INFECTION AND IMMUNITY, Feb. 2004, p. 966–971 Vol. 72, No. 2
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.2.966–971.2004
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Mechanisms of Immunity to *Ehrlichia muris*: a Model of Monocytotropic Ehrlichiosis

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Received 11 August 2003/Returned for modification 16 September 2003/Accepted 13 November 2003

Ehrlichia **species can cause life-threatening infections or chronic persistent infections. Mechanisms of protective immunity were examined in an** ***Ehrlichia muris*** **mouse model of monocytotropic ehrlichiosis. C57BL/6 mice possessed strong genetic resistance to** ***E. muris*** **of an undetermined mechanism. CD8 T lymphocytes were particularly important, as revealed by 81% fatalities for** ***E. muris*****-infected, major histocompatibility complex class I gene knockout mice compared with no deaths for wild-type C3H mice. Moreover, 80% of C3H mice depleted of CD8 and CD4 cells died of** ***E. muris*** **infection compared with only 44% of CD4 cell-depleted mice. CD8 T lymphocytes were demonstrated for the first time in an** ***Ehrlichia*** **infection to exhibit cytotoxic T-lymphocyte activity against** ***Ehrlichia*****-infected target cells. Both gamma interferon and tumor necrosis factor were shown to play synergistic roles in protective immunity in vivo for the first time, as demonstrated by 75% fatalities when both cytokines were neutralized compared with minimal mortality when they were depleted separately. Passive transfer of antibodies, but not Fab fragments, to** ***E. muris*** **protected C3H/SCID mice against lethal infection. The mechanism of increased susceptibility (22% lethality) of C57BL/6 major histocompatibility complex class II gene knockout mice and CD4 cell-depleted C3H mice (i.e., through a gamma interferon or antibody mechanism), as well as the more important role of CD8 T lymphocytes (in the form of cytotoxic T-lymphocyte activity and/or gamma interferon production), remains to be elucidated. Protective immunity against monocytotropic** ***E. muris*** **is mediated by a combination of CD8 and CD4 T lymphocytes, gamma interferon, tumor necrosis factor alpha, and antibodies.**

Human monocytotropic ehrlichiosis (HME) caused by *Ehrlichia chaffeensis* is a prototypic emerging infectious disease (33). First reported as an infectious disease of humans in 1987, HME very likely has a high prevalence throughout the rural southeastern and south central states where white-tailed deer and lone-star ticks are prevalent (29, 32). Despite dramatic susceptibility to doxycycline, the case fatality rate is a significant 3% owing to misdiagnosis and delayed or ineffective treatment (29). A similar disease in Russia has been associated with *E. muris* in *Ixodes persulcatus* ticks and development of antibodies to antigens of the closely related *E. chaffeensis* (37, 46).

Five species of *Ehrlichia* are associated with human or veterinary diseases: *E. chaffeensis*, *E. ewingii*, *E. canis*, *E. ruminantium*, and *E. muris*. Ehrlichiae cause a persistent infection in their natural hosts (e.g., *E. ruminantium* in certain wild African ruminants and *E. chaffeensis* in white-tailed deer) (9, 35). Infection of some accidental hosts results in a severe toxic shock-like illness (e.g., *E. chaffeensis* in humans and an unnamed *Ehrlichia* from Japanese *Ixodes ovatus* ticks [IOE] in experimentally inoculated mice) (13, 41, 42). Although persistent or prolonged human infections with *E. chaffeensis* have been described, it is not known what proportions of untreated human infections develop persistent infections (7, 40).

