

## Reactivation of Persistent *Chlamydia trachomatis* Infection in Cell Culture

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**Gamma interferon induces persistent chlamydial infections in cell culture. These infections are characterized by altered morphologic and biochemical features of the pathogen. These persistent forms are abnormally large and noninfectious and undergo unusual structural and functional changes, including production of a paucity of outer envelope constituents and normal levels of the chlamydial hsp60, an immunopathological antigen. The current investigation evaluates the events that occur during reactivation of infectious *Chlamydia trachomatis* from persistently infected cell cultures. Transfer of persistent chlamydial organisms to gamma interferon-free medium resulted in recovery of infectivity accompanied by an increase in levels of structural membrane proteins and reorganization of aberrant organisms to morphologically typical elementary bodies. In addition, reactivation of infectious organisms from persistent chlamydiae that were maintained in culture for several weeks was demonstrated. These studies show that persistent *C. trachomatis* maintains viability for extended periods, illustrate the reversibility of immunologically mediated persistent infections, and characterize reactivation at the ultrastructural and biochemical levels.**

*Chlamydia trachomatis* is a medically important bacterium that is responsible for a wide range of human infections and diseases. This obligate intracellular pathogen has evolved a unique developmental cycle involving a conversion between two distinct morphological forms. The infectious process begins with the attachment of the elementary body (EB) to a susceptible eukaryotic cell. Following ingestion into a host-derived endosome, the EB differentiates to the noninfectious, metabolically active reticulate body (RB). The RB multiplies by binary fission within the enlarging endosome, with the microcolony becoming visible as a chlamydial inclusion. By 48 to 72 h after infection, RBs reorganize to the metabolically inactive, infectious EBs, which are released from the host cell and can initiate new infectious cycles. This orderly developmental cycle is well characterized in cell culture systems. However, recent studies provide evidence to suggest that alternative forms that are difficult to recognize may be present during disease (8, 27, 28, 32), and studies have been initiated to characterize atypical intracellular chlamydial forms in vitro (6).

*C. trachomatis* infects the mucosal epithelium of the eyes and genital tract. Ocular infection can lead to conjunctival scarring, distortion of the lids, and trachoma, the leading cause of preventable blindness worldwide. Chlamydial infections of the urogenital tract are frequently asymptomatic but can result in severe complications, particularly in women, in whom they are manifested as fallopian tube damage and infertility. Inapparent infections in both ocular and genital tissues have been described (14, 16, 24, 33). *C. trachomatis* infections in which chlamydial antigens and nucleic acids can be identified in the absence of cultivable organisms have been reported (8, 27, 28, 32). The inability to detect *C. trachomatis* by routine culture

indicates that chlamydiae may be present but at levels undetectable by these techniques. Residual chlamydial antigen or nucleic acid also may reflect the presence of nonviable organisms. Alternatively, chlamydiae may persist as a nonculturable form that maintains viability.

Although the concept of persistent chlamydial infections is not new (21), the factors that may lead to persistence during the course of an actual infection are unknown. Recently, we characterized a gamma interferon (IFN- $\gamma$ )-mediated persistent infection in vitro (6). Those studies revealed that when infected cells are treated with low levels of IFN- $\gamma$ , chlamydial inclusions develop but do not result in the production of infectious progeny. Persistent infection is characterized by enlarged, abnormal chlamydial forms that are ultrastructurally similar to those induced by penicillin and depletion of nutrients (11, 18, 20). Evaluation of persistently infected cells reveals altered steady-state levels of key chlamydial antigens with a paucity of several outer membrane constituents, including the major outer membrane protein (MOMP), a 60-kDa outer envelope protein, and lipopolysaccharide. MOMP not only is perceived as essential for chlamydial structural integrity, making up approximately 60% of the outer membrane mass, but also may serve as a porin (4) and is implicated as an antigen that induces protective immunity (30, 36, 37). In addition to the alteration of the chlamydial outer envelope during persistent growth, levels of the stress protein hsp60, the chlamydial homolog of groEL, are maintained during persistent growth. hsp60 has been implicated as an antigen that induces immunologically related tissue damage during chlamydial disease (22).

