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Cytotoxic-T-Lymphocyte-Mediated Cytolysis of L Cells Persistently Infected with *Chlamydia* spp.

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Persistent chlamydial infections have been proposed as a means whereby chlamydiae evade immune resolution of infection. Such a mechanism would require evasion not only of the humoral immune responses but also of cell-mediated immune responses. We hypothesized that if such a mechanism is important, persistently infected cells should not be recognized by cytotoxic T cells. Persistent infections were simulated in vitro by treatment of *Chlamydia trachomatis*- or *Chlamydia psittaci*-infected cells with gamma interferon (IFN-γ), penicillin, or tryptophan depletion. Cultures were examined for induction of a chlamydial stress response (measured by transcription of *groesl* RNA) and for the effects on viability, infectivity, morphology, and immune recognition. Although both IFN-γ and penicillin induced aberrant chlamydial morphology and growth, we did not find evidence that these treatments elicited a classical stress response. In addition, T-cell-mediated lysis of *Chlamydia*-infected target cells treated with IFN-γ or penicillin or grown in tryptophan-deficient media was examined. The immune cell-mediated lysis of these treated infected cells demonstrated that despite the effects of these compounds on chlamydial growth and development, the infected cells continued to be efficiently recognized and killed by cytotoxic T cells. Thus, it seems unlikely that these in vitro models of persistence represent functional mechanisms to evade immune clearance.

There are four species of *Chlamydia*: *C. trachomatis* and *C. pneumoniae*, which are predominantly human pathogens, and *C. psittaci* and *C. pecorum*, which are predominantly animal pathogens. Different species display different tissue tropisms in the natural host; however, the underlying immunohistopathological changes are remarkably similar (16). The local reaction to all chlamydial infections is characterized by heavy infiltration with inflammatory cells. During the acute stage, polymorphonuclear leukocytes, mostly neutrophils, predominate, while at subacute and chronic stages, there are many more lymphocytes present at the site of infection (16). Plasma cells, eosinophils, and macrophages are also recruited. While there may be minor histopathological variations between chlamydial strains, the general inflammatory responses are similar for infections by *C. trachomatis* (16), *C. psittaci* (20), *C. pneumoniae* (31), and *C. pecorum* (8). The mechanism by which *Chlamydia* spp. cause inflammation and subsequent long-term complications remains unclear. What is known is that repeated or chronic chlamydial infections lead to an immune system-mediated response which often progresses to scarring, such as occurs in trachoma or pelvic inflammatory disease caused by *C. trachomatis* (16) and in experimental *C. psittaci* conjunctivitis (20).

The progression of chlamydial disease, and the observation that chlamydial DNA or antigen can sometimes be detected in patients from whom viable organisms could not be recovered, led to the hypothesis that there may be a persistent in vivo state in which the chlamydial cycle is arrested, but antigen remains as a stimulus to the immune response (26). Persistence has been defined as a long-term association between chlamydiae and their host in which organisms remain viable but are not cultivable (7). Such a condition implies the existence of an altered state, distinct from the typical developmental cycle of a productive infection.

The interaction of the immune system with such persistent or altered organisms is not understood. Indeed, the concept of protective versus deleterious immune responses in chlamydial infections is unresolved. Both humoral and cell-mediated immunity have been shown to be involved in resolution of chlamydial infection, although it seems that antibody is not essential. In a model system, congenitally athymic nude mice were unable to resolve infection (24), whereas B-cell-deficient mice resolved infection (23), implicating the cell-mediated immune system as being critical for resolution of infection. Furthermore, the adoptive transfer of *Chlamydia*-specific T-cell lines to chronically infected athymic mice resulted in clearance of infection (22). Both $CD4^+$ and $CD8^+$ cells are activated in a primary response to chlamydial infection (27). $CD4^+$ cells secrete cytokines which activate other lymphocytes, including both nonspecific effector cells and antigen-specific effector cells such as B lymphocytes and $CD8^+$ T cells. The primary function of $CD8^+$ T cells is to lyse infected target cells, and these cells have been previously demonstrated to be important in immunity against viruses and intracellular bacterial pathogens such as *Listeria* and *Mycobacterium* species (15, 17). Recently, we have characterized cytotoxic T-lymphocyte (CTL) responses to chlamydiae in unselected spleen cells from immune mice (3). Starnbach et al. (28) showed that a *Chlamydia*-specific $CD8^+$ cell line was partially protective when adoptively

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transferred into infected mice. The finding that $CD8^+$ cells appear to contribute to resolution of chlamydial infection, albeit in conjunction with $CD4^+$ cells (18, 22), raises some intriguing questions regarding the role of persistence in chlamydial pathology. For organisms to persist, not only would they have to escape antibody-mediated neutralization, but they would also have to evade $CD8^+$ T-cell recognition.