Because adequate reagents and inbred or gene knockout (KO) animals are not available for critical studies of mechanisms of immunity to *E. canis* in dogs, *E. chaffeensis* in deer or dogs, or *E. ruminantium* in sheep, goats, or cattle, investigation of mechanisms of immunity relies upon four murine models of infection with *E. chaffeensis*, IOE, *E. ruminantium*, and *E. muris*. *E. chaffeensis* causes a transient subclinical infection in immunocompetent mice without reported pathology (9, 16, 17, 35, 38, 39, 49). Although SCID mice infected with *E. chaffeensis* have been utilized to identify a protective epitope and Toll receptor 4 and major histocompatibility class II gene KO mice develop prolonged infection, the model offers very limited opportunities to elucidate mechanisms of immunity against ehrlichial disease (14, 21, 43). IOE is a superb model of fatal HME (42). However, the disease is so severe that the model is difficult to utilize to elucidate protective immunity. An interesting mouse model of *E. ruminantium* has been developed (8), but the U. S. Department of Agriculture does not permit research on these organisms in the United States, except at Plum Island. *E. muris* causes systemic infection in immunocompetent mice in association with clinical signs of illness and establishes a persistent lifelong infection (18). Its level of virulence in immunocompetent mice is ideal for investigation of mechanisms of immunity with mice in which particular immune-related genes are inactivated or immune mechanisms are neutralized.

The objectives of this study were to determine the mechanisms of control of acute monocytotropic *E. muris* infection in mice by studies designed to evaluate the importance of the roles of CD4 and CD8 T-lymphocyte subsets, major histocompatibility complex (MHC) class I and class II, gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and antibodies. Each component was demonstrated to play an effector role in the immune protection against *E. muris*.

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MATERIALS AND METHODS

Ehrlichia. *Ehrlichia muris* was a gift from Y. Rikihisa (Ohio State University, Columbus, Ohio) (48). The 10% (wt/vol) infected spleen suspension on day 9 after intraperitoneal inoculation did not kill immunocompetent mice and was used as stock. The 50% lethal dose (LD_{50}) of this stock for SCID/B6 mice was $10^{-3.5}$ (1 ml/mouse administered intraperitoneally). The 50% tissue culture infectious dose ($TCID_{50}$) of the 10% fresh infected spleen suspension in the P388D_1 mouse macrophage-like cell line (catalogue no. T1B3; American Type Culture Collection [ATCC], Manassas, Va.) was $10^{-2.5}$.

Mice. C3H/HeN and Swiss Webster mice were purchased from Harlan Sprague Dawley (Indianapolis, Ind.). BALB/c, C57BL/6, DBA/2, AKR/J, SWR/J, and immunodeficient or gene KO mice were all purchased from Jackson Laboratories (Bar Harbor, Maine). All of the mice were 6- to 8-week-old males, except those used for the DBA/2, AKR/J, and SWR/J mouse experiments, for which 4-week-old male mice were used.

Polyclonal antibody. The mouse anti-*E. muris* polyclonal antibody was produced by intraperitoneal inoculation of a 10% suspension of *E. muris*-infected spleen from the same strain of mice (1 ml/mouse). At 1 month later the mice were given booster injections with the same dose of *E. muris*. On the next day after challenge, the mice were inoculated with Sarcoma 180 cells (a gift from R.B. Tesh, University of Texas Medical Branch, Galveston, Tex.) (5×10^6 cells/mouse administered intraperitoneally). Between 10 days and 2 weeks later, the mice began to produce large amounts of ascites. The ascites was collected, and the antibody was precipitated with 50% saturated ammonium sulfate. After dialysis and filtration, the product was used in these experiments. The antibody titer for *E. muris* was 1:1,280, as determined by immunofluorescence assay (IFA). The control ascites were from mice inoculated with Sarcoma 180 cells only. Fab fragments of the antibody were prepared using a Fab Fragment kit (Pierce, Rockford, Ill.). The method was performed according to the instructions of the manufacturer.

Monoclonal antibodies. Anti-*E. muris* monoclonal antibodies were developed as previously described (6). In brief, BALB/c splenic lymphocytes were obtained from mice inoculated with *E. muris* and given booster injections 3 to 4 days before the fusion. The fusion partner was the SP2/0-Ag14 (ATCC CRL 8006) (34) myeloma cell line. The anti-ehrlichial antibody-secreting clones were identified by IFA screening and characterized by Western immunoblotting after being subcloned three times by limiting dilution.

