Characterization of *Chlamydia pneumoniae* Persistence in HEP-2 Cells Treated with Gamma Interferon

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Infection with *Chlamydia pneumoniae* has been implicated as a potential risk factor for atherosclerosis. This study demonstrated the effects of gamma interferon (IFN-γ)-mediated indoleamine 2,3-dioxygenase activity on *C. pneumoniae* persistence in HEP-2 cells, inclusion morphology, and ultrastructure. *C. pneumoniae* replication showed a dose-dependent decrease when treated with increasing concentrations of IFN-γ and a phenotypic switch resulting in a decrease in typical inclusions with an increase in smaller, less-dense atypical inclusions. Ultrastructural analysis of IFN-γ-treated *C. pneumoniae* revealed atypical inclusions containing large reticulolate-like aberrant bodies with no evidence of redifferentiation into elementary bodies.

Seroepidemiologic studies have shown increased *Chlamydia pneumoniae* antibody titers in patients with coronary artery disease (23, 30, 31). Recently, the organism has been detected in atheromatous lesions by PCR, electron microscopy, and immunocytochemistry (8, 19) and, more importantly, recovered as a viable organism from atheromatous lesions (15, 28). In vitro studies support the argument for *C. pneumoniae* as a risk factor for atherosclerosis, demonstrated through growth in endothelial cells, macrophages, and aortic smooth muscle cells (11, 12). Evidence also includes *C. pneumoniae*-induced activation of chemokines and transendothelial migration of leukocytes (24), enhancement of endothelial infection when cocultured with monocytes (20), induction of cellular oxidation of low-density lipoproteins (16), and induction of macrophage foam cell formation (17).

A bacterial infection could contribute to the initial damage of the endothelium, creating a local and systemic inflammatory response (25), thus being a potential risk factor for atherosclerosis. Atherosclerosis is primarily a chronic inflammatory event, in which growth factors and cytokines play an active role in the protective mechanisms involved with inflammation and repair. Therefore, a specific immunomodulatory cytokine, such as gamma interferon (IFN-γ), would be essential for influencing bacteriostatic events. On a cellular level, IFN-γ induces host-cell indoleamine 2,3-dioxygenase (IDO) enzyme activity, which catalyzes the insertion of molecular oxygen into l-tryptophan to form N-formylkynurenine and 1-kynurenine (14). This mechanism is considered to be directly responsible for antimicrobial activities due to a depletion of intracellular tryptophan pools, as shown with the inhibition of *Chlamydia trachomatis*, *Chlamydia psittaci*, and *Toxoplasma gondii* replication (1–3, 6, 13, 26, 29, 33).

Persistence is defined as a long-term association between *Chlamydia* and the host in which the organism remains viable, but in a culture-negative state (5). Previous studies in our laboratory demonstrated inhibitory effects of IFN-γ on *C. pneumoniae* replication (22, 32), suggestive of IDO activity, which resulted in persistence. Upon the addition of excess tryptophan, normal inclusions were recovered (22). Similar work in our laboratory with human aortic smooth muscle cells revealed an arrest in *C. pneumoniae* replication upon IFN-γ stimulation, with relief of inhibition following addition of a competitive inhibitor of IDO, 1-methyl-DL-tryptophan (1-MT) (27). Few studies have examined whether IFN-γ affects *C. pneumoniae* morphologically; therefore, we sought to characterize morphologically and ultrastructurally *C. pneumoniae* persistence in HEP-2 cells that is induced through the inhibitory effects of IFN-γ-mediated IDO activity.

