Protective Cytotoxic T Lymphocytes Are Induced During Murine Infection with *Chlamydia trachomatis*

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T cell responses are often an important component in immunity to organisms that replicate intracellularly. Cytotoxic T lymphocyte (CTL) recognition of peptide Ag in the context of MHC class I molecules results in lysis of infected cells and the release of cytokines including IFN-γ. Members of the genus *Chlamydia* are obligate intracellular pathogens that cause blindness and sexually transmitted disease worldwide. Even though it replicates within a membrane-bound vacuole, *Chlamydia trachomatis* may elicit a CTL response if *Chlamydia* Ags are present in the cytoplasmic compartment where they can be processed for presentation and bound by MHC class I. In this study, we characterized a CTL line derived from mice infected with *C. trachomatis*. This CTL line is specific for, and able to lyse, *Chlamydia*-infected cells. The peptide epitope recognized by this CTL line is present on infected cells, and is presented to the CTL by the classical MHC class I molecule H-2. Adoptive transfer of this CTL line into an infected mouse affords protection, and this protection requires the activity of IFN-γ. *The Journal of Immunology, 1994, 153: 5183.*

*Chlamydia trachomatis* is the most common cause of sexually transmitted disease in the United States and a major cause of preventable blindness worldwide. The diseases caused by *C. trachomatis* range from salpingitis and conjunctivitis to the systemic infection lymphogranuloma venereum. All known *Chlamydia* species are obligate intracellular parasites. The infectious particle, the elementary body, binds to epithelial cells and macrophages, triggering the uptake of the organism into a vacuolar compartment known as an inclusion (1). The organisms replicate intracellularly as noninfectious, metabolically active reticulate bodies, ending in the lysis of the host cell with the release of large numbers of elementary bodies.

Infection with *C. trachomatis* can confer protection against subsequent infection by the same serovar (2, 3). The basis of this immunity is the subject of extensive study. Abs have been identified that recognize the major outer membrane protein of *Chlamydia*, and several of those are able to block attachment and internalization of the organism (2). Abs have been proposed as immunotherapeutic agents, and although there has been some success with this approach, protection seems to be serovar specific (2). The role of T cells in immunity to *C. trachomatis* has also been explored. Passive transfer of immune splenocytes into naïve mice has been shown to protect those mice against subsequent *Chlamydia* challenge (3, 4). Transfer of immune sera in these experiments had no protective effect. Athymic *nullu* mice are more susceptible to *C. trachomatis* than heterozygous littermates, and T cells from immunized heterozygous mice can provide protection when transferred into susceptible athymic littermates (5, 6).

CD4+ T helper cells recognize Ags processed and presented by specialized APC such as macrophages. The Ags are generally derived from vacuolar contents and are presented as peptide fragments bound to surface MHC class II molecules (7). Recognition of cognate Ag by CD4+ T cells results in the release of cytokines that locally activate nonspecific elements of the immune system as well as other lymphocytes. Although *Chlamydia* epitopes recognized by T helper cells have been identified, the role of those T cells in protection has yet to be demonstrated (8).

The role of CD8+ CTL in immunity to *Chlamydia* has been the subject of less study. CTL can directly lyse infected cells and produce cytokines, including IFN-γ. CTL recognize peptide Ag in the context of MHC class I, expressed on all nucleated cells (9). Generally, the peptides
presented to CTL are derived from the cytoplasmic compartment of the cell (10). It is unknown which, if any, Chlamydia Ags escape from or traffic across the vacuolar membrane. Such Ags would be likely candidates for processing and presentation to CTL. Murine models of Chlamydia infection have demonstrated an important role for CTL in immunity to Chlamydia. Splenocytes of mice previously infected with Chlamydia psittaci have been shown to lyse Chlamydia-infected cells, suggesting a cytotoxic subpopulation (11). In another study (5), mixed T cell populations from an immune mouse were transferred into an infected nude mouse and the organisms were cleared. T cells recovered from the spleen after resolution of this infection were predominantly CD8⁺. The authors speculate that this enrichment of CD8⁺ T cells represents selection of the T cells important in the resolution of infection. Another recent report (12) convincingly demonstrated that in vivo depletion of CD8⁺ T cells in immune mice abrogates protection upon challenge with C. psittaci, whereas depletion of the CD4⁺ T cell population did not affect protection.

