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Murine Cytotoxic T Lymphocytes Induced following *Chlamydia trachomatis* Intraperitoneal or Genital Tract Infection Respond to Cells Infected with Multiple Serovars

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The obligate intracellular bacterium *Chlamydia trachomatis* is associated with human diseases ranging from blinding trachoma to sexually acquired genital infections and the systemic disease lymphogranuloma venereum (LGV). We have previously reported the isolation and culture of protective murine cytotoxic T lymphocytes (CTL) following intraperitoneal infection with *C. trachomatis* serovar L2, a serotype associated with human LGV. In this report, we now demonstrate that CTL can also be primed following introduction of *C. trachomatis* serovar L2 into the uterus or ovarian bursa of mice. We also describe *Chlamydia*-specific CTL lines isolated following murine infection with a typical human urogenital isolate of *C. trachomatis* (serovar D) and show that such CTL can be primed by intraperitoneal, intrauterine, or intrabursal infection. Last, we demonstrate that these murine CTL lines respond to multiple serovars, recognizing and lysing cells infected with *C. trachomatis* serovars B, C, D, F, J, K, L2, and L3, representative of organisms causing blinding trachoma, genital infection, and LGV.

Chlamydia trachomatis is a major cause of sexually transmitted disease in the developed world and the leading cause of preventable blindness worldwide. Members of the *Chlamydia* genus are obligate intracellular pathogens, undergoing a biphasic life cycle in which organisms replicate intracellularly in a noninfectious form called the reticulate body, resulting in the lysis of the host cell (11). Prior to lysis, the organisms undergo conversion to a metabolically inert but highly infectious form known as the elementary body.

C. trachomatis strains were originally divided into 15 serovars, and each serovar is associated with one of three diseases, blinding trachoma, sexually acquired disease of the genital tract, and lymphogranuloma venereum (LGV) (2, 20). The serovar determinant is the *Chlamydia* major outer membrane protein (MOMP) (1). MOMP contains the dominant antibody epitopes, and most antibodies against MOMP are limited in the serovars with which they will cross-react (19). Immunity against *Chlamydia* species following primary infection is of short duration and is afforded only against the infecting serovar (10). Antibody against *C. trachomatis* has been shown to neutralize elementary body infection of tissue culture cells (17), and most of the vaccine strategies which have been attempted for chlamydiae have been directed at stimulating a protective antibody response against MOMP (10). Vaccine strategies designed to stimulate a neutralizing antibody response against MOMP have had some success, but the antibodies generated have not been able to neutralize all serovars (12).

Our work has focused on the intracellular phase of the life cycle. We have been interested in determining the extent to which *Chlamydia* antigens are presented by infected cells to cytotoxic T lymphocytes (CTL). One role of CTL is to respond to cells infected with intracellular pathogens. CTL recognize peptide epitopes from the infecting organism which are processed by intracellular pathways and presented in a complex with host cell major histocompatibility complex (MHC) class I molecules (9). CTL recognition of cognate antigen results in lysis of the infected presenting cell, proliferation of that particular CTL clone, and secretion by the CTL of cytokines able to activate local effectors of specific and nonspecific immunity.

In a previous report (16), we described the isolation and characterization of a murine CTL line specific for *C. trachomatis*-infected cells. These CTL were obtained from mice infected with a *C. trachomatis* serovar (L2) causing LGV in humans. After adoptive transfer of these CTL into *C. trachomatis*-infected mice, a reduction in *Chlamydia* organisms in the spleens of these animals was measured. In this report, we extend these findings to show that CTL can be isolated from mice infected not only intraperitoneally, but also in the genital tract. In addition, we show that *Chlamydia*-specific CTL can be primed by infection with serovars of *C. trachomatis* causing typical human genital tract infection following either intraperitoneal or genital infection. Last, we demonstrate that several of the CTL lines that we have isolated respond to cells infected with multiple *C. trachomatis* serovars.

MATERIALS AND METHODS

Mice. Female BALB/cByJ mice ($H\text{-}2^d$) were obtained from Jackson Laboratories (Bar Harbor, Maine) and used at 8 to 12 weeks of age.

