Critical Role of Cytotoxic T Lymphocytes in Immune Clearance of Rickettsial Infection

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Cytotoxic T-lymphocyte (CTL) activity developed against the major infected target cells of rickettsial infections, endothelial cells and macrophages. Spleen cells from mice immune to Rickettsia conorii exerted specific major histocompatibility complex (MHC) class I-matched CTL activity against R. conorii-infected SVEC-10 endothelial cells, with peak activity on day 10. Similarly, spleen cells from Rickettsia australis-immune mice exerted specific CTL activity against an R. australis-infected macrophage-like cell line. Gamma interferon (IFN-γ) gene knockout mice were more than 100-fold more susceptible to R. australis infection than wild-type C57BL/6 mice. MHC class I gene knockout mice were the most susceptible, more than 50,000-fold more susceptible to a lethal outcome of R. australis infection than wild-type C57BL/6 mice. These results indicate that CTL activity was more critical to recovery from rickettsial infection than were the effects of IFN-γ. The observation that perforin gene knockout mice were more than 100-fold more susceptible than wild-type C57BL/6 mice indicates that perforin-mediated activity accounts for a large component, but not all, of the CTL-mediated antirickettsial effect. CTL activity was expressed by immune CD8 T lymphocytes. Adoptive transfer of immune CD8 T lymphocytes from IFN-γ gene knockout mice into R. australis-infected IFN-γ gene knockout mice dramatically reduced the infectious rickettsial content in the organs, confirming that CD8 T lymphocytes provide immunity against rickettsiae besides that provided by the secretion of IFN-γ. CTLs appear to be crucial to recovery from rickettsial infection.

Rickettsial diseases, including the life-threatening infections caused by Rickettsia rickettsii, R. conorii, R. sibirica, R. australis, R. prowazekii, and R. typhi, are acute infectious diseases (3, 21, 24, 26, 29, 31, 32). Either patients control the growth and spread of the obligately intracellular bacteria, often with the assistance of timely administered bacteriostatic antimicrobial drugs, or they die. The immune mechanisms by which the host controls rickettsial infection are highly dependent on cellular immunity, with a critical role identified for T lymphocytes (6, 7, 12, 13, 16, 23). An important contribution to protective immunity involved in the control of rickettsial infection is provided by cytokines, especially gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α). These cytokines act in concert to activate endothelial cells, the major target cells of rickettsial infections, as well as other minor target cells to kill intracellular organisms via a nitric oxide synthesis-dependent mechanism (5, 27, 28). The sources of these protective cytokines are hypothesized to be the T lymphocytes and macrophages that infiltrate the perivascular space surrounding the vessels with infected endothelium (7, 9).

Evaluation of the roles of the T-lymphocyte subsets revealed that CD8 T lymphocytes are crucial to the clearance of R. conorii in a disseminated endothelial target mouse model of infection (6, 7, 25). Although adoptive transfer of either CD4 or CD8 immune T lymphocytes controlled the infection and led to survival, depletion of only CD8 T lymphocytes altered the outcome of infection. CD4 and CD8 T lymphocytes are both potential rich sources of IFN-γ, which could have activated endothelial rickettsicidal activity and tipped the balance in favor of survival. However, depletion of CD4 cells had no observed effect on the course or outcome of infection. In contrast, CD8-depleted mice infected with an ordinarily sublethal dose of R. conorii remained persistently infected and ill, and a high proportion of these animals died of uncontrolled rickettsial infection and its consequent pathologic effects.

The presently reported investigations were undertaken to determine the mechanis(s) by which CD8 T lymphocytes control rickettsial infection. Because cytotoxic T-lymphocyte (CTL) activity had been observed among a population of immune spleen cells exposed to major histocompatibility complex class I (MHC-I)-matched fibroblasts (L-929 cells) infected with R. typhi, we hypothesized that rickettsia-infected endothelial cells, and to a lesser extent rickettsia-infected macrophages, are the targets of CTL-mediated clearance of the infection (20). The experiments reported here employed R. conorii- and R. australis-infected endothelial cells and macrophages as targets to assess CTL activity in vitro, endothelial cell target animal models, purified CD8 T-lymphocyte populations, MHC-I, IFN-γ, and perforin gene knockout mice, and adoptive transfer of CD8 T lymphocytes to evaluate the roles of CTL activity and cytokine production by CD8 T lymphocytes in immunity to spotted fever group (SFG) rickettsiae.