In this study we demonstrate that CTL are induced during murine infection with C. trachomatis and characterize the activity of a CTL line specific for C. trachomatis-infected cells. We show that this CTL line recognizes, in the context of MHC class I, a natural epitope found on infected cells. In addition, protection can be seen after adoptive transfer of this CTL line into infected mice, and IFN-γ is required for this protection.

Materials and Methods

**Mice**

Female BALB/cByJ mice (H-2d) were obtained from The Jackson Laboratory (Bar Harbor, ME) and used at 8 to 12 wk of age.

**Tissue culture**

The medium used for all tissue culture was RP-10, consisting of RPMI 1640 supplemented with l-glutamine, 50 μM 2-ME, antibiotics (except where noted), and 10% FCS. All cultured cells were maintained at 37°C in 7.0% CO₂. The cell lines used in this study are J774A.1 (H-2d), derived from a BALB/c macrophage tumor; BALB/3T3 (H-2d), derived from a BALB/c embryo; and P815 (H-2d), from a DBA2 mastocytoma. Untransfected L-929 cells (H-2b) and L-929 cells individually irradiated (2000 rads) were used as targets in CTL assays. Uninfected 5774 cells were prepared as described above for the preparation of target BALB/3T3 cells. In addition to the substitution of J774 cells for BALB/3T3 cells, infected cells were incubated for 24 h and the suspended cells were washed twice with PBS after removal from the tissue culture plate. The cells were then processed as described below. The number of infected cells used was 7.2 × 10⁶ per extraction.

**Growth, isolation, and detection of C. trachomatis**

*C. trachomatis* lymphogranuloma venereum serovar L2 (434) has been described previously (13). Elementary bodies were propagated on human epithelial (HeLa) monolayers and isolated by using standard protocols (14). The isolated elementary bodies were titrated on McCoy cells (15), and stored at −70°C in a medium containing sucrose, phosphate, and glutamate (SPG) (3). Aliquots of *C. trachomatis* elementary bodies were thawed at 37°C immediately before use, diluted into sterile SPG, and injected into mice or used to infect monolayers. To quantitate *C. trachomatis*, spleens from infected mice were homogenized, sonicated, diluted, and applied to McCoy cell monolayers. Inclusions were counted by immunofluorescent microscopy 48 h after infection and reported as inclusion forming units (IFU). (15).

**Infection of tissue culture monolayers with C. trachomatis**

J774 cells used for stimulation of *C. trachomatis*-specific CTL were seeded at a density of 2 × 10⁶ cells per well in a 24-well tissue culture plate in RP-10 without antibiotics. The following day the media was removed from each well and an aliquot of thawed *C. trachomatis* containing 10⁷ IFU was added to each well. To facilitate infection, the plates were centrifuged at 1200 × g for 1 h at 37°C. The inoculum was then removed by aspiration and replaced with 2 ml of RP-10 without antibiotics. After incubation for 24 h at 37°C, the cells were rinsed twice with RP-10 containing antibiotics and used in CTL cultures. All subsequent additions to the CTL cultures contained antibiotics.

Infected BALB/3T3 cells were used as targets in CTL assays. Cells were seeded into 6-well tissue culture plates at a density of 10⁶/well in RP-10 without antibiotics and incubated overnight. The media was then removed and replaced with 5 × 10⁷ IFU of *C. trachomatis*. The plates were centrifuged as above, the inoculum was removed, and 5 ml of RP-10 without antibiotics was added to each well. These Chlamydia-infected cells were incubated for 18 h, then removed from the tissue culture plate with PBS containing 0.1 M EDTA. The suspended cells were washed twice by centrifugation with RP-10 containing antibiotics and used as target cells in CTL assays. Uninfected J774 cells were prepared exactly as infected cells by using SPG without *C. trachomatis*.