In persistently infected cells, aberrant chlamydial forms are noninfectious; however, viability is maintained, as shown by the recovery of infectious progeny following the removal of IFN- $\gamma$  from the system (6). This observation suggests that either the enlarged forms of chlamydiae undergo reorganization events to generate typical infectious EBs or a small number of viable RBs and/or EBs remain and grow to levels that can be detected

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after IFN- $\gamma$  has been replaced with fresh medium. To distinguish between these two possibilities, the current study was undertaken to characterize the events that occur during the reactivation of persistent chlamydiae to typical infectious forms. Ultrastructural changes during the recovery process were evaluated to determine if the recovery of infectious organisms can be correlated to the development of typical EBs emanating from enlarged persistent chlamydial forms. Evaluation of altered expression and steady-state levels of key chlamydial proteins during reactivation also are presented. In addition, reactivation of infectious chlamydiae from long-term persistently infected cultures is documented.

## MATERIALS AND METHODS

**Organism and cell culture.** *C. trachomatis* serovar A/HAR-13 was grown in HeLa 229 cells, and EBs were purified by discontinuous density gradient centrifugation in Renografin (E. R. Squibb and Sons, Princeton, N. J.) (7). HeLa cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum (MEM-10) at 37°C in 5% CO<sub>2</sub>.

**Preparation of IFN- $\gamma$ -treated cultures.** HeLa 229 cells in MEM-10 were plated at a density of  $1.5 \times 10^5$  cells per well in 24-well culture plates. At 18 to 24 h later, the cell monolayers were washed with Hanks balanced salt solution (HBSS) and treated for 15 min at room temperature with HBSS containing DEAE-dextran (45  $\mu$ g/ml). The monolayers were washed with 1 ml of HBSS and 0.2 ml of 0.25 M sucrose–10 mM sodium phosphate–5 mM L-glutamic acid (SPG) (pH 7.2) containing  $3 \times 10^5$  inclusion-forming units of *C. trachomatis* serovar A/HAR-13 was added to each well. Cultures were incubated for 2 h at 37°C on a rocker platform. At 2 h postinfection, the inoculum was removed and replaced with 0.5 ml of MEM-10 or MEM-10 containing 0.5 ng of recombinant human IFN- $\gamma$  (Biogen, Cambridge, Mass.) per ml. Infected monolayers were incubated until the times indicated in individual experiments. To analyze events occurring following the removal of IFN- $\gamma$ , infected cells were cultured with low levels of IFN- $\gamma$  (0.5 ng/ml) for the indicated times. The medium was then removed and replaced with fresh MEM-10.

**Assay for infectivity.** At the indicated times, infected and treated cell monolayers were washed three times with HBSS, scraped from culture dishes into 0.5 ml of SPG, and frozen until all samples were collected. Samples were sonicated to disrupt the HeLa cells and release infectious EBs. These samples then were used to inoculate fresh monolayers of HeLa cells as described above. Infected monolayers were cultured for 48 h in MEM-10 containing 1  $\mu$ g of cycloheximide per ml, washed with HBSS, and fixed with methanol, and inclusions were visualized by indirect immunofluorescence with an anti-MOMP monoclonal antibody (A20) (37). To determine if infectious chlamydiae could be recovered from IFN- $\gamma$ -treated samples, infected cells were cultured with 0.5 ng of IFN- $\gamma$  per ml for the indicated times. The medium was then removed and replaced with fresh MEM-10. At the indicated times following removal of IFN- $\gamma$ , cells were harvested and assayed for infectious chlamydiae as described above.

**Immunofluorescence assays.** For indirect immunofluorescent labeling, monolayers were fixed with methanol, washed with phosphate-buffered saline (PBS), and reacted with mouse monoclonal antibodies to MOMP (A20) or hsp60 (A57-B9) (35, 37). Samples were washed with PBS–3% bovine serum albumin (BSA) and reacted with fluorescein isothiocyanate-labeled goat anti-mouse antibodies. Inclusion-containing cells were visualized with a Nikon fluorescence microscope.