MATERIALS AND METHODS

Rickettsiae. R. australis (Cutlack strain) was a gift from C. Pretzman (Department of Health Laboratory, Columbus, Ohio) and was passaged three times in Vero cells (ATCC CCL81; American Type Culture Collection, Manassas, Va.) and four times in embryonated chicken yolk sacs in our laboratory. The 10% yolk sac suspension stock contained 10⁶ PFU per ml. R. conorii (Malish 7 strain), a human isolate from South Africa, was obtained from the American Type Culture Collection (ATCC VR-613). The 10% yolk sac suspension stock contained 4 × 10⁶ PFU per ml.
Mice, C3H/HeN (H-2k) mice were purchased from Harlan Sprague-Dawley (Indianapolis, Ind.) BALB/c (H-2b) and C57Bl/6 (H-2b) mice and H-2k, IFN-γ, and perforin gene knockout mice (with a C57Bl/6 background) were all purchased from Jackson Laboratory (Bar Harbor, Maine). All mice were 6 to 8 weeks of age and were housed in specific pathogen-free conditions.

Determination of the relative resistance of wild-type C57BL/6 mice MHC-I gene knockout mice, IFN-γ gene knockout mice, and perforin gene knockout mice to R. australis infection. Because wild-type C57Bl/6 mice are highly resistant to R. conorii, R. australis was used as the SFG rickettsial agent to investigate the relative importance of IFN-γ and CTLs in IFN-γ gene knockout and MHC-I gene knockout mice, respectively. The C57Bl/6 mouse-R. australis model is a valuable tool of human SFG rickettsiosis because it has disseminated endothelial infection. However, R. australis is more invasive than R. rickettsii in humans and than R. conorii in C3H/HeN mice. Mice (30 wild-type C57BL/6, 30 IFN-γ gene knockout, 33 MHC-I gene knockout, and 22 perforin gene knockout) were inoculated intravenously with different concentrations of R. australis and observed daily for illness and death.

RESULTS

Increased susceptibility of MHC-I or IFN-γ gene knockout mice to R. australis infection compared to that of wild-type C57Bl/6 mice. The C57BL/6 strain is among the most highly resistant of mouse strains to rickettsial infection (1). The LD<sub>50</sub> of R. australis for wild-type C57Bl/6 mice was 3.6 × 10<sup>4</sup> PFU. In IFN-γ gene knockout mice, the LD<sub>50</sub> of R. australis was 195 PFU, indicating that susceptibility of the IFN-γ gene knockout mice was more than 100-fold greater than that of wild-type C57BL/6 mice. The MHC-I gene knockout mice with the C57BL/6 background were even more highly susceptible to rickettsial infection. The LD<sub>50</sub> was as little as 0.5 PFU per mouse (more than 50,000-fold greater than for wild-type C57BL/6 mice), as shown in Table 1. The MHC-I gene knockout mice infected with R. australis died between days 12 and 19 after rickettsial inoculation. All the ill mice uniformly showed severe subcutaneous edema and pleural and peritoneal effusions.

Histopathology and immunohistologic observations of R. australis. C57BL/6 mice infected intravenously with R. australis developed disseminated infection involving endothelial cells in the brain, the appropriate target since it is a critical target in human rickettsioses. There were also prominent infections in the lung, liver, testis, epididymus, adrenal fat, and kidney and milder infection in the spleen. There was only focal rickettsial infection extending to the peritoneal lining. The histopathologic lesions included vasculitis with venous thrombosis involving the testicular adrenal fat pad.

