Chlamydia species infect epithelial cells at mucosal surfaces, and are major causes of sexually transmitted diseases. Infection is characterized by inflammation which is exacerbated upon reinfection, ultimately leading to tissue damage and scarring. Although central for the development of disease manifestations, little is known about the mechanisms that initiate and sustain the inflammatory response to Chlamydia. Infection of cervical and colonic epithelial cells with Chlamydia trachomatis and Chlamydia psittaci is shown in the present studies to upregulate mRNA expression and secretion of the proinflammatory cytokines IL-8, GRO alpha, GM-CSF, and IL-6. In contrast to the rapid, but transient, cytokine induction following infection with other invasive bacteria, the epithelial cytokine response to Chlamydia was delayed until 20-24 h after infection, persisted throughout the chlamydial growth cycle (2-4 d), and required bacterial protein synthesis. Moreover, epithelial cell lines and primary endocervical epithelial cells released IL-1alpha after Chlamydia infection, and increased secretion of the proinflammatory cytokines could be inhibited by anti-IL-1alpha. This suggests that IL-1alpha, released following lysis of infected epithelial cells, may amplify the inflammatory response by stimulating additional cytokine production by noninfected neighboring cells. These findings suggest a novel pathophysiologic concept wherein the acute host response to Chlamydia at mucosal surfaces is primarily initiated and sustained by epithelial cells, the first and major targets of chlamydial infection.
Secretion of Proinflammatory Cytokines by Epithelial Cells in Response to \textit{Chlamydia} Infection Suggests a Central Role for Epithelial Cells in Chlamydial Pathogenesis

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Abstract

\textit{Chlamydia} species infect epithelial cells at mucosal surfaces, and are major causes of sexually transmitted diseases. Infection is characterized by inflammation which is exacerbated upon reinfection, ultimately leading to tissue damage and scarring. Although central for the development of disease manifestations, little is known about the mechanisms that initiate and sustain the inflammatory response to \textit{Chlamydia}. Infection of cervical and colonic epithelial cells with \textit{Chlamydia trachomatis} and \textit{Chlamydia psittaci} is shown in the present studies to upregulate mRNA expression and secretion of the proinflammatory cytokines IL-8, GRO\textsubscript{\alpha}, GM-CSF, and IL-6. In contrast to the rapid, but transient, cytokine induction following infection with other invasive bacteria, the epithelial cytokine response to \textit{Chlamydia} was delayed until 20–24 h after infection, persisted throughout the chlamydial growth cycle (2–4 d), and required bacterial protein synthesis. Moreover, epithelial cell lines and primary endocervical epithelial cells released IL-1\alpha after \textit{Chlamydia} infection, and increased secretion of the proinflammatory cytokines could be inhibited by anti–IL-1\alpha. This suggests that IL-1\alpha, released following lysis of infected epithelial cells, may amplify the inflammatory response by stimulating additional cytokine production by noninfected neighboring cells. These findings suggest a novel pathophysiologic concept wherein the acute host response to \textit{Chlamydia} at mucosal surfaces is primarily initiated and sustained by epithelial cells, the first and major targets of chlamydial infection. (\textit{J. Clin Invest.} 1997. 99:77–87.) Key words: inflammation • mucosa • IL-8 • IL-1\alpha • \textit{Chlamydia trachomatis}

Introduction

\textit{Chlamydiae} are intracellular bacterial pathogens that cause a spectrum of clinically important diseases in humans. \textit{Chlamydia trachomatis} is the most common cause of sexually transmitted disease, and is the causative agent of trachoma, the leading cause of preventable blindness (1). Moreover, chlamydial genital tract infection is an important risk factor for HIV transmission (2, 3). \textit{Chlamydia psittaci}, which predominantly infects animals and causes a variety of respiratory, gastrointestinal, and urogenital diseases, also can cause pneumonia in humans (4).

Chlamydial infection is initiated by the extracellular elementary body (EB) which attaches to and invades epithelial cells at mucosal surfaces (5). Within 8 to 12 h after invasion, EBs develop into the metabolically active reticulate bodies (RBs). RBs divide by binary fission inside a host-derived endosome that eventually occupies much of the host cell cytoplasm (then termed the chlamydial inclusion) (6). RBs reorganize into EBs 18–30 h after invasion of host cells, and infected cells begin to lyse and release infectious EBs at 48–72 h postinfection (p.i.).

Little is known about the pathogenesis of acute human chlamydial infections, and our knowledge of acute infection has been derived largely from animal models. The host response to primary chlamydial infection at mucosal surfaces occurs within 1–2 d of infection and is characterized by inflammation and mucosal infiltration with neutrophils and small numbers of monocytes (7). In animal models, the host response can control the infection, i.e., eliminate or substantially reduce the bacterial load (8–11). Both innate and acquired immunity appear to contribute to this outcome. Thus, neutrophils are recruited in large numbers to the site of infection, and are capable of killing accessible EBs, probably due to efficient fusion of lysosomes with \textit{Chlamydia}-containing phagosomes, and release of myeloperoxidase and oxidants (12, 13). In addition, T cells accumulate at the site of \textit{Chlamydia} infection and play a critical role in controlling the infection (8, 14), whereas B cells do not appear to be important for resolving infection in some models (15). In most cases, the host response to primary infection is transient and not associated with long-term tissue damage (7).