**Ultrastructural analysis.** Monolayers were washed with PBS, and cells were removed by gentle scraping with a sterile disposable syringe plunger, pelleted by centrifugation, and fixed with 2% glutaraldehyde in 10 mM phosphate buffer for 2 h at 22°C. Following three washes with PBS, the samples were fixed for 1 h in 1% osmium tetroxide in PBS. The samples were then dehydrated in a graded series of ethanol and embedded in Durcupan (Polysciences Inc., Warrington, Pa.). Sections of 80 to 90 nm were cut, stained with uranyl acetate and lead citrate, and viewed with a Hitachi transmission electron microscope.

**Detection of DNA in condensed regions of persistent organisms.** HeLa cells were infected and treated as described above. At 6 h prior to each sampling time, 10  $\mu$ M bromodeoxyuridine (BrdU) was added to cultures. Samples were fixed and embedded for transmission electron microscopy as described above. Thin sections were collected on Formvar-coated nickel grids and treated with 4 M HCl for 30 min. The samples were then reacted with anti-BrdU mouse monoclonal antibodies (Becton Dickinson, San Jose, Calif.) or the following negative controls: PBS alone, omitting the primary antibody, or an irrelevant antibody of the same isotype. Samples were washed with PBS–3% BSA and reacted with goat anti-mouse immunoglobulin G conjugated to 15-nm colloidal gold (Amersham, Arlington Heights, Ill.). Immunolabeled grids were stained with uranyl acetate and lead citrate as described above.

**SDS-PAGE and immunoblotting.** At the indicated times, infected and treated cell monolayers in 24-well culture plates were washed with HBSS and harvested in 0.2 ml of Laemmli sample buffer (19). Samples were solubilized by boiling and 80  $\mu$ l of each sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% polyacrylamide) as described by Dreyfus

et al. (13). Following electrophoretic transfer to nitrocellulose, proteins were probed with either anti-MOMP (A20) or anti-hsp60 (A57-B9) monoclonal antibody as described previously (6).

**Intrinsic labeling.** Monolayers of infected and treated cells were cultured in 24-well plates. At 50 min prior to each sampling time, medium was removed and replaced with cysteine-deficient MEM containing 20  $\mu$ g of emetine per ml. Then, 30 min later, 25  $\mu$ Ci of [<sup>35</sup>S]cysteine was added to each well for a 20-min labeling period. The cells were then washed with MEM-10 containing an excess of cold cysteine and harvested for SDS-PAGE as described above.

**Long-term maintenance of persistence.** At specified time points, triplicate HeLa cell cultures were infected and treated with persistence-inducing concentrations of IFN- $\gamma$  (0.5 ng/ml). At 48 h postinfection, immunofluorescence and immunoblot analysis were used to confirm the establishment of chlamydial persistence as defined previously (6). The culture medium was then removed and replaced with medium supernatant from 24-h-old cultures of uninfected HeLa cells in MEM-10 supplemented with 0.2 ng of IFN- $\gamma$  per ml (24-h conditioned medium). Conditioned medium was replaced every 48 h for the duration of the experiment. Maintenance of persistence by this method was necessary to maintain host cell viability, which declined more rapidly when cultures were supplemented with IFN- $\gamma$  directly. Samples were collected every 2 days for immunofluorescence and infectivity assays.