**Infection of J774 cells for extraction of antigenic peptides** was as described above for the preparation of target BALB/3T3 cells. In addition to the substitution of J774 cells for BALB/3T3 cells, infected cells were incubated for 24 h and the suspended cells were washed twice with PBS after removal from the tissue culture plate. The cells were then processed as described below. The number of infected cells used was 7.2 × 10⁶ per extraction.

**Stimulation and maintenance of C. trachomatis-specific CTL**

Spleen cells from immunized mice were washed in RP-10, and cultures containing 4 × 10⁶ splenocytes and 2 × 10⁶ irradiated (2 × 10⁶ rads) *C. trachomatis*-infected J774 cells were established in 2 ml of RP-10 in 12 wells of a 24-well tissue culture plate. Infected J774 cells used for stimulation were prepared as described above. Primary cytotoxic effector populations were harvested from the 12 wells after 7 days, and half of the recovered cells were restimulated again in 12 wells. Primary CTL cultures were prepared in 2 ml of RP-10. Infected J774 cells were used as targets in CTL assays. Cells were seeded into 6-well tissue culture plates at a density of 10⁶/well in RP-10 without antibiotics and incubated overnight. The media was then removed and replaced with 5 × 10⁷ IFU of *C. trachomatis*. The plates were centrifuged as above, the inoculum was removed, and 5 ml of RP-10 without antibiotics was added to each well. These *Chlamydia*-infected cells were incubated for 18 h, then removed from the tissue culture plate with PBS containing 0.1 M EDTA. The suspended cells were washed twice by centrifugation with RP-10 containing antibiotics and used as target cells in CTL assays. Uninfected J774 cells were prepared exactly as infected cells by using SPG without *C. trachomatis*.

**Infection of J774 cells for extraction of antigenic peptides** was as described above for the preparation of target BALB/3T3 cells. In addition to the substitution of J774 cells for BALB/3T3 cells, infected cells were incubated for 24 h and the suspended cells were washed twice with PBS after removal from the tissue culture plate. The cells were then processed as described below. The number of infected cells used was 7.2 × 10⁶ per extraction.

**CTL assays**

The activity of CTL was determined by ⁵¹Cr release assay. Target cells were resuspended in 100 μl to which 100 μCi sodium ⁵¹chromate was added. The cells were incubated at 37°C for 1 h, washed three times with RP-10, and diluted for use at 10⁶ cells per assay well in 96-well plates. Serial dilutions of CTL were added to the assay wells such that the final assay volume was 200 μl in RP-10. To test peptide preparations for targeting ability, the peptide was incubated with the target cells in the assay wells for 45 min before the addition of effector cells. Spontaneous release was determined in wells with target cells but without CTL. Maximum release was determined by the addition of detergent to wells containing target cells. After a 4-h incubation at 37°C, 100 μl of supernatant from Con A-stimulated rat spleen cells and 50 mM α-methyl mannoside.

% Specific Lysis = 100 ×

Release by CTL − Spontaneous Release

Maximal Release − Spontaneous Release
were cells of a C. trachomatis-immunized mouse. Target cells were collected, lyophilized, resuspended in RPMI 1640, diluted in RPMI 1640, and used in targeting assays.

Results

Infection with C. trachomatis can prime a specific CTL response

To determine if a CTL response is primed during infection with C. trachomatis, BALB/c mice were infected i.p. with 10⁶ IFU of C. trachomatis serovar L2. The mice were killed 14 days after infection and spleen cells were stimulated on C. trachomatis-infected J774 cells. Several CTL lines were obtained, and one, designated line 69, was chosen for further study. This line was restimulated weekly on infected J774 cells, and after the fourth restimulation the T cells were >95% CD8⁺ as determined by flow cytometry (data not shown). To test whether this CTL line, stimulated from an infected mouse, was specific for Chlamydia, infected cells, we tested its ability to specifically lyse BALB/3T3 cells infected with C. trachomatis. As shown in Figure 1, the CTL line specifically lysed cells infected with C. trachomatis. To make certain that Chlamydia-infected cells were not susceptible to lysis by nonspecific CTL, infected cells were tested by using this line (data not shown). Lysis of Chlamydia-infected cells by the other CTL lines was not significantly different than that seen with line 69, and Chlamydia-specific CTL have been reproducibly induced by following this protocol (data not shown).