\begin{itemize}
\item 1. Abbreviations used in this paper: EB, elementary body; IFU, inclusion-forming unit; p.i., postinfection; RB, reticulate body; RT, reverse transcription; SN, supernatant.
\end{itemize}
During reinfecion or reactivation of a persistent infection, the initial inflammatory response occurs with a similar magnitude, whereas T cells infiltrate more rapidly and in larger numbers compared with the host response to a primary infection. The recurrent inflammatory reaction ultimately culminates in fibrosis and scarring, which can lead to blindness, tubal infertility, ectopic pregnancy, and chronic pelvic pain (10, 16). The specific pathways that underlie this tissue damaging response to infection are not known. Chlamydial heat shock proteins have been proposed to be involved in inducing inflammation at the site of infection (17), but this proposal is controversial (18, 19).

Epithelial cells at mucosal surfaces can secrete chemottractant and proinflammatory cytokines in response to infection with pathogenic bacteria, suggesting that these cells act as an early warning system for immune and inflammatory cells in the underlying mucosa (20). The epithelial cytokine response to pathogenic bacteria depends on several factors, including the site of infection and the specific pathogen. For example, some noninvasive pathogenic bacteria, such as *Pseudomonas aeruginosa* (21, 22), uropathogenic *Escherichia coli* (23), and *Helicobacter pylori* (24, 25), induce proinflammatory cytokine secretion by epithelial cells at sites that normally harbor few or no bacteria, e.g., lungs, bladder, and stomach. In contrast, bacterial invasion appears to be required for epithelial cytokine induction at sites which are physiologically exposed to an abundant bacterial flora, i.e., the colon, because only invasive bacteria induce cytokine secretion by colon epithelial cells (26–28). Furthermore, toxins released by some pathogenic bacteria may play an additional role in inducing or modulating epithelial cytokine responses (29, 30).

In the present studies, the concept of epithelial cells as an important component of the early host response to infection with pathogenic bacteria was applied to understanding the pathogenesis of chlamydial disease. We show that in vitro infection of cervical and colonic epithelial cells with *Chlamydia* induces the secretion of an array of proinflammatory cytokines that have chemotactic and proinflammatory functions, including IL-8, GROα, GM-CSF, IL-6, and IL-1α. Moreover, in contrast to invasion by other bacteria that induce a rapid but transient proinflammatory cytokine response upon entry (26), chlamydial invasion alone was not sufficient for this response. Rather, intracellular chlamydial growth was required, and the epithelial cytokine response was sustained throughout the chlamydial growth cycle. These findings suggest a new basis for understanding the mechanism of chlamydial pathogenesis, wherein the acute host inflammatory response to *Chlamydia* is mediated by the local production of proinflammatory cytokines from infected epithelial cells.

**Methods**

*Chlamydia* strains. *C. trachomatis* serovar L2/434/Bu was propagated in L929 suspension cultures. *C. trachomatis* serovars D/W3/ Cx and D/UW12/Ur, and *C. psittaci* API were propagated in L929 cell monolayers, as described previously (31). Briefly, cultures were grown in RPMI growth medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 60 μg/ml vancomycin, and 10 μg/ml gentamicin) at 37°C in 95% air/5% CO2. Cultures infected with *C. trachomatis* L2 or *C. psittaci* API were grown for 48 h, and those infected with *C. trachomatis* serovars D or I for 72 h, before harvesting. Infected monolayers were detached by scraping, and then pelleted and sonicated to lyse the host cells. Cell debris was removed by differ-
RNA extraction and reverse transcription (RT)-PCR analysis of cytokine mRNA levels. Total cellular RNA was extracted from epithelial cells using an acid guanidinium thiocyanate-phenol-chloroform method (28). Qualitative RT-PCR analysis and quantitative RT-PCR analysis using internal RNA standards of mRNA levels for human IL-8, GROα, GM-CSF, IL-6, IL-1α, TGFβ1, and β-actin was performed as described previously (28). This method was sensitive to <10^3 mRNA molecules/μg total RNA.

Statistical analysis. The statistical significance of the differences in cytokine secretion from control and infected cultures was determined by two-tailed t test assuming unequal variances between groups. Significance levels are indicated in the figure and table legends.

Results

*C. trachomatis* infection induces IL-8 secretion by HEp-2 cells. Acute chlamydial infections are characterized by intense inflammation and infiltration of the mucosa predominantly with neutrophils, but also other inflammatory cells (7). Little is known about the signals responsible for this response. We asked whether chlamydial infection of epithelial cells, which are the major targets of infection, induces the production and secretion of chemoattractant and proinflammatory cytokines by the infected cells. HEp-2 epithelial monolayers were infected with *C. trachomatis*, and cultured for up to 5 d to encompass at least one entire cycle of chlamydial development. The initial studies focused on the potent neutrophil chemoattractant IL-8. Infection of HEp-2 cells with *C. trachomatis* increased IL-8 secretion by >50-fold compared with mock infected control cells (Fig. 1A). Increased levels of IL-8 in the culture supernatants were first detected 20–24 h p.i., reached a maximum 3 d p.i., and remained elevated thereafter. Moreover, infection with as few as 1 IFU of *C. trachomatis* per 10 epithelial cells increased IL-8 secretion when measured 3 d after infection (Fig. 1B). In contrast, no increase in IL-8 secretion was detected early after infection (6-10 h p.i.), even if a high multiplicity of infection was used, e.g., up to 1,000 IFUs/cell (Fig. 1B).