## RESULTS

**Morphological evaluation of recovery from IFN- $\gamma$ -induced persistence.** Treatment of chlamydia-infected cells with low levels of IFN- $\gamma$  (0.5 ng/ml) postinfection results in the loss of chlamydial infectivity despite the presence of chlamydial inclusions containing large abnormal RBs (6). Removal of IFN- $\gamma$  results in the recovery of infectious chlamydiae (6). Extensive analysis by transmission electron microscopy of chlamydia-infected cells cultured in the presence of IFN- $\gamma$  failed to reveal the presence of typical EB forms, suggesting that the enlarged, aberrant RBs reorganize to infectious EBs when subsequently cultured in the absence of IFN- $\gamma$ . Morphological development of infectious chlamydiae from aberrant forms following the removal of IFN- $\gamma$  was analyzed. Figure 1 shows sequential changes that occurred during the recovery process. Aberrant chlamydial forms demonstrating internal fragmentation and extensive budding were observed early in the recovery process (Fig. 1A to C). In addition, single aberrant organisms displaying multiple nucleoid-like regions of condensation were observed late in the recovery process (Fig. 1D). Internal reorganization of the atypical forms to numerous relatively normal-size chlamydiae was evident at both 24 and 48 h (Fig. 1E) following IFN- $\gamma$  removal. These different morphological features of the recovery process could be observed within a single chlamydial inclusion and were characteristic of the population of infected cells. By 48 h following the removal of IFN- $\gamma$ , few abnormal forms were present and chlamydial inclusions contained predominantly morphologically typical EBs and RBs (data not shown). This process was concomitant with the recovery of infectious progeny as reported previously (6) (data not shown). These studies provided evidence that persistent chlamydial forms maintained viability and that recovery of infectious progeny after IFN- $\gamma$  removal was a result of reorganization of persistent forms and not the presence of small numbers of viable RBs and/or EBs that remained undetected by culture during IFN- $\gamma$  treatment.

**Detection of DNA in condensed regions of persistent organisms.** During the normal chlamydial developmental cycle, RBs multiply by binary fission and are characterized by the presence of dispersed chromatin. Differentiation of RBs to infectious EBs is accompanied by the formation of a dense nucleoid-like structure. The condensation of the chlamydial nucleoid is concomitant with developmentally regulated expression of proteins homologous to the eukaryotic protein H1 histone (3). Therefore, the multiple dense structures within a single aberrant form observed in the present studies (Fig. 1D) appeared to be condensed nucleoid masses, and their presence indicated



