxnard, Calif.) at 500 × *g* for 10 min at 4°C. Differential cell counts were made on Wright-stained cell smears prepared by cytocentrifugation (Cytospin centrifuge, Shandon Southern Instruments, Camberly, England) The cells were suspended in RPMI 1640 (Microbiological Associates) with 10% fetal bovine serum. Washed peritoneal suspensions were adjusted to 10^6 macrophages per ml.

Infection of PC in vivo. Duplicate cell smears of peritoneal exudate cells from infected animals ad-

justed to 10^6 macrophages per ml were stained with Giemsa and examined for percent macrophages or lymphocytes containing intracellular rickettsiae. One hundred cells of each type were examined per sample.

Infection of resident peritoneal macrophages in vitro. PC harvested from uninfected BALB/c and C3H/He mice were adjusted to 10^6 macrophages per ml, and aliquots were dispensed in polypropylene tubes (12 by 75 mm; Falcon Plastics) for 2 h at 37°C and 5% CO_2 in moist air. Cells were then exposed to Gilliam rickettsiae at approximately 5 PFU per macrophage for 1 h at room temperature with occasional shaking. Samples were removed immediately after the adsorption period and 24 and 48 h later. Duplicate cell smears of two samples from each time period were stained with Giemsa and examined for percent macrophages infected and number of intracellular rickettsiae in infected cells. A total of 400 macrophages were observed for each time period.

Inflammatory response to rickettsial infection. The inflammatory response to i.p. injection of 1,000 PFU of Gilliam rickettsiae in BALB/c and C3H mice was monitored by estimating numbers of PC and numbers of peroxidase-positive macrophages per mouse generated in response to infection. Cytocentrifuged cell smears of harvested PC were stained for peroxidase by the method of Kaplow (5) and counterstained with Wright stain.

Lymphokine supernatants. Animals were inoculated with 1,000 PFU of Gilliam rickettsiae and sacrificed 1 to 8 days after infection. Spleens were aseptically removed and passed through stainless-steel sieves (50 mesh) into tissue culture medium (RPMI 1640). Single-cell suspensions, obtained by serial aspirations through 19- and 23-gauge needles, were treated with NH_4Cl lysis buffer to remove erythrocytes. Spleen cells were centrifuged at 250 × *g* for 10 min at 4°C and resuspended to a concentration of 5 × 10^6 viable cells per ml in medium with 5% heat-inactivated fetal bovine serum (Microbiological Associates).

A 20-ml amount of spleen cell suspension with either 10^6 heat-killed Gilliam rickettsiae (10) or 5 μg of concanavalin A per ml was incubated in 75-cm^2 plastic tissue culture flasks (Falcon Plastics) for 48 to 60 h at 37°C. Supernatant fluids from replicate cultures were pooled, centrifuged at 450 × *g* for 15 min at 4°C, and divided into aliquots. Concanavalin A-stimulated spleen cell supernatants were adsorbed with 10 μg of Sephadex G-10 per ml before storage; antigen-stimulated supernatants were filtered to remove rickettsiae. Aliquots were stored at 4°C until use (14).

In certain experiments, peritoneal exudate lymphocytes were tested for their ability to produce macrophage-activating lymphokines (MAF). PC were harvested from mice injected i.p. 1 to 8 days previously with 1,000 PFU of Gilliam: cells were adjusted to 5 × 10^6 PC per ml and dispensed in 1-ml aliquots in polypropylene tubes (12 by 75 mm; Falcon Plastics) with or without heat-killed rickettsiae (10^6 per ml). Cultures were incubated for 48 to 60 h at 37°C. Supernatant fluids of replicate cultures were pooled, centrifuged at 450 × *g* for 15 min at 4°C, and filtered to remove rickettsiae. Aliquots were stored at 4°C until use.

Reference supernatants containing MAF were generously supplied by Monte S. Meltzer, National Cancer Institute, Bethesda, Md.

Activation of macrophages for rickettsiacidal activity in vitro. PC were exposed to 5 PFU of Gilliam rickettsiae per macrophage for 1 h at room temperature. Cells were washed once at 400 × *g* and resuspended in medium (controls) or various dilutions (1/10 to 1/270) of lymphokine-containing supernatants of spleen cells from *Mycobacterium bovis* strain BCG-infected mice stimulated with purified protein derivative in vitro (obtained from M. S. Meltzer). Cultures were incubated at 34°C in 5% CO in moist air for 24 h. Giemsa-stained cell smears were examined microscopically for percent infected macrophages, and rickettsiacidal activity of macrophages treated with lymphokines was determined by the following formula: 100 × [(percent infected control macrophages − percent infected treated macrophages)/percent infected control macrophages].

Activation of macrophages in vivo. Background levels of infected macrophages in C3H/He mice injected i.p. with *R. tsutsugamushi* Gilliam (progressive infection to 40% by day 10) prevented use of the rickettsiacidal assay to monitor development of activated macrophages during the course of rickettsial infections. Tumor cytotoxicity, which correlates both in vivo and in vitro with the development of rickettsiacidal activity (9–11), was used as an independent measurement of macrophage activation. PC were harvested from mice inoculated i.p. 1 to 9 days previously with 1,000 PFU of Gilliam and adjusted to 10^6 macrophages per ml in RPMI 1640 plus 10% fetal bovine serum, and 0.5-ml aliquots were placed in tissue culture wells (Cluster[24], Costar, Cambridge, Mass). Cultures were incubated for 2 h at 37°C and 5% CO_2 in moist air. Nonadherent PC were removed by repeated washing of culture wells, and [^{3}H]thymidine-prelabeled tumor mKSA TU-5 cells were added (4 × 10^4 target cells per well). PC-tumor cell cultures were incubated for 48 h. Labeled tumor cell monolayers digested with 0.5% sodium dodecyl sulfate in water were used to estimate total incorporated counts. Macrophage-mediated tumor cytotoxicity was estimated by the release of [^{3}H]thymidine into the culture supernatants of triplicate cultures at 48 h and was expressed as percent total incorporated counts.