Tissue culture. The medium used for tissue culture (unless otherwise noted) was RP-10, consisting of RPMI 1640 supplemented with L-glutamine, 50 μM 2-mercaptoethanol, antibiotics (penicillin, streptomycin, and gentamicin, except where noted), and 10% fetal calf serum (FCS). All cultured cells were maintained at 37°C in 7.0% CO_2. The cells infected for stimulation of *Chlamydia*-specific CTL were J774A.1 ($H\text{-}2^d$), derived from a BALB/c monocyte-macrophage tumor. BALB/3T3 ($H\text{-}2^d$) cells derived from a BALB/c embryo were infected to test the antigen specificity of the CTL lines.

Growth, isolation, and detection of *C. trachomatis*. The organisms used to infect J774 cells for stimulation of *C. trachomatis* CTL were *C. trachomatis* LGV serovar L2 (434/Bu) (20) and a genital isolate of *C. trachomatis*, serovar D (UW-3/Cx). Elementary bodies of these serovars were propagated in Eagle's minimal essential medium (MEM) with 10% FCS on human epithelial cell (HeLa) monolayers and purified by differential centrifugation in 30% Renografin (4). The organisms used to prepare infected BALB/3T3 target cells were *C.*

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trachomatis serovars B (UW1000), C (TW-3/OT), F (UW-6/Cx), J (UW-36/Cx), K (UW-31/Cx), and L3 (404/Bu). Elementary bodies of these serovars were propagated on McCoy cell monolayers in Eagle's MEM and purified as described above. All isolated elementary bodies were counted on McCoy cells by immunofluorescent microscopy approximately 42 h after infection, and the results are reported as inclusion-forming units (IFU) per milliliter (15). Organisms were stored at −70°C in a medium containing sucrose, phosphate, and glutamate (SPG) (5). Aliquots of *C. trachomatis* elementary bodies were thawed at 37°C immediately prior to use, diluted into sterile SPG, and injected into mice or used to infect monolayers.

Infection of tissue culture monolayers with *C. trachomatis*. J774 cells were used for stimulation of *C. trachomatis*-specific CTL. The cells were seeded at a density of 2×10^5 cells per well in a 24-well tissue culture plate in RP-10 without antibiotics. The following day, the medium was removed from each well and replaced with an aliquot of thawed *C. trachomatis* (10^7 IFU). The plates were centrifuged at $1,200 \times g$ for 1 h at 37°C to enhance contact of the organisms with the monolayer. 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 with RP-10 containing antibiotics and used in CTL cultures. All subsequent additions to the CTL cultures contained antibiotics.

Infected BALB/3T3 cells used as targets in CTL assays were seeded into six-well tissue culture plates at a density of 10^6/well in RP-10 without antibiotics and incubated overnight. The medium was then removed, and an aliquot of *C. trachomatis* containing 5×10^7 IFU was added to each well. The plates were centrifuged as above, and the inoculum was removed and replaced with 5 ml of RP-10 without antibiotics. The cells infected with the individual *Chlamydia* serovars were incubated for 18 h and then removed from the tissue culture plate with phosphate-buffered saline (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 cells were prepared exactly like infected cells with SPG without *C. trachomatis.*

Stimulation and maintenance of *C. trachomatis*-specific CTL. Spleen cells from immunized mice were washed in RP-10, and cultures containing 4×10^6 splenocytes and 2×10^5 irradiated (2×10^4 rads) *C. trachomatis*-infected J774 cells were established in 2 ml of RP-10 in 12 wells of a 24-well tissue culture plate. The J774 cells used for stimulation were infected and 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 of a new 24-well tissue culture plate. In addition to the effector cells, each culture well contained 4×10^6 irradiated (2,000 rads) syngeneic spleen cells and 2×10^5 irradiated (2×10^4 rads) *C. trachomatis*-infected J774 cells. Subsequent weekly stimulations were carried out with 2-ml cultures in multiple wells of a 24-well tissue culture plate. Each culture consisted of 10^5 responder cells, 4×10^6 irradiated (2,000 rads) syngeneic spleen cells, and 2×10^5 irradiated (2×10^4 rads) *C. trachomatis*-infected J774 cells in a medium containing RP-10 supplemented with 5% supernatant from concanavalin A-stimulated rat spleen cells and 50 mM α-methyl mannoside. Each of the stimulated cell populations contained >95% $CD8^+$ cells after 3 weeks of in vitro stimulation, as determined by flow cytometry.

