Rickettsial infection in murine models activates an early anti-rickettsial effect mediated by NK cells and associated with production of gamma interferon

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Abstract. Natural killer (NK) cell activity was significantly increased on days 2–6 of infection in the Rickettsia conorii-infected C3H/HeN mice and on day 2 in the Rickettsia typhi-infected C57BL/6 mice. Depletion of NK cell activity utilizing anti-NK1.1 monoclonal antibody enhanced the susceptibility of normally resistant C57BL/6 mice to infection with R. typhi, and depletion of NK cell activity with antibody to asialo GM1 enhanced the susceptibility of C3H/HeN mice to infection with R. conorii. Serum gamma interferon was increased in R. conorii-infected C3H/HeN mice compared with NK cell-depleted, infected mice during the early course of infection. Additionally, the NK cell activating cytokine IL-12 was elevated in the sera of infected mice during the time period representing enhanced NK cell activity compared with uninfected mice. Thus, it appears that NK cells contribute to the early anti-rickettsial immune response, likely via a mechanism involving gamma interferon.

INTRODUCTION

Rickettsial diseases are notorious for their ability to threaten the lives of healthy persons. The most pathogenic rickettsiae are Rickettsia rickettsii, Rickettsia prowazekii, R. conorii, and Rickettsia typhi, the etiologic agents of Rocky Mountain spotted fever, epidemic louse-borne typhus, boutonneuse fever, and murine typhus, respectively. Virulence determinants in rickettsiae have yet to be identified, and the host factors underlying increased susceptibility in older age groups and males have not been elucidated. Studies of experimentally infected animals have identified some of the critical components of the host response, including gamma interferon (IFN-γ), tumor necrosis factor-α (TNF-α), and CD8 cells. However, the only rickettsial factor that has been implicated is the infectious dose. Larger doses result in greater numbers of foci of rickettsia-infected endothelium throughout the body, including vital organs such as the brain and lungs. Growth of rickettsiae in these more numerous foci reaches a lethal level of injury before the immune system can control the rickettsial growth.

Innate immune responses can limit rickettsial growth in a naive host prior to development of specific anti-rickettsial immunity. A preliminary report suggested an antirickettsial role for natural killer (NK) cells. We therefore evaluated the role of NK cells in two animal models of rickettsial infection. The R. conorii-C3H/HeN mouse model was used because it is the best available and most extensively investigated model for the study of immunity and pathogenesis of spotted fever group (SFG) rickettsiosis. Because the NK cells of C3H/HeN mice do not express any known specific cell membrane protein, antibodies to asialo GM1 were used at a concentration that depletes principally NK cell activity. Because NK cells of C57BL/6 mice have a specific protein on their surface that allows their depletion by anti-NK1.1 monoclonal antibody, we desired to study the effects of NK cells on rickettsial infection in this mouse strain. As C57BL/6 mice are highly resistant to infection with R. conorii, we infected these mice with R. typhi. Our results demonstrate an association between NK cell activation, elevated IFN-γ levels and reduction in rickettsial titers in infected organs.

MATERIALS AND METHODS

Rickettsiae. Rickettsia conorii (Malish 7 strain) is a human isolate from South Africa with an unknown number of passages in the yolk sacs of embryonated chicken eggs. Rickettsia typhi (Wilmington strain) is a human isolate with four embryonated chicken egg passages, eight Vero cell passages, and one plaque purification in Vero cells. Both strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA; VR-613, and VR-144, respectively).

Cell lines. All cell lines were obtained from ATCC. YAC-1 cells (ATCC#TIB160) derived from a murine Moloney-virus-induced T-lymphocyte lymphoma were used for NK cell cytotoxic activity assays. Vero cells (ATCC CCL 81) derived from kidneys of African green monkeys were used in rickettsial plaque assays. PK 136 (ATCC #HB-191), a hybridoma cell line, secretes an IgG2 monoclonal antibody (mAb) directed against the surface antigen, NK1.1, found on NK cells from C57Bl/6 mice, but not BALB/c or C3H mouse strains.