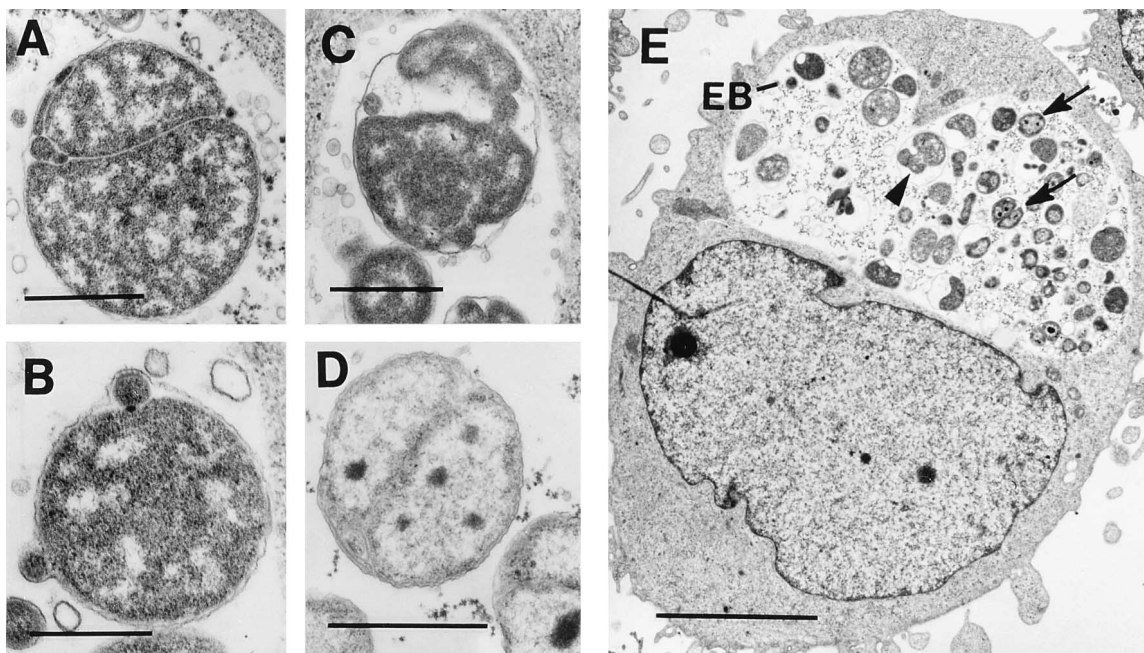


FIG. 1. Ultrastructural analysis by electron microscopy of rescue of infectious chlamydial forms from cells treated with IFN- $\gamma$  for 48 h after infection and subsequently cultured in the absence of IFN- $\gamma$ . (A and B) At 12 h following the removal of IFN- $\gamma$ , internal fragmentation of aberrant forms (A) and budding from enlarged RBs (B) were observed. (C) By 24 h following the removal of IFN- $\gamma$ , several additional events were observed, including internal reorganization of aberrant forms within the chlamydial membrane. (D) Nucleoid-like structures within aberrant forms were observed at both 24 and 48 h following the removal of IFN- $\gamma$ . (E) The different features of a typical chlamydial inclusion 24 h after the removal of IFN- $\gamma$  include internal reorganization within aberrant forms (arrowhead), multiple nucleoid-like structures (arrows), and the reappearance of typical chlamydial elementary bodies (EBs). Bars, 1  $\mu$ m (A to D) and 5  $\mu$ m (E).

that the reorganization of genetic material occurred during the recovery process. This was confirmed by labeling with the thymidine analog BrdU, which was used to detect the presence of DNA. Immunoelectron microscopy with anti-BrdU-specific antibodies was done to identify and localize DNA. Specific labeling was associated with aberrant chlamydiae and smaller budding forms (Fig. 2A) at 12 h after IFN- $\gamma$  removal and with dense nucleoid structures by 24 h after IFN- $\gamma$  removal (Fig. 2B). Thus, the localization of chlamydial DNA to nucleoid-like masses suggested that multiple genomes were present in persistent forms and underwent reorganization to produce multiple infectious EBs from a single aberrant form of the organism.

**Analysis of protein levels during recovery.** IFN- $\gamma$ -mediated induction of persistence has a pronounced effect on the steady-state levels of several important chlamydial antigens. Treatment of infected cells with low concentrations of IFN- $\gamma$  is accompanied by a substantial decrease in the major constituents of the chlamydial outer membrane (6). A reduction in the levels of MOMP, the 60-kDa outer envelope protein, and LPS corresponds to altered chlamydial growth and differentiation. To determine if recovery of infectivity following the removal of IFN- $\gamma$  results in increased synthesis of outer membrane components, immunoblot analyses were performed. Treatment of infected cells with 0.5 ng of IFN- $\gamma$  per ml resulted in a reduction in the steady-state levels of MOMP (shown as a doublet) and the 60-kDa outer envelope protein compared with those in untreated controls (Fig. 3). When IFN- $\gamma$ -containing medium was replaced at 48 h with IFN- $\gamma$ -free medium, an increase in the levels of MOMP and the 60-kDa outer envelope protein was detected 12 h later. The levels of MOMP and the 60-kDa outer envelope protein approached those in untreated samples at 24 and 48 h, respectively, following the removal of IFN- $\gamma$ . In contrast, normal levels of hsp60 were present during IFN- $\gamma$  treatment and the recovery period. Thus, as expected, recovery

of infectivity following the removal of IFN- $\gamma$  is accompanied by an increase in the steady-state levels of key structural chlamydial components.