CTL assays. The activity of the CTL lines was determined by a chromium release assay. Target cells were resuspended in 100 μl to which 100 μCi of sodium 51-chromate was added. The cells were incubated at 37°C for 1 h, washed three times with RP-10, and diluted for use at 10^4 cells per assay well in 96-well plates. Serial dilutions of CTL were added to the assay wells so that the final assay volume was 200 μl in RP-10. Spontaneous release was determined in wells with target cells but without CTL. Maximum release was determined by addition of detergent to wells containing target cells. Following a 4-h incubation at 37°C, 100 μl of supernatant was evaluated on a Wallac 1470 Wizard gamma counter. Specific lysis was determined as follows: % specific lysis = 100 × [(release by CTL − spontaneous release)/(maximal release − spontaneous release)]. In all experiments, spontaneous release was less than 40% of maximal release by detergent. As expected, *C. trachomatis*-infected target cells were not lysed by CTL lines specific for epitopes from *Listeria monocytogenes* or influenza virus.

Statistics. Each CTL line was tested for recognition of target cells infected with selected *C. trachomatis* serovars in at least two independent experiments. Significance levels were determined by a standard paired *t* test and a pooled estimate of the (residual) variance (pooled over all CTL-serovar combinations). First, the difference between the percent lysis for each CTL-serovar combination and its paired control was calculated. Then, the mean difference was computed by averaging over the (two or three) replicates of each CTL-serovar combination. To test the hypothesis that the percent lysis for each CTL-serovar combination is not different from that of its CTL-uninfected-cell control, this mean difference is compared with zero by a *t* test. The variance needed for the *t* test was estimated by pooling the within-combination CTL-serovar residual sums of squares and dividing by the degrees of freedom (for four CTL lines against each of four serovars with two replicates each plus one CTL line against four serovars with three replicates each, giving a total of 24 degrees of freedom). One-tailed *P* values are reported because the assays are designed to measure preferential lysis of infected cells over lysis of uninfected cells.

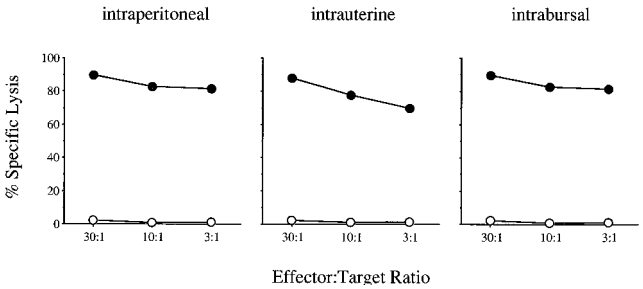

FIG. 1. Lysis of cells infected with *C. trachomatis* LGV serovar L2 by CTL in a ^{51}Cr release assay. The CTL lines were stimulated from spleen cells of mice following infection with *C. trachomatis* by the route of infection shown above each panel. The CTL lines used were line 69 (16), generated by intraperitoneal infection; line 83, generated by intrauterine infection; and line 89, generated by intrabursal infection. The target cells were *C. trachomatis* serovar L2-infected (●) or uninfected (○) BALB/3T3 cells.