Antibodies. Rabbit anti-asialo GM1, antiseraum (WAKO Pure Chemical Industries Ltd., Osaka, Japan) containing IgG, IgA, and IgM immunoglobulin isotypes reacts with mouse NK cells, monocytes, and T-lymphocytes, and depletes mainly NK cell activity when given intravenously or intraperitoneally at the dose employed. Anti-NK1.1 mAb was prepared as ascites in pristane-primed BALB/c mice inoculated intraperitoneally with PK 136 hybridoma cells, and was enriched for immunoglobulin by precipitation with a half saturated solution of ammonium sulfate followed by dialysis with phosphate buffered saline (pH 7.2).

NK cell cytotoxic activity assays. Effector cells. Murine splenocytes were separated into a single cell suspension in 10 ml Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS. The concentration of mononuclear cells was adjusted to 1 × 10⁶/ml.

Target cells. 5 × 10⁴ YAC-1 cells were labeled with 100 μCi sodium chromate [⁵¹Cr] (Amersham, Piscataway, NJ) for 60 minutes at 37°C with occasional gentle shaking. After labeling, the cells were washed three times in DMEM containing 10% FBS, resuspended in 1 ml of medium, and assessed for viability by trypan blue dye exclusion. Labeled
YAC-1 cells were resuspended in DMEM with 10% FBS at a concentration of 1 × 10^7 cells/ml.

Murine NK cell activity was measured using a 4 hr ^51Cr release assay. Splenocytes were suspended in DMEM with 10% FBS at an initial concentration of 1 × 10^7 cells/ml. Three-fold serial dilutions were made, and 100 μl of effector cell suspension plus 100 μl of labeled YAC-1 target cell suspension were dispensed into duplicate wells of a 96-well microtiter plate with an effector:target ratio ranging from 0.3 to 100:1. Maximum release values were determined by suspending labeled targets in 200 μl of 2% NP-40. Plates were centrifuged at 190 g for 1 min at 25°C, and then incubated at 37°C for 4 hr. The plate was centrifuged again at 190 g for 1 min at 25°C; then 25 μl of the supernatant was removed and measured for ^51Cr release using a γ-counter (Top Count, Packard Instrument Co., Downers Grove, IL). NK cell activity was measured as % cytotoxicity according to the following equation:

\[
\% \text{ cytotoxicity} = \frac{(\text{cpm experimental} - \text{cpm spontaneous})}{(\text{cpm maximum} - \text{cpm spontaneous})} \times 100
\]

Experimental design. Mice were infected intravenously with a sublethal dose of either R. conorii (a SFG rickettsia) or R. typhi (a TG rickettsia). PBS-inoculation was employed for uninfected controls. Each of three repeated SFG rickettsiosis experiments utilized 18 mice divided into three groups: group 1, R. conorii-infected, NK-depleted; group 2, R. conorii-infected, sham-depleted; group 3, uninfected controls. R. conorii-infected C3H/HeN mice were either depleted of NK cells or underwent sham-depletion. NK cell depletion was achieved by intravenous injection of rabbit anti-asialoGM1, in a 200 μl volume prepared by mixing 50 μl of Ab containing 7.8 mg of total protein with 150 μl of phosphate buffered saline. Ab was injected intravenously two days before rickettsial inoculation and intraperitoneally 1 day after rickettsial inoculation according to the instructions of the manufacturer.10,11 Sham-depletion involved injecting the same volume of normal rabbit serum by the same routes at the same time intervals. Each of the three repeated TG rickettsiosis experiments used 18 mice divided into three groups: group 1, R. typhi-infected, NK-depleted; group 2, R. typhi-infected, sham-depleted; group 3, uninfected controls. Similarly R. typhi-infected C57BL/6 mice were depleted of NK cells by the same regimen of timing and routes of inoculation of anti-NK1.1 monoclonal antibody-containing ascites fluid (200 μg of total protein).16

Two mice per group in each experiment were sacrificed on days 2, 4, and 6 after infection, and spleens were removed to determine NK cell activity by YAC-1 cell cytotoxicity assay, and to titrate the infectious rickettsial content by plaque assay as previously described.9 Sera were collected from C57BL/6 and C3H/HeN mice for assays of IL-12 and IFN-γ concentrations by enzyme immunoassay kits (Genzyme, Cambridge, MA) according to the manufacturer’s instructions. BALB/c mice congenic for the Cmv1 gene conferring resistance to murine cytomegalovirus (MCMV) (generously provided by Dr. Wayne Yokoyama, Washington University, St. Louis, MO) were infected with Rickettsia sibirica.12 The maintenance and care of experimental animals complied with the National Institutes of Health guidelines for the humane use of laboratory animals.