Peptides can be eluted from infected cells that can target APC for lysis by C. trachomatis-specific CTL

Unlike protein Ag, which must be introduced into the cytoplasm of the APC for processing and presentation by MHC class I, antigenic peptide can be exogenously added to surface MHC molecules (18). To assess the diversity of antigenic peptides presented to this Chlamydia-specific CTL line, peptides were acid eluted from tissue culture cells infected with C. trachomatis. The eluted peptides were then fractionated by HPLC and each of the fractions was tested for targeting activity on P815 cells using the C. trachomatis-specific CTL line. As seen in Figure 2, the majority of the targeting activity resided in a single peak contained in fractions 30 to 31. Other fractions also seem to contain smaller quantities of antigenic peptides or less reactive antigenic peptides. Similar less reactive peptides may also contribute to the peak centered on fractions 30 to 31.

The Chlamydia-specific CTL recognize a peptide in the context of H-2 Ld

To determine which MHC class I molecule presents the antigenic peptide contained in fractions 30 to 31, these fractions were pooled and applied to untransfected L-929 cells (H-2k), as well as to L-929 cells transfected individually with genes encoding the Dd, K₁, or Ld molecules. As shown in Figure 3, the pooled fractions were only able to

![FIGURE 1. Lysis of C. trachomatis-infected cells by CTL in a ⁵¹Cr release assay. The CTL line was stimulated from spleen cells of a C. trachomatis-immunized mouse. Target cells were C. trachomatis-infected (---) or uninfected (--O--) BALB/3T3 cells.](image1)

![FIGURE 2. Targeting activity of HPLC-fractionated peptides for Chlamydia-specific CTL. Peptides were acid eluted from MHC molecules on C. trachomatis-infected J774 cells and fractionated by reverse-phase HPLC. The relevant part of the gradient is shown as a dashed line. Each fraction was lyophilized, resuspended in tissue culture media, and tested in a ⁵¹Cr release assay for the ability to target P815 cells for recognition by the Chlamydia-specific CTL line (---). As a control for toxicity of cellular components that might co-elute with peptides, each fraction was also tested for the ability to cause chromium release in the absence of CTL (-----). The E:T ratio used in each assay was 5:1.](image2)
FIGURE 3. Determination of the MHC class I molecule presenting Ag to the Chlamydia-specific CTL line. Pooled peptide samples from fractions 30 to 31 were tested for the ability to target cells for lysis by C. trachomatis-reactive CTL in a $^{51}$Cr release assay. The cells were tested without pooled peptides (hatched bars) and with pooled peptides (solid bars). Target cells shown at the bottom of each pair of bars are L-929 (H-2$k$) cells individually transfected with the genes for D$d$, K$d$, or L$d$; L-929 cells alone as a negative control; and P815 (H-2$d$) cells as a positive control.

Adoptive transfer of Chlamydia-reactive CTL into infected mice reduces Chlamydia load

To determine if these C. trachomatis-specific CTL are able to alter the severity of Chlamydia infection, we infected mice i.v. with $10^6$ IFU of C. trachomatis followed 30 min later with $10^7$ C. trachomatis-specific CTL. Control mice received either no CTL or CTL specific for an Ag expressed on influenza virus-infected cells. Animals were killed 3 days later and infection was assessed by determining the number of C. trachomatis IFU per spleen. As shown in Figure 4, adoptive transfer of Chlamydia-specific CTL reduced the number of organisms in the spleen 10-fold compared with control mice, which received no CTL or those that received influenza virus-specific CTL. This suggests that CTL may play a role in limiting the establishment of Chlamydia infection or limiting replication within the mammalian host.

