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Immunoelectron-Microscopic Quantitation of Differential Levels of Chlamydial Proteins in a Cell Culture Model of Persistent *Chlamydia trachomatis* Infection

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Electron immunolabeling techniques were used for quantitative evaluation of alterations in the steady-state levels of chlamydial antigens in persistent *Chlamydia trachomatis* cultures. Gamma interferon-mediated persistent chlamydial development correlated with an increase in the levels of the chlamydial heat shock protein (hsp60) and with a significant reduction in the levels of the major outer membrane protein.

An in vitro cell culture system for gamma interferon (IFN-γ)-mediated persistent *Chlamydia trachomatis* infection has been established (3). Induction of persistent chlamydial development occurs when IFN-γ is present during infection (1) and is characterized by the presence of enlarged, atypical chlamydial forms that are noninfectious but that retain viability as shown by the reactivation of infectious elementary bodies (EB) upon the removal of IFN-γ (3, 4). These organisms exhibit alterations in key chlamydial components with a reduction in the synthesis (4) and steady-state levels of the major outer membrane protein (MOMP), a protective antigen (10), while normal levels of hsp60, an immunopathologic antigen (8), are apparently maintained (3, 4). Because an overall reduction of chlamydial growth accompanies development of the persistent state (i.e., inclusions are smaller) in addition to morphological alterations in the organism itself (i.e., enlarged, aberrant chlamydiae), it is difficult to determine if the resulting differential protein levels are a result of an increase in the levels of hsp60, a decrease in the levels of MOMP, or an alteration in the levels of both proteins. In addition, IFN-γ treatment causes a slight decrease in both the infected cell number and the host cell number. Because of these intervening factors it is difficult to directly compare chlamydial protein levels in IFN-γ-treated cultures and untreated controls.

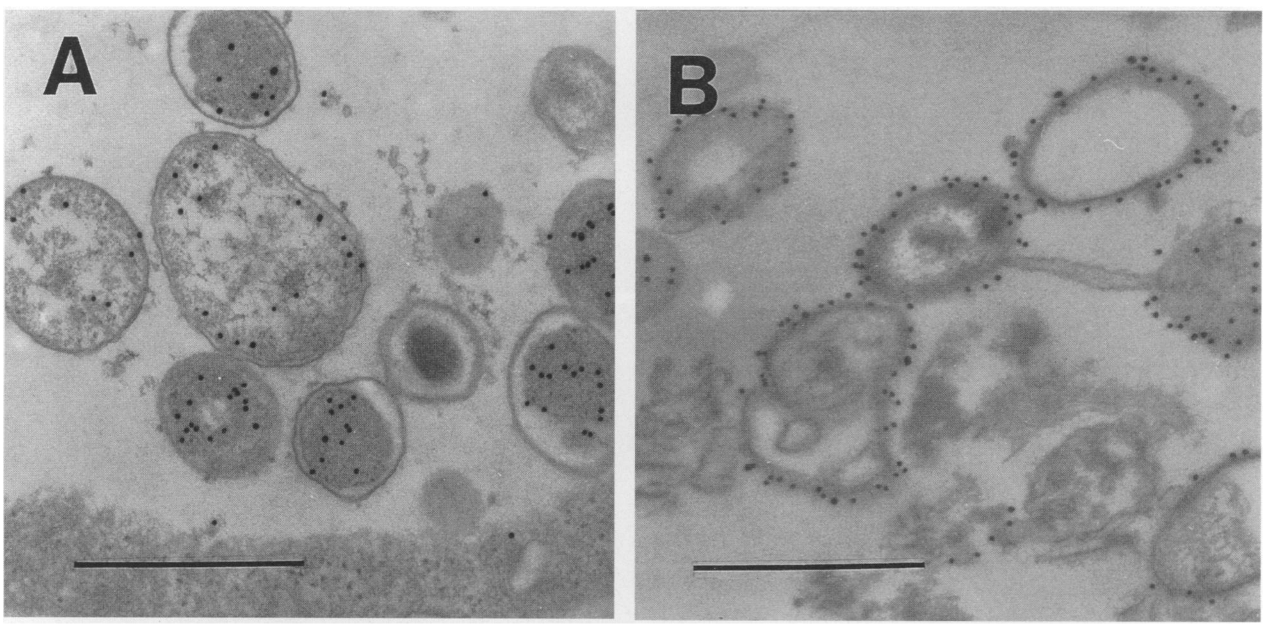

FIG. 1. Immunoelectron microscopy of *C. trachomatis*, colloidal gold labeled with anti-hsp60 primary antibody postembedding (A) and with anti-MOMP primary antibody preembedding (B). Samples were embedded in Durcupan. Bar = 0.5 μm.