Several methods have been used to produce atypical chlamydial organisms that are potentially useful as in vitro models of persistent infection. These include treatment with gamma interferon (IFN-γ) (6), penicillin (10, 29), or amino acid depletion (5, 12). These models of persistence probably function to induce persistent forms via different mechanisms. The response of chlamydiae subjected to penicillin treatment or amino acid depletion has been less well characterized than the response to IFN-γ; however, aberrant forms are produced in all cases. The IFN-γ model for persistence suggests that a critical concentration of IFN-γ results in viable but noninfectious chlamydial forms which have aberrant morphology and display reduced levels of the major outer membrane protein (MOMP) and increased amounts of heat shock protein 60 (hsp60) (6). It has been suggested that in this form, chlamydiae are able to evade immune clearance because persistence within the cell protects the organisms from antibody-mediated neutralization. It is unclear which if any of these three models are useful approximations of the in vivo situation, nor is it clear whether all chlamydial strains would respond similarly to such persistence-inducing treatments. This issue is particularly important for the IFN-γ model, as it is believed that IFN-γ acts by depleting tryptophan (30). However, chlamydial strains differ with respect to their tryptophan requirements (1), and the majority of work to date has focused on IFN-γ treatment of a tryptophan-dependent strain. Thus, an important criterion in assessing the relevance of persistence models to the in vivo situation is the applicability to all chlamydial strains.

If in vitro aberrant forms are a satisfactory model for in vivo persistence and subsequent pathogenesis, then (i) similar responses should be found in the majority of chlamydial strains and (ii) the aberrant forms should not only evade the humoral immune response but also be resistant to cell-mediated killing. We tested this hypothesis by evaluating several models of persistent infection to determine whether persistently infected cells were susceptible to CTL-mediated cytolysis.

MATERIALS AND METHODS

***Chlamydia* culture.** *C. psittaci* (AP1), *C. trachomatis* serovar A (HAR-13), and *C. trachomatis* serovar B (TW-5) were routinely cultured in L929 cell monolayers, and *C. trachomatis* serovar L2 (434/Bu) was cultured in L929 suspension cultures. Cultures were grown in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone, Logan, Utah), 60 μg of vancomycin (Sigma, St. Louis, Mo.) per ml, and 10 μg of gentamicin (Sigma) per ml. Infected cells were pelleted by centrifugation and sonicated to release infectious chlamydiae. These organisms either were used to infect L929 or HeLa monolayers at a multiplicity of infection of 1 to 5 or were further purified over a discontinuous 30 to 44% Renografin (Squibb Diagnostics, Princeton, N.J.) gradient for mouse infections as described previously (4).

Morphology. Chlamydial morphology was assessed by staining 24- or 48-h monolayers of infected cells with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies directed against the MOMP (Syva, Palo Alto, Calif.) and the lipopolysaccharide (LPS) (Meridian, Cincinnati, Ohio) and viewing them at a magnification of ×1,000. Additionally, inclusion size and number were quantitated by culturing chlamydiae in 24-well plates for 48 h and then staining them with an FITC-labeled monoclonal antibody directed against the LPS. Monolayers were viewed with an inverted microscope with a 10× objective lens and quantitatively analyzed by using a charge-coupled device series 200 camera (Photometrics, Tucson, Ariz.) with IP Lab Spectrum 2.4.1 image analysis software (Signal Analytics, Vienna, Va.). The software program enumerated inclusions on the basis of both the intensity and the area of the FITC stain. Mean inclusion size was determined by measuring the total area of inclusions per field and dividing by the number of inclusions in that field.

Infectivity and viability. Infectivity after various treatments was determined by harvesting chlamydial organisms from infected monolayers by sonication at 48 h and then inoculating them onto fresh L929 cell monolayers. Viability was determined by replacing the original media with new growth media at 48 h, incubating the samples for a further 24 h to allow the chlamydiae to recover, and then harvesting the infected monolayer and inoculating onto fresh monolayers.