Analysis of lymphokines in supernatants of antigen- or mitogen-stimulated spleen cells and PC. The tumor cytotoxicity assay (described above) was used to analyze supernatants from spleen cells and PC for the presence of MAF.

RESULTS

We examined a number of parameters involved in defense against infectious diseases to gain some insight into the basis of host susceptibility to i.p. inoculation of the Gilliam strain of *R. tsutsugamushi*.

Inflammatory response to i.p. inoculation of Gilliam. The initial host response to invading microorganisms is recruitment of immunocompetent cells to the site of infection. Since i.p. inoculation of *R. tsutsugamushi* initi-

ates a localized infection of the peritoneal cavity (3, 6), we examined the inflammatory response in C3H and BALB/c mice after i.p. inoculation of *R. tsutsugamushi* strain Gilliam. Figure 1A shows the total number of PC recovered per mouse in two separate experiments. A slight increase (twofold, at maximum) occurred in the number of PC recovered from BALB/c mice 2 to 8 days after inoculation of rickettsiae, but the numbers of PC returned to control levels by day 10. In contrast, C3H mice were slower to produce an inflammatory exudate, but the numbers of PC increased dramatically on days 6 and 10. The total number of macrophages recovered per mouse (Fig. 1B) in both BALB/c and C3H mice reflected the pattern seen with total PC (Fig. 1A). In both BALB/c and C3H strains of mice, neutrophil response to inflammation induced by inoculation of rickettsiae followed the kinetics of inflammatory responses to a variety of infectious or sterile agents: the number of neutrophils increased from 2 to 20% in 24 h and decreased to control levels by 48 h.

Changes in percentage of peroxidase-positive macrophages, a cytochemical index of mononuclear phagocyte maturation (8), is a nonspecific sequela of inflammation. Immature, peroxidase-positive macrophages are more responsive to lymphokines (soluble T-lymphocyte products) inducing tumoricidal and microbicidal activity than the well-differentiated resident macrophages (14). We examined the number of macrophages recruited in response to rickettsial inoculation in both BALB/c and C3H mice (Fig. 2). Total numbers of peritoneal macrophages recovered from BALB/c mice remained approximately 10^6 per mouse, whereas peroxidase-staining macrophages increased 6- to 10-fold ($3–5 \times 10^6$ to 30×10^6 per mouse) by 8 days after infection. The influx of peroxidase-positive macrophages into the peritoneum of infected C3H mice was more erratic. The percentage of macrophages staining positively for peroxidase was actually depressed below control levels in infected C3H mice through day 6 after inoculation of rickettsiae. Numbers of peroxidase-positive macrophages increased on day 6; however, close examination of total macrophages recovered per mouse (Fig. 2A) shows that the relative proportion of peroxidase-positive to peroxidase-negative macrophages in infected animals remained similar to that in control animals. Infected animals had a fivefold increase in both number of macrophages and number of peroxidase-positive macrophages compared with those of the controls. The percentage of immature macrophages in the total PC population began increasing in peritoneal exudates of C3H mice on day 8, and by day 10, 43% of the macrophages present in the inflammatory exudate stained positively for peroxidase. C3H mice were capable of producing an inflammatory response after i.p. inoculation of rickettsiae. However, influx of peroxidase-pos-

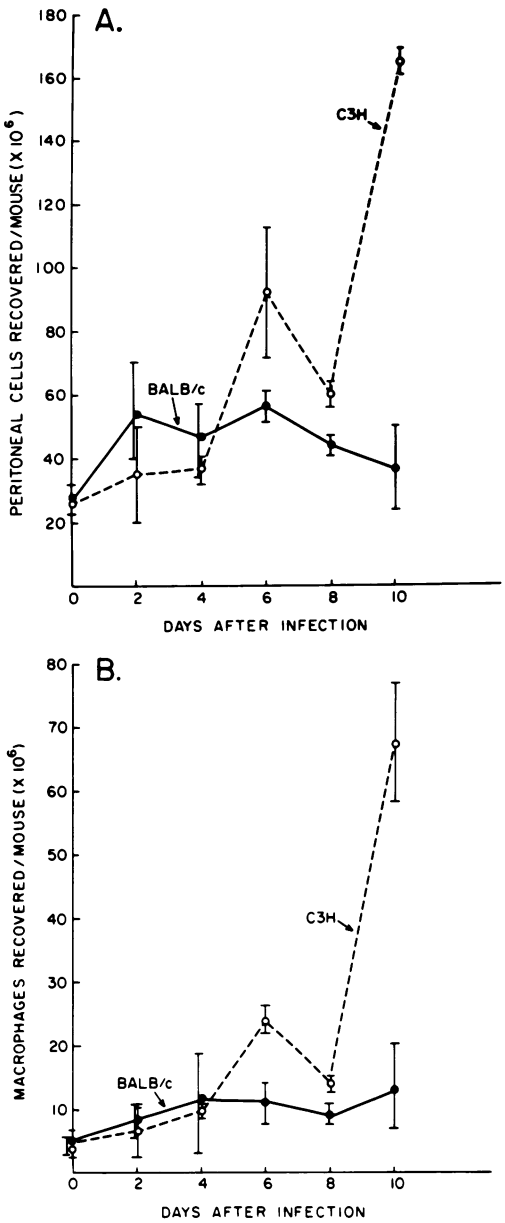

FIG. 1. *Inflammatory response of BALB/c and C3H mice after inoculation of 1,000 PFU of Gilliam: numbers of PC (A) and macrophages (B) recovered per mouse. PC were harvested by peritoneal lavage, total cells were counted in a hemacytometer, and differential counts were performed on Wright-stained cell smears. Results are expressed as the mean ± standard error for five mice in each of two experiments.*