The maintenance of HEP-2 cells (ATCC CCL 23), propagation and infection of *Chlamydia pneumoniae* isolate (A-03, ATCC VR-1452), and IFN-γ stimulation assays were performed as previously described (22, 24, 27). To determine the kinetics of IDO activity, confluent HEP-2 monolayers were treated with IFN-γ (200 U/ml) and incubated at 37°C in 5% CO₂. At indicated time points (0, 6, 12, 24, 48, and 72 h), medium containing IFN-γ was removed and monolayers were pulse treated and tryptophan catabolism was measured as previously described (27). The specificity of IDO activity was measured by pretreating HEP-2 monolayers with increasing concentrations of (0 to 50 mM) of the IDO competitive inhibitor, 1-MT (Aldrich, Milwaukee, Wis.) (7) at 37°C for 1 h, followed by IFN-γ stimulation (25 U/ml) for 48 h, followed by measurement of tryptophan catabolism. The infectivity of IFN-γ-treated *C. pneumoniae* was determined by harvesting infected monolayers, titration of lysates onto fresh HEP-2 monolayers, and incubation without IFN-γ for 48 h. To examine *C. pneumoniae* inclusion morphology, IFN-γ-treated (25 U/ml) or untreated infected HEP-2 monolayers were fixed and stained at 48 h with fluorescein isothiocyanate (FITC)-labeled *Chlamydia* anti-lipopolysaccharide (anti-LPS) or mouse anti-*C. pneumoniae* major outer membrane protein (anti-MOMP) (Accurate Chemical and Scientific Corporation, Westbury, N.Y.) followed by sheep anti-mouse immunoglobulin G FITC-la-
beled antibody (StressGen, Victoria, British Columbia, Canada) and inclusion morphology was examined by epifluorescence microscopy (magnification, ×400). Quantitation of inclusions was assessed by counting the number of inclusion bodies per 10 fields at a magnification of ×400. To confirm inclusion morphology, additional monolayers were stained by using the immunoperoxidase mouse ABC Staining System (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) according to the manufacturer’s recommendations. To examine ultrastructural morphology of IFN-γ-treated C. pneumoniae inclusions, HEP-2 cells were grown on glass coverslips and infected with high titer C. pneumoniae, treated with 25 U/ml IFN-γ or untreated for 48 h. Samples for transmission electron microscopy (TEM) were processed according to the method of Beatty et al. (4), with the exception of being embedded in LX112 plastic. Samples for immunogold labeling were fixed and processed as previously described (4), and grids were reacted with Chlamydia anti-LPS (diluted in PBS), for 2 h at room temperature, followed by washing and reaction with 10-nm-diameter colloidal gold anti-mouse immunoglobulin G (Accurate Chemical and Scientific Corporation). Processed grids for TEM and immunoelectron microscopy (IEM) were examined with a Philips CM10 transmission electron microscope.

Statistical analysis was conducted using one-way analysis of variance with Tukey’s multiple comparison and with a P value of <0.05 used to determine statistical significance.

The kinetics of IDO activity in HEP-2 cells were examined in confluent monolayers treated with IFN-γ (200 U/ml), and tryptophan catabolism was measured over a 72-h period. IDO enzyme activity peaked between 12 and 24 h, with 85.4% ± 0.8% tryptophan catabolism, and then decreased steadily over the next 48 h, reaching 23.7% ± 4.8% catabolism at 72 h (Fig. 1A). Tryptophan catabolism was reduced to 7.4% ± 1.9% (P < 0.05) when monolayers were pretreated with increasing concentrations of 1-MT (0 to 50 mM), followed by IFN-γ stimulation (25 U/ml) for 48 h (Fig. 1B).

In the absence of IFN-γ, C. pneumoniae replication produced an average of 89.0 ± 16.0 inclusions per 10 high power fields (hpf). However, in the presence of increasing concentrations of IFN-γ (25 to 500 U/ml) (Fig. 1C), replication was markedly decreased in a dose-dependent manner, showing a significant linear downward trend of 25.5 ± 9.5 inclusions per 10 hpf when treated with IFN-γ at 500 U/ml (75.0% inhibition; P < 0.05). The direct role of IFN-γ-mediated IDO activity on C. pneumoniae replication was shown through pretreatment of infected monolayers with 1-MT, which resulted in no significant reduction in inclusion numbers in the presence of increasing concentrations of IFN-γ (Fig. 1C). The passage of infected monolayers onto fresh HEP-2 cells was performed to determine the infectivity of elementary bodies (EBs) contained within IFN-γ-treated inclusions (Fig. 1D). Inclusions containing infectious EBs were recovered from infected HEP-2 lysates that had been treated with IFN-γ (0 to 600 U/ml) with decreasing titers of 1,225.0 ± 85.4 to 450.0 ± 95.7 inclusion-forming units per/ml. However, harvested lysates treated with 800 U of IFN-γ per ml were unable to produce infectious progeny.