The time course of the epithelial IL-8 response to *C. trachomatis* depends on the infectious dose. Because cumulative IL-8 secretion reached a maximum 3 d after *C. trachomatis* infection and cell lysis began to occur at this time, the relation of IL-8 secretion rates to the number of viable cells in the infected cultures was characterized. For these experiments, cultures supernatants were harvested and replaced every 24 h for 4 d after infection, and IL-8 concentrations were determined for each 24-h period. In parallel, the number of viable cells present in the monolayers was determined at the end of each 24-h period. As shown in Fig. 2, most of the increase in IL-8 secretion occurred before substantial cell lysis was noted. At later times after infection (>3 d), IL-8 secretion decreased and this was paralleled by lysis of the majority of host cells, suggesting that the decrease in IL-8 secretion was caused by the decrease in the number of viable cells in the infected monolayers.

The time course of increased IL-8 secretion and cell lysis was dependent on the dose of *C. trachomatis* in the inoculum (Fig. 2A). At the highest inoculum used (~2 IFUs/cell), maximal IL-8 secretion was observed 2 to 3 d p.i., whereas at the lowest inoculum (approximately 0.02 IFUs/cell), increased IL-8 secretion was not observed until after 3 d after infection, and maximal IL-8 secretion not until the end of the 4-d observation period. Despite a trend towards earlier IL-8 secretion at higher inocula, no increase in IL-8 secretion was observed early after infection (6–10 h p.i.), even at very high inocula (Fig. 1B). In contrast to the effect of the inoculum dose on the time of maximal IL-8 secretion, the inoculum dose had a minor effect on...
the maximal levels of IL-8 secretion observed during the 4-d period of the experiment, because these were comparable at the three inoculum doses tested, i.e., over a 100-fold inoculum range (Fig. 2 A).

**Increased secretion and mRNA levels of an array of proinflammatory cytokines in HeLa cervical epithelial cells infected with C. trachomatis.** We next asked whether proinflammatory cytokines other than IL-8 were upregulated in response to *C. trachomatis* infection of epithelial cells. As shown in Table I, infection of HeLa cells with *C. trachomatis* increased secretion of GROα, GM-CSF, and IL-6 by > 70-fold compared with controls, as determined 3 d after infection. Moreover, a three- to fourfold increase in the levels of IL-1α was also detected in the supernatants of *C. trachomatis*-infected HeLa cells. Increased secretion of IL-8, GROα, GM-CSF, IL-6, and IL-1α in response to *C. trachomatis* infection was accompanied by increased mRNA levels for these cytokines, as determined 2 d after infection of HeLa cells with 1 IFU/cell (Table II). In contrast, mRNA levels for TGFβ1, a cytokine with known antiinflammatory activity (32), and the housekeeping gene β-actin were not affected, or decreased, after chlamydial infection of HeLa cells (Table II). As shown in a time course analysis, mRNA levels for IL-8 and IL-1α increased within 24 h after infection of HeLa cells, and reached a maximum 2 d after infection (Fig. 3).

**Different chlamydial strains induce IL-8 secretion by various cultured human cervical and colon epithelial cell lines.** In addition to *C. trachomatis* L2, we tested the ability of two other *C. trachomatis* strains representative of another *C. trachomatis* biovar (serovars I and D) and of *C. psittaci* (AP1) to induce IL-8 secretion by HeLa epithelial cells. As shown in Table III, infection of HeLa cells with *C. trachomatis* increased secretion of IL-8, GROα, GM-CSF, IL-6, and IL-1α in response to *C. trachomatis* infection was accompanied by increased mRNA levels for these cytokines, as determined 2 d after infection of HeLa cells with 1 IFU/cell (Table II). In contrast, mRNA levels for TGFβ1, a cytokine with known antiinflammatory activity (32), and the housekeeping gene β-actin were not affected, or decreased, after chlamydial infection of HeLa cells (Table II). As shown in a time course analysis, mRNA levels for IL-8 and IL-1α increased within 24 h after infection of HeLa cells, and reached a maximum 2 d after infection (Fig. 3).

### Table I. Increased Cytokine Secretion by HeLa Cells Infected with *C. trachomatis*<sup>a</sup>

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control</th>
<th>+ <em>C. trachomatis</em> L2</th>
<th>Ratio Infected/Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>0.11±0.01</td>
<td>33.22±2.10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>302</td>
</tr>
<tr>
<td>GROα</td>
<td>0.06±0.01</td>
<td>9.32±0.30&lt;sup&gt;3&lt;/sup&gt;</td>
<td>155</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>&lt;0.01</td>
<td>0.71±0.07&lt;sup&gt;3&lt;/sup&gt;</td>
<td>&gt;71</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.13±0.01</td>
<td>12.51±0.51&lt;sup&gt;1&lt;/sup&gt;</td>
<td>96</td>
</tr>
<tr>
<td>IL-1α</td>
<td>&lt;0.03</td>
<td>0.11±0.02&lt;sup&gt;3&lt;/sup&gt;</td>
<td>&gt;3.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Monolayers of HeLa cells in 24-well plates were infected with approximately 1 IFU/cell *C. trachomatis* L2 for 2 h, after which monolayers were washed and further incubated for 2 d. Medium was removed, fresh medium was added, and cultures were incubated for an additional 24 h. Culture supernatants were collected, and cytokine concentrations were determined by ELISA. Values are means±SD of the values of triplicate cultures from one experiment. A second experiment showed comparable results, e.g., ratios of cytokine secretion in infected cultures to controls were 273, 84, 77, and 77 for IL-8, GROα, GM-CSF, and IL-6, respectively. Differences between means were evaluated by ANOVA (P ≤ 0.005, except IL-1α for which P ≤ 0.05).