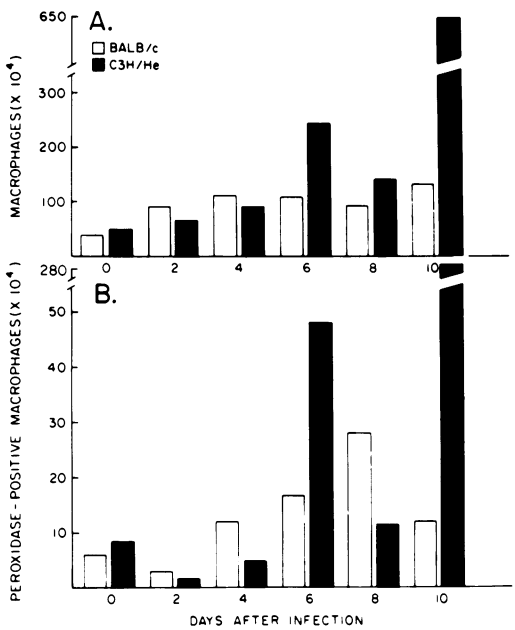

FIG. 2. *Inflammatory response of BALB/c and C3H mice after inoculation of 1,000 PFU of Gilliam: numbers of peroxidase-positive macrophages in total number of macrophages recovered per mouse. PC were harvested by peritoneal lavage, and differential counts were performed on peroxidase-stained cell smears counterstained with Wright stain. Results are expressed as the mean number of macrophages or peroxidase-positive macrophages in duplicate samples from mice inoculated for 1 to 10 days with rickettsiae.*

itive macrophages, which may be effector cells of antirickettsial immunity (10), was delayed several days in susceptible C3H compared with resistant BALB/c mice.

Infection of peritoneal macrophages. Examination of PC smears from infected animals revealed a progressive infection of C3H macrophages, whereas BALB/c macrophages remained relatively free of rickettsiae throughout the 10 days (Fig. 3). The percentage of C3H macrophages containing intracellular rickettsiae increased steadily, until 40% of the macrophages observed on day 8 after infection contained rickettsiae. In contrast, BALB/c macrophages were minimally infected (1 to 2%) over the same period of observation. When peroxidase-stained cell smears were examined for infected macrophages, the percent macrophages containing rickettsiae remained the same. Peroxidase activity is located in the perinuclear region of the macrophage, a region where *R. tsutsugamushi* preferentially replicates after infection (11). Peroxidase staining (Fig. 4A) would obscure the presence of rickettsiae. No change in the percent macrophages infected per 100 macrophages observed in peroxidase-stained cell smears suggested that the differentiated tissue macrophage, rather than the immature peroxidase-positive cell, was becoming infected with rickettsiae in C3H/He mice. Representative PC smears from day 8-infected BALB/c and C3H mice are contained in Fig. 4B and C. C3H peritoneal exudate cells were heavily infected with rickettsiae, whereas BALB/c macrophages appeared to be free of intracellular organisms and highly vacuolated.

We have previously reported that resident peritoneal macrophage populations from BALB/c mice support the growth of *R. tsutsugamushi* strain Gilliam in vitro (10, 11). The possibility existed that C3H macrophages in vitro either (i) were more susceptible to Gilliam infection or (ii) supported a more rapid growth of rickettsiae than BALB/c macrophages. The infection of BALB/c and C3H macrophages with *R. tsutsugamushi* Gilliam in vitro is shown in Fig. 5. Under the conditions of this experiment, 26% of both BALB/c and C3H macrophages contained intracellular rickettsiae 1 h after exposure to Gilliam. We noted no significant differences in either the percent infected macrophages or the number of intracellular rickettsiae in BALB/c or C3H macrophages over 48 h. C3H resident peritoneal macrophages were not more susceptible to Gilliam infection and did not support a more rapid growth of rickettsiae in vitro.

Development of activated macrophages. In previous studies, BALB/c macrophages activated in vitro with lymphokines (10) or in vivo during scrub typhus infection (11) were nonspecifically tumoricidal and refractory to rickettsial infection in vitro. Since macrophages of C3H mice were progressively infected with rickettsiae after scrub typhus infection, it was possible that activated rickettsiacidal macrophages did not develop in C3H animals. We compared the ability of BALB/c and C3H resident peritoneal macrophages to respond to lymphokines in vitro with development of rickettsiacidal activity (Fig. 6). The dose-response curves of macrophages from C3H and BALB/c mice were identical, suggesting that macrophages from both strains of mice were equally capable of responding to lymphokines for intracellular killing of rickettsiae.

More relevent to the in vivo situation was the development of activated macrophages in both strains of mice after i.p. inoculation of rickettsiae. Tumor cytotoxicity was used as the index of macrophage activation since (i) background levels of rickettsial-infected macrophages in

C3H mice were too high to permit accurate assessment of rickettsiacidal activity, and (ii) under a wide variety of in vitro and in vivo situations, tumor cytotoxicity paralleled rickettsiacidal activity in activated macrophages (9–11). Mice were inoculated with 1,000 PFU of rickettsiae i.p. on 9 successive days, and macrophages were harvested from all infected and control mice on day 10. Macrophages from both strains of mice were activated to non-specifically kill tumor cells by 4 days after inoculation of rickettsiae, and BALB/c macrophages remained activated (Fig. 7). In contrast, C3H macrophages became transiently activated on days 4 and 5, had reduced tumoricidal activity on days 6 and 7, and were activated again on day 8 through the death of the animals (day 10). Although the number of peroxidase-positive macrophages (most responsive to MAF) present in C3H mice on day 6 (Fig. 2) was more than three times the number present in BALB/c mice, C3H macrophages were not activated (Fig. 7). The lack of activation of C3H macrophages on days 6 to 7 after infection was reproducible with inocula of both 1,000 and 100 50% lethal doses of rickettsia. The presence of peroxidase-positive macrophages, in combination with data that C3H macrophages respond as well to lymphokines as do BALB/c macrophages (Fig. 6), suggested that the lack of C3H macrophage activation on day 6 was not due to a defect in the macrophage itself, but was possibly due to decreased production of MAF in the peritoneal cavity at this time after rickettsial inoculation.

