Differential Clearance and Immune Responses to Tick Cell-Derived versus Macrophage Culture-Derived *Ehrlichia chaffeensis* in Mice\(^\dagger\)

Roman R. Ganta,\(^1\)* Chuanmin Cheng,\(^1\) Elizabeth C. Miller,\(^2\) Bridget L. McGuire,\(^2\) Lalitha Peddireddi,\(^1\) Kamesh R. Sirigireddy,\(^1\) and Stephen K. Chapes\(^2\)

Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine,\(^1\) and Division of Biology, College of Arts and Sciences,\(^2\) Kansas State University, Manhattan, Kansas 66506

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Human monocytic ehrlichiosis is caused by a tick-transmitted rickettsia, *Ehrlichia chaffeensis*. We recently reported that *E. chaffeensis* grown in tick cells expresses different proteins than bacteria grown in macrophages. Therefore, we tested the hypothesis that immune responses against *E. chaffeensis* would be different if the mice are challenged with bacteria grown in macrophages or tick cells. We assessed the *E. chaffeensis* clearance from the peritoneum, spleen, and liver by C57BL/6J mice using a TaqMan-based real-time reverse transcription-PCR assay. Macrophage-grown *E. chaffeensis* was cleared in 2 weeks from the peritoneum, whereas the pathogen from tick cells persisted for nine additional days and included three relapses of increasing bacterial load separated by three-day intervals. Tick cell-grown bacteria also persisted in the livers and spleens with higher bacterial loads compared to macrophage-grown bacteria and fluctuated over a period of 35 days. Three-day periodic cycles were detected in T-cell CD62L/CD44 ratios in the spleen and bone marrow in response to infections with both tick cell- and macrophage-grown bacteria and were accompanied by similar periodic cycles of spleen cell cytokine secretions and nitric oxide and interleukin-6 by peritoneal macrophages. The *E. chaffeensis*-specific immunoglobulin G response was considerably higher and steadily increased in mice infected with the tick cell-derived *E. chaffeensis* compared to DH82-grown bacteria. In addition, antigens detected by the immunoglobulins were significantly different between mice infected with the *E. chaffeensis* originating from tick cells or macrophages. The differences in the immune response to tick cell-grown bacteria compared to macrophage-grown bacteria reflected a delay in the shift of gene expression from the tick cell-specific Omp 14 gene to the macrophage-specific Omp 19 gene. These data suggest that the host response to *E. chaffeensis* depends on the source of the bacteria and that this experimental model requires the most natural inoculum possible to allow for a realistic understanding of host resistance.

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TABLE 1. Inoculum doses used in mouse infections with *E. chaffeensis* grown in DH82 or ISE6 cells

<table>
<thead>
<tr>
<th>Expt</th>
<th>Source of bacteria (cell line)</th>
<th>Inoculum size (10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DH82</td>
<td>NT*</td>
</tr>
<tr>
<td>2</td>
<td>DH82</td>
<td>3.2</td>
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<tr>
<td>3</td>
<td>DH82</td>
<td>3.5</td>
</tr>
<tr>
<td>4</td>
<td>DH82</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>DH82</td>
<td>2.0</td>
</tr>
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</table>

* NT, not tested.

mated number of bacteria used as an inoculum per mouse in each experimental infection group. Mice used for infection studies were 6 to 8 weeks old at the beginning of each experiment, and they were injected intraperitoneally (i.p.) with an average of 3.5 × 10^6 bacteria from DH82 cells and 2.3 × 10^6 bacteria from ISE6 cells. The numbers of bacteria from each treatment group were not statistically different from one another (P > 0.1 by matched Student t test or P > 0.05 for the Wilcoxon signed-rank test). The first experiment used bacteria derived only from tick cells (3.2 × 10^6), whereas in experiments 2 to 5 mice received *E. chaffeensis* grown in DH82 and tick cells cultures. The inoculum size varied from as low as 0.7 × 10^6 to as high as 4.2 × 10^6 bacteria for the organisms originating from tick cells (the inoculum size varied over a range of sixfold) and the DH82 culture-derived bacteria used as the inoculum ranged from 1.0 × 10^6 to 7.5 × 10^6 per mouse (the inoculum size varied in about the sevenfold range).