**Effect of rickettsial infection on NK cell activity.** In two different mouse models of disseminated endothelial-target rickettsial infection, a spotted fever group (R. conorii) and a typhus group (R. typhi) rickettsiosis, NK cell activity was increased during the early part of the course of infection. Activation of NK cells in spleens from infected sham-depleted C3H/HeN mice was significantly higher than in the NK cell-depleted mice on days 2 (P = 0.004), 4 (P = 0.001), and 6 (P = 0.008) (Figure 1). The peak of NK cell activity
A more prolonged effect was observed in C3H/HeN mice, which are intrinsically more susceptible to rickettsial infection. C3H/HeN mice depleted of NK cells by antibodies to the surface antigen, asialoGM1, were susceptible to infection with R. conorii. NK cell depletion in the R. conorii-mouse model resulted in increased rickettsiae in the spleens of NK cell-depleted mice on days 4 and 6 after inoculation (Figure 3).

Effect of depletion of NK cells on IFN-γ levels in rickettsial infection. Early in the immune response R. conorii-infected, sham-depleted C3H/HeN mice developed higher levels of serum IFN-γ than NK cell-depleted infected animals (Figure 4). The difference in serum IFN-γ concentrations was absent later in the course of infection, after the adaptive immune response developed. There was no significant difference in the serum IFN-γ concentrations between depleted and sham-depleted C57BL/6 mice. IFN-γ was not detected in sera from uninfected controls.

Effect of rickettsial infection and NK cell depletion on serum IL-12 levels. Serum IL-12 (p70) levels were significantly increased (171.0 ± 11.2 pg/ml) in sham-depleted C3H/HeN mice on day 2 of infection compared with 0 ± 0 pg/ml in uninfected mice and decreased to lower concentrations on day 4 (10.2 ± 6.0 pg/ml) and day 6 (8.2 ± 8.4 pg/ml) of infection (Figure 5A). IL-12 levels in NK cell-depleted C3H/HeN mice were lower on day 2 (43 ± 36 pg/ml), and were similar to non-depleted mice on day 4 (28 ± 13 pg/ml) and day 6 (18 ± 18 pg/ml). The concentration of

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**TABLE 1**

<table>
<thead>
<tr>
<th>Day after inoculation with R. typhi</th>
<th>Sham-depleted group</th>
<th>NK-cell depleted group</th>
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<tbody>
<tr>
<td></td>
<td>NK1.1/CD3^+ cells x 10^6 per spleen</td>
<td>NK1.1/CD3^+ cells x 10^6 per spleen</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1.4 ± 0.4</td>
<td>4.9 ± 0.2†</td>
</tr>
<tr>
<td></td>
<td>0.58 ± 0.04</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.73 ± 0.2*</td>
<td>2.7 ± 0.6*</td>
</tr>
<tr>
<td></td>
<td>0.26 ± 0.1</td>
<td>0.52 ± 0.02‡</td>
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</tbody>
</table>
| Normal uninfected control          | 2.1 ± 0.3           | 0.53 ± 0.4            | 5.5 ± 0.2             | 0.5 ± 0.2

* In comparison with the sham-depleted mice on the same day after infection with R. typhi, the quantity of cells in the anti-NK1.1-treated group was significantly decreased (P < 0.05).
† In comparison with normal uninfected mice, the NK cells were increased significantly in the rickettsiae-infected group (P < 0.05).
‡ Treatment with anti-NK1.1 MAb induced a decrease of NK1.1/CD3^+ cells on the day 4 after R. typhi infection (P < 0.05) compared with sham-depleted, infected mice.
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ANTI-RICKETTSIAL ACTIVITY OF NK CELLS