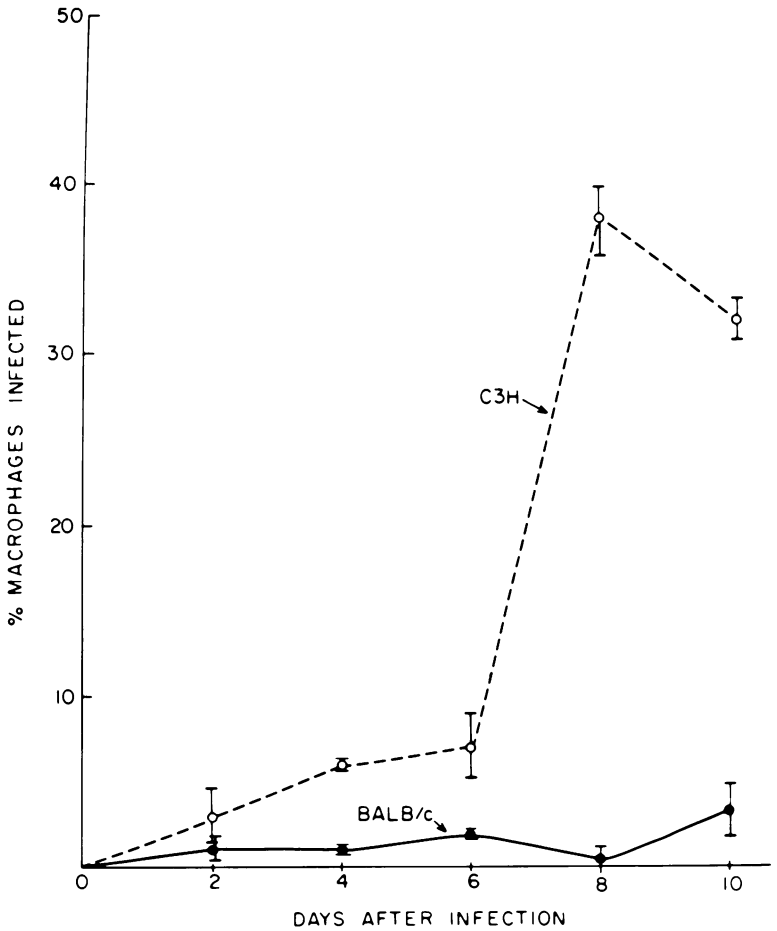

FIG. 3. *Peritoneal macrophages containing intracellular rickettsiae after i.p. inoculation of R. tsutsugamushi Gilliam. PC from four mice were pooled, washed, and adjusted to 10^6 macrophages per ml. Giemsa-stained cell smears were examined microscopically for percent infected macrophages. Results are expressed as the mean ± standard error for duplicate samples of PC harvested from BALB/c or C3H mice 1 to 10 days after inoculation of rickettsiae.*