### Table II. Increased Cytokine mRNA Levels in *C. trachomatis*-infected HeLa Cells<sup>b</sup>

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Number of mRNA molecules/μg cellular RNA</th>
<th>Ratio Injured/Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>3.5×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.3×10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>GROα</td>
<td>4.0×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.1×10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>&lt;10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.7×10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-6</td>
<td>7.5×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.6×10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-1α</td>
<td>1.9×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.9×10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>7.0×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>9.0×10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-actin</td>
<td>1.1×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.5×10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>b</sup>Monolayers of HeLa cells in 75-cm² flasks were infected with approximately 1 IFU/cell of *C. trachomatis* L2 for 2 h, washed, and further incubated for 48 h. Total cellular RNA was extracted, and levels of mRNA transcripts were determined by quantitative RT-PCR using internal RNA standards, as described in Methods.
HeLa cells after cm tion could be caused by the extracellular presence of cm tion. and SiHa cells. Thus, the cytokine response to chlamydial in-
the relative increase was lower than that observed for HeLa
trachomatis tested. As shown in Table III, HT-29 cells also responded to
response to Figure 3. RNA was extracted at the indicated times after infection. (serovar L2), washed, and further incubated for up to 72 h. Total
and SiHa cells. Thus, the cytokine response to chlamydial in-
nRNA was omitted from reverse transcription and PCR amplification.
maximize the differences between samples. One-sixth vol of each
PCR reaction was size-fractionated on a 1% agarose gel, which was
stained with ethidium bromide, and photographed. As a control,
RNA was omitted from reverse transcription and PCR amplification.
(B) Quantitative RT-PCR analysis. mRNA levels for IL-8 ( ), IL-1α ( ), and β-actin ( ) were determined by quantitative RT-PCR using internal RNA standards, and are expressed as a ratio of the mRNA levels in C. trachomatis-infected cells to the levels in uninfected control cells. The absolute values of the mRNA levels for IL-8, IL-1α, and β-actin in control cells are shown in Table II.

Figure 3. Time course of increased IL-8 and IL-1α mRNA levels in HeLa cells after C. trachomatis infection. HeLa cell monolayers in 75-
cm² flasks were infected for 2 h with ~ 1 IFU/cell of C. trachomatis (serovar L2), washed, and further incubated for up to 72 h. Total RNA was extracted at the indicated times after infection. (A) Qualitative RT-PCR analysis. 25–50 ng total cellular RNA were reverse-transcribed, and amplified by PCR for 32–34 cycles, as appropriate to maximize the differences between samples. One-sixth vol of each PCR reaction was size-fractionated on a 1% agarose gel, which was stained with ethidium bromide, and photographed. As a control, RNA was omitted from reverse transcription and PCR amplification. (B) Quantitative RT-PCR analysis. mRNA levels for IL-8 ( ), IL-1α ( ), and β-actin ( ) were determined by quantitative RT-PCR using internal RNA standards, and are expressed as a ratio of the mRNA levels in C. trachomatis-infected cells to the levels in uninfected control cells. The absolute values of the mRNA levels for IL-8, IL-1α, and β-actin in control cells are shown in Table II.

Table III, all four chlamydial strains induced a ≥ 20-fold increase in IL-8 secretion by HeLa cells. Furthermore, the IL-8 response to C. trachomatis was not limited to HeLa cells, because SiHa cervical epithelial cells also secreted increased levels of IL-8 following infection. Because Chlamydia infection can cause proctitis, a colon epithelial cell line (HT-29) was tested. As shown in Table III, HT-29 cells also responded to C. trachomatis infection with increased IL-8 secretion, although the relative increase was lower than that observed for HeLa and SiHa cells. Thus, the cytokine response to chlamydial infection is a conserved feature among different chlamydiae, as well as the three epithelial cell lines tested.