DISCUSSION

The role of NK cells has been investigated in viral infections, facultative intracellular bacterial infections in which macrophages and hepatocytes are the target cells, and with chlamydia in which epithelial cells are the principal target. The current study shows the importance of NK cells in obligate intracellular infection of endothelial cells by rickettsiae. In murine models of herpes simplex virus 1 and MCMV, NK cells are important in host defense. The MCMV model and the C3H/HeN mouse-R. conorii model show an increase in IL-12 secretion and in NK cell activation. Resistance of C57BL/6 mice to MCMV is associated with the Cmv1 gene, which regulates MCMV replication and is tightly linked to the NK cell gene complex. Resistance is abrogated by depletion of NK cells. In C57BL/6 mice, NK cell antiviral activity is mediated by IFN-γ in the liver and by a perforin-dependent mechanism in the spleen. Because of the sensitivity of BALB/c mice, to both SFG rickettsiae and MCMV, the resistance of C57BL/6 mice to both agents, and the similar importance of NK cells in these infections, we performed preliminary experiments to examine the role of the Cmv1 resistance gene in experimental R. sibirica infection of congenic BALB/c mice carrying Cmv1 instead of the normally present Cmv1. Rickettsia sibirica infection was studied because BALB/c mice are more susceptible to this rickettsia than to R. conorii or R. typhi. All of the BALB/c Cmv1 congenic mice died of overwhelming rickettsial disease. At the same dose of rickettsiae, the C57BL/6 mice neither became ill nor died. Thus, Cmv1 did not confer the ability to resist R. sibirica infection, implying that this gene does not control the critical NK cell function for resistance to rickettsial infection.

The role of NK cells in rickettsial infections is different from other intracellular bacterial infections, due in part to the endothelial cell target of these organisms. In mice infected with the facultatively intracellular bacteria, Salmonella and Listeria, NK cells are activated and serve as a host defense similar to that against rickettsiae. Resistance to Listeria is mediated by IFN-γ activated macrophages, and IFN-γ is produced by NK cells that are activated by IL-12.

Similarly, we propose that infected endothelial cells activated by TNF-α, presumably from macrophages, and by IFN-γ produced by IL-12 activated NK cells, inhibit the growth of intracellular rickettsiae. Although pulmonary infection of mice with the mouse pneumonitis strain of Chlamydia trachomatis activates NK cells in the lung and spleen, there was no effect on the chlamydial growth in the lung. The NK cell response to intravaginal C. trachomatis infection is rapid with NK cells appearing in the genital tract at 12 hr and peaking at 2 days. The NK cells appear to be the source of the early IFN-γ production in the chlamydial model, as both NK cells and the IFN-γ response were abrogated by treatment with antibodies to asialo-GM1. In contrast to the models of rickettsiosis in which rickettsial titers were increased in NK cell-depleted animals, chlamydial titers were not significantly different in NK cell-depleted mice.

Thus, there is substantial evidence to support a role for NK cell production of IFN-γ as a host cell-activating cytokine in rickettsioses. Indeed, mouse endothelial cells activated with IFN-γ and TNF-α kill intracellular rickettsiae by a NO-dependent mechanism. On the other hand, our studies have provided no evidence of an NK cell-mediated cytotoxic effect on rickettsiae-infected cells. A signal for NK cell activation is the lack of MHC Class I molecules on the target cell membrane.
Class I molecules (Diaz CM and others, unpublished data). In preliminary experiments, SVEC-10 endothelial cells infected by rickettsiae did not differ from uninfected endothelial cells in their susceptibility to cytotoxicity mediated by splenic NK cells (Billings AN, unpublished data). IFN-γ production by NK cells is a more likely mechanism of NK cell activity than NK cell-mediated cytoxicity. Owing to the importance of the critical level of the rickettsial load in the severity of injury by rickettsial infection, NK cell damping the growth of rickettsial early in the course of infection has the potential to affect the ultimate outcome of infection.3

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