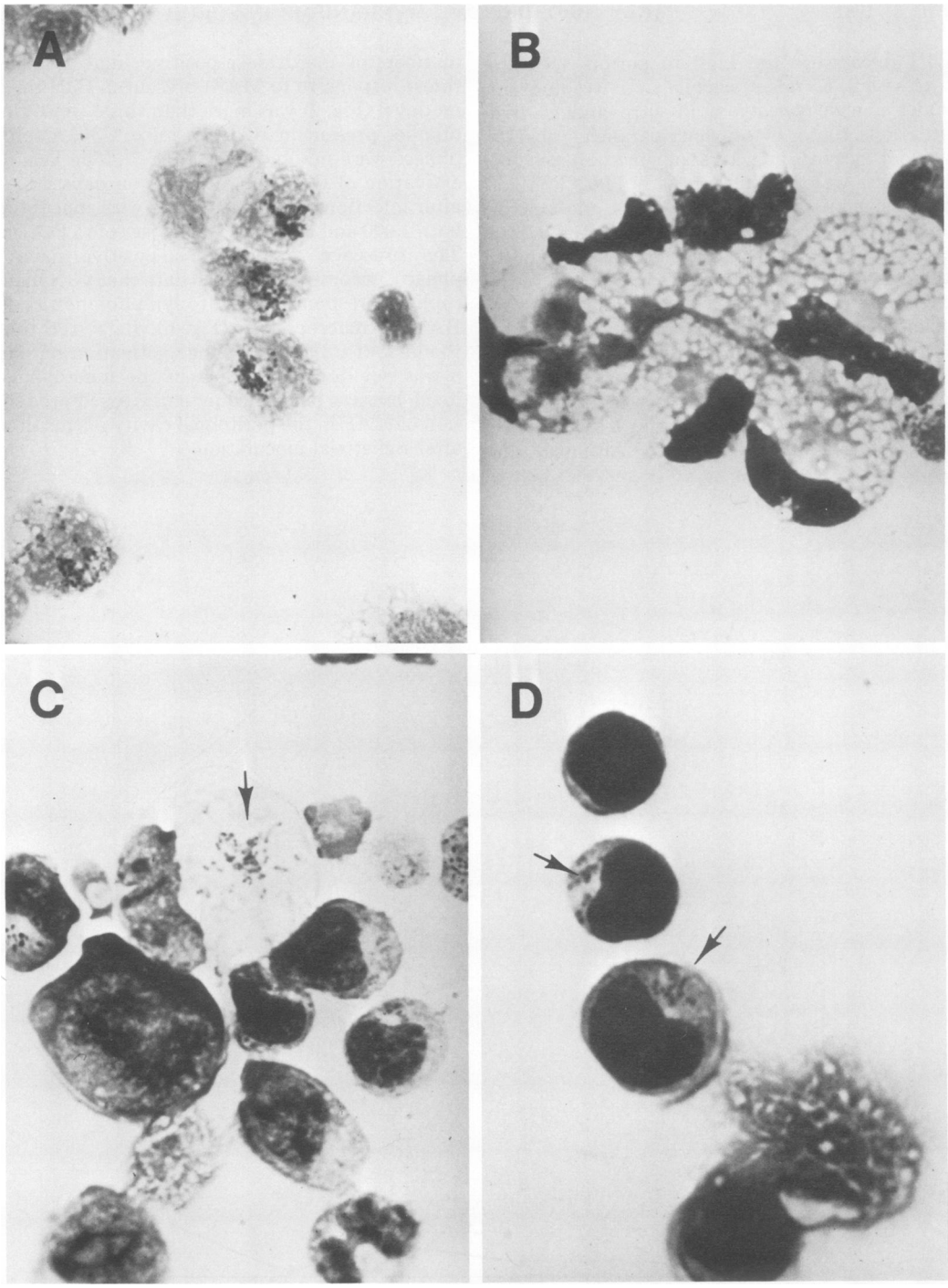

FIG. 4. *Intracellular rickettsiae in PC recovered from BALB/c or C3H/He mice 8 days after infection with 1,000 PFU of R. tsutsugamushi strain Gilliam. (A) PC cytocentrifuge smears stained with peroxidase and counterstained with Wright stain: peroxidase activity is in perinuclear region of immature macrophages; (B) PC cytocentrifuge smears stained with Giemsa from BALB/c mice infected for 8 days with 1,000 PFU of R. tsutsugamushi (note extensive vacuolization and absence of rickettsiae); (C) cytocentrifuge smears stained with Giemsa from C3H/He mice infected for 8 days with 1,000 PFU of R. tsutsugamushi (arrows indicate rickettsiae in infected macrophages); (D) PC cytocentrifuge smears stained with peroxidase and counterstained with Giemsa from C3H/He mice infected for 8 days with 1,000 PFU of R. tsutsugamushi (arrows indicate rickettsiae in infected lymphocytes).*

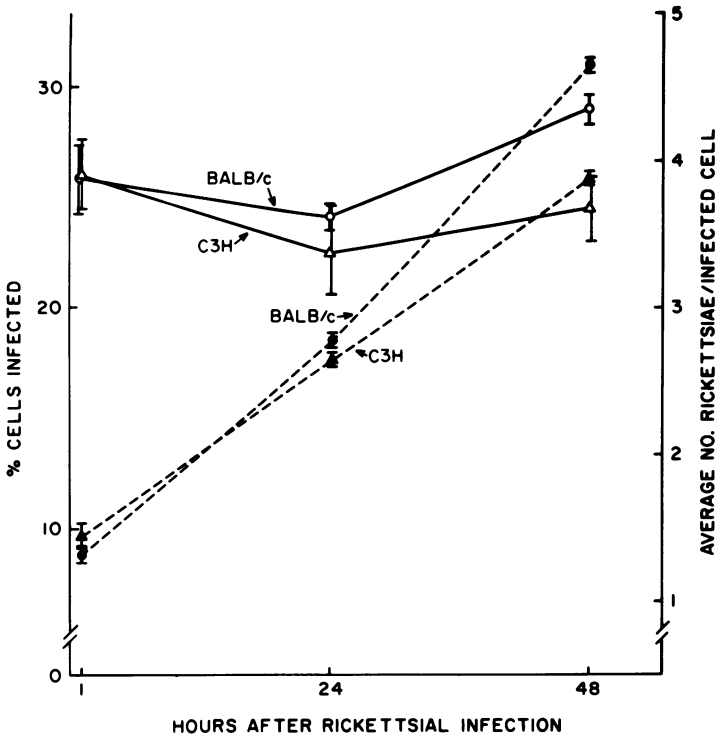

FIG. 5. *Infection of resident peritoneal macrophages of BALB/c and C3H/He mice with R. tsutsugamushi Gilliam. PC were harvested from 10 mice, pooled, washed, adjusted to 10^6 macrophages per ml, and exposed to 5,000 PFU of Gilliam rickettsiae per macrophage for 1 h. Cultures were incubated at 37°C, and samples were removed immediately after exposure to rickettsiae and 24 and 48 h later. Samples were stained with Giemsa and examined microscopically for the percent macrophages infected and average number of intracellular rickettsiae per infected macrophage. Results are expressed as the mean percent infected macrophages ± standard error for duplicate samples (400 macrophages observed) or mean number of rickettsiae ± standard error in infected macrophages (dashed line; 200 infected macrophages observed).*

Lymphocyte production of MAF. We first examined the production of MAF by spleen cells from infected animals exposed to antigen (heat-killed Gilliam rickettsiae, 10^6 per ml) or concanavalin A, a nonspecific T-cell mitogen. With the exception of day 2 in BALB/c mice, MAF production by spleen cells of both infected mouse strains stimulated with antigen in vitro was comparable to that of the control spleen cells (Fig. 8, shaded regions). Certainly, MAF levels achieved with mitogen stimulation were not reached with antigen stimulation. Although spleen cells from rickettsia-infected animals could be stimulated to produce MAF by mitogens, response to rickettsial antigens (which have been shown [10] to stimulate production of lymphokines by spleen cells of 21-day rickettsia-infected BALB/c mice) was minimal. Activation of peritoneal macrophages during early responses to rickettsial infection may be a localized event, with lymphokines produced by resident peritoneal lymphocytes.

