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Inhibition of *Chlamydia pneumoniae* Growth in HEp-2 Cells Pretreated with Gamma Interferon and Tumor Necrosis Factor Alpha

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An in vitro culture system was used to study the effects of increasing concentrations of human cytokines on the intracellular replication of *Chlamydia pneumoniae*. HEp-2 cell monolayers, pretreated for 24 h with 200 U of human recombinant gamma interferon (IFN-γ) per ml restricted the intracellular replication of *C. pneumoniae*. Tumor necrosis factor alpha (TNF-α; 25 ng/ml) exhibited a synergistic effect with IFN-γ by reducing the concentration of IFN-γ necessary to restrict intracellular growth to 100 U/ml. The addition of 200 μg of tryptophan per ml significantly reversed the inhibitory effects of IFN-γ and TNF-α, suggesting involvement of the indoleamine-2,3-dioxygenase pathway in the restriction process.

Chlamydia pneumoniae is an obligate intracellular bacterium which can cause upper and lower respiratory tract infections (5), as well as sinusitis (6) and otitis media (11), in humans. There is also evidence that infection by *C. pneumoniae* may be an important risk factor for coronary artery disease (9, 13), adult-onset asthma (7), and reactive airway disease in children (4).

Cytokines have been demonstrated to restrict the growth of many intracellular bacterial pathogens and are significant activators of host cellular immune responses to infection. Gamma interferon (IFN-γ) can be important to both recovery from and protection against infection by these pathogens. IFN-γ has been shown to activate cell-mediated responses that lead to the resolution of systemic disease due to *Chlamydia psittaci* in experimental animals (10), and there is evidence of IFN-γ-mediated inhibition of the intracellular replication of *C. psittaci* (14). This last activity can be attributed to host cell tryptophan catabolism, which in turn is due to the induction of the enzyme indoleamine-2,3-dioxygenase (3). In addition to IFN-γ, tumor necrosis factor alpha (TNF-α) is an important mediator of inflammation and inhibits the intracellular replication of *Chlamydia trachomatis* (15).

The antibody response to *C. pneumoniae* infection has been well documented (6). Recently, Surcel et al. (16) suggested a role for a cell-mediated immune response in four patients with *C. pneumoniae* infection, as measured by an in vitro lymphocyte blast transformation assay. Their data point towards an important role for certain human cytokines in activating the cellular immune response to this infection. In the present study, we examined the effects of IFN-γ and TNF-α on the intracellular replication of *C. pneumoniae* in HEp-2 cell monolayers.

HEp-2 cells were obtained from the American Type Culture Collection (ATCC CCL 23) and maintained in Iscove's Modified Eagle's Medium (BioWhittaker, Walkersville, Md.) supplemented with 10% fetal bovine serum, nonessential amino acids, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, and antibiotics (gentamicin and vancomycin). No cycloheximide was included in any media during these studies. Cells were grown in 75-cm^2 flasks (Costar, Cambridge, Mass.), harvested with trypsin-EDTA, washed, and resuspended until they attained a density of 3×10^5 cells per ml. Flat-bottomed, 96-well tissue culture plates (Costar) were seeded with 0.1 ml of HEp-2 cell suspension and allowed to adhere for 24 h prior to use. Cells were pretreated with 25 to 400 U of human recombinant IFN-γ (Promega Corp., Madison, Wis.) per ml for an additional 24 h prior to infection with *C. pneumoniae*. In some experiments, HEp-2 cell monolayers were pretreated with 25 to 400 U of IFN-γ per ml along with the addition of 25 ng of TNF-α (Promega) per ml. Other experiments involved the addition of a 200-μg/ml final concentration of tryptophan (Sigma) to monolayers pretreated with 25 to 400 U of IFN-γ per ml alone or with 25 ng of TNF-α per ml, followed by infection with *C. pneumoniae*. Control wells containing no IFN-γ, TNF-α, or tryptophan were included in each experiment.