Extracellular chlamydial LPS is not responsible for increased epithelial cytokine secretion following Chlamydia infection. Increased IL-8 secretion in response to chlamydial infection could be caused by the extracellular presence of Chlamydia, i.e., mediated by chlamydial cell wall components such as LPS, or by events associated with chlamydial invasion, or may require later events in the chlamydial life cycle involving bacterial protein synthesis and growth. To address the possibility that the extracellular presence of chlamydial LPS was responsible for the induction of IL-8, the colon epithelial cell line SW620 was used, which was previously shown to respond to relatively low levels of LPS with increased IL-8 secretion (33). Monolayers of SW620 cells that were infected with approximately 4 IFUs/cell of viable C. trachomatis L2, and incubated for 24 h, showed a sixfold increase in IL-8 secretion (2.26 ng IL-8/ml after C. trachomatis infection, 0.36 ng IL-8/ml in uninfected controls). In contrast, when the same inoculum was heat inactivated at 75°C for 10 min before coculture, a treatment which interferes with bacterial invasion but does not affect chlamydial LPS (34, 35), IL-8 secretion by SW620 monolayers did not increase (0.36 ng IL-8/ml after addition of heat-treated C. trachomatis, 0.36 ng IL-8/ml in controls). Furthermore, no increase in IL-8 secretion by SW620 cells was observed after addition of up to an equivalent of 100 IFUs/cell of heat-inactivated C. trachomatis. As a positive control, LPS from E. coli O111 efficiently induced high levels of IL-8 secretion by SW620 cells (14.41 ng IL-8/ml after stimulation with 100 ng/ml LPS for 24 h, 1.39 ng IL-8/ml after stimulation with 1 ng/ml LPS, and 0.32 ng IL-8/ml in control cultures).

Bacterial protein synthesis is required for the induction of IL-8 by epithelial cells following chlamydial infection. To determine whether bacterial protein synthesis is required for the epithelial IL-8 response, epithelial monolayers were infected with Chlamydia for 2 h to allow invasion to occur, and treated with chloramphenicol to inhibit bacterial protein synthesis, growth, and replication (36). Chloramphenicol prevented the increase in IL-8 secretion from HeLa cells infected with C. trachomatis or C. psittaci (Fig. 4), and from HT-29 cells infected with C. trachomatis (C. trachomatis-infected 3.5 ± 0.2 ng IL-8/ml, C. trachomatis-infected + chloramphenicol 1.0 ± 0.1 ng/ml, controls 0.6 ± 0.1 ng/ml; supernatants were harvested 48 h after infection, data are mean ± SD of triplicate cultures). In contrast, chloramphenicol had no effect on increased IL-8 secretion following IL-1α stimulation of HeLa cells (data not shown).

We next asked whether differentiation of Chlamydia from RBs to EBs is important for IL-8 induction, because maximal IL-8 secretion was observed 2–3 d after infection, a time period that coincides with the condensation from RBs to EB differentiation. Penicillin inhibits RB division and RB to EB differentiation, but has no known effect on RB metabolism (37, 38). As shown in Fig. 4, addition of penicillin to freshly infected HeLa monolayers had little effect on the IL-8 response of these cells to C. trachomatis infection following IL-1α stimulation of HeLa cells (data not shown).

As a positive control, LPS from E. coli O111 efficiently induced high levels of IL-8 secretion by SW620 cells (14.41 ng IL-8/ml after stimulation with 100 ng/ml LPS for 24 h, 1.39 ng IL-8/ml after stimulation with 1 ng/ml LPS, and 0.32 ng IL-8/ml in control cultures).
Table III. Different Chlamydia Strains Induce IL-8 Secretion by Human Cervical and Colon Epithelial Cells*

<table>
<thead>
<tr>
<th>Cells</th>
<th>Chlamydia strains</th>
<th>Inoculum (IFUs/well)</th>
<th>IL-8 secreted (ng/ml)$^{b}$</th>
<th>Ratio Infected/Control</th>
<th>Number of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>C. trachomatis</td>
<td>L2/434/Bu</td>
<td>0.2±0.1</td>
<td>57.0±3.3$^{a}$</td>
<td>285</td>
</tr>
<tr>
<td></td>
<td>C. psittaci</td>
<td>API</td>
<td>0.3±0.1</td>
<td>151.4±20.2$^{a}$</td>
<td>505</td>
</tr>
<tr>
<td>SiHa</td>
<td>C. trachomatis</td>
<td>L2/434/Bu</td>
<td>0.6±0.2</td>
<td>13.5±0.9$^{a}$</td>
<td>23</td>
</tr>
<tr>
<td>HT-29</td>
<td>C. trachomatis</td>
<td>L2/434/Ur</td>
<td>&lt;0.2</td>
<td>35.6±2.1$^{a}$</td>
<td>178</td>
</tr>
</tbody>
</table>

*Confluent monolayers of human cervical (HeLa and SiHa) and colon (HT-29) epithelial cells in 24-well plates were infected for 2 h with the indicated doses of Chlamydia, washed, and further incubated for 48–72 h. Medium was removed, fresh medium was added, and cultures were incubated for an additional 24 h. Culture supernatants were collected, and IL-8 concentrations were determined by ELISA. HeLa cell cultures infected with C. trachomatis D/UW3/Cx were incubated for 96 h without a change in media before supernatants were collected. Results are means±SD of the values of triplicate cultures from representative experiments. The number of total experiments performed is given in the last column.$^{a}$Values are significantly different from the respective controls (P ≤ 0.01, except SiHa for which P ≤ 0.05).