Treatment of chlamydial cultures. Cultures were treated with heat, penicillin, IFN-γ, and amino acid depletion as follows. *C. psittaci* AP1 was cultured in 25-cm^2 flasks for 20 to 24 h and then exposed to 45°C for 0, 5, 10, and 30 min and 4 h. At each of these time points, viability was determined and RNA was extracted. RNA was also extracted from 20- to 24-h cultures of *C. trachomatis* serovars A, B, and L2 after 5 min of heat treatment. *C. psittaci* AP1 was cultured for 20 h, then penicillin (Sigma) was added to give a concentration of 100 U/ml, and the organisms were incubated for a further 10 min, 30 min, 4 h, and 24 h. *C. trachomatis* serovar L2 was cultured in the presence of 100 U of penicillin per ml for 24 h. At these time points, infectivity was assessed and RNA was extracted. *C. psittaci* AP1 and *C. trachomatis* serovars L2, A, and B were grown in L929 or HeLa 229 monolayers in 24-well plates for 24 to 48 h in the presence of 0, 0.6, 1.2, 2.5, 5, and 10 U of IFN-γ per ml. Recombinant murine IFN-γ (Genzyme, Cambridge, Mass.) was used for L929 cell cultures, and recombinant human IFN-γ (Genzyme) was used for HeLa 229 cell cultures. At 24 h (serovar L2 and strain AP1) or 48 h (serovars A and B), cultures were examined for inclusion number, inclusion size, and chlamydial morphology. Infectivity and viability were assessed after a further 24 h of incubation. Chlamydiae were grown in 25-cm^2 flasks in the presence or absence of 2.5 U of IFN-γ per ml, and RNA was extracted at 24 h (serovar L2 and strain AP1) or 48 h (serovars A and B). Additionally, *C. psittaci* was grown for 20 h, then IFN-γ was added to 2.5 U/ml, the organisms were incubated for a further 10 min, 30 min, 4 h, and 24 h, RNA was extracted, and viability was determined at each time point. *C. psittaci* AP1 and *C. trachomatis* serovars A, B, and L2 were cultured for 24 h (AP1 and L2) or 48 h (A and B) in Selectamine minimal essential medium (Gibco, Grand Island, N.Y.) containing 0, 10, 50, and 100% of the standard tryptophan concentration (10 mg/liter). Inclusion number and chlamydial morphology were assessed.

RNA extraction and hybridization. Chlamydiae were grown in 25-cm^2 monolayers of L929 cells and exposed to the treatments described above. RNA was subsequently extracted by an acid phenol-guanidinium extraction procedure (11). RNA was electrophoresed through 1.5% formaldehyde agarose gels and transferred to Hybond N^+ (Amersham, Arlington Heights, Ill.) in 50 mM NaOH. Membranes were incubated in prehybridization solution (4× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA; pH 7.7], 0.5% blocking agent [Boehringer Mannheim, Indianapolis, Ind.], 1% sodium dodecyl sulfate [SDS], 100 μg of denatured calf thymus DNA per ml) for 2 h at the hybridization temperature. Labeled *omp1*, *groesl*, or 16S rRNA probe (100 ng) was added, and the probe was hybridized overnight at 65°C for *omp1* and *groesl* or at 45°C for 16S rRNA. Membranes were then washed once at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS, followed by three washes in 0.1× SSC at 65°C for *omp1* and *groesl* or in 2× SSC at 45°C for 16S rRNA. Filters were air dried and exposed to X-ray film for 1 to 10 days. Band intensities were compared by using image analysis.

The MOMP gene probe was generated by amplifying a fragment of approximately 1 kb from both *C. psittaci* and *C. trachomatis omp1* genes (25). The *groesl* probe was generated by amplifying a fragment of approximately 730 bp from the *C. psittaci* and *C. trachomatis groesl* operons, using primers HS2 (5′-CCGCAT GCTCAGATCAAGCAACGACC-3′) and HS3 [5′-GGGAATTCATC(AG)A CAAC(AG)AC(CT)TT-3′]. The amplified fragments were column purified (Promega, Madison, Wis.) and labeled with [α-^{32}P]dCTP by random priming (Pharmacia, Alameda, Calif.). An oligonucleotide designated G2, specific for the chlamydial 16S rRNA gene (21), was used to detect 16S rRNA to standardize RNA concentrations. This oligonucleotide was end labeled with [α-^{32}P]dCTP by using 3′-terminal transferase (Pharmacia).