On day 16, in the R. australis-infected MHC-I gene knockout mice, persistent rickettsial infection, reflecting failure to control rickettsial growth and eliminate the infectious organisms, was observed in vascular foci in the brain (Fig. 1) and subarachnoid space, and extensive spread of the infection to the
inflammatory exudate in the peritoneal and pleural cavities was also observed. Immunohistochemical studies also revealed a disseminated vasculocentric infection with many rickettsiae in the endothelia and perivascular cells of perforin gene knockout animals sacrificed on day 12 postinfection and in IFN-γ gene knockout mice sacrificed on day 6 postinfection. The rickettsiae were observed mainly in spleen, liver, and lung tissues, with lesser amounts in brain and kidney tissues. The lesions in the infected MHC-I, IFN-γ, and perforin gene knockout mice included mononuclear cell-rich vasculitis and perivasculitis, interstitial pneumonitis, hepatic granulomas, cell death of hepatocytes, mononuclear cell-rich peritonitis, and submesothelial inflammation.

Identification and kinetics of CTL activity of T lymphocytes of rickettsia-infected mice. CTL activity was observed in both the C3H/HeN-R. conorii and the BALB/c-R. australis animal models of rickettsial infection. In the C3H/HeN mouse model, the CTL activity was detected on days 9 to 12 after R. conorii infection. At its peak on day 10, this CTL activity was detected only in rickettsia-infected, MHC-matched target cells, not in uninfected target cells or with MHC-unmatched immune effector cells (BALB/c mice immunized with R. conorii) (Fig. 2B). During the first week of infection, the cytotoxicity studies revealed a relatively high nonspecific reaction (Fig. 2A). The specific CTL activity had decreased significantly at 3 weeks after infection (Fig. 2C). In the R. australis-infected BALB/c mouse model, the highest specific CTL activity was observed on day 12 after rickettsial infection (Fig. 3). The results for CTL activity with purified CD8 T lymphocytes were similar to those with unpurified T lymphocytes.

CTL activity of CD8 T cells plays an important role in the control of the rickettsial content in R. australis-infected IFN-γ gene knockout mice. CD8 T lymphocytes have two major functions: CTL activity and production of cytokines, such as IFN-γ. It has been established that IFN-γ plays an important role in controlling rickettsial infection (5, 27, 28). This experiment was designed to determine whether the remarkable susceptibility of MHC-I gene knockout mice was due to a lower level of IFN-γ production or a lack of CTL activity. To study the role of CTL activity in conferring immunity against rickettsial infection, we chose IFN-γ gene knockout mice to exclude the possibility of immune function induced by IFN-γ. The adoptively transferred immune or normal CD8 T cells were also derived from IFN-γ gene knockout mice. All three groups of mice became ill on day 5 whether they were subjected to adoptive transfer with immune or nonimmune CD8 T lymphocytes (10^7/mouse) or were not subjected to adoptive transfer with any cells after inoculation with 5 LD_{50} of R. australis. On day 6, one of the mice in the non-adoptive-transfer group was moribund. As shown in Table 2, mice that were subjected to transfer with immune CD8 T lymphocytes from IFN-γ gene knockout mice had dramatically lower rickettsial infectivity titers than the mice given normal CD8 T lymphocytes or the group that received no cells.

Role of perforin in antirickettsial CTL activity. Among the perforin gene knockout mice, the LD_{50} of R. australis was 31 PFU. Thus, although these mice were much more susceptible to R. australis than immunocompetent C57BL/6 mice, they were not as susceptible as the MHC-I gene knockout mice. It can be concluded that perforin is an effector of antirickettsial activity in this model but that it does not account for all of the CTL activity directed against R. australis-infected cells.

Investigation of the role of apoptosis mediated by CD8 T lymphocytes adoptively transferred from immune or naive mice into R. australis-infected animals. The number of apoptotic cells in the liver 8 h after infection with R. australis and adoptive transfer of naive and immune CD8 T cells was significantly different between the two groups (0.6 apoptotic cell/50 HPF in the naive CD8 recipients versus 23.6 apoptotic cells/50 HPF in the immune CD8 recipients, P < 0.001). The number of apoptotic cells decreased at 18 and 24 h but the differences between the groups receiving nonimmune and immune CD8 T lymphocytes were still statistically significant (3.6 versus 10.4 apoptotic cells/50 HPF, respectively, at 18 h, P = 0.002; 4.6 versus 7.8 apoptotic cells/50 HPF, respectively, at 24 h, P < 0.017).