**Materials and Methods**

**In vitro cultivation of *E. chaffeensis***. The *E. chaffeensis* Arkansas isolate was cultivated in either the canine macrophage cell line DH82 at 37°C (9) or in the tick cell line ISE6 at 34°C (39). Cultures from T-75 flasks with 80 to 90% infectivity were used for experimental infection studies.

**Experimental infections.** *E. chaffeensis* bacteria from infected macrophages or tick cells were dispersed by vortexing the cells in the presence of glass beads. The infectivity were used for experimental infection studies.

**Tissue collection and processing.** Blood, peritoneal exudate cells, spleens, livers, and bone marrow were harvested from normal or infected mice (both treatment groups) at different times postinfection. The peritoneal exudate cells and spleen and liver samples were used to assess the presence of *E. chaffeensis*. Plasma recovered from blood was used for antibody analyses. Spleen and bone marrow cells were used for mapping cell phenotypes. Spleen and peritoneal macrophages were also used to measure cytokine production. Blood was collected from the retro-orbital sinus into a heparinized micropipette and transferred to a microcentrifuge tube containing 25 μl of heparin (10,000 IU/ml). The blood was centrifuged at 3,000 × g, and the plasma was stored at −70°C. Spleens were divided in half. One-half was used to prepare RNA to quantitate bacteria (see below). The second half was homogenized, erythrocytes were lysed, and the cells were counted. A total of 5 × 10^6 spleen cells per 60-mm plate were cultivated in Dulbecco modified Eagle medium containing 2% fetal bovine serum (D-MEM), and gentamicin (50 μg/ml). Supernatants were collected after a 20-h incubation at 37°C and frozen at −20°C until cytokine assays were performed (described below). A fraction of the spleen cells was analyzed with flow cytometry (see below). Bone marrow cells were washed from the femora and humeri and were used for culture or flow cytometry. About 100 mg of liver tissue was homogenized and used to isolate RNA according to the Tri-reagent method (see below). Peritoneal exudate cells containing predominantly macrophages were collected from *E. chaffeensis*-infected and unaffected control mice by peritoneal lavage with 20 ml of ice-cold, sterile PBS. Approximately 2 × 10^6 cells per sample were seeded into wells of 24-well culture plates in volume of 2 ml. Cells were incubated for 20 h, culture supernatants were collected, and cell-free supernatant was stored at −20°C until the cytokine assays were performed. A fraction of the peritoneal wash cells was also used for RNA isolation to determine the presence of bacteria.

**RNA isolation.** Total RNA from a 0.5-ml portion of in vitro cultures or cell pellets collected from 5 ml of peritoneal wash cells or from about 50 mg each of spleen or 100 mg of liver tissue was isolated by using the Tri-Reagent RNA isolation kit according to the manufacturer’s protocol (Sigma Aldrich, St. Louis, MO). Purified RNA pellets from in vitro cultures or peritoneal wash cells were resuspended in 50 μl of TE (10 mM Tris, 1 mM EDTA [pH 8.0]), and the pellets formed were or liver tissues were resuspended in 50 μl of TNE (10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 7.4) and concentration of the RNA was assessed using a spectrophotometer (NanoDrop Technologies, Wilmington, DE) by calculating the ratio between the optical densities at 260 and 280 nm. The absorbance ratio for all samples ranged between 1.6 and 2.0. The quality of RNA for a subset of samples was also confirmed by resolving them on a 1.5% formaldehyde agarose gel (38).

**Real-time quantitative RT-PCR.** The master mixture for RT-PCR assay is 25 μl in volume containing 2 μl of RNA, 5 pmol each of the TaqMan forward and reverse primers, 3.75 pmol of *E. chaffeensis*-specific TaqMan probe, 5 nmol of deoxynucleoside triphosphates, 125 nmol of MgCl₂, and 1 μl of SS-III and Taq mix (SuperScript-III, One-Step RT-PCR system with platinum Taq DNA polymerase, Invitrogen Technologies, Carlsbad, CA). The temperature cycles used for the assay were one cycle each at 48°C for 30 min and 95°C for 5 min, followed by 45 cycles of 95°C for 15 s, 50°C for 30 s, and 60°C for 60 s. The product formation was monitored in real-time by measuring the emitted fluorescence in the extension phase of the PCR cycles using a SmartCycler system (Cepheid, Sunnyvale, CA). The reaction is regarded as positive for the presence of a template when amplified product formation results in the detection of 10 fluororescent units. The temperature cycle at which this occurs is regarded as the C_T value that is template concentration dependent (48).

