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Morphologic and antigenic characterization of interferon γ-mediated persistent *Chlamydia trachomatis* infection *in vitro*

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ABSTRACT **An *in vitro* cell culture system was used to study the effect of interferon γ (IFN-γ) on *Chlamydia trachomatis* growth and differentiation. The effect of IFN-γ on chlamydiae was dose-dependent. IFN-γ at 2 ng/ml completely inhibited chlamydial growth and differentiation; however, persistent infection was established when chlamydiae were cultured with IFN-γ at 0.2 ng/ml. Persistent infection was characterized by the development of noninfectious atypical chlamydial forms from which infectious progeny could be recovered only when IFN-γ was removed from the culture system. Analysis of persistently infected cells by immunofluorescent microscopy and immunoblotting with specific antibodies revealed that the atypical chlamydial forms had near-normal levels of the 60-kDa heat shock protein, an immunopathologic antigen, and a paucity of the major outer membrane protein, a protective antigen. Furthermore, steady-state levels of other outer membrane constituents, such as the 60-kDa cysteine-rich outer membrane protein and lipopolysaccharide, were greatly reduced. If IFN-γ causes similar events to occur *in vivo*, then persistently infected cells could augment the pathogenesis of the chronic inflammatory sequelae that follow chlamydial infection by serving as depots of antigen capable of stimulating a sustained inflammatory response.**

Chlamydia trachomatis is an obligate intracellular parasite that has a distinctive biphasic growth cycle. Chlamydiae exist as two cell types with distinct morphological and functional properties: the elementary body (EB) is the metabolically inert, infectious extracellular form, and the reticulate body (RB) is the noninfectious, metabolically active intracellular form. Infections caused by chlamydia are among the most prevalent causes of genital and ocular diseases worldwide. Chlamydial infections can progress to chronic inflammatory sequelae that lead to blindness or infertility. The immune mechanisms that augment chronic inflammation are ill-defined, but repeated exposure to chlamydial antigens is thought to contribute to the disease process. Although the importance of repeated infection in the development of the chronic inflammatory sequelae of chlamydial disease has been established (1), persistent infection might also serve as a source of antigen for chronic inflammatory responses.

Documentation of *in vivo* persistent chlamydial infection remains controversial, but several studies provide indirect evidence that persistent infections occur and, thus, might contribute to the pathogenesis of chlamydial disease (2–7). These studies have led to renewed interest in defining conditions that result in persistence, how persistent infections can be maintained given the relatively rapid host cell turnover time, and how persistence could alter disease status. A mechanism by which persistence could augment the disease process is by exacerbating hypersensitivity to chlamydial antigens. Factors that lead to persistence are not well-defined, but previous studies demonstrate that treatment of host cells with interferon γ (IFN-γ) before infection with chlamydiae results in the formation of atypical RBs that fail to differentiate into infectious progeny (8–12). Because persistent infections have been proposed as a source of antigen for the stimulation of immunopathology that leads to blindness and infertility, we evaluated whether infectious chlamydiae could be rescued from the aberrant RB forms that follow IFN-γ treatment and whether the levels of important chlamydial immunogens were altered during abnormal growth.

MATERIALS AND METHODS

Organism. *C. trachomatis* serovar A/Har-13 was grown in HeLa 229 cells, and EBs were purified by discontinuous density centrifugation in Renografin (Squibb) (13).