Immunoelectron microscopy is a standard method for ultrastructural localization of antigens on cells and tissues and

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TABLE 1. Quantitation of chlamydiae by electron microscopy[a]

HeLa 229 cells	No. of chlamydiae/ inclusion[b]	Diam of inclusion (μm)	Diam of chlamydia (μm)	Estimated area of chlamydia (μm^2)[c]
Untreated	131.2 (4.44)	10.81 (0.59)	0.354 (0.016)	0.395
IFN-γ treated	14.4 (1.17)	7.94 (0.41)	1.07 (0.135)	3.600

[a] Data are means (and standard errors of the means).
[b] Determined from the analysis of 20 different chlamydial inclusions.
[c] Area was estimated by using the mean diameter of chlamydiae.

permits a relative quantitative evaluation of antigenic distribution. This method was used to extend previous studies of differential levels of chlamydial proteins observed under conditions of persistent chlamydial development (3, 4). The present study demonstrated a significant reduction in the level of MOMP under conditions of IFN-γ-mediated chlamydial persistence and an increase in the level of chlamydial hsp60 when levels of these antigens were expressed as the number of colloidal gold particles present as a function of the total *C. trachomatis* surface area.

C. trachomatis serovar A/Har-13 was grown in HeLa 229 cells, and EB were purified by discontinuous density centrifugation in Renografin (E. R. Squibb and Sons, Princeton, N.J.) (5). For the preparation of persistently infected cultures, HeLa 229 cells in minimal essential medium with 10% fetal bovine serum (MEM-10) were infected with *C. trachomatis* as described previously (3). Two hours postinfection, the inoculum was removed and replaced with MEM-10 alone or MEM-10