To determine the inoculum to be used for experimental infections, RNA was isolated from an aliquot of each suspension used for injection, and the amount of bacterial 16S RNA was determined by real-time RT-PCR assay (48). The C_T values were compared to a standard curve that was prepared by diluting known concentrations of bacterial 16S rRNA and determining the individual C_T values for each dilution. The minimum detection sensitivity was estimated at one...
organism based on the assumption that there are approximately 100 molecules of 16S rRNA present per one organism for DH82 derived E. chaffeensis (17, 48). We also estimated the ratio of 16S rRNA to RNA gene in tick cell-derived E. chaffeensis, and it is similar to that found in DH82 culture-derived E. chaffeensis, i.e., ~1:10. Thus, we estimated bacterial numbers by translating 100 copies of 16S rRNA molecules as equal to one bacteria.

Quantitative ELISA to map IgG subclasses. Quantitative enzyme-linked immunosorbent assay (ELISA) using whole-cell antigen (20 ng/well) was performed to determine the concentrations of E. chaffeensis-specific immunoglobulin G (IgG) subclass antibodies by following a protocol described previously (24, 25). Antigens used for ELISAs for macrophage-derived E. chaffeensis-infected mouse plasma were made from E. chaffeensis purified from DH82 cultures. Similarly, homologous antigens were used for ELISAs to map IgG subclass antibodies in mice infected with tick cell-derived E. chaffeensis.

Western blot analysis. The whole-cell protein extract (3 µg/well) of purified E. chaffeensis Arkansas isolate cultured in either macrophage- or tick cell-derived organisms was resolved on a sodium dodecyl sulfate–12% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and were probed with the plasma from E. chaffeensis-infected mice diluted at 1:128 for the presence of E. chaffeensis-specific antibody (24, 25).

Nitric oxide and cytokine assessment. As a measure of nitric oxide (NO) production, we estimated peritoneal macrophage secretion of NO, a stable end product of the NO synthesis pathway (50) using the Griess reaction (51). The sensitivity of the assay was approximately 200 nM. Macrophage cytokines were measured peritoneal cells were isolated from mice injected with bacteria from either type of cell. Each mouse sample was measured independently, and multiple time points were tested in each experiment. The adherent macrophages were removed free of adherent cells, and they were cultured ex vivo for 24 h. Supernatants were collected and stored for assay. For spleen cells, interleukin-2 (IL-2), IL-6, and IL-10 and gamma interferon (IFN-γ) were assayed by using capture ELISAs in 96-well polystyrene plates as detailed previously (8). The sensitivities of these assays were 50 pg/ml, 20 pg/ml, 30 pg/ml, and 2 ng/ml for IL-2, IL-6, IL-10, and IFN-γ, respectively. The spleen cell supernatants were combined according to harvest date and type of infection. Supernatants produced by different cell types were also assessed by using cytokine beads and assayed for IL-1α, IL-2, IL-5, IL-6, IL-10, IFN-γ, tumor necrosis factor alpha (TNF-α), granulocyte-macrophage colony-stimulating factor, IL-4, and IL-17 by using a Flow Cytometry Multiplex mouse Th1/Th2 10plex kit (Bender MedSystems, Vienna, Austria).

The kinetics of the secretion of NO and IL-6 by peritoneal macrophages were very similar among the different experiments conducted. However, the peak secreted amounts of NO and IL-6 varied among the four experiments. Therefore, to illustrate the kinetics of the response, we normalized the data as follows. After we calculated how much cytokine was made by cells from each mouse at each time point, the peak amount of cytokine made in an experiment by a particular sample was valued at 100% of the maximum cytokine made. All other samples were valued at ≤100% of the maximum sample. Samples from the same time point were averaged, and the values at each time for four experiments were used for the analysis.

Flow cytometry. Mouse spleen and bone marrow cells were resuspended in DME2 and 106 cells were incubated for 30 min in the wells of 96-well plates in 50 µl of goat serum diluted 1:2 in Hanks buffered salt solution. Cells were stained with 10 µl of mouse-specific, anti-Cd62L-APC antibodies (BD Pharmingen, Franklin Lakes, NJ) and CD44-PE (eBiosciences, San Diego, CA) and separately stained with 5 µl of mouse-specific, anti-CD3-APC (BD Pharmingen) antibodies. Cells were incubated for 1 h, washed two times in Hanks balanced salt solution, and fixed with 250 µl of PBS containing 1% formalin. The fluorescence intensity was measured on a FACSCalibur analytical flow cytometer (Becton Dickinson, San Jose, CA). Splenic lymphocytes were identified in forward-scatter versus side-scatter plots and assessed for CD44 and CD62L. In the bone marrow, the percentage of CD3+ cells was determined in each population visible on forward-scatter versus side-scatter plots. The population with the highest number of CD3+ cells was used in the analyses of CD44 and CD62L.