Preparation of IFN-γ-Treated Cultures and Assay for Infectivity. HeLa 229 cells in minimal essential medium with 10% fetal bovine serum (MEM-10) were plated at a density of 1.5×10^5 cells per well in 24-well culture plates and maintained at 37°C in 5% CO_2. Eighteen to 24 hr later the cell monolayers were washed once with Hanks' balanced salt solution (HBSS) and then treated for 15 min at room temperature with HBSS containing DEAE-dextran (45 μg/ml), followed by two washes with HBSS. The final wash was removed and replaced with 0.2 ml of 0.25 M sucrose/10 mM sodium phosphate/5 mM L-glutamic acid (SPG), pH 7.2, containing 3×10^5 inclusion-forming units (IFU) of *C. trachomatis* serovar A (HAR-13), and incubated for 2 hr at 37°C on a rocker platform. Two hours after infection the inoculum was removed and replaced with 0.5 ml of either MEM-10 or MEM-10 containing recombinant human IFN-γ at 0.05, 0.2, or 2.0 ng/ml (Biogen). At the indicated times the monolayers were washed three times with HBSS, and cells were scraped from the culture dishes into 0.5 ml of SPG solution and frozen until all samples were collected. Samples were briefly sonicated to disrupt the HeLa cells and release the chlamydial EBs. The disrupted cell suspensions were diluted in SPG solution and used to inoculate fresh monolayers of HeLa cells, as described above. Infected monolayers were cultured in MEM-10 containing cycloheximide at 1 μg/ml for 48 hr, then washed once with HBSS, and fixed with methanol; inclusions were visualized by indirect immunofluorescence with an anti-major outer membrane protein (MOMP) monoclonal antibody (mAb) (A-20) (14, 15).

In experiments to determine whether infectious chlamydiae could be rescued from the growth inhibitory effects of IFN-γ, infected cells were cultured with inhibitory concentrations of IFN-γ (0.2 and 2.0 ng/ml) (see Table 1) for 48, 72, 96, or 120 hr. The medium was then removed and replaced

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Abbreviations: IFN-γ, interferon γ; hsp60, 60-kDa heat shock protein; MOMP, major outer membrane protein; EB, elementary body; RB, reticulate body; IFU, inclusion-forming units; mAb, monoclonal antibody.

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Table 1. Effect of IFN-γ on production of infectious chlamydiae

IFN-γ, ng/ml	Chlamydiae after infection, IFU				
	24 hr	48 hr	72 hr	96 hr	120 hr
0.0	0	5870 ± 813	35,200 ± 2800	1670 ± 880	595 ± 218
0.05	0	5360 ± 874	33,700 ± 4040	2490 ± 623	667 ± 184
0.2	0	0	0	0	0
2.0	0	0	0	0	0

The effect of IFN-γ on recovery of infectious *C. trachomatis* was assessed at the indicated times after infection. Data are presented as mean IFU counts of triplicate cultures ± SEM of duplicate experiments.

with MEM-10, and at 0, 24, 48, and 72 hr after the removal of IFN-γ, cells were harvested in SPG solution, sonicated, and assayed for infectious chlamydiae, as described above.

Ultrastructural Analysis. HeLa cells were plated at a density of 1×10^6 cells per well in 6-well culture plates, and infections were done as described above. Forty-eight hours after infection untreated or IFN-γ treated (0.2 ng/ml) monolayers were washed twice with phosphate-buffered saline (PBS), pH 7.2. The cells were removed from the culture dishes by gentle scraping with a sterile disposable syringe plunger, pelleted by centrifugation ($200 \times g$), and fixed with 2% glutaraldehyde/10 mM phosphate buffer for 2 hr at 22°C. After three washes with PBS, the samples were postfixed for 4 hr with 0.1% osmium tetroxide/PBS at 22°C. Samples were then dehydrated in a graded series of ethanol and embedded in Durcupan (Polysciences). Blocks were cut into sections of 80- to 90-nm thickness, stained with uranyl acetate and lead citrate, and viewed by using a Hitachi transmission EM.

Indirect Immunofluorescence Assays. For indirect immunofluorescent staining, 48-hr cultures were fixed with methanol and treated with either anti-MOMP (A-20) or anti-60-kDa heat shock protein (hsp60) (A57-B9) mAb, as described (14, 15). The exposure time was identical for all photomicrographs.

SDS/PAGE and Immunoblotting. SDS/PAGE (16) and immunoblotting (17) were done on cell monolayers harvested with Laemmli sample buffer (18) and probed with either a mixture of monospecific rabbit anti-hsp60 antiserum and mouse anti-MOMP (mAb A-20), or polyclonal rabbit anti-60-kDa cysteine-rich outer membrane protein, or mouse anti-lipopolysaccharide (mAb EVI-H1). Autoradiographs were analyzed with a scanning densitometer.