RESULTS

Genital infection with an LGV serovar of *C. trachomatis* can prime a specific CTL response. In a previous report (16), we demonstrated that *Chlamydia*-specific CTL are primed following intraperitoneal infection with *C. trachomatis* serovar L2. In order to determine if specific CTL are also primed following genital infection, we attempted to culture *Chlamydia*-specific CTL from mice following either intrauterine or intrabursal infection. BALB/c mice were infected by surgical introduction of *C. trachomatis* serovar L2 into either the uterus (10^5 IFU in 100 μl) or the ovarian bursa (10^4 IFU in 10 μl), simulating lower and upper genital tract infections, respectively. The mice were allowed to recover for 14 days, after which the mice were killed. The spleen cells were then stimulated in vitro on *C. trachomatis*-infected J774 cells for 3 weeks, and the resulting CTL lines were tested for their ability to lyse cells infected with *C. trachomatis.* As shown in Fig. 1, the CTL primed by either route of genital infection were able to lyse cells infected with *C. trachomatis.* No lysis of the uninfected controls was apparent. In addition, we were unable to generate long-term *C. trachomatis*-specific CTL lines by stimulating spleen cells from uninfected mice. These data demonstrate that a *Chlamydia*-specific CTL response can be primed by genital infection in addition to the intraperitoneal route, as shown previously.

Atypical genital *Chlamydia* serovar can also prime a specific CTL response. In order to determine whether *Chlamydia*-specific CTL are stimulated in response to infection with a typical human genital serovar of *C. trachomatis*, we infected BALB/c mice by three routes of infection: intraperitoneal infection (10^8 IFU in 300 μl), intrauterine infection (10^5 IFU in 100 μl), and intrabursal infection (10^4 IFU in 10 μl). The mice were killed 14 days after infection, and spleen cells from these animals were stimulated on *C. trachomatis* (serovar D)-infected J774 cells. After 3 weeks of culture in vitro, the stimulated CTL lines were tested for their ability to lyse cells infected with *C. trachomatis.* As shown in Fig. 2, the CTL primed following infection by all routes were able to lyse cells infected with *C. trachomatis.* No lysis of the uninfected controls was seen. These data suggest that CTL epitopes are also present on cells infected with serovars associated with diseases other than LGV.

***Chlamydia*-specific CTL lines recognize cells infected with multiple *C. trachomatis* serovars.** To determine the extent to which the CTL lines described above are able to recognize cells infected with serovars other than the one used to prime the response, we tested the ability of selected lines to lyse cells

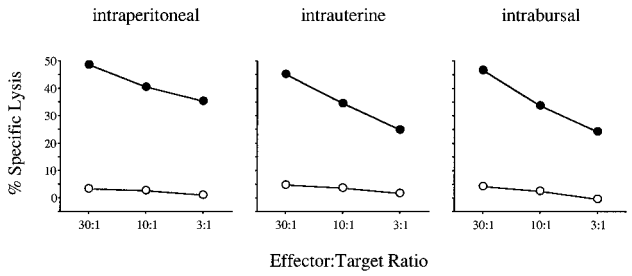

FIG. 2. Lysis of cells infected with *C. trachomatis* LGV serovar D by CTL in a ^{51}Cr release assay. The CTL lines were stimulated from spleen cells of mice following infection with *C. trachomatis* by the route of infection shown above each panel. The CTL lines used were line 92, generated by intraperitoneal infection; line 95, generated by intrauterine infection; and line 97, generated by intrabursal infection. The target cells were *C. trachomatis* serovar D-infected (●) or uninfected (○) BALB/3T3 cells.

infected with several human *C. trachomatis* serovars. The serovars tested were L2 and L3 (LGV serovars); serovars B and C, serovars which cause trachoma; and serovars D, F, J, and K, which are associated with *Chlamydia* genital infections. As shown in Table 1, each of the CTL lines tested was able to lyse cells infected with any of the *C. trachomatis* serovars (≥98% confidence level), suggesting that the CTL lines recognize epitopes common to all the serovars tested.

DISCUSSION

This study demonstrates that CTL can be primed following both intraperitoneal and genital infection with either *C. trachomatis* serovar L2, an LGV serovar, or serovar D, which is associated with human genital tract infections. Of greater interest is that all these CTL lines recognize cells infected with multiple serovars. In addition to those described here, we have generated many *C. trachomatis*-specific CTL lines, and all of those tested have shown the same ability to lyse cells infected with multiple serovars. Furthermore, all *C. trachomatis* serovars that we have tested have been lysed by each CTL line tested. The serovars examined cause trachoma, urogenital infection, and LGV, and each has a distinct MOMP sequence. This suggests that the CTL epitope does not lie within a variable domain of MOMP.