Passive protection. Anti-*E. muris* intact polyclonal antibody, its Fab fragment, monoclonal antibodies, or control ascites was inoculated into SCID/B6 mice, with 0.5 ml administered intravenously and 1 ml intraperitoneally per mouse. At 24 to 48 h later the mice were challenged intraperitoneally with 5 LD_{50} of *E. muris*. The mice were observed daily for mortality for 1 month.

Depletion of CD4 T lymphocytes, both CD4 and CD8 T lymphocytes, TNF-α, IFN-γ, or both TNF-α and IFN-γ. The method of depletion of CD4 or both CD4 and CD8 lymphocytes in C3H/HeN mice was performed as described previously (11). Briefly, 1 mg of purified monoclonal antibody against mouse CD4 from hybridoma GK1.5 (ATCC T1B 207) and/or hybridoma 53-6.72 (ATCC T1B 105) directed against mouse CD8 was inoculated intravenously (0.5 ml per mouse) after *E. muris* infection on days −2, 0, 2, 7, 14, 21, 28, and 35. Depletion of IFN-γ and/or TNF-α was performed according to a method described previously (10) except that the period of treatment with the monoclonal antibody was longer (until day 35).

Cytotoxic T-lymphocyte (CTL) activity assay. The CTL assay was performed according to a standard method (41). Target cells (P388D_1 cells) were inoculated with *E. muris* and incubated for 3 to 4 days, when approximately 50% of the cells were infected as determined by the observation of morulae in Diff Quik-stained cells. The cells were scraped off the flask, and a single-cell suspension was prepared, labeled by incubation with 200 μCi of ^{51}Cr (Amersham, Piscataway, N.J.) for 1 h, and then washed three times with Dulbecco modified Eagle medium containing 10% fetal bovine serum without phenol red and aliquoted into 96-well plates at a concentration of 2×10^4 cells/well.

Effector cells were prepared from DBA/2 mice immunized with a 10% suspension of *E. muris*-infected DBA/2 spleen cells. P388D_1 cells were derived from DBA/2 mice and thus have the same MHC class I. At 1 month later the mice were sacrificed, and a single-spleen-cell suspension was prepared and adjusted to a concentration of 5×10^6/ml and stimulated with Renografin-purified *E. muris* antigen (30 μg/ml). The antigen-presenting cells were used at a proportion of 10^5/ml of irradiated dendritic cells (36) (5,000 rads) and 5×10^6/ml of normal DBA spleen cells (2,300 rads). At 5 days later the CD8 T lymphocytes were separated by use of a mouse CD8 subset column (R & D, Minneapolis, Minn.). The CD8 T cells were adjusted to a concentration of 2×10^6/ml and used as effector cells. The CTL assay was performed for 10 h at effector cell-to-target cell ratios of 10:1, 3:1, and 1:1. The percentage of target cell lysis was calculated as follows: (sample counts per minute [cpm] − spontaneous release cpm)/(maximum release cpm − spontaneous release cpm) × 100.

TABLE 1. Importance of CD4 and CD8 T-lymphocyte subsets in immunity to *E. muris*

Mouse strain	Gene KO or depletion	No. of mice	% Fatalities (*P* value in comparisons with wild-type results)
C57BL/6	MHC class I KO	9	0
	MHC class II KO	9	22 (0.509)
	Wild-type C57BL/6	9	0
C3H	MHC class I KO	16	81 (<0.001)
	Wild-type C3H	9	0
	CD4 depleted	9	44 (0.092)
	CD4 + CD8 depleted	10	80 (0.002)

RESULTS

***E. muris* caused a nonlethal infection of immunocompetent mice.** A total of 20 mice each of strains Swiss Webster, C3H/HeN, and C57BL/6 were inoculated intraperitoneally with a fresh homologous 10% suspension of *E. muris*-infected spleen. All of the mice had developed splenomegaly at 10 days after inoculation. No mice died.