Briefly, cell monolayers were established in 24-well culture plates, infected with chlamydiae, and treated with IFN-γ, as described above. At 24, 48, 72, and 96 hr after infection the culture medium was removed, and the cell monolayers were harvested in 200 μl of Laemmli sample buffer. The samples were then solubilized by boiling, and 50 μl of each sample was analyzed by SDS/PAGE and immunoblotting.

RESULTS

Effect of IFN-γ on Production of Infectious Chlamydiae. Initial studies were done to determine the effect of IFN-γ on chlamydial growth and the production of infectious progeny. IFN-γ inhibited the production of infectious *C. trachomatis* EBs in a dose-dependent manner when added to cultures shortly after infection (Table 1). IFN-γ at 0.05 ng/ml had no effect, whereas IFN-γ at 0.2 and 2.0 ng/ml completely inhibited the production of infectious progeny. Although infected cells treated with IFN-γ at 0.2 ng/ml did not produce infectious progeny, chlamydial inclusions were observed by phase-contrast microscopy (data not shown). Examination of those cells by EM confirmed the presence of inclusions that contained large aberrant RB forms (Fig. 1). Thus chlamydial

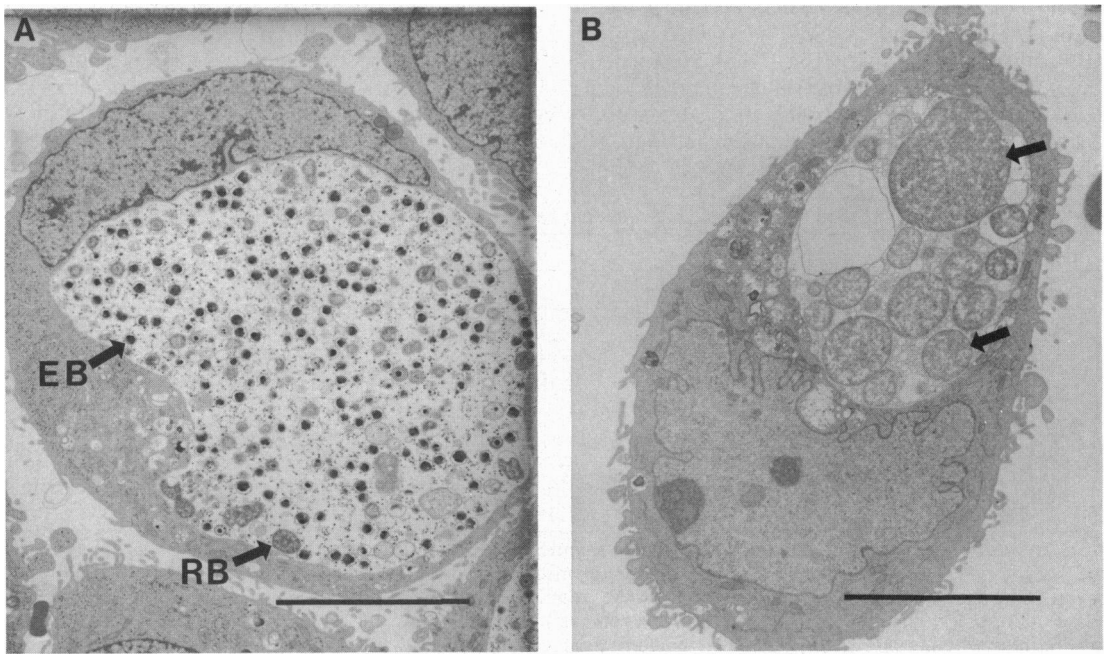

FIG. 1. Ultrastructural analysis by EM of untreated (*A*) and IFN-γ-treated (0.2 ng/ml) (*B*) cells 48 hr after infection. Note the typical RB and EB forms in inclusions of untreated cells. The IFN-γ-treated cells do not contain typical chlamydial forms, but instead the inclusions are characterized by large atypical RB forms (indicated by arrow). (Bar = 5 μm.)

growth was altered but was not completely inhibited by postinfection treatment with IFN-γ.