To determine the quantity of typical versus atypical inclusions, increasing concentrations of IFN-γ (0 to 800 U/ml) were used to stimulate C. pneumoniae-infected HEP-2 cells. Inclusions were observed by reacting infected monolayers with FITC anti-LPS antibody. As the IFN-γ concentration increased to 200 U/ml, the number of typical inclusions decreased from 32.2 ± 4.5 inclusions per 10 hpf to no detectable inclusions (P < 0.05) (Fig. 1E). Atypical inclusions were observed when monolayers were treated with 25 U of IFN-γ per ml (16.8 ± 6.3) (Fig. 1F) and continued to increase up to 22.7 ± 4.5 (P < 0.0) per 10 hpf when the IFN-γ concentration reached 200 U/ml. Higher concentrations up to 800 U/ml yielded declining inclusion numbers (Fig. 1F). However, IFN-γ-induced atypical inclusions never reached levels equal to typical inclusion numbers when grown in the absence of IFN-γ.

In order to determine morphological differences between untreated and IFN-γ-treated C. pneumoniae inclusions, monolayers were reacted with FITC labeled Chlamydia anti-LPS or anti-MOMP antibodies and viewed by epifluorescence microscopy or stained by immunoperoxidase assay and viewed by light microscopy. Figure 2A and B depict FITC labeled inclusions with morphological differences when C. pneumoniae is grown in the presence of IFN-γ. Normal inclusions stained with Chlamydia anti-LPS (Fig. 2A) or anti-MOMP (Fig. 2B) appeared as typical, large densely stained, round fluorescent inclusions, in contrast to atypical inclusions, which appeared smaller in size and demonstrated a less-dense fluorescent staining intensity. To confirm morphological differences in IFN-γ-treated and untreated C. pneumoniae inclusions, an immunoperoxidase assay was used after reacting monolayers with antibodies to LPS (Fig. 2C) or MOMP (Fig. 2D). Similar results were observed in IFN-γ-treated inclusions, demonstrating a phenotypic alteration of a typical inclusion into a smaller atypical inclusion.

To detect ultrastructural alterations in IFN-γ-treated C. pneumoniae inclusions, monolayers were examined by TEM and IEM. C. pneumoniae-infected HEP-2 cells at 48 h contained large normal inclusions with multiple EBs, reticulate...
bodies (RBs) and intermediate bodies, located near the host cell nucleus (Fig. 2E). When C. pneumoniae-infected HEp-2 cells were treated with 25 U of IFN-γ per ml, inclusions at 48 h demonstrated atypical ultrastructural morphology (Fig. 2F). These induced atypical inclusions appeared be generally smaller in diameter and contained fewer bacteria than typical inclusions. Atypical inclusions also contained reticulate-like, pleomorphic, aberrant bodies (ABs), which were generally larger in diameter than typical RBs, with a sparse densitometric appearance, and no evidence of redifferentiation into EBs. Immunogold labeling confirmed that these atypical inclusions were C. pneumoniae, due to the reactivity of gold-labeled Chlamydia anti-LPS with the ABs (Fig. 2F, insert).

IFN-γ has the ability to induce host cell IDO enzyme activity, thus creating an oxygen-independent mechanism for the inhibition of intracellular pathogens (18). Our previous studies using aortic smooth muscle cells (27) detailed IFN-γ-induced C. pneumoniae persistence in a cell-line important for the progression of atherosclerosis. This previous study also confirmed involvement of the IDO pathway; however, there was no examination of the morphological effect of IFN-γ-mediated induction of persistence. The present studies demonstrated similar IFN-γ-mediated inhibition of C. pneumoniae replication in HEp-2 cells due to IDO activity, creating a dose-dependent effect on replication where inclusion numbers decreased as IFN-γ concentrations increased. We demonstrated that replication was not inhibited in the presence of 1-MT, where treatment presumably relieved catabolism of intracellular tryptophan pools, creating sufficient amounts of this essential amino acid to support chlamydial growth.