CD4 and CD8 T lymphocytes were both important in immunity to *E. muris*. Initially, the importance of CD4 and CD8 T lymphocytes was evaluated by the use of gene KO mice, nearly all of which have the C57BL/6 genetic background (Table 1). It was observed that with the exception of MHC class II gene KO mice (22% fatalities), none of the C57BL/6 gene KO mice died after infection with *E. muris*. The critical importance of CD8 T lymphocytes in the only relevant gene KO mice available on the C3H genetic background, namely, MHC class I gene KO mice (81% fatalities compared with no deaths in wild-type C3H/HeN mice), was apparent.

Because of the lack of informativeness of the C57BL/6 gene KO experiments and the lack of availability of other relevant gene KOs on the C3H genetic background, studies involving depletion of CD4 and CD8 T lymphocytes were performed. Depletion of CD4 T lymphocytes resulted in the death of 44% of *E. muris*-infected mice compared with no deaths among the sham-depleted, infected mice. To determine whether the importance of CD8 T lymphocytes (as already demonstrated clearly in the 81% mortality of MHC class I gene KO mice) was redundant with, or additional to, the contribution of CD4 T lymphocytes, both CD8 and CD4 T lymphocytes were depleted in C3H/HeN mice infected with *E. muris*. The increase to of the level of fatalities to 80% indicated that both CD4 and CD8 T lymphocytes contributed significantly to immune protection against *E. muris*.

Immune CD8 lymphocytes showed cytotoxic activity against *E. muris*-infected target cells. CD8 T lymphocytes from immune DBA/2 mice showed cytotoxic activity with MHC class I-matched, *E. muris*-infected P388D_1 cells (Fig. 1). No CTL activity by immune CD8 T lymphocytes against uninfected P388D_1 cells or by naïve CD8 cells against infected or uninfected target cells was observed. The observations were demonstrated to be reproducible. Studies utilizing gene KO mice

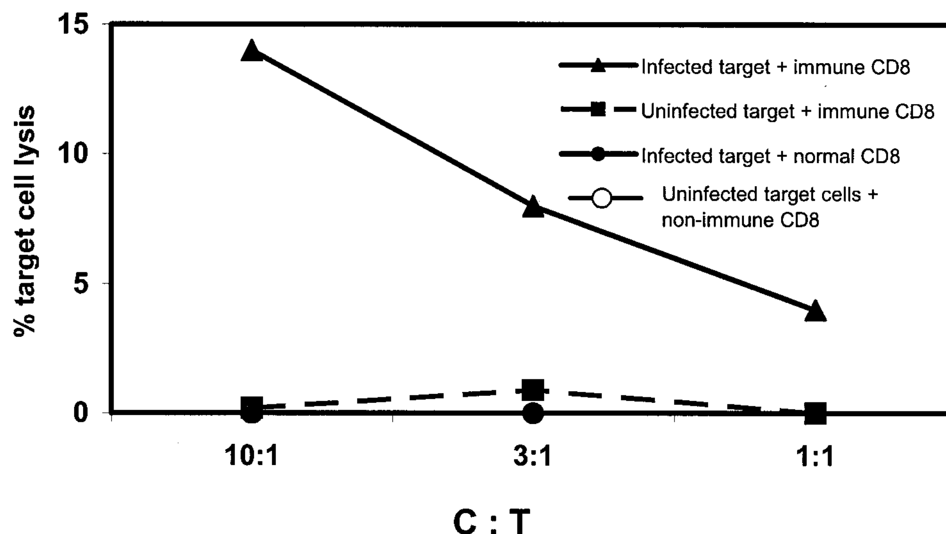

FIG. 1. CTL activity of CD8 lymphocytes expressed as percent target cell lysis at effector-to-target ratios of 10:1, 3:1, and 1:1. Interaction of uninfected target cells with normal CD8 T lymphocytes resulted in no target cell lysis.