Rescue of Infectious Chlamydiae from IFN-γ-Treated Cells. If treatment with IFN-γ resulted in persistence, then recovery of infectious EBs would be expected upon removal of IFN-γ. Therefore, rescue experiments were done by culturing chlamydiae-infected cells for either 48, 72, 96, or 120 hr with IFN-γ concentrations that caused atypical growth (0.2 and 2.0 ng/ml). When IFN-γ-containing medium was replaced after 48 hr with medium without IFN-γ, infectious progeny were recovered from cells grown in the presence of 0.2 ng of IFN-γ per ml but were not recovered from those in 2.0 ng/ml (Table 2). Infectious chlamydiae were recovered at 24 hr after removal of IFN-γ, and the number of infectious EBs increased throughout the times tested. Furthermore, infectious chlamydiae were rescued from cells treated with IFN-γ at 0.2 ng/ml for 72, 96, and 120 hr, although the number of infectious chlamydiae recovered decreased with longer IFN-γ treatments. Therefore, IFN-γ produced persistent chlamydial infection characterized by inclusions containing atypical RBs, which upon removal of IFN-γ reverted to normal growth patterns with differentiation into infectious progeny.

Altered Ratios of Chlamydial Antigens During Persistent Infection. Experiments were done to document biochemical changes associated with the development of the atypical persistent forms. Because two chlamydial antigens have been implicated as playing important roles in immune responses elicited after chlamydial infection, these were initially selected for evaluation. Antibodies to MOMP, the principle surface protein of *Chlamydia*, neutralize infectivity, suggesting a role for MOMP in protective immunity (17, 19). In contrast, the chlamydial hsp60 elicits a local inflammatory response characterized by lymphofollicular hyperplasia and mononuclear cellular infiltration of the submucosa, which characterize chronic chlamydial infection (20–25). The presence of atypical RB forms in IFN-γ-treated cells led to the examination of the levels of chlamydial antigens during persistent infection. For these studies immunofluorescence and immunoblotting were used to compare the amounts of hsp60 and MOMP in IFN-γ-treated and untreated cells. Differential levels of chlamydial hsp60 and MOMP were observed. mAbs specific to hsp60 and MOMP stained normal inclusions with comparable intensity (Fig. 2 *A* and *C*, respectively), whereas chlamydiae cultured in the presence of IFN-γ at the concentration found to cause persistence (0.2 ng/ml) were stained intensely with anti-hsp60 mAb (Fig. 2*B*) but were only sparsely stained with anti-MOMP mAb (Fig. 2*D*). The different staining pattern also was observed by using rabbit polyclonal antisera to MOMP and hsp60, implying that the weak staining with anti-MOMP mAb was not simply the result of altered epitope expression (data not shown).

To confirm differences in protein levels in untreated and IFN-γ-treated cells, immunoblot analyses were done (Fig. 3). In samples analyzed 24 hr after infection differences in hsp60 or MOMP were not observed between untreated and IFN-γ treated cells (Fig. 3*A*). However, at 48 hr after infection altered steady-state levels of chlamydial proteins were observed in cells treated with IFN-γ at 0.2 ng/ml. Densitometer tracings of immunoblots of cells 48 hr after infection indicated nearly 1/1 ratio of hsp60 to MOMP (4.085 mm^2 vs. 4.450 mm^2, respectively, lane 3) in untreated cells, but a 5/1 ratio of hsp60 to MOMP (1.636 mm^2 vs. 0.313 mm^2, respectively, lane 7) was observed in cells treated with IFN-γ at 0.2 ng/ml (data not shown). Interestingly, other outer membrane constituents (the 60-kDa outer membrane protein and lipopolysaccharide) were similarly reduced in IFN-γ-treated cells (Fig. 3 *B* and *C*). Cells harvested 72 and 96 hr after infection continued to display a similarly altered protein profile. The altered pattern of protein levels observed with IFN-γ treatment was dose-dependent; 0.05 ng/ml had no detectable effect, and 2 ng/ml had a pronounced effect.