CTL assays. CTL assays were done as previously described (3) that except additional cultures were grown in the presence of IFN-γ (2.5 U/ml) or penicillin (100 U/ml) for the initial 24 h. Neither IFN-γ nor penicillin was included during the assay incubation period. Briefly, ICAM-1-transfected L929 cells were infected with *C. trachomatis* serovar L2, with and without penicillin or IFN-γ, and incubated for 24 h. In addition, cells were infected with serovar A and incubated either in medium with reduced tryptophan or in complete medium. Fresh spleen cells were prepared from C3H mice which had been immunized intravenously three times with live *C. trachomatis* serovar L2. Spleen cells were tested for the ability to kill control L2- or A-infected L929 cells, L2-infected L929 cells which had been cultured in penicillin or IFN-γ, and A-infected L929 cells which had been cultured with reduced tryptophan. Killing was assessed by a standard ^{51}Cr release assay and expressed as a percentage of the maximum lysis.

RESULTS

The concept of persistence in chlamydial infections is a popular one because it may contribute to understanding pathogenesis of disease. Three methods were used to induce persistent chlamydial forms in vitro: IFN-γ treatment, penicillin treat-

TABLE 1. Effect of IFN-γ (2.5 U/ml) on chlamydial inclusion morphology in HeLa cells

Organism	Expression			% of untreated control		
	Atypical	MOMP antigen[a]	LPS antigen[a]	Inclusion no.	Inclusion size	Infectivity
C. trachomatis						
A and B	+	+/−	+	60	10	0
L2	−	++	++	100	50	20
C. psittaci AP1	−	++	++	100	50	10

[a] As detected by staining cultures with anti-MOMP and anti-LPS monoclonal antibodies.

ment, and tryptophan depletion. These treatments were evaluated first for their abilities to induce persistence, as defined by the production of viable but noninfectious organisms, and second for the induction of aberrant morphology, both of which are proposed to play important roles in evading the immune response. It has been suggested that in such a persistent state, chlamydiae produce increased levels of hsp60 and may serve as an antigen depot which stimulates the deleterious components of the immune system (6). The production of hsp60 (measured by the quantity of the *groesl* RNA transcript) was therefore also assessed. Finally, it was determined whether these persistent or aberrant forms were susceptible to CTL-mediated lysis.

IFN-γ treatment. IFN-γ was tested because it is induced during chlamydial infections (2) and thus is a relevant physiological stress to which chlamydiae would be exposed. Further, it has been suggested to play a role in persistence-mediated pathogenesis via its postulated effect on increased hsp60 expression (6).

C. trachomatis serovars A and B produced large aberrant forms when infected HeLa cell cultures were treated with IFN-γ. These aberrant forms stained weakly with an anti-MOMP monoclonal antibody but could be readily visualized with an anti-LPS monoclonal antibody (Table 1). The inclusions were decreased in both size and number, and progeny were noninfectious (Table 1; Fig. 1A). In contrast, *C. trachomatis* serovar L2 and *C. psittaci* AP1 displayed normal morphology and stained strongly with both anti-MOMP and anti-LPS monoclonal antibodies when grown in the presence of IFN-γ (Table 1). Although the inclusion size decreased, the number of inclusions was not altered (Fig. 1B). There was a substantial drop in infectivity, but this did not decrease to zero (Table 1).

Amino acid depletion. One of the mechanisms by which IFN-γ affects human cells is tryptophan degradation via induction of the enzyme indoleamine 2,3-dioxygenase. We reasoned that the difference in IFN-γ susceptibility between chlamydial strains described above may be related to differing requirements for exogenous tryptophan (1). Tryptophan requirements of these strains were determined, both to confirm that IFN-γ susceptibility correlated with tryptophan requirement and as a functionally analogous means of inducing persistence in IFN-γ-sensitive strains. Thus, a persistence model could be tested in mouse cell lines, which have a mechanism for IFN-γ susceptibility different from that of HeLa cells (19). Selectively omitting tryptophan from the medium inhibited the growth of *C. trachomatis* serovars A and B but had no observable effects on either inclusion size or infectious progeny of *C. trachomatis* serovar L2 or *C. psittaci* AP1 (data not shown).