In vitro and in vivo monitoring of changes in gene expression of the p28-Omp locus genes 14 and 19. To evaluate changes in the gene expression, ISE6 tick cell-derived E. chaffeensis was purified (46) and used to infect several T-25 flasks containing DH82 (macrophage) or ISE6 (tick cell) cultures. Similarly, DH82 culture-derived E. chaffeensis was purified and used to infect flask containing DH82 cultures. Total RNA was isolated from cell cultures at several time points postinoculation until the culture reached 80 to 100% infectivity (0.5 to 5 days). Total RNA was recovered by using the Tri-Reagent RNA method (described above). We recently reported that the p28-Omp 14 transcript is the predominant transcript seen in tick cell-derived E. chaffeensis with low-level expression from the p28-Omp gene 19, whereas the macrophage-derived cultures had a predomin-
Both infection groups developed *E. chaffeensis*-specific IgG responses with predominant expression (>90%) of the complement-fixing IgG molecules IgG2a, IgG2b, and IgG3 for all postinfection day samples (Fig. 3). The IgG2 and IgG3 concentrations of mice injected with bacteria grown in ISE6 tick cells were usually at least twice as high as those in mice injected with bacteria grown in macrophages.

Western blot analysis using whole-cell *E. chaffeensis* antigens showed considerable variation in the antigens recognized by the immunoglobulins made in response to tick cell-derived *E. chaffeensis* compared to those made against macrophage-derived *E. chaffeensis* (Fig. 4). Antibodies from macrophage- and tick cell-derived *E. chaffeensis*-infected mice recognized considerably more antigens from the bacteria they were immunized with (homologous antigens) compared to antigens detected in bacteria grown in the other cell line (heterologous antigens). The antibodies recognized few shared antigens and also recognized several unique antigens. Mouse-to-mouse variation within the same experimental group was also visible in their ability to bind antigens and the concentration of the antibodies made (Fig. 4). The intensity of detection signals resulting from antigen and antibody complex to homologous antigens was also higher than those with heterologous antigens.

**Peritoneal macrophage activation in *E. chaffeensis*-infected mice.** The kinetics of the secretion of NO and IL-6 in peritoneal macrophages were very similar for the normalized data for four independent experiments (Fig. 5). Peritoneal macrophages from mice infected with tick cell-grown *E. chaffeensis* secreted peak amounts of nitric oxide at about the same time as that observed for macrophage-grown pathogen, except that the magnitude of the peak response was lower at days 7, 10, 20, and 22 (P < 0.05; Fig. 5A). Macrophages from both treatment
groups showed two peaks of secretion, one each at 10 to 12 days and at 20 to 22 days after infection.

Macrophages from mice challenged with both types of bacteria also secreted IL-6 with both treatment groups having peak secretions at approximately 19 to 21 days after infection. Macrophages from mice challenged with DH82-grown bacteria produced significantly more IL-6 than macrophages from mice challenged with ISE6 tick cell-grown bacteria at 12, 19, 20, and 21 days after infection ($P < 0.05$; Fig. 5B).

**Lymphocyte assessment for CD62L and CD44.** To determine the progression of the immune response to *E. chaffeensis*, the expression of cell surface molecules CD62L and CD44 was investigated. We analyzed both molecules simultaneously, and the data are presented in Fig. 6 as a ratio of the percent CD62L to the percent CD44 on lymphocytes in the spleen and bone marrow to give a measure of “effector memory” (29). The CD62L/CD44 ratio was very similar for both infection groups in the spleen and decreased until about 15 days after experi-
mential challenge for mice injected with either tick cell-grown or macrophage-grown *E. chaffeensis* (Fig. 6A). This low point immediately preceded the initial depression in the number of bacteria estimated to be in the spleen by real-time RT-PCR (Fig. 1B). By day 21 after infection, the CD62L/CD44 ratio returned to about 0.9. The CD62L/CD44 ratio then oscillated above and below the 0.8 value for the rest of the infection. The CD62L/CD44 ratio was also very similar for both infection groups for the cells isolated from the bone marrow (Fig. 6B). There was a general trend for an initial depression at around day 15 with an increase that peaked for tick cell-derived bacteria at day 21. There also was some indication for both treatment groups that the bone marrow CD62L/CD44 ratio oscillated with time.