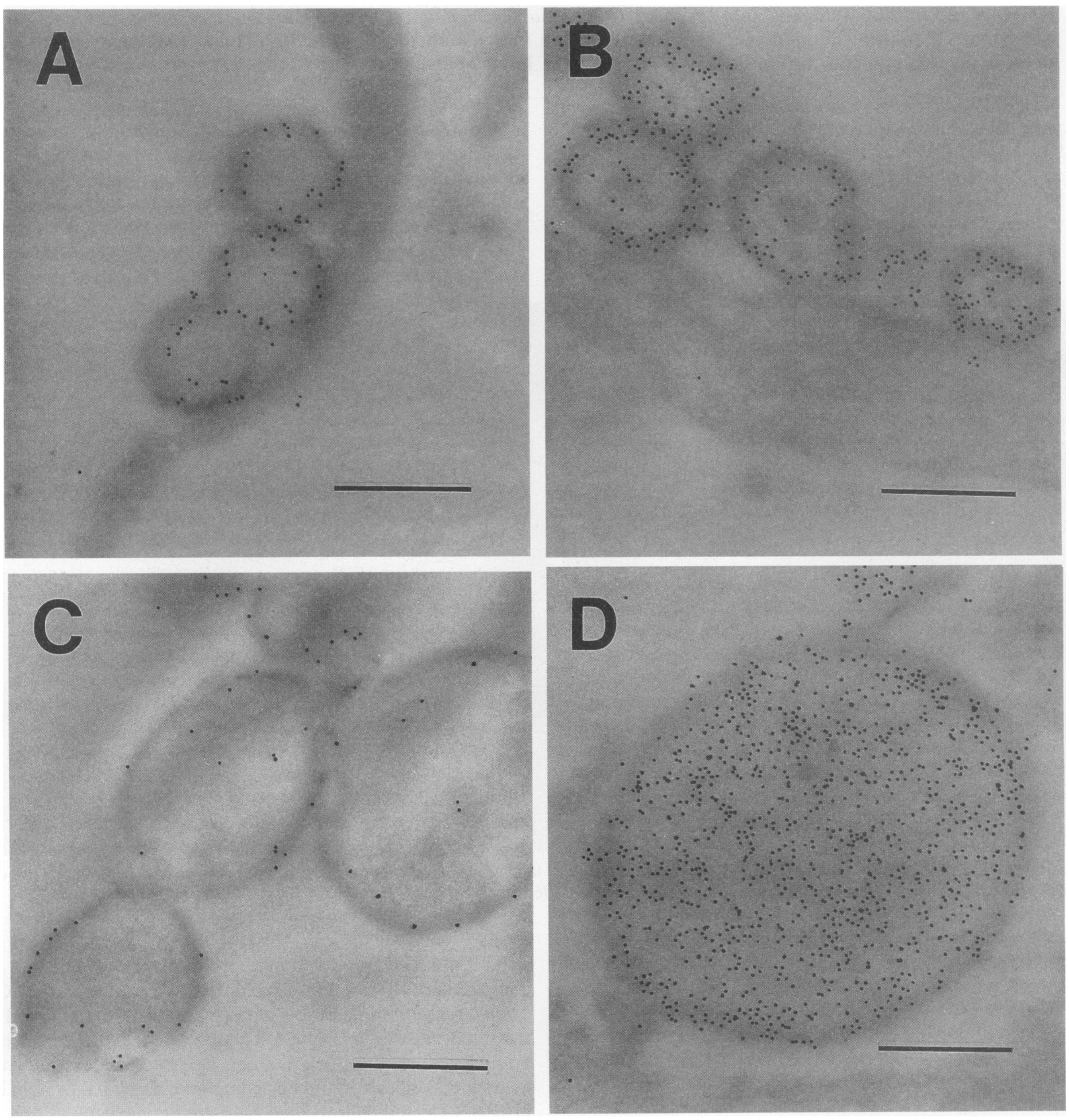

FIG. 2. Immunoelectron-microscopic analyses of untreated (A and B) and IFN-γ-treated (0.5 ng/ml) (C and D) cells, colloidal gold labeled with either anti-MOMP (A and C) or anti-hsp60 (B and D) primary antibodies. Samples were embedded in Lowicryl. Bar = 0.5 μm.

containing 0.5 ng of recombinant human IFN-γ (Biogen, Cambridge, Mass.) per ml.

For electron microscopy analyses, 48-h-infected monolayers were washed with phosphate-buffered saline (PBS), and the cells were removed by gentle scraping with a sterile syringe plunger and pelleted by centrifugation. In specimen preparation we were confronted with the problem of retaining the antigenicity of the proteins of interest. Previously we have used a procedure encompassing glutaraldehyde fixation followed by secondary fixation in osmium tetroxide and embedding in Durcupan (Polysciences Inc., Warrington, Pa.) (3, 4). This procedure provided ultrastructural preservation with excellent morphological detail. In addition, the preparations retained antigenicity for immunolabeling with antibodies to chlamydial hsp60 (Fig. 1A). However, the antigenicity of MOMP was lost, as shown by the inability to be labeled with a number of MOMP-specific monoclonal antibodies recognizing epitopes in different regions of the protein. Techniques allowing for the penetration of immunological reagents into fixed and permeabilized cells prior to embedding resulted in the labeling of chlamydial membranes with anti-MOMP-specific antibodies (Fig. 1B). However, these preparations failed to maintain intact chlamydial inclusions.

A variety of conditions for fixation, embedding, and antibody application were tested. Eventually, Lowicryl K4M resin (Chemische Werke Lowi, Waldkraiburg, Germany) was employed and found to be satisfactory for analysis (6). Briefly, infected cells were washed with 0.1 M Sorenson's buffer and fixed in 2% paraformaldehyde–0.25% glutaraldehyde. The samples were dehydrated in 70% methanol for 5 min at 4°C followed by 90% methanol for 30 min at −20°C. The samples were infiltrated with increasing concentrations of Lowicryl and embedded by UV photopolymerization of Lowicryl for 24 h at −20°C followed by 48 h at room temperature. Gold and/or silver sections were cut with a diamond knife and collected on nickel grids.

For immunolabeling, the sections were placed on drops of blocking buffer (PBS, 3% bovine serum albumin) for 30 min at room temperature. The sections were then reacted for 2 h with either anti-MOMP (A20) or anti-hsp60 (A57-B9) antibody diluted in PBS (9, 11). Monoclonal antibody A20 is serovar-specific, recognizing an epitope in variable domain 1 of MOMP (2). Monoclonal antibody A57-B9 reacts with the carboxyl terminus of hsp60 and is not cross-reactive with eukaryotic hsp60 (in HeLa 229 cells) (9). In addition, sample sections exposed to PBS alone, without the primary antibody, or PBS with an irrelevant antibody of the same isotype were used as negative controls. After being washed three times with blocking buffer, the samples were reacted with 15-nm colloidal gold-conjugated secondary antibody for 2 h. The sections were washed in blocking buffer, rinsed with deionized water, and stained with uranyl acetate and lead citrate prior to being viewed on a Hitachi transmission electron microscope.