Increased cytokine secretion by Chlamydia-infected epithelial cell lines is partially mediated by the cellular release of IL-1α. Chlamydial growth causes lysis of the host cells 2–5 d after infection, and previous studies have shown that HeLa and other epithelial cells contain preformed bioactive IL-1α that can be released upon cell lysis (39). Because IL-1α is a potent stimulus for IL-8 secretion, the possibility was tested that the observed induction of IL-8 and other cytokines after chlamydial infection is mediated by the release of IL-1α. HeLa, SiHa, or HT-29 cells were infected with 1 IFU/cell C. trachomatis L2 and cultured for up to 4 d. During this time, the supernatants were replaced every 24 h with fresh media containing anti–IL-1α or control antibodies. As shown in Fig. 5, addition of anti–IL-1α inhibited up to 85% of the increase in IL-8 secretion by HeLa and SiHa cells following chlamydial infection compared with Chlamydia-infected control cultures containing no added antibodies, or antibodies of irrelevant specificity. The anti–IL-1α–mediated inhibition of IL-8 secretion was observed within 24 h after infection, and the relative magnitude of the inhibition was similar throughout the 4-d period of the experiment (Fig. 5, and data not shown). In control experiments, an identical concentration of anti–IL-1α to that used in the experiments with Chlamydia-infected cultures completely inhibited (>99%) the increase in IL-8 secretion by HeLa and SiHa cells following stimulation with 1 ng/ml IL-1α. Furthermore, addition of anti–IL-1α inhibited >95% of the increase in the secretion of GM-CSF, IL-6 following infection of HeLa cells with C. trachomatis (data not shown), indicating that the release of IL-1α from infected cells plays an important role in the Chlamydia-induced induction of all of the cytokines tested.

Figure 4. Effect of chloramphenicol and penicillin on Chlamydia-induced IL-8 secretion in HeLa cells. Monolayers of HeLa cells in 24-well plates were infected for 2 h with approximately 1 IFU/cell of C. trachomatis, serovar L2 (top), or C. psittaci AP1 (bottom), washed, and fresh growth medium was added containing 68 μg/ml chloramphenicol (△), or 100 U/ml penicillin G (○), or neither of these antibiotics (▼). Mock-infected cultures (●) were used as controls. Cultures were incubated for 4 d, during which supernatants were collected and replaced every 24 h with fresh media containing the appropriate antibiotics. IL-8 concentrations in the 24-h supernatants were determined by ELISA. Data represent means±SD of the results of triplicate cultures from one representative out of a total of three (C. trachomatis) or two (C. psittaci) experiments. Values of untreated infected cultures were significantly increased compared with uninfected controls at 2, 3, and 4 d after infection for both chlamydial strains (P ≤ 0.01). No significant increase was found between chloramphenicol-treated infected cultures and uninfected controls at any time point (P > 0.05). The efficacy of the antibiotic treatments was confirmed by microscopic examination of monolayers stained with antibodies against chlamydial LPS.

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Added antibodies (*C. trachomatis*) 24-well plates were infected for 2 h with approximately 1 IFU/cell of

2 and 3 for SiHa cells (*control Ig*-treated infected cultures at days 2, 3, and 4 for HeLa and days

IL-1β determined by ELISA. Data represent means ± SD of the results of triplicate cultures. Values represent means ± SD of the results of triplicate cultures. Values of untreated infected cultures were significantly increased compared with uninfected controls at 2, 3, and 4 d after infection for all three cell lines (*P* ≤ 0.02). Values from anti–IL-1β–treated infected cultures were significantly different from control Ig-treated infected cultures at days 2, 3, and 4 for HeLa and days 2 and 3 for SiHa cells (*P* ≤ 0.02), whereas no significant difference was found between anti–IL-1β–treated and control Ig–treated infected HT-29 cultures at any time point (*P* > 0.05). Two additional experiments with HeLa and HT-29 cells confirmed these results. Thus, HeLa cell cultures infected with C. psittaci for 72 h contained 35.6 ng IL-8/ml, while uninfected controls had 0.2 ng/ml. Addition of anti–IL-1β and control Ig to the C. psittaci-infected HeLa cultures resulted in 20.6 and 36.2 ng IL-8/ml, respectively, indicating a 42% inhibition of IL-8 secretion by anti–IL-1β. In HT-29 cells, *C. trachomatis* infection increased IL-8 secretion to 2.57 ng/ml 48 h after infection compared with 0.62 ng/ml in uninfected controls. Addition of anti–IL-1β and control Ig had no significant effect on the increase in IL-8 secretion (2.33 and 3.34 ng/ml, respectively) after infection.

**Increased IL-8 secretion by primary endocervical epithelial cells in response to *C. trachomatis* infection.** To verify that the IL-8 response to *C. trachomatis* was not limited to transformed epithelial cell lines, primary human epithelial cell cultures were established from normal endocervix, and infected with *C. trachomatis*. As shown in Table IV, IL-8 secretion by primary endocervical epithelial cell cultures increased 1.5- to 3.4-fold after *C. trachomatis* infection. In experiments which showed a relatively lower increase in IL-8 secretion after infection, the levels of IL-8 secretion by control cells tended to be greater than those in control cells in experiments in which greater IL-8 induction was observed (compare experiments 1 and 2, subject 3 in Table IV, and data not shown). Thus, maximal IL-8 levels after *C. trachomatis* infection were similar in most experiments, while IL-8 secretion in control cultures was more variable between experiments. Stimulation of primary endocervical epithelial cell cultures with a combination of the agonists IL-1β and TNFα increased IL-8 secretion by 1.7- to 10.9-fold (Table IV), suggesting that IL-8 secretion by control cultures was relatively close to maximal in some cases. The findings in primary endocervical epithelial cells are consistent with the observations in the cervical epithelial cell lines, although the relative magnitude of the IL-8 response to *C. trachomatis* was lower in the primary cells due to a higher level of background secretion in the control cultures. The lower relative magnitude of the IL-8 response in infected primary epithelial cell monolayers may also be related to the finding that only 10–30% of cells contained chlamydial inclusions, whereas in HeLa cell monolayers infected with *C. trachomatis* under comparable conditions > 90% of cells had chlamydial inclusions.