The protective effect of Chlamydia-specific CTL is IFN-$\gamma$ dependent

IFN-$\gamma$ has been shown to be important in resistance to a number of intracellular pathogens. To determine whether IFN-$\gamma$ plays a role in the protective effect seen after adoptive transfer of CTL into C. trachomatis-infected mice, we conducted these same adoptive transfer experiments in the presence and absence of a neutralizing Ab against IFN-$\gamma$. Two days before infection, those mice receiving the Ab were given 200 $\mu$g of H22.1, a hamster mAb that efficiently neutralizes mouse IFN-$\gamma$ (19). The level of infection was assessed by enumerating C. trachomatis IFU per spleen 3 days after infection. Again, adoptive transfer of the Chlamydia-specific CTL before infection afforded protection against the organism (Fig. 5, A and B). Pretreatment of the mice with the anti-IFN-$\gamma$ mAb abrogated the protective effect of the adoptively transferred CTL (Fig. 5, C and D). These data suggest that IFN-$\gamma$ may play a role in the protection against C. trachomatis by CTL.
immunity to Chlamydia (11, 12), previous attempts to isolate and characterize CTL specific for C. trachomatis have been unsuccessful (20, 21). Here we report the isolation of a long-term CTL line from a mouse infected with C. trachomatis. The CTL line specifically lyases cells infected with C. trachomatis and recognizes a peptide epitope present on infected cells in the context of the classical MHC class I molecule L^d. Adoptive transfer of the CTL line into mice is able to protect the mice against a challenge with C. trachomatis, and this protection seems to be dependent on IFN-γ.

Many aspects of Chlamydia infection are striking in their similarity to viral infections. The organism is an obligate intracellular parasite, requires host ATP, survives in the intracellular environment, and has a small genome size (1). Protection against viral infection often requires CTL activities such as direct lysis of infected cells and/or the release of cytokines such as IFN-γ. As with viral infections, several roles for CTL can be envisioned in the protection against Chlamydia. Direct lysis of infected cells such as epithelial cells of the urogenital tract, the conjunctiva, or circulating macrophages, could limit the replication of the organism. Organisms released early in the replication cycle are more likely to be reticulate bodies, unable to infect neighboring cells. The concomitant release of IFN-γ from the activated CTL may play a role in the activation of local macrophages, stimulating them to destroy remaining organisms (22, 23). In addition, many in vitro studies have shown that treatment of infected cells with IFN-γ limits the replication of Chlamydia (24–28).

Previous experiments examining the course of C. trachomatis infections in mice treated with neutralizing anti-IFN-γ Ab showed a pronounced increase in organisms recovered from multiple organs (4, 29).

We are in the process of defining further the mechanism by which CTL protect against Chlamydia. In this study, administration of a neutralizing Ab before adoptive transfer abrogated the protective effect of this CTL line, showing that protection requires the activity of INF-γ. Although the interpretation of these data are complicated by the pleiotropic effect of systemic neutralization, one explanation is that this CTL line exerts its in vivo protective effect by the secretion of INF-γ. To specifically test the role of IFN-γ production by CTL in protection, we have obtained a strain of mice in which the gene for IFN-γ has been eliminated. After this mutation is bred onto BALB/c mice, we will infect these IFN-γ−/− mice with C. trachomatis and culture from them Chlamydia-specific CTL. If adoptive transfer of these IFN-γ-deficient CTL into infected syngeneic IFN-γ−/− mice protects against infection, then other cytokines produced by CTL or the lytic effect of CTL are sufficient for protection.

We have compared the level of protection seen after adoptive transfer of CTL with the protection afforded by previous infection with C. trachomatis. Three days after infection, mice that had previously resolved a C. trachomatis infection contained fewer IFU per spleen than those that had not been previously infected (mean log difference of 1.7, data not shown). The protection by the CTL line shown in Figure 4 represents a mean log difference of 1.0. This might suggest that immunity against Chlamydia requires several elements of the immune system; CTL alone may not be able to completely resolve infection. Alternatively, this difference could result from the experimental system. Cultured CTL returned i.v. to the mice may not efficiently home to the site of infection. It is also possible that different lines reactive to the same Ag or lines reactive to other Ags may afford greater protection.