**Analysis of protein synthesis during recovery.** To analyze chlamydial protein expression during recovery, infected-cell cultures were treated with emetine to inhibit host cell protein synthesis and then pulse-labeled with [ $^{35}$ S]cysteine for 20 min prior to the indicated samples times. The use of [ $^{35}$ S]cysteine is a particularly useful measure of expression of chlamydial outer envelope proteins since the three major outer envelope proteins (MOMP and the 60- and 12-kDa outer envelope proteins) contain an abundance of cysteine residues (2, 12, 23). Initial analysis of autoradiographs revealed an overall lower level of chlamydial protein expression in the presence of 0.5 ng of IFN- $\gamma$  per ml when samples were analyzed up to 72 h postinfection. A prominent doublet was observed at approximately 37 to 40 kDa in untreated samples at 48 h after infection (Fig. 4, lane 2) and was identified as MOMP by immunoblotting of two-dimensional gels (results not shown). In IFN- $\gamma$ -treated samples there was only low-level synthesis of the doublet (lane 4). When IFN- $\gamma$  was removed from the system at 48 h following infection and replaced with medium without IFN- $\gamma$ , an increase in the synthesis of MOMP occurred corresponding to the increase in steady-state levels of MOMP shown by immunoblotting (Fig. 3). The increase in MOMP synthesis was evident at 12 h, and synthesis was comparable to that in untreated samples by 24 h after the removal of IFN- $\gamma$ .

Other prominent protein bands in untreated samples at 48 h after infection were observed at approximately 60 kDa, shown as a doublet by [ $^{35}$ S]cysteine labeling (Fig. 4, lane 2). Immunoblot analyses of two-dimensional gels identified these proteins as hsp60 and the 60-kDa outer envelope protein. In the presence of IFN- $\gamma$  at 48 h, only hsp60 was identified by cysteine labeling (lane 4), corresponding to immunoblot data showing