DISCUSSION

This report established for the first time that rickettsia-infected endothelial cells and macrophages are targets of CTL activity. The pioneering work of Rollwagen and colleagues had

TABLE 1. Median LD_{50} of R. australis in wild-type C57BL/6 and MHC-I, IFN-γ, and perforin gene knockout mice

<table>
<thead>
<tr>
<th>Strain of mice</th>
<th>Dilution of R. australis</th>
<th>No. of survivors/total no. of mice</th>
<th>LD_{50} (PFU/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC-I KO*</td>
<td>10^{-5}</td>
<td>0/6</td>
<td>10^{-6.54} (0.5)</td>
</tr>
<tr>
<td></td>
<td>10^{-6}</td>
<td>4/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10^{-7}</td>
<td>8/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10^{-8}</td>
<td>7/7</td>
<td></td>
</tr>
<tr>
<td>IFN-γ KO</td>
<td>10^{-3}</td>
<td>0/10</td>
<td>10^{-3.71} (195)</td>
</tr>
<tr>
<td></td>
<td>10^{-4}</td>
<td>7/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10^{-5}</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>Perforin KO</td>
<td>10^{-3}</td>
<td>0/8</td>
<td>10^{-4.51} (31)</td>
</tr>
<tr>
<td></td>
<td>10^{-4}</td>
<td>1/7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10^{-5}</td>
<td>5/6</td>
<td></td>
</tr>
<tr>
<td>Wild-type C57BL/6</td>
<td>10^{-1}</td>
<td>3/10</td>
<td>10^{-1.44} (3.6 × 10^6)</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>8/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>10/10</td>
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</tr>
</tbody>
</table>

*KO, knockout.
previously demonstrated the existence of T-lymphocyte-mediated CTL activity against MHC-I-matched, *R. typhi*-infected fibroblasts (20). In addition, the present report strongly suggested for the first time that CTL activity is a more critical function of CD8 T lymphocytes in the clearance of rickettsiae than the production of IFN-γ is. It is important to consider that CD4 and NK cells can secrete IFN-γ, possibly replacing the function of IFN-γ production. However, only CD8 T lymphocytes provide MHC-I-restricted CTL activity.

The specific CTL activity of immune T lymphocytes, particularly the CD8 subset, derived from two different animal models of SFG rickettsial infection was directed against *R. conorii*-infected, MHC-I-matched endothelial cells and against *R. australis*-infected, MHC-I-matched macrophages. These cell types are those which are infected in human rickettsioses and in these mouse models. That they are susceptible to CTL activity is an important consideration implying that MHC-I molecules are not significantly downregulated during rickettsial infection and that particular rickettsial epitopes are recognized by CD8 T lymphocytes in the context of MHC-I molecules. Indeed, MHC-I molecules are not downregulated in *R. conorii*-infected SVEC-10 mouse endothelial cells as assayed by flow cytometry (M. Diaz, J. P. Olano, and D. H. Walker, unpublished data). CTL activity is a potential mechanism of elimination of rickettsia-infected cells. Consistent with this hypothesis is the persistence of *R. conorii* infection in mice depleted of CD8 T lymphocytes (7). If the effect of CTLs on infected cells is induction of apoptosis, the rickettsia-containing apoptotic bodies could hypothetically be phagocytosed rapidly by adjacent cells (e.g., perivascular macrophages) and degraded in their phagolysosomes.