DISCUSSION

IFN-γ had a pronounced effect on the growth of *C. trachomatis* and the steady-state levels of several important chlamydial antigens. Treatment with low concentrations (0.2 ng/ml) of IFN-γ after infection caused a loss of chlamydial infectivity despite the presence of chlamydial inclusions. EM analysis revealed morphological changes in the chlamydiae characterized by the presence of large abnormal RBs and an absence of infectious EBs. Similar atypical chlamydial morphology has also been observed when infected cells are treated with penicillin (26, 27), but the antigenic characteristics of those forms have not been described. The atypical chlamydial forms that develop after IFN-γ treatment exhibited different steady-state levels of hsp60 relative to three major constituents of the chlamydial outer membrane; MOMP, 60-kDa outer membrane protein, and lipopolysaccharide. The reduction in these components may correspond to a loss in structural integrity resulting in aberrant chlamydial growth. The inability to culture infectious chlamydiae from cells treated with IFN-γ indicated that the aberrant form of the organism was noninfectious, but viability was main-

Table 2. Rescue of chlamydiae from persistently infected cells

IFN-γ		Chlamydiae after removal of IFN-γ,* IFU			
Treatment,† hr	ng/ml‡	0 hr	24 hr	48 hr	72 hr
48	0.2	0	1590 ± 128	6020 ± 524	9660 ± 524
	2.0	0	0	0	0
72	0.2	0	1220 ± 139	3770 ± 621	7040 ± 621
	2.0	0	0	0	0
96	0.2	0	521 ± 104	1420 ± 234	2640 ± 249
	2.0	0	0	0	0
120	0.2	0	0	560 ± 127	993 ± 98
	2.0	0	0	0	0

Data are presented as mean IFU count of triplicate samples ± SEMs of duplicate experiments.

*At 24-hr intervals after replacement of IFN-γ-containing medium with fresh MEM-10, cells were harvested and assayed for infectious chlamydiae. Indicated time points range from 2 days (48-hr IFN-γ treatment, 0 hr after IFN-γ removal) to 8 days (120-hr IFN-γ treatment, 72 hr after IFN-γ removal) after infection.

†To determine whether infectious chlamydiae could be rescued from the growth inhibitory effects of IFN-γ, infected cells were cultured for the indicated times with inhibitory concentrations of IFN-γ (0.2 and 2.0 ng/ml). IFN-γ-containing medium was removed at these indicated times and replaced with fresh MEM-10.

‡The quantitative recovery of chlamydiae from cells not treated with IFN-γ is shown in Table 1 (IFN-γ concentration of 0 ng/ml).