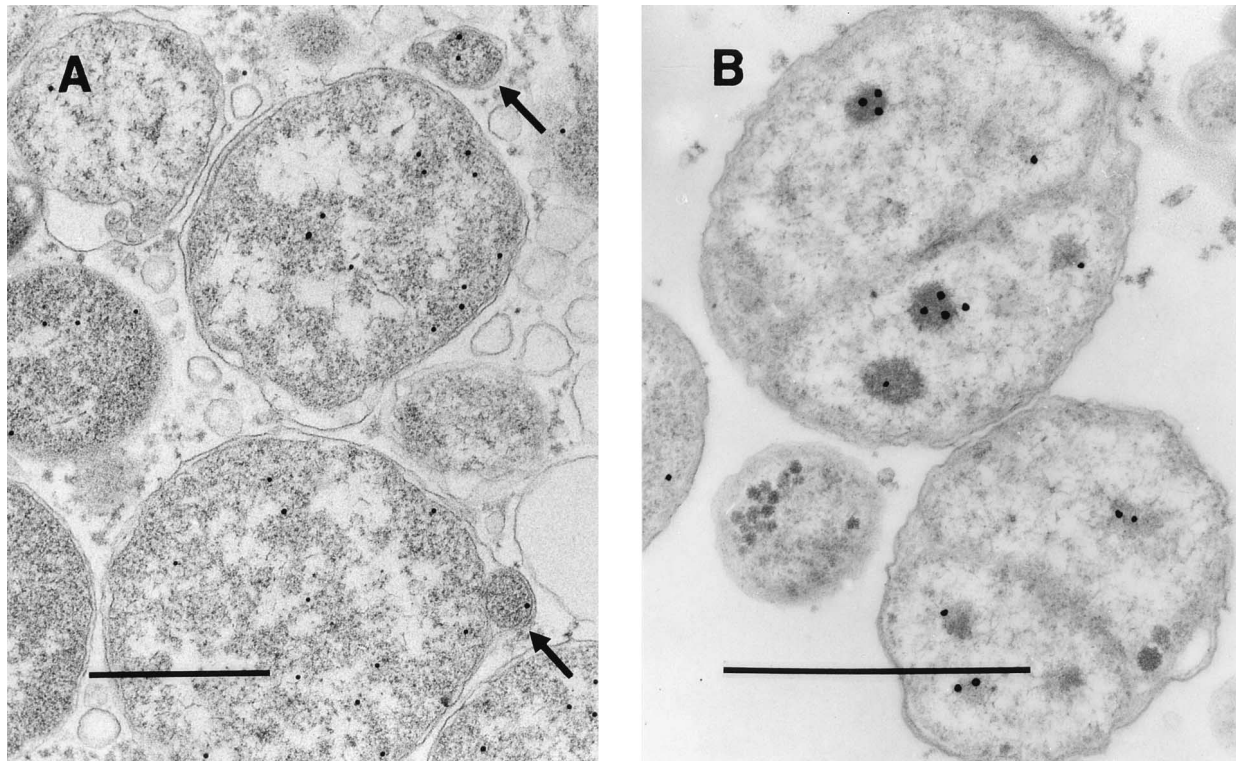


FIG. 2. Immunoelectron microscopy of BrdU incorporation during the recovery process. (A) At 12 h after removal of IFN- $\gamma$ , specific labeling was associated with aberrant chlamydiae and with smaller budding forms (indicated by arrows). (B) At 24 h after removal of IFN- $\gamma$ , anti-BrdU antibodies localized DNA synthesis to dense nucleoid masses. Bar, 1  $\mu$ m.

near-normal levels of hsp60 and reduced levels of 60-kDa envelope protein in persistently infected cells compared with untreated controls (Fig. 3). Synthesis of the 60-kDa outer envelope protein was evident at 24 h following the removal of IFN- $\gamma$ , as shown by the reappearance of a doublet (Fig. 4). The higher levels of MOMP synthesis than of 60-kDa outer envelope protein synthesis (8 versus 24 cysteines) (2, 12) can be explained by the relative abundance of these proteins at the sample times and the nature of the chlamydial developmental

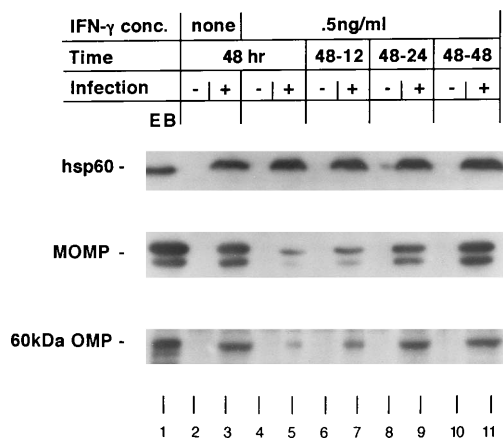


FIG. 3. Immunoblot analysis of untreated and IFN- $\gamma$ -treated cells with anti-hsp60, anti-MOMP, and anti-60-kDa outer envelope protein antibodies. 48-12, 48-24, and 48-48 refer to IFN- $\gamma$  treatment for 48 h followed by the time of incubation.

cycle. The 60-kDa outer envelope protein is developmentally regulated and expressed only late in the developmental cycle, in contrast to MOMP, one of the most abundant chlamydial proteins, which is constitutively expressed (15, 29). Pulse-chase experiments indicated that the low level of synthesis of MOMP and the 60-kDa outer envelope protein in IFN- $\gamma$ -treated cultures was not a result of degradation of these proteins in the presence of IFN- $\gamma$  (data not shown). The relatively early appearance of these proteins following the removal of IFN- $\gamma$ , compared with their appearance in a typical infected culture (data not shown), further suggested that the recovery of infectious organisms must have arisen from the enlarged atypical forms and not from low levels of EBs present.

**Long-term maintenance of persistence.** We investigated the length of time that aberrant forms could be maintained in cell culture with subsequent reactivation of infectious organisms. When infected HeLa cells were treated with persistence-inducing concentrations of IFN- $\gamma$  (0.5 ng/ml), the level of recoverable infectivity following the removal of IFN- $\gamma$  decreased with increasing length of treatment. The decrease in the level of recoverable infectious chlamydiae appeared to be due to the effect of IFN- $\gamma$  on both the chlamydiae and host cell proliferation, with complete loss of cell culture monolayers within 1 week of culture. Therefore, to maintain long-term persistently infected cells, cultures were treated with 0.5 ng of IFN- $\gamma$  per ml to induce chlamydial persistence and subsequently maintained at a lower level of IFN- $\gamma$  (0.2 ng/ml) to limit potentially detrimental effects of this cytokine on the infected-cell population. Maintenance of infected host cells required addition of fresh MEM-10 (with IFN- $\gamma$ ) every 48 h. However, low-level reactivation of chlamydiae occurred despite the presence of IFN- $\gamma$ ,