**Primary endocervical epithelial cells contain bioactive IL-1β that is released following *C. trachomatis* infection.** The studies with transformed cervical epithelial cell lines indicated that release of IL-1β is an important mechanism by which the IL-8 response of these cells is amplified following *C. trachomatis* in-
Table IV. Increased IL-8 Secretion by Primary Endocervical Epithelial Cells after C. trachomatis Infection*  

<table>
<thead>
<tr>
<th>Cells</th>
<th>Subject</th>
<th>Exp.</th>
<th>IL-8 secreted</th>
<th>+ C. trachomatis L2</th>
<th>+ IL-1α + TNFα</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ng/ml</td>
<td>Ratio Infected/Control</td>
<td>ng/ml</td>
</tr>
<tr>
<td>Primary endocervical epithelial cells</td>
<td>1</td>
<td>1</td>
<td>37.5</td>
<td>1.9</td>
<td>62.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>22.1</td>
<td>3.0</td>
<td>239.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>29.9</td>
<td>1.5</td>
<td>223.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>11.9</td>
<td>3.4</td>
<td>21.8</td>
</tr>
<tr>
<td>HeLa cervical epithelial cells</td>
<td>§</td>
<td></td>
<td>0.8</td>
<td>24.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

*Primary endocervical epithelial cell cultures were established as described in Methods. Subconfluent monolayers in 24-well plates were infected for 2 h with C. trachomatis L2, washed, and further incubated for 3 d. Parallel cultures were stimulated with 20 ng/ml each of IL-1α and TNFα. IL-8 concentrations in the supernatants were determined by ELISA. For subject 3, two different subcultures of the original primary cultures were used for two separate experiments. Cultures contained approximately 5–9 × 10⁴ cells/well. Monolayers were infected with 2 × 10⁵ IFUs/well. Infectivity was confirmed by indirect immunofluorescence with an antibody against chlamydial major outer membrane protein. Cells were stimulated with IL-1α only. HeLa cells were adapted to serum-free conditions before infection, and infection experiments were performed under identical conditions to those used for the primary cell cultures.

Discussion

Chlamydial infections are characterized by intense and persistent local inflammation that is exacerbated upon reinfection, ultimately leading to tissue damage and scarring. The mechanisms which elicit this response are unknown, but an understanding of this process is central to the prevention and mitigation of disease. The findings presented here suggest a novel concept for chlamydial pathogenesis wherein the acute host response to Chlamydia in the genital tract, and at other mucosal surfaces, is primarily initiated and sustained by epithelial cells, the first and main targets of chlamydial infection. As shown herein, Chlamydia-infected epithelial cells expressed and secreted increased levels of the chemokines IL-8 and GROα, as well as the cytokines GM-CSF, IL-1α, and IL-6. Several of these cytokines are potent chemotaxins and activators for neutrophils, monocytes, and T lymphocytes (40), while others have pleiotropic effects on cell functions associated with acute inflammation, including the induction of adhesion molecules on endothelial cells, the secretion of additional proinflammatory cytokines by macrophages and other cells, and the induction of acute phase proteins in epithelial cells and macrophages (41, 42). Thus, the array of cytokines produced by epithelial cells after chlamydial infection is consistent with the nature of the host inflammatory response in vivo.

In addition to the epithelial proinflammatory cytokine response, other mechanisms may be involved in the innate immune response to Chlamydia. For example, chlamydial EBs are chemotactic for neutrophils which, in part, is due to activation of the neutrophil chemotaxtractant C5a (43). In addition, chlamydial LPS can stimulate proinflammatory cytokine secretion by macrophages (44). However, chlamydial LPS is much less potent than LPS of other gram-negative bacteria (44, 45), macrophages typically are not a major target of Chlamydia infection, and epithelial cells of the genital and gastrointestinal tracts often are poorly responsive to LPS (26, 33). The conclusion that chlamydial LPS is not important in eliciting the production of proinflammatory cytokines from epithelial cells is further supported by the present finding that an epithelial cell line, SW620, known to be LPS-sensitive (33) failed to respond to high doses of heat-inactivated Chlamydia.