**Spleen cell cytokine profiles.** When we measured spleen cell cytokine responses after infection, we detected measurable levels of IL-1α, IL-4, IL-6, and IL-10 (Fig. 7), but IFN-γ and IL-2 were undetectable by capture ELISA or by flow cytometry. Secretion of the four detectable cytokines also followed a cyclical pattern similar to that observed for the bacterial load, the CD62L/CD44 ratio in the spleen, and other measurements. The cyclical cytokine expression trends remained very similar for both of the infection groups, except that the secretion levels were significantly lower (*P* < 0.05 unless indicated otherwise) for the tick cell-derived *E. chaffeensis*.

**Expression of p28-Omp locus genes 14 and 19 in vitro and in vivo.** The antibody responses to tick cell-derived bacteria were different from the antibody responses raised against macrophage-grown bacteria. We hypothesized that these differences were caused by the differential antigen expression, such as Omp gene 14 in ISE6 tick cells compared to Omp gene 19 expression in DH82 macrophages and the level of expression of these genes when bacteria were introduced into a new host environment. To test this hypothesis, bacteria grown in ISE6 cells or DH82 cells were assayed for the transcripts for p28-Omp 14 and 19 genes after the bacteria were used to infect new cells (Fig. 8A). When DH82 cells were infected with *E. chaffeensis* from ISE6 cells, the expression of gene 14 was high after 12 h and was expressed in a declining pattern for an additional 3 days. On day 4, the expression of genes 14 and 19 equalized, and by day 5, the p28-Omp gene 14 became the dominantly expressed form (Fig. 8A). Bacteria maintained in consecutive DH82 passages maintained high-level expression of gene 19, whereas bacteria maintained in ISE6 cells retained high-level expression of gene 14 (Fig. 8A).

To determine whether similar delays in gene shifts occurred in mouse macrophages in vivo, we examined RNA samples from B6 mouse peritoneal macrophages that tested positive for *E. chaffeensis* after mice were infected with DH82 or ISE6 cell-derived bacteria (Fig. 8B). In B6 mice infected with ISE6 cell-derived *E. chaffeensis*, the transcripts for gene 14 were present at high levels 1 and 3 days after experimental challenge similar to what we observed in vitro. The expression of gene 19
increased and the expression of gene 14 decreased between days 7 and 17 postinfection. A similar analysis was not possible using B6 mice infected with DH82-derived E. chaffeensis after day 7 because the bacteria were not detectable in peritoneal exudate cells beyond this day postinfection. Because B6 mice cleared infections of DH82 derived-bacteria so quickly, we did similar experiments in C2D mice. These mice do not cure the infection (25) and allowed us to isolate bacteria for long periods of time (Fig. 8C). In these experiments, gene 14 transcripts remained high in C2D mice infected with ISE6-derived E. chaffeensis for 3 days after i.p. experimental challenge. By 5 days, transcription of genes 14 and 19 were equal. However, on subsequent days postinfection, we detected more gene 19 transcripts than those for gene 14.

DISCUSSION

Human monocytic ehrlichiosis is an emerging zoonotic infection that has doubled in incidence since 1983 (40). In the present study, we presented data showing that E. chaffeensis grown in tick cells induces an immune response in mice that is distinct from that observed for the pathogen grown in macrophages. Although E. chaffeensis grown in both cell types is cleared by the murine host and induced a similar range of macrophage and T-cell cytokines, the host responses to tick cell-grown bacteria were slower, and the concentrations of cytokines measured in the present study.
were lower. In addition, there were distinct humoral immune responses detected.