tent infection characterized by large atypical RBs and an absence of infectious EBs in a cell culture model (6). IFN- $\gamma$  alters the chlamydial developmental cycle such that production of infectious progeny is inhibited but viability is maintained, as indicated by the recovery of infectivity following the removal of IFN- $\gamma$ . Alteration in chlamydial growth and differentiation was concomitant with a decrease in the levels of three major structural constituents of chlamydiae in the presence of IFN- $\gamma$ : MOMP, the 60-kDa outer envelope protein, and LPS. Indoleamine 2,3-dioxygenase induction is an IFN- $\gamma$ -mediated activity essential for the development of chlamydial persistence (5). It is not clear how this activity directly relates to differential regulation of chlamydial protein synthesis, but it is known, for example, that chlamydial hsp60 contains no tryptophan residues (12) whereas serovar A MOMP contains several tryptophans, which are present only in conserved regions of the molecule (2). The present study indicated that the recovery of infectious chlamydiae following the removal of IFN- $\gamma$  is accompanied by an increase in both the synthesis and steady-state levels of the chlamydial components MOMP and the 60-kDa outer envelope protein that were down-regulated during the persistent state. The rates at which these proteins were synthesized following IFN- $\gamma$  removal suggested that synthesis arises from reorganization of aberrant chlamydial forms and not from low levels of viable EBs present during IFN- $\gamma$  treatment.

Ultrastructural analysis of *Chlamydia* cells following the removal of IFN- $\gamma$  revealed reorganization of atypical RBs to morphologically typical developmental forms of chlamydiae concomitant with the recovery of infectious organisms. Internal fragmentation and budding events similar to those reported during the recovery from penicillin-induced aberrant chlamydial growth (20) were observed. Features similar to those observed during the recovery process in this study have been identified *in vivo* in an ultrastructural analysis of *C. trachomatis* infection of murine oviducts (25).

Another feature observed during recovery from IFN- $\gamma$ -induced persistence was the development of multiple dense structures within a single aberrant form. Similar observations were observed in typical chlamydia-infected cells (34) and more recently in studies of induction of aberrant chlamydial growth by nutrient deprivation (11). Immunoelectron microscopy localized chlamydial DNA to the dense nucleoid-like masses. These observations suggested that endopolygony occurred, in which several daughter cells formed within a parent organism with multiple nuclear divisions possibly occurring before progeny formation.

It was also found that IFN- $\gamma$ -mediated persistent *Chlamydia* cells could be maintained for extended periods in cell culture and were viable and that infectious organisms were recoverable after the immunological pressure was relieved. Because *C. trachomatis* infects mucosal epithelial cells of the eyes and genital tract, it has long been thought that chlamydiae are unlikely to persist because of the high turnover rate of these epithelial cells. *C. trachomatis* also may gain access to sites in the subepithelial tissue (8), thus providing a tissue site where chlamydiae could reside for extensive periods; however, these observations remain to be confirmed. Persistence of chlamydiae during natural infection would require alterations between the development of metabolically inactive, aberrant, persistent forms and periods of active chlamydial growth.

Although animal models, cell culture, and epidemiological studies have provided evidence for the occurrence of chlamydial persistence, documentation of such a state in natural infections remains to be established conclusively. Studies reported here defined biological characteristics associated with reactivation of persistent *C. trachomatis* infections. Further work in

*vitro* and *in vivo* will improve the understanding of damaging versus protective immunity and develop a more complete picture of the pathogenesis of chlamydial disease based on the unique characteristics of persistent *Chlamydia* organisms as defined in this cell culture model.

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