The effects of a CTL response detrimental to the host must also be considered. It has been proposed that IFN-γ plays a role in the establishment of chronic Chlamydia infections (26). Chlamydia-infected cells treated with IFN-γ respond by slowing replication of the organisms along with inducing striking alterations of reticulate body and inclusion architecture (25–28, 30). The response of immune cells producing IFN-γ to infected cells may induce the organism into such a cryptic form. Persistence of Chlamydia Ags in the conjunctiva or fallopian tube and the resulting inflammatory response may contribute to the pathology of trachoma and salpingitis.

In addition to cell-mediated immunity, humoral defenses also seem to be important in overall immunity to Chlamydia (2). It seems logical that neutralizing Ab would be important in blocking binding and internalization of organisms early in infection and immediately after release from an infected cell. Ab can also play a role in the opsonization of free organisms, subjecting them to destruction by phagocytic cells (31). Once the organism has been internalized and is replicating within the cell, protection likely requires cell-mediated immune mechanisms such as CTL and T helper cells.

The stimulation of CTL during Chlamydia infection raises other interesting questions. Epitopes presented by MHC class I molecules are generally derived from cytoplasmic sources (10), yet C. trachomatis is thought to remain inside the vacuolar compartment throughout the replication process (1). The data presented here suggest that some Chlamydia proteins are able to traffic across the inclusion membrane. Such proteins or peptides may serve the organism by altering host cell structures or function, or may simply leak from the vacuolar compartment into the cytoplasm. Introduction of proteins into the cytoplasm of host cells by bacteria outside the cytoplasmic compartment is a strategy common to several pathogens (32–35). In C. trachomatis, such a function has been proposed for the Mip protein, a peptidyl-prolyl cis/trans isomerase (36). The importance of CD8+ T cells has been documented in other systems in which an intracellular pathogen remains
within the vacuolar compartment. Protection against *Toxoplasma gondii* can be achieved by adoptive transfer of CD8<sup>+</sup>-enriched spleen cells from immune mice (37). As in the system described here, pretreatment of the mice with anti-IFN-γ Ab eliminated the protective effect (38).

One goal of our continuing research is the identification of the dominant epitope recognized by this *Chlamydia*-specific CTL line. Here we report the elution of peptides from the MHC molecules of cells infected with *C. trachomatis*. After fractionation of these peptides by HPLC, we were able to identify fractions that contained a targeting peptide. This approach has been used in the analysis of other peptides naturally presented by MHC molecules (39). Several strategies have been used to sequence a peptide contained in a complex HPLC fraction that is able to target cells for lysis by T cells (39, 40). The CTL line described in this study seems to recognize peptide bound to the MHC class I molecule L<sup>d</sup>. The amino acid sequences of typical L<sup>d</sup>-binding peptides has been examined (41), and these data should prove useful in narrowing the search for the sequence of the antigenic peptide.

Here we have used a human *C. trachomatis* strain in a murine infection model, culturing CTL after i.p. infection. A mouse biovar of *C. trachomatis* has been used to infect the female genital tract of mice (5, 6), and in future studies we will attempt to prime a CTL response during genital infection. Resolution of genital infection after adoptive transfer of CTL could also be measured using this model.

Identification of *Chlamydia* proteins that localize to the cytoplasmic compartment of host cells and are able to prime a CTL response will raise the prospect of using these proteins in a subunit vaccine for humans. Such a vaccine therapy would require the delivery of the antigenic protein to the cytoplasm of host cells. Attenuated strains of viruses or other pathogens such as *L. monocytogenes* that are known to deliver proteins into the cytoplasmic compartment might be used as delivery vehicles. Identification of the antigenic protein might also allow for the stimulation and culture of *Chlamydia* reactive human CTL.

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**References**