Mice infected with tick cell-derived *E. chaffeensis* exhibited higher rickettsemia and slower clearance. This was best exemplified by the data showing that mice injected with tick cell-derived bacteria resolved peritoneal bacteremia about 9 days later than mice injected with bacteria grown in macrophage cultures. The slow clearance in mice injected with tick cell-derived bacteria was not because these mice were injected with more bacteria. In fact, although it is not statistically different, the mice consistently received more DH82-grown bacteria than tick cell-derived *E. chaffeensis* (Table 1), yet the clearance of DH82-derived bacteria was faster. In addition, we assessed the clearance kinetics in five independent experiments with inocula as low as 0.7 x 10^6 to as high as 4.2 x 10^6 for tick cell-derived *Ehrlichia* and inocula ranging from 1.0 x 10^6 to 7.5 x 10^6 for mice injected with DH82 culture-derived *E. chaffeensis*. Independent of the dose used for either group of bacteria, the general kinetics of clearance were the same. For example, the clearance of bacteria from the peritonea of mice challenged with DH82-culture-derived bacteria was consistently in the range of 14 to 17 days. Therefore, the range of challenge doses did not change the kinetics of the mouse response. More importantly, the clearance kinetics for DH82-derived bacteria was consistent with our previous reports in which we used inocula of 5 x 10^6 infected DH-82 cells (24, 25), where we did not quantitate the actual numbers of bacteria. Finally, our estimations of inocula given to the mice did not appear to be skewed because of differences in the ratio of the rRNA gene to rRNA for *E. chaffeensis* grown in the two different host cell backgrounds. Although it is possible that an organism can make different levels of rRNA in different environments (i.e., rates of rRNA transcriptions can vary in different host environments to support varying needs of protein synthesis), we did not find evidence for this based on the rRNA gene-to-rRNA ratios for tick cell- and DH82-derived *E. chaffeensis*.

The delay in clearance was more pronounced in the peritoneum than in the liver and spleen, which may reflect the fact that the peritoneum is the site of injection and that the impact of the origin of the bacteria is on the initial host response compared to responses that occur later. This hypothesis is supported by the observations that ISE6-derived bacteria do not activate macrophages to the same extent as bacteria grown in DH82 cells, as judged from the NO and IL-6 secretions by the peritoneal macrophages (Fig. 5). Macrophages are also not stimulated as well by an *Ehrlichia* injection in C3H/HeJ mice compared to wild-type mice, and this poorer stimulation delays clearance 2 to 3 weeks (24).

In the present study, we used a real-time, RT-PCR assay for the first time to assess the presence of bacteria after experimental infections. This assay is highly specific and sensitive for detecting *E. chaffeensis* (48). It allowed us to make a more sensitive quantitation of rickettsemia after the experimental challenges with *E. chaffeensis* compared to previous studies. The delayed clearance in liver and spleen compared to the peritoneum is consistent with our previous observations (24, 25). Our hypothesis for bacteria persisting longer in the liver and spleen compared to the peritoneum is supported by the observations that ISE6 culture was drained to the spleen and liver, and it takes the activation of acquired immunity to clear those organs.

An interesting observation made during the present study was that mice infected with bacteria from both treatment
groups relapsed with a periodicity of about 3 days. Although considerably longer, cyclical rickettsemia has been reported for two related tick-transmitted rickettsiales of the genus *Anaplasma*. *Anaplasma marginale* and *A. platis* rickettsemia fluctuates in vertebrate hosts acquiring infections by both natural and experimental methods (23, 28, 33). French et al. found differences in expressed outer membrane proteins during each wave of rickettsemia (23). To our knowledge, this is the first study to demonstrate cyclical rickettsemia for an *Ehrlichia* species. However, it is still not clear whether or how antigen expression contributes to the oscillations. Although we have not reported the cyclical pattern of clearance in the past, a review of our older data set reveals hints of the same type of response (24).

The host cyclical immune response to *E. chaffeensis* corresponds with the peaks of rickettsemia. The oscillatory nature of bacterial clearance, cellular activation, and cytokine secretion after infection most likely reflects the natural resonance of intracellular responses (1, 34, 35) and/or the manifestation of amplification loops of the expanding immune response (6, 36). Wodarz et al. (57) called this process “dynamic elimination.” In that model, the length of the oscillatory pulse was dependent on the magnitude of the virus infection and the strength of the cytoplasmic T-cell response. In an *E. chaffeensis* infection, it is easy to envision an initial nonspecific response to the bacteria, followed by the activation of more specific Th1 effectors (24). The generation of helper T cells and their feedback on macrophages, which form the core response against the intracellular pathogen, represent one of the quintessential demonstrations of cytokine amplification of the immune response. Th1 cells secrete IFN-γ, which upregulates MHCII expression on macrophages, which leads to enhanced antigen presentation. Indeed, when Wigginton and Kirschner modeled the clearance of *Mycobacterium tuberculosis*, another macrophage-tropic intracellular pathogen, they predicted oscillations in the clearance, cytokines secreted, macrophages isolated from alveoli and macrophage activation (54). Our data are consistent with their predictions. The recurring rickettsemia in our experiments may represent transitions in the host immune response that allow opportunities for the bacteria to reemerge.