Penicillin treatment. Because the effects of IFN-γ and amino acid depletion varied between strains, we sought a persistence-inducing model which would be consistent between strains. Penicillin treatment has been shown to decrease infectivity and to induce aberrant forms in all strains which have been examined, consistent with the operational definition of persistence. Furthermore, penicillin may be inappropriately administered to individuals with *Chlamydia* infections and is therefore relevant in vivo.

Treatment of three *C. trachomatis* serovars (A, B, and L2) as well as an avian *C. psittaci* strain (AP1) with penicillin from the time of infection induced large, aberrant forms, which could be identified by light microscopy at 24 h. These forms were noninfectious; that is, no inclusions developed after penicillin-treated chlamydiae were harvested and inoculated onto fresh monolayers (data not shown).

Induction of hsp60. It has been shown previously that a stress response can be induced in *C. trachomatis* by heating to

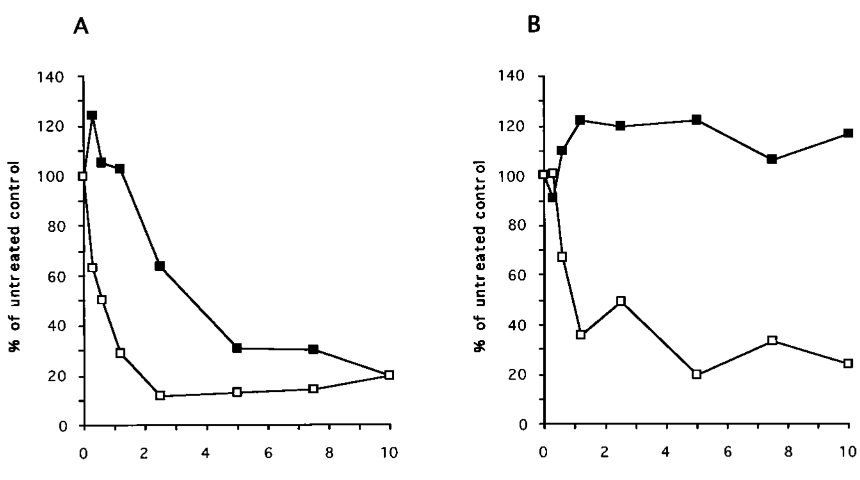

FIG. 1. Effect of IFN-γ on number of chlamydial inclusions (closed squares) and on mean inclusion size (open squares). *C. trachomatis* serovars A (A) and L2 (B) were cultured in HeLa cells for 48 h in the presence of increasing concentrations of recombinant human IFN-γ. At 48 h, monolayers were fixed and inclusions were visualized by staining with an FITC-conjugated monoclonal antibody specific for chlamydial LPS. Inclusions were quantitated by using a charge-coupled device camera and image analysis software. Note that the IFN-γ control (0 U/ml) underestimates the number of inclusions due to fusion of inclusions at the multiplicity of infection used.

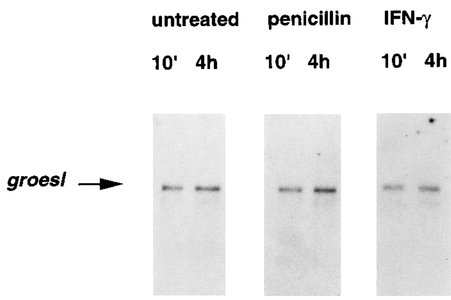

FIG. 2. Effect of stress on *groesl* expression. *C. trachomatis* L2-infected HeLa cell cultures were incubated for 24 h and then either left untreated or treated with penicillin (100 U/ml) or IFN-γ (2.5 U/ml) for 10 min and 4 h. Total RNAs from equivalent amounts of infected treated or untreated cultures were electrophoresed through denaturing gels and transferred to nylon membranes. The blots were hybridized with a PCR-generated probe specific for the chlamydial *groesl* operon.