(perforin studies, six animals; TAP studies, three animals) to examine the potential importance of anti-*E. muris* CTL activity in vivo were not revealing, as all the mice had a C57BL/6 genetic background and were completely resistant to illness.

IFN-γ and TNF-α act synergistically against *E. muris*. Infection of C57BL/6 gene KO mice for IFN-γ (9 animals), inducible nitric oxide synthase (13 animals), and phox (9 animals) studies was not informative because all were completely resistant to illness and death. The potential importance of TNF-α was suggested by the development of a mild self-limited illness (ruffled fur, less activity) in some of the nine *E. muris*-infected TNF receptor gene KO mice.

Thus, experiments were performed using C3H/HeN mice in which the activity of IFN-γ and/or TNF-α was neutralized. Depletion of both IFN-γ and TNF-α increased the mortality of *E. muris* infection in C3H/HeN mice to 75% (Table 2). Depletion of IFN-γ alone did not cause a significant effect. Only one mouse died in this group. None of the TNF-α-depleted, *E. muris*-infected mice died. Thus, IFN-γ and TNF-α acting synergistically are very important effectors of immunity against *E. muris*.

Complement was not critical to the resistance of mice to *E. muris*. Complement is an element of innate immunity and a potential factor in antibody-mediated immunity. As there were no complement gene KO mice available at the time of this study, animals genetically deficient in C5 were utilized. C5 deficiency is particularly informative, because both the classical and alternative pathways of complement activation require C5. AKR/J, SWR/J, and DBA/2 mice carry a null allele at HC (8, 10) and produce no C5.

In these experiments 4-week-old male mice were used. AKR/J mice were more susceptible (17% mortality among 23 animals) than mice of the other two C5-deficient strains (no mortality among 15 animals of each strain). Thus, the susceptibility of AKR/J mice to *E. muris* was not explained by C5 deficiency unless it must be present in combination with other still-unknown genetic factors.

Antibody played a role in immunity against *E. muris* infection. The presence of antibody was sufficient to protect the SCID/B6 mice against challenge with 5 LD_{50}s of *E. muris* (Table 3). This protection was Fc dependent, requiring intact antibody. The Fab fragments of antibodies were not protective. Two monoclonal antibodies (EM16 and EM17) directed against 28-kDa proteins (p28s) of *E. muris* are isotypes of immunoglobulin G1 (IgG1) and IgG2b, respectively. The IFA titer for *E. muris* of each antibody preparation was ≥ 1:20,480. These two monoclonal antibodies did not provide passive protection. More monoclonal antibodies need to be developed and evaluated to identify the protective B-cell epitopes. Studies of relevant gene KO mice (for studies using Fc receptor 1, three mice were used; for Fc receptor 2B studies, three mice were used; and for Fc receptor 3 studies, three mice were used)

TABLE 2. IFN-γ and TNF-α are important cytokines in immunity to *E. muris*

Group	No. of mice	% Fatalities (*P* value in comparisons with wild-type results)
IFN-γ depleted	9	11 (0.992)
TNF-α depleted	9	0
IFN-γ + TNF-α depleted	12	75 (0.003)
Wild-type C3H	9	0

TABLE 3. Passive protection of antibody against *E. muris*

Group	No. of mice	% Fatalities (range, mean [± SD] for day of death)
Polyclonal anti-*E. muris* ascites	10	0
Normal ascites	10	100 (day 14–17, 15.7 ± 1.1)
Polyclonal anti-*E. muris* Fab	8	100 (day 16–22, 18.9 ± 2.2)
Anti-*E. muris* MAb[a] (Em 16)	5	100 (day 18–25, 20.6 ± 2.9)
Anti-*E. muris* MAb (Em 17)	6	100 (day 16–22, 18.7 ± 2.3)

[a] MAb, monoclonal antibody.

were not revealing, as all mice had a C57BL/6 genetic background and were resistant to illness.