We measured CD62L and CD44 to measure the development of effector memory (37, 44). The development of effector memory cells (CD44+CD62L-) peaked just after bacteria reemerged during infection regardless of the source of bacteria. This point in time might be when the initial innate immune response is waning and the adaptive response is developing, but not fully functional. The kinetics of the peritoneal macrophage secretion of NO would support this hypothesis. The detectable NO response peaked at day 10 and then waned until day 17, when there was a second wave of activity. Interestingly, IL-6 also fluctuated once it was produced at peak amounts around day 20, and its production corresponded with the final waves of infection.

Our inability to detect IL-2 or IFN-γ after 7 days of infection with *E. chaffeensis* from either cell line appears to be inconsistent with the growing evidence that IFN-γ and a Th1-type immune response plays a role in host resistance to monocytic *ehrlichiosis* (5, 18, 24, 25, 31). We did detect Th1 cytokines in plasma within 10 h after infection, and we have observed transcriptional activation of a number of Th1-associated genes immediately after infection with both types of bacteria (data not shown). Therefore, we feel that the difference between our system and other investigations has to do with when we are looking for the cytokine. We are measuring the host response at a time when it is transitioning from early Th1 responses to the production of Th2-type cytokines later in the response (32). It is also important to point out that Bitsaktsis et al. (5) demonstrated a Th1 response to *E. chaffeensis* by showing IFN-γ production at the single-cell level. Their technique allows for the detection of small amounts of cytokine that might be missed when screening plasma or culture supernatants the way we did.

There was very low ex vivo secretion of TNF-α by spleen cells or peritoneal macrophages from mice injected with either type of bacteria and culture of cells in vitro. This was similar to our recent study on experimental infections in several mouse strains (25). Other researchers have suggested that TNF-α is necessary for the resolution of *Ixodes ovatus Ehrlichia* infection (5, 18). However, different methods were used to make the inference. For example, these methods either used antibody to deplete cytokine or knockout animals to deduce an impact. Alternatively, Bitsaktsis et al. measured TNF-α after a 4-day in vitro sensitization assay which makes it possible to detect small amounts of cytokine (5). It is also possible that the difference is due to the fact that *Ixodes ovatus Ehrlichia* is more pathogenic than *E. chaffeensis* (31).

The lower concentrations of cytokine production by mice challenged with tick cell-grown bacteria and the distinct differences in the development of the humoral response in those mice have significant implications about the model system currently being used by investigators studying *E. chaffeensis*. The poorer responses in the absence of tick saliva suggest that host cell environment (macrophage versus tick cells) plays a role in activating the host response. The distinct kinetics of antibody production and the specificity of the antibodies made against *E. chaffeensis* antigens by mice from the two treatment groups are also intriguing. It is not clear why tick cell-grown bacteria induce a more prolonged IgG response. Our in vitro and in vivo analyses of the changes in gene expression suggest that the transition from tick-specific antigen expression to macrophage-specific antigen expression is a very slow process for *E. chaffeensis*. The delayed clearance of tick cell-derived *E. chaffeensis* with distinct immune responses may reflect the host’s inability to alter its immune response to the changing antigenic makeup of the bacteria. This differential protein expression might be one of the mechanisms used by *E. chaffeensis* to persist longer in vertebrate hosts. A number of groups have used DH82 cells to produce *E. chaffeensis* for in vivo experimental challenges, including our own (24, 25). Perhaps tick cell-derived bacteria would be a better source of inoculum to assess the true host immune responses against *E. chaffeensis*. In addition, although it appears that there is no difference in the immune response to various isolates of *E. chaffeensis* (25), it is not clear whether isolates other than the Arkansas strain of *E. chaffeensis* will behave in a similar fashion. Additional studies will be necessary to resolve this issue. Nevertheless, it is clear that much more remains to be determined about this intriguing intracellular pathogen.
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