One of these CTL lines (line 69, Table 1) has been shown to reduce *Chlamydia* infection in a murine model. This raises the exciting possibility that a vaccine strategy which incorporates epitopes recognized by these CTL lines may provide immunity which is independent of the serovar of the infecting organism. Certainly a major thrust of this work in the future will be the identification of the epitopes (and the proteins from which they are derived) recognized by these CTL. A vaccine intended to provide protection in the human population, with its great diversity of MHC class I types, would require the incorporation of a full-length protein and, ideally, several full-length proteins.

Identification of the proteins recognized by these CTL will also enhance our understanding of the *Chlamydia*-host cell interaction. Proteins which are processed for presentation on MHC class I molecules to CTL are generally derived from the cytoplasm of the presenting cell (9). Chlamydiae are vacuolar pathogens, thought to remain within a vacuolar compartment throughout their life cycle. Therefore, proteins processed into CTL epitopes may have evolved the ability to translocate across the vacuolar membrane into the cytoplasm of host cells. Such proteins may aid the organism by altering host cell struc-

TABLE 1. CTL lysis of cells infected with various serovars of *C. trachomatis*[a]

CTL line (reference)	Priming *C. trachomatis* serovar	Route of infection	Mean % specific lysis (*P*) with target serovar:								
			B (trachoma)	D (genital infection)	F (genital infection)	L2 (LGV)	C (trachoma)	J (genital infection)	K (genital infection)	L3 (LGV)	None (uninfected cells)
69 (16)	L2	Intraperitoneal	52 (<0.0001)	29 (0.01)	47 (0.0001)	62 (<0.0001)	49 (<0.0001)	41 (<0.0001)	31 (0.0003)	46 (<0.0001)	4
83 (this report)	L2	Intrauterine	34 (0.002)	27 (0.01)	45 (<0.0001)	58 (<0.0001)	N/D[b]	N/D	N/D	N/D	5
92 (this report)	D	Intraperitoneal	44 (<0.0001)	25 (0.008)	48 (<0.0001)	25 (0.009)	N/D	N/D	N/D	N/D	2
95 (this report)	D	Intrauterine	49 (<0.0001)	26 (0.008)	47 (<0.0001)	22 (0.02)	N/D	N/D	N/D	N/D	3

[a] Selected CTL lines were tested for their ability to recognize and lyse cells infected with the *C. trachomatis* serovars shown. Although the mean percent specific lysis values for each CTL-serovar pair are shown, comparison of the degree of lysis is of limited value. Factors such as differential infection levels with each serovar, differences in ^{51}Cr labeling, and differences in percent spontaneous release make comparison of these numbers difficult. Shown in parentheses is the *P* value for CTL lysis of the infected cells compared with lysis of uninfected cells. The effector-to-target cell ratio used in each assay was 30:1.

[b] N/D, not done.

tures or functions. An alternative explanation is that these peptide epitopes may simply leak from the vacuolar compartment into the cytoplasm of host cells. Alternative pathways of antigen processing and presentation to CTL are an area of current investigation in several laboratories (3, 7, 8, 13, 14). Although we have no evidence that these pathways are responsible for presentation of *Chlamydia* antigens, such possibilities are intriguing.

It is also interesting to consider potential detrimental effects of the immune response to chlamydiae, as infections with *C. trachomatis* can result in immune or autoimmune pathology. Patients recovering from *Chlamydia* infections are predisposed to reactive arthritis, a disease associated with a particular MHC class I molecule, HLA-B27 (6). Women with *C. trachomatis* genital tract infections are at increased risk for pelvic inflammatory disease, a major cause of ectopic pregnancy and infertility which is thought to have a significant immunopathologic component (18). It is conceivable that CTL responding to cells infected with chlamydiae play a role in these diseases.

In this report, we have demonstrated that there are antigens recognized by the immune system following intraperitoneal or genital infection with *C. trachomatis* which are common to multiple serovars. The identification and characterization of these antigens will aid in the study of the immune response to these pathogens, which are responsible for considerable morbidity worldwide.

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