C. pneumoniae BAL-37 (obtained from Margaret Hammerschlag, Health Sciences Center at Brooklyn, Brooklyn, N.Y.) was passed in HEp-2 cell monolayers, titrated, and stored at −70°C until needed. A 100-μl aliquot (1.7×10^5 cells per ml) was added to each well containing the HEp-2 cell monolayers, and the plates were centrifuged at $800 \times g$ for 1 h at 37°C prior to an additional 30-min incubation at 37°C. The inoculum was then aspirated and replaced with fresh medium, and all the plates were incubated for 72 h at 37°C. Cell monolayers were then fixed for 10 min in methanol and stained with a genus-specific fluorescein isothiocyanate-labelled antibody (Pathfinder; Kallestad, Chaska, Minn.) for 30 min at room temperature. Wells were examined by epifluorescence microscopy at a magnification of ×200 for the presence of inclusion body formation. The average numbers of inclusions per high-powered field (HPF) were calculated from 10 fields per well. The data presented represent the results from three experiments. The statistical significance ($P < 0.05$) of differences between results

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TABLE 1. Inhibition of *C. pneumoniae* BAL-37 replication in HEp-2 cells by IFN-γ and TNF-α

Treatment	Replication[a] at IFN-γ concn (U/ml) of:					
	0	25	50	100	200	400
IFN-γ	104.5 ± 3.6	112.0 ± 4.4	103 ± 3.1	104 ± 5.1	0	0
IFN-γ + tryptophan	121.3 ± 2.6	101.2 ± 2.10	98.6 ± 4.6	82.3 ± 3.3	80.0 ± 3.7	81.3 ± 1.0
IFN-γ + TNF-α	95.2 ± 4.5	101.1 ± 4.1	100 ± 3.7	0	0	0
IFN-γ + TNF-α + tryptophan	100.0 ± 2.1	109.7 ± 5.0	97.7 ± 4.2	78.1 ± 3.3	77.0 ± 3.6	80.1 ± 2.2

[a] Average number of inclusions ± standard error per 10 HPF.

was calculated by an analysis of variance with a Newman-Keuls posttest.

As shown in Table 1, HEp-2 cell monolayers pretreated with IFN-γ for 24 h prior to infection showed a significant inhibition of *C. pneumoniae* BAL-37 replication at an IFN-γ concentration of 200 U/ml. The average number of inclusions per HPF ± the standard error was reduced from 104.5 ± 3.6 at an IFN-γ concentration of 0 U/ml to 0 inclusions at 200 U/ml ($P < 0.05$).

Pretreatment of HEp-2 cell monolayers with 0 to 400 U of IFN-γ per ml combined with 25 ng of TNF-α per ml resulted in inhibition of *C. pneumoniae* BAL-37 replication at an IFN-γ concentration of 100 U/ml. The average number of inclusions per HPF was reduced from 95.2 ± 4.5 with 0-U/ml IFN-γ plus 25-ng/ml TNF-α to 0 inclusions with 100-U/ml IFN-γ plus 25-ng/ml TNF-α ($P < 0.05$). TNF-α appeared to act in a synergistic fashion with IFN-γ to reduce the critical IFN-γ concentration by 50% to 100 U/ml.

IFN-γ-mediated inhibition of *C. pneumoniae* BAL-37 replication in HEp-2 cell monolayers was significantly reversed in the presence of 200 μg of tryptophan per ml. The average number of inclusions per HPF was 121.3 ± 2.6 with 0-U/ml IFN-γ plus 200-μg/ml tryptophan and remained at 81.3 ± 1.0 with 400-U/ml IFN-γ plus 200-μg/ml tryptophan ($P < 0.05$). Significant reversal of inhibition mediated by IFN-γ plus TNF-α was likewise seen in the presence of 200-μg/ml tryptophan (100 ± 2.1 inclusions per HPF with 0-U/ml IFN-γ plus 25-ng/ml TNF-α plus 200-μg/ml tryptophan, decreasing to 80.1 ± 2.2 with 400-U/ml IFN-γ plus 25-ng/ml TNF-α plus 200-μg/ml tryptophan) ($P < 0.05$).

The addition of IFN-γ (any concentration) or TNF-α at 25 ng/ml, or the combination of IFN-γ and TNF-α, to HEp-2 cells monolayers immediately following infection failed to inhibit the rate of replication of *C. pneumoniae* compared with that of untreated monolayers (data not shown).