DISCUSSION

Although sterile immunity has not been demonstrated in infections caused by *Ehrlichia*, there is substantial evidence that the immune system plays an important role in the control of ehrlichial infection. The veterinary literature contains convincing reports that immunization of animals with inactivated *E. ruminantium* stimulates their ability to survive an ordinarily highly lethal infection (24, 25, 28). Similar enhanced survival follows vaccination with live attenuated *E. ruminantium* or deliberate infection of animals followed by treatment with tetracycline (15). DNA vaccines expressing particular antigens stimulate protective immunity against *E. ruminantium* challenge in mice (31). Conversely, immunocompromised persons appear to be more susceptible to severe HME and to infection with *E. ewingii* (23, 34). In the present reported studies, the importance of CD8 and CD4 T lymphocytes, antibodies, and IFN-γ combined with TNF-α in protective immunity of mice against *E. muris* was demonstrated for the first time. The data confirmed some principles previously proposed for immunity to ehrlichiae mediated by antibodies, Fc receptors, MHC class II, and IFN-γ and suggest novel anti-ehrlichial mechanisms involving MHC class I and CTL (1, 3, 5, 8, 14, 21, 22, 26, 44, 50).

The most interesting observations made during these experiments concerned the presence and likely importance of CD8 cytotoxic T lymphocytes. To the best of our knowledge, this is the first report of CTL activity in an ehrlichial infection. The dramatic conversion from 100% survival of *E. muris* infection in wild-type C3H mice to a fatal outcome of 81% of MHC class I KO mice and an increase from 44% fatalities in CD4 T-lymphocyte-depleted mice to 80% fatalities in mice depleted of both CD8 and CD4 T lymphocytes indicates that CD8 T-lymphocyte recognition of *E. muris* antigens in the context of MHC class I presentation is a critical factor in protective immunity against ehrlichiae. Whether CTL activity or IFN-γ secretion or both are the critical mechanisms remains to be determined (47). These interpretations are consistent with the prior observation that effective adoptive transfer of immunity to *E. ruminantium* in mice requires CD8, but not CD4, T lymphocytes (8). C57BL/6 mice possess such strong undefined genetic resistance to *E. chaffeensis* that gene KO mice for MHC class I, IFN-γ, and inducible nitric oxide synthase are completely resistant (12). C57BL/6 mice gene KOs for perforin, cytochrome B245, TAP, T-cell receptor delta chain, and Ig Fc receptors 1, 2B, and 3 were resistant even to *E. muris*, which is more pathogenic for mice than *E. chaffeensis*. These negative results reflect the genetic resistance of C57BL/6 mice more than a lack of a role for these immune system genes.

The results in the present study indicate that CD4 T lymphocytes contribute to immune protection of C3H/HeN mice against *E. muris*. Depletion of CD4 T lymphocytes converted the outcome of *E. muris* infection from 100% survival to 44% fatalities, and even C57BL/6 mice with KO of the MHC class II gene became ill and suffered 22% fatalities. Taken in conjunction with the other results of this and previous studies, it would seem likely that the major contribution of CD4 cells in immunity to *E. muris* is the secretion of IFN-γ, with additional roles in the development of the humoral immune response and providing help for CD8 T lymphocyte proliferation. Even *E. chaffeensis* is capable of establishing a persistent infection in mice with MHC class II gene KO (14). It is noteworthy that *E. ruminantium* infection of bovine endothelial cells downregulates the expression of both MHC class I and class II on the main target cells of this infection, bovine endothelial cells, which perhaps represents an ehrlichial mechanism of evading immune clearance (45).