45°C (13). We hypothesized that such a stress response may also be initiated by other factors. If persistence is a response to an environmental insult, a stress response may be involved, and this would be consistent with the notion of hsp60-mediated pathogenesis (6). Thus, two models of persistence, penicillin and IFN-γ treatment of infected cells, were chosen, and treated cultures were examined for the induction of a stress response. To develop a quantitative detection method for stress response, the response of *C. psittaci* to heat and changes in *groesl* transcript abundance was further characterized. When chlamydiae were exposed to 45°C, *groesl* mRNA was induced within 5 min. The amount of mRNA peaked between 5 and 10 min after induction, showing a 7- to 10-fold increase in *groesl* mRNA (data not shown). The amount of mRNA began decreasing between 10 and 30 min, and by 4 h after initiation of treatment, it had decreased almost to basal levels. In contrast, *omp1* mRNA levels remained constant for the first 10 min, but there was an overall reduction by the 4-h time point, while 16S rRNA remained constant throughout the entire period (data not shown).

Although heat induces a stress response in *Chlamydia* spp. within 5 min, which decreases by 4 h after initiation of treatment, there was no evidence that a stress response was induced by either penicillin or IFN-γ when cultures were treated similarly to the heat-stressed cultures, that is, cultured for 20 h and then exposed to penicillin or to IFN-γ for up to 4 h (Fig. 2). Thus, we can conclude that chlamydiae do not display an early stress response to penicillin or to IFN-γ, as they do to heat. However, both penicillin and IFN-γ require longer time periods to exert a morphological effect. It is possible that, given the lag time in either cell penetration or enzyme activity, the response may not be observed within 4 h of treatment. Thus, RNA extracted after 24 h of treatment was also examined. Again, no stress response was detectable when the cultures were incubated for 24 h in the presence of penicillin or of IFN-γ (Fig. 3). Similar results were obtained when chlamydiae were cultured in L929 or HeLa cells. Thus, while these experiments show that chlamydiae do not mount a classical stress response to penicillin or IFN-γ, it is very difficult to experimentally address the question of whether a transient stress response occurs sometime during the 48- to 72-h life cycle of chlamydiae. If such a hypothetical transient response does occur, its relevance to pathogenesis mediated by hsp60 is unclear and is not consistent with the persistence model, which requires prolonged increased expression of this antigen.

CTL lysis of infected cells. To test whether aberrant chlamydial forms could be recognized by immune T cells, penicil-

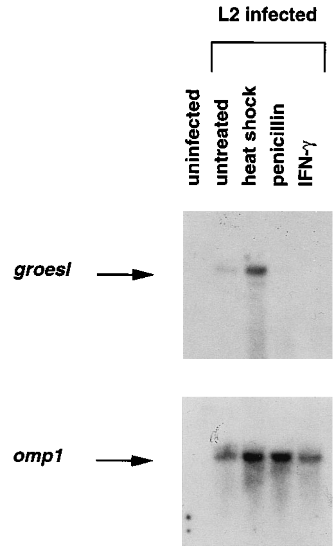

FIG. 3. Induction of *groesl* in *C. trachomatis* L2. *C. trachomatis*-infected L929 cell cultures were untreated or treated with heat (45°C for 5 min), penicillin (100 U/ml for 24 h), or IFN-γ (2.5 U/ml for 24 h). Equal amounts of total RNA from infected treated or untreated cultures were electrophoresed through denaturing gels and transferred to nylon membranes. The blots were hybridized with PCR-generated probes specific for the chlamydial *groesl* operon and the chlamydial *omp1* gene.

lin- and IFN-γ-treated L2 cultures were tested in CTL assays. Interestingly, these cultures were killed as efficiently as the untreated controls (Fig. 4A). Killing of penicillin-treated cultures was similar to that of untreated controls in the experiment shown (Fig. 4A) and in some experiments was marginally greater than that of untreated controls (data not shown). Thus, penicillin-mediated persistence or the generation of aberrant nonproductive forms did not reduce the ability of the immune system to recognize and kill the infected target cells. IFN-γ