The time course of cytokine induction following chlamydial infection was considerably delayed compared to that found previously for infection of epithelial cells with other invasive bacteria (26), suggesting that different mechanisms are involved. No increase in cytokine secretion was detected during the first 8 h after chlamydial infection, induction of maximal secretion required at least 48 h, and high-level secretion persisted throughout the bacterial growth cycle (up to 4 d). In contrast, cytokine secretion following infection with Salmo nella increases within 90 min after infection, reaches maximal levels at 3 h, and decreases rapidly thereafter (26). Consistent with the rapid cytokine response to Salmonella and other invasive bacteria, which use different invasion strategies, host cell responses closely associated with bacterial invasion appear to be crucial for the epithelial cytokine response to these bacteria (26). The relatively delayed induction of cytokine secretion, and the absence of increased cytokine secretion by Chlamydia-infected cells treated with chloramphenicol, indicate that chlamydial invasion alone is not responsible for the epithelial
Table V. Primary Endocervical Epithelial Cells Contain Bioactive IL-1α That Is Released after C. trachomatis Infection*

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Stimulators added</th>
<th>Subject</th>
<th>No inhibitor added</th>
<th>Anti-IL-1α</th>
<th>Anti-TNFα</th>
<th>Control Ig</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Epithelial cell lysate</td>
<td>3</td>
<td>4,016 ± 149</td>
<td>42 ± 9**</td>
<td>3,962 ± 481</td>
<td>4,020 ± 76</td>
</tr>
<tr>
<td></td>
<td>Epithelial cell lysate</td>
<td>4</td>
<td>1,375 ± 124</td>
<td>31 ± 4**</td>
<td>1,351 ± 113</td>
<td>1,593 ± 96</td>
</tr>
<tr>
<td></td>
<td>SN, Chlamydia-infected</td>
<td>3 (exp. 2)</td>
<td>298 ± 82</td>
<td>30 ± 5**</td>
<td>237 ± 23</td>
<td>247 ± 20</td>
</tr>
<tr>
<td></td>
<td>SN, Control</td>
<td>5 (exp. 2)</td>
<td>62 ± 2</td>
<td>20 ± 4**</td>
<td>50 ± 6</td>
<td>73 ± 17</td>
</tr>
<tr>
<td></td>
<td>IL-1α</td>
<td></td>
<td>3,588 ± 257</td>
<td>25 ± 6**</td>
<td>3,581 ± 340</td>
<td>4,276 ± 746</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td></td>
<td>42 ± 15</td>
<td>36 ± 2</td>
<td>39 ± 8</td>
<td>42 ± 12</td>
</tr>
<tr>
<td>2</td>
<td>SN, Chlamydia-infected</td>
<td>2</td>
<td>748 ± 22</td>
<td>173 ± 16**</td>
<td>671 ± 80</td>
<td>601 ± 67</td>
</tr>
<tr>
<td></td>
<td>SN, Control</td>
<td>2</td>
<td>332 ± 8</td>
<td>149 ± 13**</td>
<td>285 ± 31</td>
<td>245 ± 37</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td></td>
<td>693 ± 38</td>
<td>626 ± 9</td>
<td>178 ± 14</td>
<td>666 ± 20</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td></td>
<td>198 ± 22</td>
<td>150 ± 9</td>
<td>172 ± 19</td>
<td>194 ± 14</td>
</tr>
</tbody>
</table>

*Confluent HeLa cell monolayers in 96-well plates were stimulated for 1 h with cell lysates or supernatants from primary endocervical epithelial cell cultures (50 µl/well). Cultures were washed three times, and further incubated for 4 h with fresh medium (75 µl/well). IL-8 concentrations in the supernatants were determined by ELISA. Results are means ± SD of the results of triplicate cultures. Preliminary experiments had shown that a 1 h pre-stimulation period with IL-1α is sufficient to obtain maximal IL-8 secretion in the subsequent 4 h culture period whether or not IL-1α was present during the 4-h period. To obtain cell lysates from primary endocervical epithelial cells, cultures were established as described in Methods, and subconfluent monolayers in 24-well plates were scraped off into 1 ml medium, and sonicated for 5 s on ice. Lysates were used at 25% final concentration. Supernatants (SN) from C. trachomatis-infected and uninfected control cultures were obtained as described in the legend to Table IV, and were used at 25% final concentration. IL-1α was used at 1 ng/ml, and TNFα was used at 2 ng/ml. Subject and experiment numbers correspond to those in Table IV. Goat antibodies against human IL-1α or TNFα, or normal goat IgG as a control, were added to supernatants or cell lysates at 25 µg/ml. The mixtures were incubated for 30 min at room temperature, and used to stimulate HeLa cell monolayers. None of the control Ig values is significantly different from the respective no inhibitor controls (P > 0.05). **Values are significantly different from the respective no inhibitor controls (P ≤ 0.05).
such as macrophages and fibroblasts, in the underlying mucosa. The resulting inflammation presumably would have both protective and deleterious effects, because the inflammatory response can help to resolve the infection as well as contribute to fibrosis and tissue scarring (50). An important, yet unresolved, question is whether mitigation of the epithelial cytokine response, and the ensuing acute inflammation, would decrease the damaging sequelae of chlamydial infection or, alternatively, promote the spread of infection and increase tissue damage in the host. The epithelial cytokine response to Chlamydia infection reported herein offers a new perspective in understanding chlamydial pathogenesis, because it can explain acute inflammation, as well as the inflammation in repeated infections. A corollary of these findings is that vaccination directed towards developing serum antibodies and antigen-specific T lymphocytes against Chlamydia may not prevent acute inflammation, because the epithelial cytokine response would not be diminished unless the vaccine prevented initial epithelial cell infection. This may, at least in part, explain the outcome of chlamydial vaccination trials, in which vaccination did not prevent mucusal inflammation and, in some cases, was associated with more severe disease following chlamydial reinfection (16).

Acknowledgments

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References