The effect of depletion of both IFN-γ and TNF-α was an increase of lethality to 75%. This is the first direct in vivo evidence for the important of IFN-γ and TNF-α as host defenses against *Ehrlichia* species. Elegant in vitro studies of *E. chaffeensis* infection of a human monocytic cell line (THP-1 cells) had previously demonstrated that intricate interactions have evolved between the host and parasite. *E. chaffeensis* infection upregulates mRNA for transferrin receptors, which increase on the ehrlichial cytoplasmic inclusions in monocytes providing iron that is essential for ehrlichial growth (2). The effect of IFN-γ on human macrophages (when IFN-γ is present prior to or early in the infection) is to kill intracellular *E. chaffeensis* by limitation of the availability of iron transferrin (1). Conversely, *E. chaffeensis* has evolved a mechanism to activate protein kinase A, which blocks IFN-γ-induced tyrosine phosphorylation of Jak 1, Jak 2, and Stat 1 and their subsequent antiehrlichial effect (20). These highly evolved interactions emphasize the probable importance of IFN-γ as a host defense against *Ehrlichia* organisms. The data reported in the present study indicate that, in vivo, IFN-γ and TNF-α act synergistically.

IFN-γ also appears to play a role in host defenses against *E. ruminantium*. Bovine endothelial cells activated by IFN-γ inhibit the growth of intracellular *E. ruminantium* in association with the production of nitric oxide and without evidence for a role for TNF-α (26, 27, 30, 44). Moreover, DBA/2 mice are protected against *E. ruminantium* by adoptive transfer of splenic T lymphocytes (which secrete a significant concentration of IFN-γ, but not TNF-α, when stimulated by *E. ruminantium* antigens) (5). The reasons for the apparent difference with regard to the importance of TNF-α are not clear, but the experiments reported herein are the only in vivo studies of the role of TNF-α in *Ehrlichia* infection.

Moreover, the antibody-dependent, Fc-receptor-dependent protection of SCID mice against fatal *E. muris* infection confirmed the principle (established by Winslow et al. for *E. chaffeensis* infections in mice) that antibodies play a role in the clearance of ehrlichiae in vivo (50). Further extension of their model identified an epitope within peptides 61 to 90 of the first hypervariable region of the *E. chaffeensis* p28 (p28-19) as a target for protective immunity conferred most effectively by IgG monoclonal antibodies of picomolar affinity levels (21). Although 40% of the 100 *E. chaffeensis*-specific monoclonal antibodies characterized in that study were directed against p28-19, neither of the two anti-*E. muris* p28 monoclonal antibodies that we evaluated were protective. Further development and investigation of anti-*E. muris* monoclonal antibodies are required for identification of the protective target proteins and epitopes.

Antibody opsonization of monocytotropic ehrlichiae may

also enhance the host defenses by inducing the production of the proinflammatory cytokines interleukin-1 beta (IL-1β), IL-6, and TNF-α by macrophages which opsonized the ehrlichiae encounter (19). Curiously, infectivity of *E. ruminantium* for bovine endothelial cells has been reported to be neutralized by polyclonal antibodies in vitro and yet no protection was observed in the *E. ruminantium*-DBA/2 mouse model (4).

Altogether, these studies emphasize the utility of the *E. muris* mouse model for elucidation of the protective immune mechanisms against monocytotropic ehrlichiae. Because of the close genetic and antigenic relationships between *E. chaffeensis* and *E. muris*, there are excellent prospects for greater progress in the evaluation of candidate vaccine antigens as well as dissection of the orchestration of the events in the development of protective immunity through the use of the murine *E. muris* model.

ACKNOWLEDGMENTS

We express our gratitude to Kelly Cassity and Melissa Yancey for expert secretarial assistance in the preparation of the manuscript.

This research was supported by a grant from the National Institute of Allergy and Infectious Diseases (AI 31431).

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Editor: W. A. Petri, Jr.