For each quantitative evaluation, 10 infected cells were photographed at a magnification of ×5,000 and analyzed at a final magnification of ×13,750. The diameters of chlamydiae and chlamydial inclusions and the numbers of chlamydiae and colloidal gold particles were determined manually.

Analysis of the specimen preparations revealed that antigenicity was preserved and that localization of the gold label was specific for chlamydiae. Morphology was compromised, however, with the omission of osmium tetroxide, resulting in poor contrast, particularly of membranes. In addition, it was difficult to definitively distinguish EB and reticulate bodies (RB); therefore, these developmental forms were not differentiated during quantitation of samples embedded in Lowicryl. However, analysis of parallel samples prepared with Durcupan revealed roughly a 1:2 ratio of EB to RB in untreated samples 48 h postinfection (data not shown). The ratio of hsp60 to MOMP in EB compared with that in RB is not conclusively known. However, results of immunoblotting at various times postinfection suggest that the ratio of these proteins remains relatively constant throughout the developmental cycle (3). Electron-microscopic analysis of infected cells treated with IFN-γ revealed the development of enlarged, aberrant RB forms with no detectable EB at 48 h postinfection. Morphological evaluation and quantification of IFN-γ-treated infected cells embedded in Lowicryl revealed smaller inclusions containing nearly a 10-fold decrease in the number of chlamydial organisms compared with that in untreated controls (Table 1). In contrast, the average estimated area (in square micrometers) in a cross section of the IFN-γ-induced aberrant forms was almost 10 times that in untreated controls. Therefore, the total cross-sectional area of chlamydiae per inclusion was identical for untreated and IFN-γ-treated samples. The patterns of colloidal gold distribution revealed that anti-MOMP antibodies principally localized to the outer envelope of chlamydiae (Fig. 2A and C) (7), whereas anti-hsp60 labeling was more uniformly distributed in the cytoplasm of the organisms (Fig. 2B and D). Very little labeling was observed in the inclusion lumen or on the infected host cell, and this low background was similar under all conditions tested.

When colloidal gold beads were counted and expressed as the number of particles per square micrometer of chlamydiae, the intensity of hsp60 labeling was found to be 137.9 particles per μm² for typical organisms and 191.7 particles per μm² for persistent organisms (Table 2). The intensity of anti-MOMP labeling was 51.7 particles per μm² for typical chlamydiae and 8.8 particles per μm² for persistent organisms. These data indicate that the levels of hsp60 increased slightly in persistent organisms and that MOMP levels decreased approximately sixfold as determined when cross sections were analyzed at 48 h postinfection. When the density of colloidal gold beads specific for MOMP was expressed as the number of particles per micrometer of cross-sectional chlamydial envelope, the intensity of labeling decreased twofold in persistent organisms (data not shown). In addition, a threefold decrease in the surface-to-volume ratio of the enlarged, aberrant persistent organisms was observed when the ratio was compared with that of typical chlamydial forms. Therefore, the sixfold decrease in

TABLE 2. Immunolabeling with chlamydia-specific antibodies[a]

HeLa 229 cells and antibody	No. of colloidal gold particles/ chlamydia[b]	No. of colloidal gold particles/ inclusion[c]	No. of colloidal gold particles/ μm² of chlamydiae[d]
Untreated			
anti-hsp60	52.9 (3.97)	6,940.5	137.9 (11.05)
anti-MOMP	19.0 (1.76)	2,492.8	51.7 (6.78)
IFN-γ treated			
anti-hsp60	670.0 (105.4)	9,648.0	191.7[e] (27.6)
anti-MOMP	29.5 (3.29)	424.8	8.8[f] (1.01)

[a] Data are means (and standard errors of the means).
[b] Determined from the analysis of 10 different chlamydial inclusions.
[c] Determined from the mean numbers of colloidal gold particles per chlamydia and the mean numbers of chlamydiae per inclusion.
[d] Determined from the numbers of colloidal gold particles per square micrometer of chlamydiae and the estimated areas of chlamydiae.
[e] $P > 0.05$ by a two-tailed t test.
[f] $P < 0.0005$ by a two-tailed t test.

MOMP levels correlated with a reduction in both the density of MOMP molecules and the total length of the cross-sectional chlamydial envelope per inclusion. Similar results were obtained when monoclonal antibodies recognizing epitopes in other regions of MOMP and hsp60 were used.

These experiments further substantiated the presence of differential levels of two key chlamydial antigens in IFN-γ-mediated persistent *C. trachomatis* infection and proved to be essential in defining the chlamydial antigenic composition and distribution as functions of persistence.

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