Cytokines have been well described as potentiators of the cellular immune response and the subsequent inhibition of replication of several species of *Chlamydia*. IFN-γ has been shown to inhibit the intracellular growth of *C. psittaci* 6BC in L cells and monocyte-derived macrophages (14). McCafferty et al. (10) reported an important role for IFN-γ in defense against early infection with *C. psittaci* of experimentally infected mice. Beatty et al. (1) reported that the treatment of HeLa 229 cell monolayers with 0.2 ng of IFN-γ per ml completely inhibited the replication of *C. trachomatis* serovar A/Har-13. No effect was seen, however, with 0.05 ng of IFN-γ per ml. IFN-γ and TNF-α have been identified as human cytokines which can restrict the intracellular replication of *C. trachomatis* (14, 15). IFN-γ, added to L cells at 25 U/ml 18 to 24 h prior to infection with the lymphogranuloma venereum biovar of *C. trachomatis*, reduced the inclusion count by 50%. Shemer-Avni et al. (15) have shown that TNF-α, added to HEp-2 cell monolayers 24 h prior to infection with *C. trachomatis* at concentrations as low as 0.13 ng/ml, can reduce the infectious yield by 50%. An increase in the concentration of TNF-α to 200 ng/ml resulted in a 99% reduction in infectious yield. Those authors also have described a synergism which exists between IFN-γ and TNF-α in suppressing chlamydial multiplication.

The data presented here support the possibility of a similar role for these cytokines in inhibiting the intracellular multiplication of *C. pneumoniae* in HEp-2 cells. The addition of IFN-γ at 200 U/ml 24 h prior to infection was capable of completely inhibiting the formation of detectable inclusion bodies in the HEp-2 cell monolayers. There appeared to be no dose-dependent inhibitory effect over the IFN-α concentration range of 0 to 400 U/ml, which may indicate that an optimum concentration is necessary for IFN-α to exert its actions. The addition of TNF-α alone at 25 ng/ml had no ability to suppress chlamydial replication. However, when TNF-α was combined with IFN-γ, we saw a synergistic effect in which the critical concentration of IFN-γ needed to completely suppress *C. pneumoniae* inclusion body formation was decreased to 100 U/ml. Of interest is the fact that a preincubation period of 24 h is required for these cytokines to exhibit their effects. This observation is in contrast to the data presented by Beatty et al. (2), who found that when IFN-γ was added following the inoculation step it was capable of restricting the subsequent growth of *C. trachomatis*, as determined by titration of EB concentrations. In the present study, no attempt was made to determine the infectivity of the EB contained within these inclusion bodies. Inclusion body numbers were simply counted following a 72 h incubation period.

Byrne and coworkers have published a series of articles which strongly support the role of tryptophan depletion by the host cell as a mechanism of IFN-mediated inhibition of *C. psittaci* and *C. trachomatis* replication (3, 12). Furthermore, the ability of *C. trachomatis* to establish a persistent infection of HeLa 229 cells in vitro has been attributed to the action of subinhibitory levels of IFN-γ (1). In the present study, the addition of 200 μg of tryptophan per ml to HEp-2 monolayers caused a significant reversal in IFN-γ- and TNF-α-mediated inhibition of *C. pneumoniae* inclusion body formation. These data strongly suggest a role for the indoleamine-2,3-dioxygenase pathway in suppressing *C. pneumoniae* intracellular replication. Inclusion body formation in these tryptophan-treated monolayers appeared morphologically normal when compared with untreated control monolayers. The idea of a persistent *C. pneumoniae* infection in vivo becomes an important consideration when one contemplates the potential involvement of this organism with human atherosclerosis and coronary heart disease (8, 9). It seems that a long-term persistent chlamydial infection of coronary arterial vessel walls could lead to atheroma formation.

Overall, in light of the involvement of the cellular immune response to *C. pneumoniae* infections (16), this study demonstrates that IFN-γ and TNF-α can alter the intracellular fate of this organism and thus may play an important role in the pathogenesis of human infections.

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