It is well known that C. trachomatis can be induced into a persistent form via treatment with IFN-γ, antibiotics, or tryptophan starvation; and these persistent forms have been well defined by TEM and IEM (3, 4, 9, 10). However, persistent C. pneumoniae infections have been less well defined. The developmental cycle of C. pneumoniae was reported by Wolf et al. (34), who described detailed ultrastructural events, including endocytosis, differentiation, and formation of infectious progeny. The time course in which C. pneumoniae EBs invade a host cell and differentiate into an RB with evidence of replication is within 12 to 19 h postinfection (34). When a host cell is stimulated with IFN-γ, IDO activity peaks within 12 h (Fig. 1A); therefore, for persistence to be established, an infectious EB must invade a host cell prior to induction of IDO. In the event that these sequential events occur, where invasion is followed by IFN-γ stimulation, it is suggested that further replication and differentiation of the inclusion is arrested in a persistent state due to IDO catabolism of intracellular tryptophan pools. Our data showed that IFN-γ-induced atypical C. pneumoniae inclusions contain EBs able to infect new HEp-2 cells. This does not follow the classic definition of persistence, in which bacteria remain viable but in a culture-negative state (5). However, inclusions from the highest IFN-γ concentration tested by these studies failed to produce infectious progeny when passed onto fresh HEp-2 cells in the absence of IFN-γ. This observation indicates an irreversible effect of IFN-γ on C. pneumoniae replication. A most likely explanation for the presence of infectious EBs in IFN-γ-treated C. pneumoniae inclusions was that demonstrated by Wolf et al. (34), describing the developmental cycle to be asynchronous at 48 h postinfection, creating a mixture of RBs and EBs. This would also support the mixture of typical and atypical inclusions seen during the described morphological switch, which occurs at low levels of IFN-γ treatment. This creates a possibility of a percentage of inclusions being further advanced in the replicative stage of development; therefore, more mature EBs and RBs would be unaffected by depleted tryptophan pools, allowing infectious progeny to complete the developmental cycle. Inclusions containing primarily RBs in the early stages of development would be arrested in a persistent state due to the unavailability of tryptophan, resulting in the inability of RB-like aberrant bodies to further develop into normal EBs.

C. trachomatis inclusions have been evaluated under persistent conditions using TEM and IEM, which described inclusions containing uniformly enlarged RB forms (3, 4). However, we observed C. pneumoniae persistent inclusions as ultrastructurally distinct from that of C. trachomatis (Fig. 6), with no typical EB or RB morphology, i.e., with only pleomorphic RB-like structures of various sizes within small inclusions. A recent report, by Mathews et al. (21), described the TEM ultrastructure of IFN-γ-induced C. pneumoniae persistence as containing a mixture of normal and abnormal inclusions, in combination with smaller abnormal inclusions, with considerably lower numbers of bacteria. This later study induced persistence under a low IFN-γ concentration, compared to our observations using higher units of IFN-γ, which may account for the absence of typical inclusions from our data. The C. pneumoniae persistence induced by ampicillin has also been reported and these forms were described as abnormal, large single-cell RBs, in addition to vesicles of unknown origin (34).

It is clear from our results and the work of others that under altered environmental conditions in cell culture, an inducible C. pneumoniae persistent form exists, which will serve as the basis for future studies focusing on molecular and immunologic characterization. Recently, evidence has emerged that C. pneumoniae up-regulates transcription of specific genes, such as ompA, ompB, pyk, nlpD, and Cpn0585, in response to IFN-γ treatment compared to normal cultures (21). This indicates an altered host cell environment, which may create a nutritionally stressed condition in C. pneumoniae that leads to persistence.

FIG. 2. HEp-2 cells were infected with C. pneumoniae and treated with IFN-γ (25 U/ml) for 48 h and stained with anti-Chlamydia LPS (A) or anti-C. pneumoniae (MOMP) antibody (B) and viewed under ×400 epifluorescence microscopy. Additional monolayers were stained using the ABC immunoperoxidase assay after reacting with anti-Chlamydia-LPS (C) or anti-C. pneumoniae (MOMP) (D) and viewed by light microscopy (×400). Typical and atypical inclusions are indicated with arrows. HEp-2 cells were infected with C. pneumoniae, treated with IFN-γ (25 U/ml) for 48 h, and fixed for TEM or IEM. (E) TEM of a typical C. pneumoniae inclusion containing EBs and RBs and intermediate bodies (IBs). (F) IFN-γ-treated C. pneumoniae inclusion, reacted with anti-Chlamydia-LPS and labeled with 10-nm-diameter gold-conjugated second-affinity antibodies. IEM of an atypical inclusion containing large reticulate-like ABs. Magnifications: ×10,000 (E) and ×9,800 (F). The insert depicts increased magnification of immunogold-labeled AB (×23,000).
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The events leading up to and including persistence may create a chronic inflammatory response, which is consistent with the response-to-injury hypothesis in the development and advancement of atherosclerosis (25). Future in vitro studies will provide more accurate information of host cell environmental conditions, which induce C. pneumoniae into a persistent form. This will enable an examination of C. pneumoniae gene and protein expression, allowing a better understanding of natural persistent infections.

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