Peritoneal exudate lymphocytes from Gilliam-resistant animals transfer complete protection against rickettsial challenge at an earlier time after infection than spleen cells (2). Examination of peroxidase-stained PC smears from infected animals in our study revealed a progressive infection (Fig. 9) of small, mononuclear, peroxidase-negative cells from C3H mice which were morphologically similar to lymphocytes (Fig. 4D). BALB/c cells of a similar morphological and cytochemical nature were minimally (1 to 2%) infected throughout the 10 days (Fig. 9). The possibility existed that localized activation of macrophages by peritoneal exudate lymphocytes was deficient in C3H mice at a critical time after rickettsial inoculation. When we examined the ability of resident and inflammatory peritoneal exudate lymphocytes of both mouse strains to produce MAF during rickettsial infection (Fig. 10), there was essentially no difference. Peak activity of MAF production with antigen stimulation occurred on day 7 after inoculation of

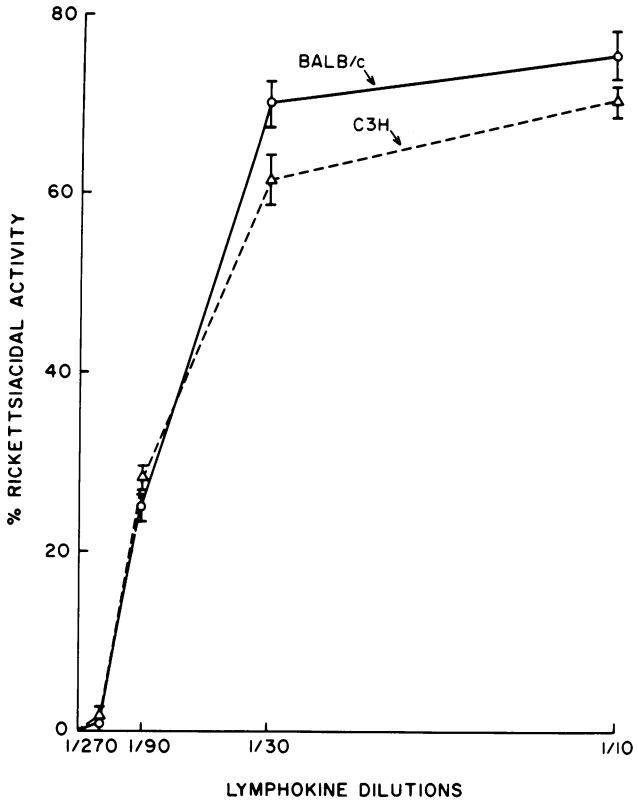

FIG. 6. *Treatment of resident peritoneal macrophages from BALB/c and C3H/He mice with lymphokines in vitro. PC were harvested from 10 mice of each strain, pooled, washed, adjusted to 10^6 macrophages per ml, and exposed to 5,000 PFU of R. tsutsugamushi Gilliam for 1 h. Cultures were washed and treated with medium or lymphokine dilutions for 24 h at 37°C. Duplicate cell smears stained with Giemsa were examined microscopically for the percent infected macrophages, and rickettsiacidal activity of lymphokine-treated cultures was determined by the following formula: 100 × [(percent infected control macrophages − percent infected treated macrophages)/percent infected control macrophages]. Results are expressed as the mean percent rickettsiacidal activity ± standard error for triplicate cultures at each lymphokine dilution.*

rickettsiae for both BALB/c and C3H peritoneal exudate lymphocytes.

Production of circulating antibody. Sera from mice infected i.p. with Gilliam strain *R. tsutsugamushi* were examined by the indirect fluorescent-antibody test (1) for anti-Gilliam antibodies (Table 1). By this technique, we could detect no circulating antibody in sera from either BALB/c or C3H mice before day 10 after infection. Resistant BALB/c mice began to develop detectable circulating antibody (titers > 1/20) by 14 days after infection. These data would suggest that circulating antibody plays little, if any, role in the initial immune response to scrub typhus.

DISCUSSION

The relative contribution of humoral and cellular immunity in resistance to infections with *R. tsutsugamushi* is unknown. Several lines of evidence suggest that the role of humoral immunity is minimal in the early stages of infection (15). Our data on circulating antibody in resistant and susceptible mouse strains support these findings: antibody was not detected in serum from BALB/c or C3H mice until late in infection, just before death of C3H animals. Nonspecific immune responses, however, particularly those related to the macrophage, do appear to be important early in infections with *R. tsutsugamushi.* Protection against lethal infections can be induced by passive transfer of immune T-cell-enriched lymphocyte populations (15) or inoculation of susceptible animals with macrophage-activating agents such as *M. bovis* BCG (Nacy et al., Fed. Proc. **38**:1078, 1979). That the macrophage is the effector cell in these nonspecific responses is suggested by several reports (3,

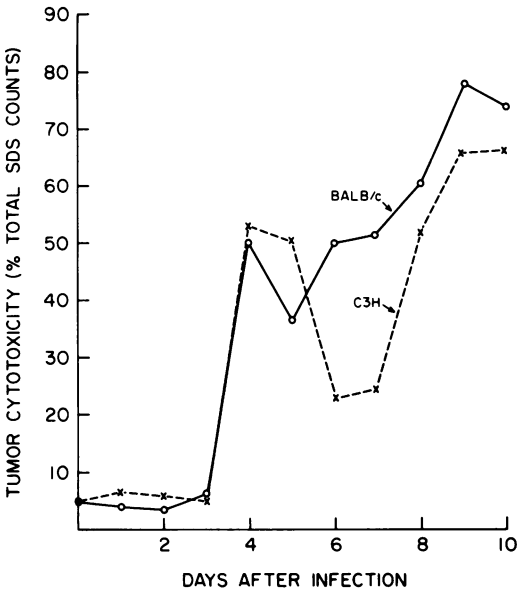

FIG. 7. *Tumor cytotoxicity by adherent PC from peritoneal exudates of BALB/c and C3H mice infected with 1,000 PFU of R. tsutsugamushi Gilliam. Groups of mice were infected on 9 sequential days, and PC were harvested from infected and control animals on day 10. Aliquots of PC, 10^6 macrophages per ml, were plated in tissue culture wells and allowed to adhere at 37°C for 2 h, and nonadherent cells were removed by repeated washing. [^{3}H]thymidine-prelabeled tumor cells were added, 4×10^4 per well, and cultures were incubated for 48 h at 37°C. Labeled tumor cell monolayers digested with 0.5% sodium dodecyl sulfate were used to estimate total incorporated counts. Results are expressed as the mean percent total incorporated counts for triplicate cultures.*