The demonstration that apoptosis is significantly greater in the livers of *R. australis*-infected mice subjected to adoptive transfer with immune CD8 T lymphocytes than in infected mice subjected to adoptive transfer with naïve CD8 T lymphocytes suggests that infected cells are eliminated by apoptosis associated with CTL activity. Further evidence supporting a role for apoptosis induced by CD8 T lymphocytes was derived from investigation of tissues performed in a previously reported study involving depletion of CD8 T lymphocytes (7). *R. conorii*-infected C3H/HeN mice depleted of CD8 T lymphocytes had significantly less apoptosis than infected, sham-depleted animals in the livers (4.8 apoptotic cells/50 HPF in the CD8-depleted animals versus 28.6 apoptotic cells/50 HPF in the sham-depleted animals, *P* < 0.001) and in the lungs (7.4 apoptotic cells/50 HPF versus 14.6 apoptotic cells/50 HPF, respectively; *P* = 0.001) on day 5 of infection (J. P. Olano, D. H. Walker, and H.-M. Feng, unpublished data).

Moreover, the kinetics of the CTL activity paralleled rickettsial clearance in the well-studied *R. conorii*-C3H/HeN mouse model with the demonstration of CTLs and evidence for an effect on rickettsial growth on day 6, peak of CTL activity and clearance of *R. conorii* on day 10, and downregulation of CTLs after rickettsiae are no longer present on day 15 (25). The C57BL/6-*R. australis* model showed a somewhat prolonged peak of the CTL activity, correlating with greater perivascular invasiveness of *R. australis* and the consequently longer period required for clearance.

The studies with MHC-I and IFN-γ gene knockout mice revealed that CD8 T lymphocytes contribute to protective im-
Immunity to SFG rickettsiae by both CTL activity and production of IFN-γ. It is remarkable that mice with the C57BL/6 background associated with resistance to rickettsial infection appeared to suffer a fatal course of infection when inoculated with a dose that approximated a single PFU of rickettsiae in the absence of MHC-I molecules. Moreover, adoptive transfer of R. australis-immune CD8 T lymphocytes incapable of producing IFN-γ into IFN-γ gene knockout mice provided protective immunity as demonstrated by marked reduction in rickettsial content in the lungs, liver, and spleen, most likely due to a CTL-dependent mechanism. The importance of IFN-γ in immunity to rickettsiae is emphasized by the observation that the IFN-γ gene knockout mice with the C57BL/6 background were more than 100 times more susceptible to a lethal outcome of R. australis infection than wild-type C57BL/6 mice. Mice can survive rickettsial infection without IFN-γ, but they cannot survive without MHC-I.

In other studies, CD8 T-lymphocyte clones directed against R. conorii were established (H.-M. Feng and D. H. Walker, unpublished data). Among 15 R. conorii-reactive CD8 T lymphocyte clones that were evaluated for their ability to confer protection via adoptive transfer, 5 clones protected mice from a lethal rickettsial infection. Four of the protective clones pro-
duced a high concentration (ranging from 3,495 to 10,814 PFU) of IFN-γ when stimulated with R. conorii antigen. Thus, even though CTL activity is of greater importance in immunity to rickettsiae, the fact that CD8 T lymphocytes contribute to immunity also via secretion of IFN-γ is confirmed by the demonstration that the ability to produce IFN-γ when stimulated by a single epitope of R. conorii can provide a decisive edge to immunologically naïve mice with an intact immune system. It is unfortunate that during the period when the CD8 clones were available the CTL assay was not established in our laboratory.

The use of two different animal models was required by the desire to study rickettsial infection in gene knockout mice that were available only with the C57BL/6 background, which are highly resistant to R. conorii infection. The R. australis-C57BL/6 mouse model resembled the R. australis-BALB/c mouse model, which was the original endothelial target mouse model of rickettsial infection (4). In C57BL/6 mice, endothelial cells were the primary target; then highly invasive infection followed. The model allowed more definitive conclusions regarding the roles of MHC-I and IFN-γ in immunity to rickettsiae.

This work extends the knowledge of the role of CTL in intracellular infection. The CTL was originally described for lymphocytic choriomeningitis virus and has been noted for its importance mainly for viral infections. Studies of CTLs in bacterial infections have focused primarily on facultatively intracellular organisms that parasitize macrophages, such as Listeria monocytogenes and Mycobacterium tuberculosis (8, 11, 14, 30). For obligately intracellular bacteria and viral infections with target cells other than macrophages, few investigations have been reported.