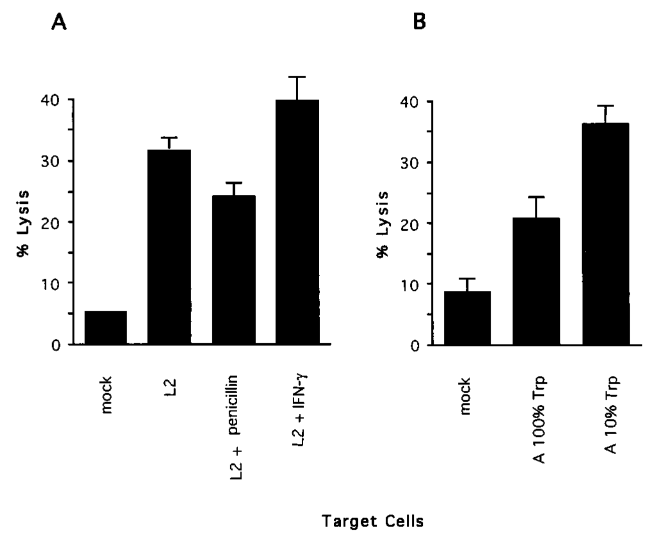

FIG. 4. CTL killing of *Chlamydia*-infected L929 cells is not decreased by persistence-inducing treatments. (A) CTL killing of mock-infected, L2-infected, penicillin-treated L2-infected, and IFN-γ-treated L2-infected cells; (B) CTL killing of mock-infected and serovar A-infected L929 cells with and without tryptophan depletion. The effector/target ratio in the experiments shown was 100:1. Results are expressed as mean ± standard error of the mean (n = 3) from representative experiments.

treatment of infected cells reproducibly led to significantly increased killing compared with the untreated control (Fig. 4A). The increase in cytolysis was partially due to non-immune system-mediated lysis of IFN-γ-treated infected cells, as 5 to 15% lysis was also observed in the presence of naive spleen cells or in the absence of spleen cells. However, it was apparent that specific immune killing accounted for a large part of the lysis, indicating that persistent forms are still recognized efficiently. Finally, when serovar A (a tryptophan-dependent serovar) was grown in tryptophan-deficient medium, fewer and smaller inclusions were formed. Surprisingly, the CTL killing of these cells was reproducibly increased over that of the untreated control culture (Fig. 4B). In contrast, it has been our experience that decreased lysis is generally observed when cells are infected with decreased amounts of chlamydial inoculum or when cultures are assayed at earlier time points when inclusions are smaller (data not shown).

DISCUSSION

A proposed mechanism for chlamydial pathogenesis is that *Chlamydia* spp. are able to persist in an altered state in which they are resistant to clearance by the immune system (26). It has further been suggested that such a persistent state can be modeled by treatment with IFN-γ or penicillin treatment and that the aberrant chlamydial forms thus generated produce increased levels of hsp60 and may serve as antigen depots which stimulate the deleterious components of the immune system (6). Despite the fact that cell-mediated immunity is known to be critical for clearance of chlamydial infection (24) and may even be capable of resolving infection in the absence of antibody (23), the potential role of cellular immunity in recognizing aberrant or persistent chlamydial forms has not been examined experimentally. The recent demonstration of *Chlamydia*-specific CTLs allowed us to address the question of whether persistent or aberrant chlamydial forms could be recognized and killed by CTLs. To establish a persistence model amenable to answering this question, we initially characterized the responses of different chlamydial strains to treatments which have been used previously as models for persistent infection.

The data demonstrated that the ability of IFN-γ to induce aberrant forms, as described previously for serovar A (6), is limited to a few strains. Specifically, *C. trachomatis* serovars A and B, but neither *C. trachomatis* serovar L2 nor *C. psittaci* AP1, produce aberrant forms after IFN-γ treatment of infected HeLa cells. In contrast to serovars A and B, serovar L2 and strain AP1 stained strongly with anti-MOMP monoclonal antibodies after growth in HeLa cells treated with IFN-γ and did not demonstrate a reduction in the number of inclusions. This difference in outcome may be explained by the mechanism by which IFN-γ exerts its effect. In human cells, IFN-γ induces the enzyme indoleamine 2,3-dioxygenase, which degrades tryptophan to *N*-formyl-kynurine and kynurenine (30). Selective omission of tryptophan from the chlamydial growth medium markedly inhibits growth of *C. trachomatis* serovars A to C but not of serovars D to K or L2 or several *C. psittaci* strains (1). We also showed significantly greater inhibition of *C. trachomatis* serovars A and B than of *C. trachomatis* serovar L2 or of *C. psittaci* AP1 when tryptophan was omitted from the growth medium. Thus, IFN-γ-mediated induction of aberrant forms correlated with tryptophan requirements.