9, 11). Use of inbred mouse strains provided an additional tool for studying the role of macrophages in the early response to scrub typhus. In the present study, analysis of nonspecific host defense mechanisms in a resistant mouse strain (BALB/c) supported the concept of macrophages as antirickettsial effector cells: (i) inflammatory response (appearance of peroxidase-positive macrophages) occurred early in response to infection; (ii) macrophages were minimally (1 to 2%) infected; and (iii) macrophages became nonspecifically activated early and remained activated throughout the infection.

Inflammatory reactions during acute immune (14) or delayed hypersensitivity (7, 8) responses are characterized by a rapid immigration of peroxidase-positive mononuclear phagocytes. In BALB/c mice infected with *R. tsutsugamushi*, the number of peroxidase-positive macrophages began increasing 4 days after inoculation of rickettsiae. In C3H mice, however, the numbers of macrophages increased on day 6 after infection with *R. tsutsugamushi*, but most macrophages present were peroxidase negative. Recruitment of inflammatory macrophages to the site of infection was delayed in C3H mice, and macrophages were apparently recruited from sources other than peripheral circulation. Not until day 8 after infection did the proportion of peroxidase-positive macrophages begin increasing in C3H mice.

It is interesting to note that rickettsiae proliferated in peroxidase-negative macrophages. The increase in total numbers of peroxidase-negative macrophages, which occurred from days 4 to 6, may have influenced the increase in the percent cells infected with rickettsiae, which occurred from days 6 to 8, by supplying large numbers of macrophages that are susceptible to infection.

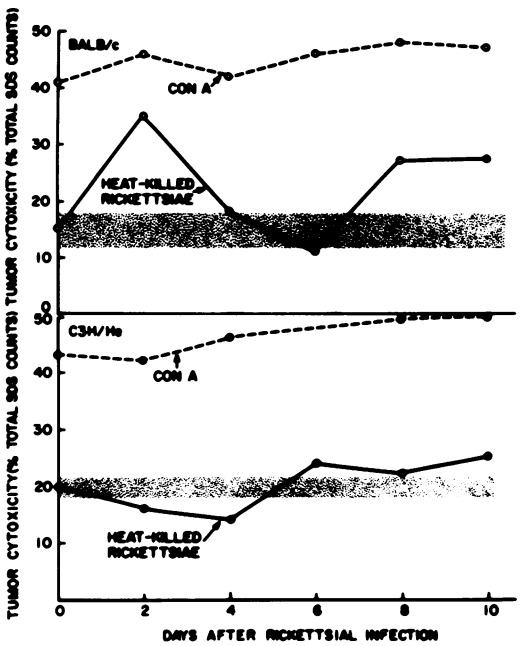

FIG. 8. *MAF produced by spleen cells stimulated with concanavalin A (Con A) or heat-killed rickettsiae from BALB/c or C3H mice infected with R. tsutsugamushi. Resident peritoneal macrophages from C3H mice were exposed to culture supernatants of antigen- or mitogen-stimulated spleen cells for 4 h, and [^{3}H]thymidine-prelabeled tumor target cells were added. Cultures were incubated for 48 h at 37°C. Prelabeled tumor cell monolayers digested with 0.5% sodium dodecyl sulfate were used to estimate total incorporated counts. Results are expressed as the mean percent total sodium dodecyl sulfate counts for triplicate cultures. Stippled area represents the mean ± standard deviations for uninfected mouse spleen cells treated with heat-killed rickettsiae in vitro.*

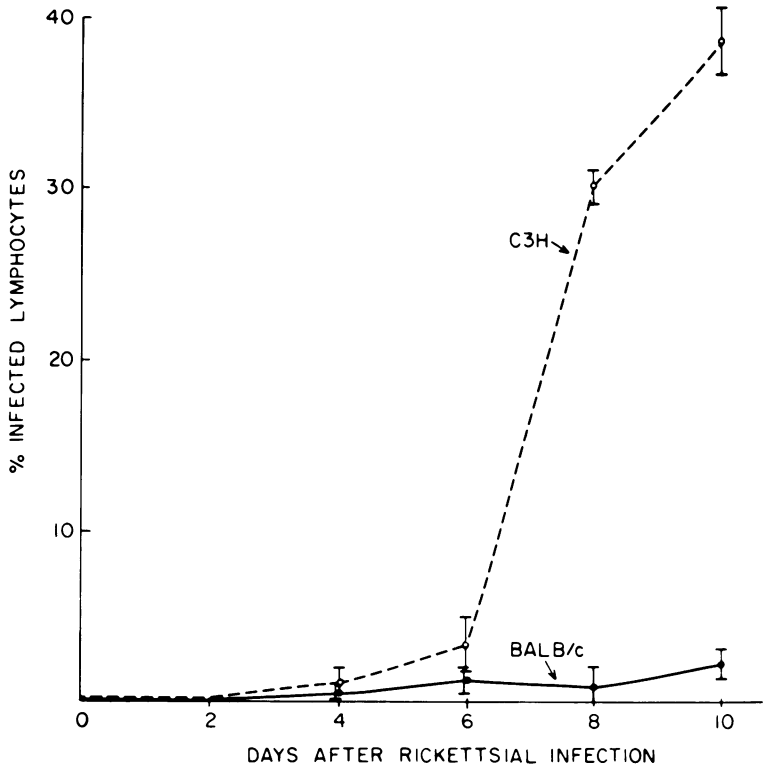

FIG. 9. *Infection of peritoneal exudate lymphocytes of BALB/c and C3H mice inoculated with R. tsutsugamushi strain Gilliam for 1 to 10 days. PC were harvested from infected and control animals, washed, and adjusted to 10^6 macrophages per ml. Cell smears were stained for peroxidase activity and counterstained with Giemsa. Results are expressed as the mean percent infected lymphocytes ± standard error for duplicate samples (100 cells observed per sample).*