Because of its obligate intracellular parasitism principally of epithelial cells, infection with Chlamydia is of particular interest for comparison with rickettsial infection principally of endothelial cells. Somewhat surprisingly, the T-lymphocyte subset of greater importance for chlamydial is different from that for rickettsiae. Adoptive transfer of a CD4 cell-enriched T-cell line provided more effective immunity than a CD8 T-cell line in clearing Chlamydia trachomatis from the genital tracts of persistently infected nude mice (18). In the same model, one of two CD8 T-lymphocyte clones provided the ability to clear the chlamydial infection. The protective clone was directed against a genus-specific antigen and produced TNF-α and IFN-γ (10).

In a pulmonary infection model of C. trachomatis (mouse pneumonitis strain), depletion of CD4 T lymphocytes produced a greater effect, with higher levels of chlamydial growth and mortality, than did depletion of CD8 T lymphocytes (15). The importance of CD8 T lymphocytes was more apparent in MHC-I gene knockout mice, in which chlamydial growth and mortality were increased, but survivors had resolved the infection by day 34. Our results for rickettsial infection differ from these chlamydial models in that immune clearance of rickettsial infection was more dependent on CD8 T lymphocytes, and there is evidence that CTL activity is a critical factor.

Currently, our framework of knowledge of immunity to rickettsiae includes major contributions of cellular immunity, particularly nitric oxide-dependent killing of rickettsiae in endothelial cells activated by IFN-γ and TNF-α followed by the

**TABLE 2. Infectious content**

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Adoptive transfer</th>
<th>10⁴ PFU of R. australis/g of tissue from the:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>1</td>
<td>Immune CD8</td>
<td>29.6 ± 1.5</td>
</tr>
<tr>
<td>2</td>
<td>Naive CD8</td>
<td>4,814 ± 758</td>
</tr>
<tr>
<td>3</td>
<td>No transfer</td>
<td>2,816 ± 189</td>
</tr>
</tbody>
</table>

*Mean of two repeated experiments. ± standard deviation.

Immune IFN-γ gene knockout CD8 T-lymphocyte recipients’ organs contained significantly lower R. australis PFU than the corresponding organs of recipients of naïve IFN-γ gene knockout CD8 T lymphocytes (P < 0.05) or those of mice with no transfer (P < 0.05).
elimination of the remaining rickettsia-infected cells by CTL activity. From the results of infection of perforin gene knock-out mice, it can be concluded that perforin secretion was one of the mechanisms of elimination of R. conorii-infected cells by CTLs, but it was not the only mechanism since the LD50 for MHC-I gene knockout mice was only 0.5 PFU of R. australis, nearly 2 orders of magnitude lower than the LD50 of R. australis in the perforin gene knockout mice. As of yet, the roles of Fas-Fas ligand or granulysin in antirickettsial CTL activity have not been determined. It should be noted that all of the mice studied contained CD4 T lymphocytes, B cells capable of antibody production, and innate immunity including NK cells and complement and that these components very likely contributed to antirickettsial immunity although less critically than CD8 T lymphocytes and antibody produced by B cells in the perforin gene knockout mice. As of yet, the roles of Fas-Fas ligand or granulysin in antirickettsial CTL activity have not been determined. It should be noted that all of the mice studied contained CD4 T lymphocytes, B cells capable of antibody production, and innate immunity including NK cells and complement and that these components very likely contributed to antirickettsial immunity although less critically than CD8 T lymphocytes and antibody produced by B lymphocytes, are even more susceptible to R. australis than MHC-I gene knockout mice (H. M. Feng and D. H. Walker, unpublished data).

The discovery that endothelial cells infected with rickettsiae are a target of CTL activity that seems necessary for the host's survival raises other questions related to the role of endothelial cells as antigen-presenting cells in the development of the immune response, the identification of CD8 T-lymphocyte epitopes of Rickettsia species, and the potential contribution of CTLs to the pathogenesis of rickettsial diseases.

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