Taken together, these results indicate that there may be two mechanisms by which IFN-γ exerts its effect in these cells. One mechanism occurs in the serovar and strain less sensitive to tryptophan depletion (serovar L2 and strain AP1) and results in undiminished numbers of inclusions, which retain normal morphology, but decreased numbers of infectious progeny are obtained. IFN-γ may affect *C. trachomatis* serovars A and B in a similar manner, but either it has more pronounced effects which result in morphological changes or there may be an additional mechanism which leads to the aberrant morphology in these serovars. However, most chlamydial strains are not sensitive to the omission of tryptophan from the growth media (1); therefore, if this IFN-γ-mediated persistent state does contribute to pathogenesis, it is unclear that this would be true for all chlamydial strains. As all chlamydial infections are similar in pathogenesis, it is difficult to envision that different mechanisms would apply for the different strains.

Regardless of the mechanism by which aberrant chlamydial forms are produced, the critical issue from the viewpoint of pathogenesis is whether such aberrant organisms would still be recognized and cleared efficiently by the immune system. Although tryptophan-dependent serovars (A, B, and C) may potentially be recognized with less efficiency by MOMP-specific antibodies, this work demonstrated that it is unlikely the remaining 15 serovars would show poorer antibody binding. Moreover, the host defense against intracellular pathogens seldom relies solely on antibody neutralization. The CTL response is important in the clearance not only of viral pathogens but also of intracellular bacteria (15). Chlamydial infections elicit CTLs which are functional in vitro (3, 28), and there is also evidence suggesting these cells may play a role in vivo (28). Thus, it is important to determine whether cells infected with aberrant, or viable but noninfectious, chlamydial forms would be recognized by specific CTLs.

When the ability of *Chlamydia*-specific CTLs to kill IFN-γ-treated, L2-infected cells was tested, increased lysis in comparison with the untreated, infected control was demonstrated. This was predominantly due to specific CTL-mediated lysis, although a portion of this increase may be attributed to the increase in nonspecific lysis of *Chlamydia*-infected cells which is observed following treatment with IFN-γ (9). It is difficult to reconcile a role for IFN-γ in promoting long-term persistence, in light of both the IFN-γ-mediated lysis previously described (9) and the specific CTL killing that we have demonstrated, both of which would function to reduce the chlamydial load. Therefore, even presuming that IFN-γ does produce aberrant persistent forms in vivo, these forms would not preferentially escape clearance by the cell-mediated immune system.

Use of tryptophan depletion enabled a *Chlamydia* persistence model to be tested in the absence of increased background lysis. Interestingly, even though growth of *C. trachomatis* serovar A was decreased when tryptophan was depleted, the efficiency of lysis was reproducibly increased. This finding was intriguing, because generally the percentage lysis increases throughout the chlamydial life cycle or at higher multiplicities of infection (unpublished data), both of which may be presumed to feed more antigen into the processing pathway. The increased lysis demonstrated with tryptophan limitation has relevance in vivo, because physiologic concentrations of amino acids are lower than those found in culture media and may lead to induction of abnormal forms (14). There appears to be some mechanism which compensates for the decreased sensitivity expected to be seen with decreased growth to keep the lysis at the same or an increased level. The block in the metabolic cycle may allow the preferential accumulation of specific antigens that are recognized by CTLs. Alternatively, starvation may increase the availability of these antigens to the endogenous processing pathway by impairing inclusion integrity.

A further demonstration of the ability of CTLs to recognize and kill aberrant chlamydial forms was provided by the efficient killing of penicillin-treated cells infected with chlamydiae. Once again, the killing of the treated cultures was consistently equal to the killing in the untreated cultures despite the fact that different mechanisms probably function to create aberrant forms in penicillin-treated cells and IFN-γ-treated cells.

The important conclusion from these experiments is that regardless of whether chlamydial forms are typical or aberrant, they are efficiently killed by specific CTLs. Thus, there is no direct evidence that such aberrant forms are capable of completely evading the immune response. An interesting and potentially important question is whether such a response would be advantageous or deleterious. One could envision a scenario in which the majority of chlamydial organisms were successfully neutralized by antibody, and all infected cells, whether or not aberrant or persistent forms were present, would be killed by CTLs, leading to resolution of infection. Alternatively, though not mutually exclusively, CTLs may contribute to disease pathogenesis mediated by lysing infected cells, even those with nonproductive or aberrant chlamydial forms.

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