In response to rickettsial inoculation, macrophages from C3H mice become non-specifically activated for tumor cytotoxicity on day 4, had reduced cytotoxic activity on days 6 and 7, and became activated again on days 8 to 10. Although 40% of macrophages harvested from C3H mice on day 8 contained intracellular rickettsiae, macrophage populations were as cytotoxic for tumor cells as were BALB/c macrophages (Fig. 7). Infection of macrophages, then, cannot account for the lack of activated C3H macrophages on days 6 and 7 after rickettsial inoculation.

Although we did not demonstrate that the appropriate macrophage activation stimulus was present in vivo during the course of scrub typhus infection, lymphocytes from C3H mice infected with rickettsiae did produce MAF when exposed to heat-killed rickettsiae in vitro. The ability of C3H lymphocytes to produce lymphokines was similar to that of BALB/c lymphocytes, even though a number of C3H lymphocytes contained intracellular rickettsiae starting day 6 after rickettsial inoculation. Infection of lymphocytes may not impair normal functions of the cell or, alternatively, infection of lymphocytes may be specific for cell type, and T cells which secrete lymphokines in response to antigenic stimulus may not be affected. In either case, lymphocytes which respond to rickettsial antigens for production of lymphokines were present in C3H mice at a time when macrophages were not activated.

What factors might contribute to loss of macrophage function at a critical time after infection? Lack of macrophage activation could reflect the nature of cells recruited to the site of infection in C3H mice: peroxidase-negative macrophages are less responsive to MAF than are inflammatory macrophages newly arrived from peripheral circulation (14). Alternatively, loss of macrophage activation may be the result of suppressive factors released during inflammation by any of several cell types present during an immune response, including macrophages. A third possibility is that antigen presentation to appropriate lymphocytes for production of lymphokines is impaired in infected C3H mice. Al-

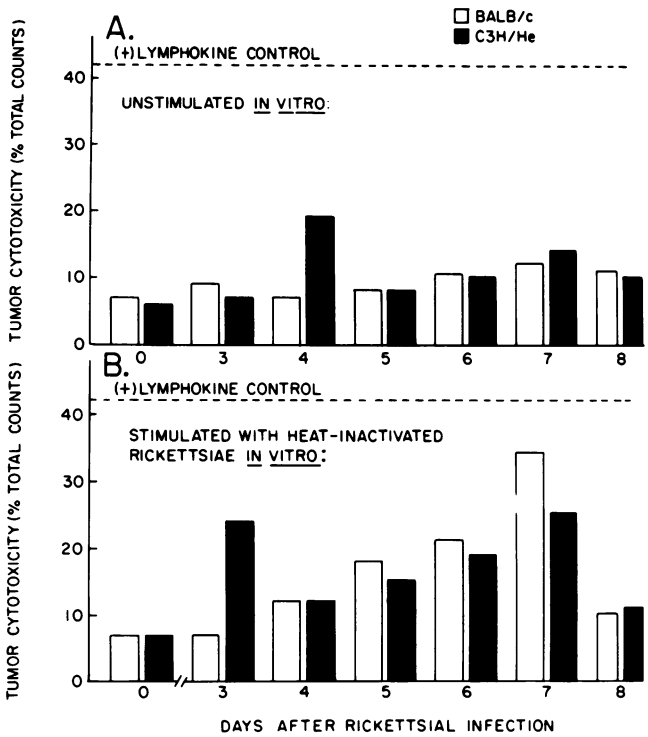

FIG. 10. *MAF produced by peritoneal exudate lymphocytes (A) or peritoneal exudate lymphocytes stimulated with heat-killed rickettsiae (B) in vitro. Culture supernatants of PC were assayed on monolayers of resident peritoneal macrophages from uninfected C3H mice. Macrophages were treated with supernatants for 4 h before addition of [^{3}H]thymidine-prelabeled tumor target cells. Macrophage-tumor target cell cultures were incubated for 48 h at 37°C. Tumor cell monolayers digested with 0.5% sodium dodecyl sulfate were used to estimate the total incorporated counts. Results are expressed as the mean percent total incorporated counts for triplicate cultures.*

TABLE 1. *Indirect fluorescent-antibody titers of sera collected from BALB/c and C3H mice after i.p. inoculation of R. tsutsugamushi strain Gilliam*

Day after infection	Antibody titer[a]	
	BALB/c	C3H/He
2	0	0
4	0	0
6	0	0
8	0	0
10	0	20
12	40	ND[b]
14	320	ND

[a] Antisera reacted with acetone-fixed Gilliam rickettsiae before reaction with fluorescein-conjugated anti-immunoglobulin G.

[b] ND, Not done; i.p. inoculation of C3H/He mice with 1,000 PFU resulted in death of infected animals on approximately day 10.

though the data presented in this report cannot indicate which of the possibilities relates to the genetic defect of C3H mice, we have demonstrated that the macrophage contributes significantly to survival of rickettsial infections. It is unlikely that susceptibility of C3H mice to i.p. inoculation of Gilliam *R. tsutsugamushi* is intrinsic to the macrophage itself. Obviously, interaction of cells during an immune response is dynamic and very complex. The genetic defect of C3H mice may be at the level of regulation of macrophage response to infection and could affect either (i) early recruitment of the appropriate macrophage population (i.e., inflammatory, peroxidase-containing macrophages) for expression of antirickettsial immunity at the site of infection or (ii) modulation of effector function expression during infection.

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