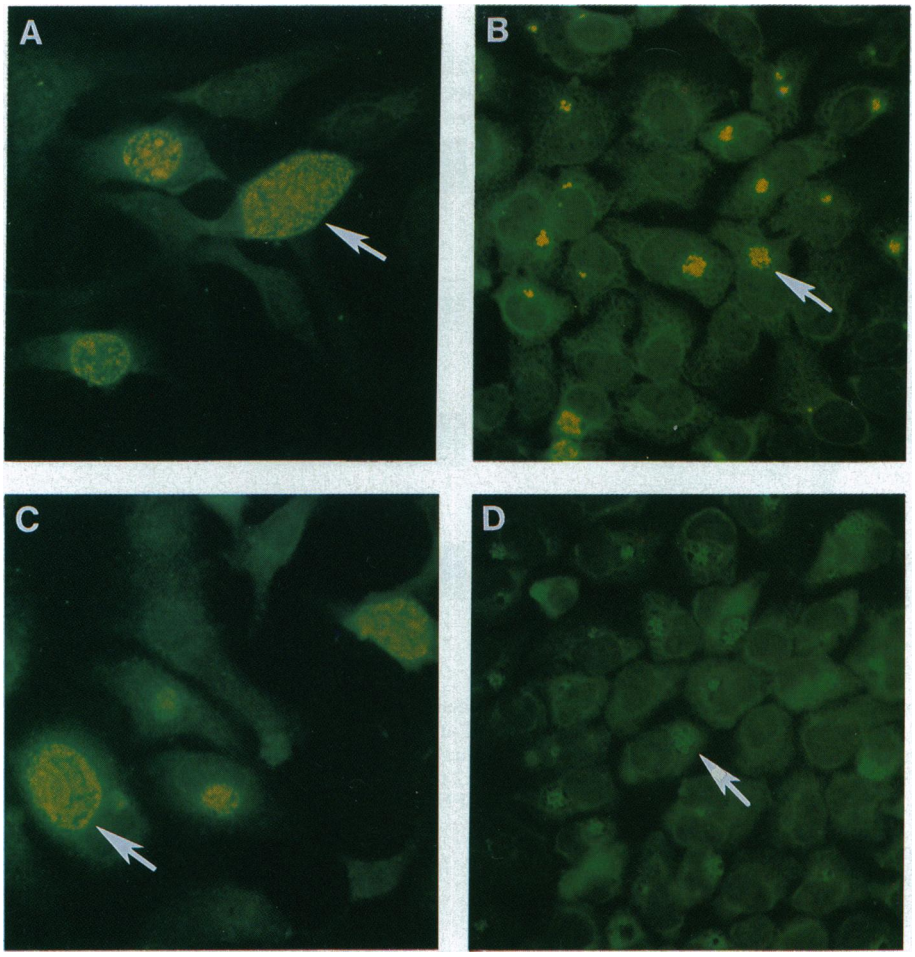

FIG. 2. Indirect immunofluorescent staining of untreated (*A* and *C*) and IFN-γ-treated (0.2 ng/ml) (*B* and *D*) monolayers with either anti-hsp60 (*A* and *B*) or anti-MOMP (*C* and *D*) mAbs. Arrows identify chlamydial inclusions.

tained because infectivity was recovered after IFN-γ removal (Table 2). *In vitro* IFN-γ-mediated persistent infection has features common to human ocular and genital chlamydial infections, such as the frequent inability to isolate infectious organisms, even though chlamydial antigen or nucleic acid are detectable (3, 5–7). Those data suggest that persistent, inapparent chlamydial infection occurs *in vivo*. If persistent chlamydial infections arise *in vivo* with features similar to the *in vitro* model described, then decreased amounts of a protective antigen, such as MOMP and near-normal levels of an immunopathogenetic antigen (hsp60), could contribute to the stimulation of the chronic inflammation that characterizes chlamydial diseases.

The concepts that persistent chlamydial infections occur *in vivo* and persistent infection is a mechanism of immunopathology are controversial and, as yet, unconfirmed. However, data from human disease and animal models of chlamydial infection are suggestive of persistent infection *in vivo* (3–6). The *in vivo* mechanism(s) of chlamydial persistence is ill-defined, but nutrient deprivation (28), antibiotic treatment (26, 27), and cytokines (9, 12, 29) all affect chlamydial growth *in vitro*. Induction of enzymes that catabolize tryptophan is thought to be a mechanism by which IFN-γ inhibits chlamydial growth (10). The effects of IFN-γ on chlamydial growth are clearly concentration-dependent and can lead to either complete inhibition of chlamydial growth (2.0 ng/ml) or to persistence (0.2 ng/ml). Those same effects might also be expressed *in vivo*. The levels of IFN-γ that we have found to be inhibitory for *C. trachomatis* serovar A growth *in vitro* are achievable *in vivo* after chlamydial infections. Arno *et al.* (30) reported concentrations of IFN-γ in cervical secretions and serum of women with chlamydial cervicitis to be ≈6 and 9 units of IFN-γ per ml, respectively (0.5 ng of IFN-γ ≈ 6 units). Much higher serum concentrations of IFN-γ (10–800 units/ml) were reported for women with pelvic inflammatory disease (31). Therefore, secretion of IFN-γ at local sites of infection could reach concentrations that would completely inhibit chlamydial growth and production of infectious progeny and, thus, resolve infection. Conversely, IFN-γ concentrations *in vivo* might inhibit chlamydial differentiation but maintain viability. Inapparent persistent infection, as assessed by negative culture, would result. Under those conditions persistent infections could be very important in the chronic inflammatory sequelae of chlamydial disease. For example, the antigenic stimulus for chronic inflammation could come from recrudescence caused by IFN-γ-mediated persistence. Alternatively, because we have shown that infected cells treated with IFN-γ contain an abundance of hsp60, a potential immunopathologic target (32), and a paucity of MOMP, a protective immune target (32), then these abnormal inclusions could function as antigen depots for the stimulation of chronic inflammation that precedes the serious sequelae of chlamydial infection.

In vitro studies of IFN-γ-mediated persistence indicate that immune reactivity to *Chlamydia* might contribute to persistence and inapparent chlamydial infections. The proposed model requires that initial infection stimulates the immune system and elicits local production of IFN-γ that activates host cells and alters chlamydial growth and differentiation while maintaining viability and hsp60 production. The *in vivo*

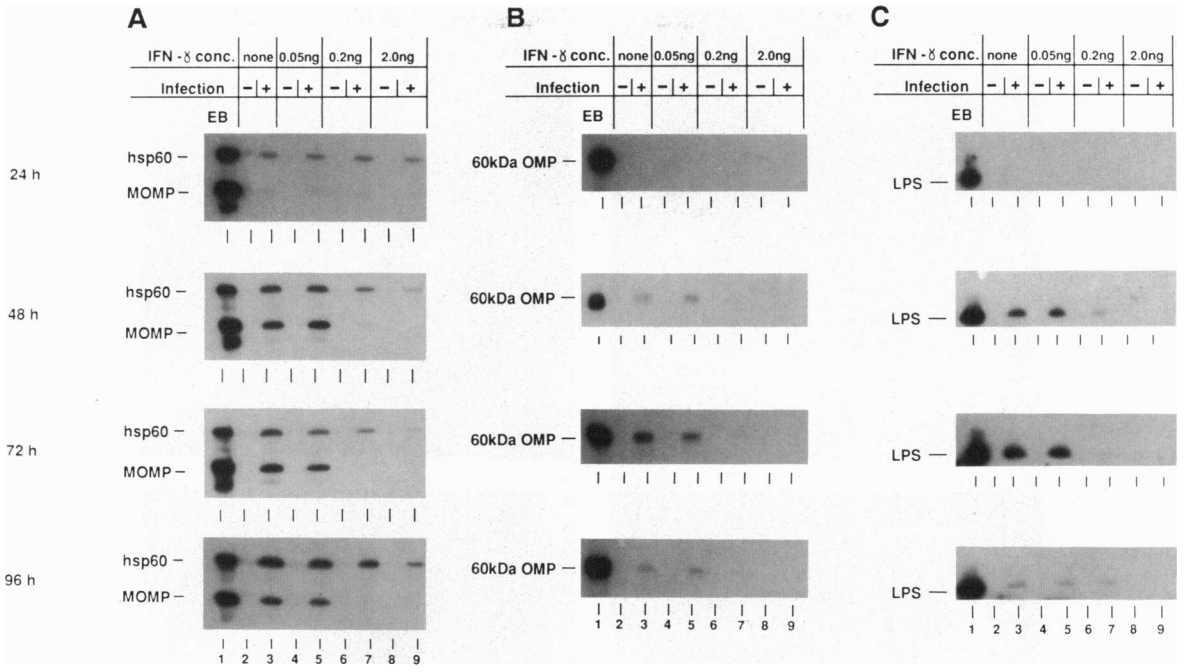

FIG. 3. Immunoblot analysis of uninfected (−) and infected (+) cells with anti-hsp60 and anti-MOMP (*A*), anti-60 kDa outer membrane protein (OMP) (*B*), and anti-lipopolysaccharide (LPS) (*C*) antibodies. Lanes: 1, *C. trachomatis* serovar A EBs; 2, uninfected cells and no IFN-γ treatment; 3, chlamydiae-infected cells and no IFN-γ treatment; 4, uninfected cells treated with IFN-γ at 0.05 ng/ml; 5, infected cells treated with IFN-γ at 0.05 ng/ml; 6, uninfected cells treated with IFN-γ at 0.2 ng/ml; 7, infected cells treated with IFN-γ at 0.2 ng/ml; 8, uninfected cells treated with IFN-γ at 2.0 ng/ml; 9, infected cells treated with IFN-γ at 2.0 ng/ml. conc., Concentration.

correlate of these findings would be chlamydial persistence and chronic hypersensitivity.

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