Persistent Chlamydial Envelope Antigens in Antibiotic-Exposed Infected Cells Trigger Neutrophil Chemotaxis

Priscilla B. Wyrick, Stephen T. Knight, Terry R. Paul, Roger G. Rank, and Claire S. Barbier

An in vitro coculture model system was used to explore conditions that trigger neutrophil chemotaxis to *Chlamydia trachomatis* infected human epithelial cells (HEC-1B). Polarized HEC-1B monolayers growing on extracellular matrix (ECM) were infected with *C. trachomatis* serovar E. By 36 h, coincident with the secretion of chlamydial lipopolysaccharide and major outer membrane protein to the surfaces of infected cells, human polymorphonuclear neutrophils (PMNL) loaded with azithromycin migrated through the ECM and infiltrated the HEC-1B monolayer. Bioreactive azithromycin was delivered by the chemotactic PMNL to infected epithelial cells in concentrations sufficient to kill intracellular chlamydiae. However, residual chlamydial envelopes persisted for 4 weeks, and PMNL chemotaxis was triggered to epithelial cells containing residual envelopes. Infected endometrial cells demonstrated up-regulation of ENA-78 and GCP-2 chemokine mRNA. Thus, despite appropriate antimicrobial therapy, residual chlamydial envelope antigens may persist in infected tissues of culture-negative women and provide one source for sustained inflammation.

*Chlamydia trachomatis* is an important genital pathogen worldwide. Serologic variants (serovars) D–K are the leading cause of bacterially acquired sexually transmitted infections, responsible for an estimated 4 million cases annually in the United States. Without antimicrobial treatment, these insidious pathogens spread from the lower to the upper genital tract to cause chronic infection and induce serious sequelae, such as epididymitis, pelvic inflammatory disease, ectopic pregnancy, and infertility [1]. Doxycycline and azithromycin have been shown to be effective in the long-term eradication of *C. trachomatis* in lower genital tract infections; however, the poor clinical cure rate has been proposed to be due to the persistence of chlamydial antigens in the endometrium or fallopian tubes of culture-negative, infertile women [2, 3].

While chlamydiae-infected men tend to experience some dysuria and urethral discharge, a significant percentage of infected women are asymptomatic. How these obligate intracellular bacteria interact with their host mucosal epithelial cells to produce such a spectrum of inflammatory events is puzzling. The focal nature of chlamydial infections makes it difficult to find chlamydial inclusions in histologic sections from endocervical biopsy specimens. When inclusions are found, they are located in the apical region of superficial columnar epithelial cells. However, a common histologic finding in such biopsies from culture-positive women is periglandular and subepithelial inflammatory cell infiltrates [4, 5]. The density and pattern of the inflammation varies considerably in both symptomatic and asymptomatic individuals and with the extent of disease. In addition, both the focal loss of surface epithelium and the enhanced recruitment of polymorphonuclear leukocytes (PMNL) in the cervix are believed to be risk factors for chlamydiae-positive women for acquiring human immunodeficiency virus (HIV) infection. Chlamydiae-recruited PMNL that contact HIV-carrying monocytes in semen have been demonstrated to trigger induction of infectious virus [6].

The purpose of our in vitro studies was to use a coculture model system to examine the conditions under which *C. trachomatis* serovar E infection of a polarized human endometrial epithelial cell line trigger PMNL chemotaxis and whether or not reduction of chlamydial infection by azithromycin alters PMNL chemotaxis.

Materials and Methods

Growth of *chlamydiae*. *C. trachomatis* E/UW-5/CX, a human urogenital isolate, was obtained from S. P. Wang and C.-C. Kuo (University of Washington, Seattle). Standardized inocula of *C. trachomatis* infectious elementary bodies (EB) were prepared from McCoy cells grown on microcarrier beads, and the progeny EB were harvested, titrated for infectivity, and stored as described previously [7].

*Human cell lines.* The HEC-1B cell line (HTB-113, American Type Culture Collection, Rockville, MD), originally derived from...
a patient with endometrial carcinoma, was used as the principal cell line for these studies. The HeLa cell line (ATCC CCL2), originally obtained from a woman with adenocarcinoma of the endocervix, was used only as a positive control for interleukin (IL)-8 secretion in response to chlamydial infection. Both cell lines were confirmed by the Hoechst staining reagent to be free of Mycoplasma contamination throughout the study. The cells were cultured in Dulbecco’s modified Eagle MEM (Life Technologies Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and 10 mM HEPES buffer, pH 7.3 (DMEM) and incubated at 35°C in an atmosphere of 5% carbon dioxide and 95% humidified air.

Isolation of PMNL. Human PMNL from healthy donors were isolated from heparinized whole blood using the one-step ficoll-hypaque separation procedure, followed by a second separation step utilizing 3% dextran [8]. The PMNL were washed and resuspended in DMEM supplemented with 5% heat-inactivated (56°C for 30 min in a water bath) normal human serum, previously determined in our laboratory to be negative for antibodies to chlamydiae. The PMNL were counted in a hemocytometer and shown to be >95% viable, as assessed by trypan blue exclusion.

In some experiments, PMNL were loaded with azithromycin by incubating them with azithromycin (25 mg/L; Pfizer, Groton, CT) for 1 h at 35°C [9–11]. The PMNL were washed three times to remove excess extracellular antibiotic and resuspended in fresh DMEM. The antibiotic-loading procedure did not affect the viability of the PMNL. Previously conducted control experiments confirmed that azithromycin was not leaking out of the antibiotic-loaded PMNL in the agarose well to diffuse into the infected epithelia. The PMNL were counted in a hemocytometer and shown to be >95% viable, as assessed by trypan blue exclusion.

Chemotaxis in the coculture model chamber. The coculture model chamber was constructed in our laboratory as described previously [11]. Briefly, a woven nylon mesh screen was positioned between and sealed with super glue to two plexiglass O-rings, making a chamber 6-mm high by 6-mm in diameter. Four channels were made in the bottom of the lower O-ring by melting the plexiglass with a hot 22-gauge needle; the channels permitted medium to flow into the lower chamber during incubation and culture. The assembled chamber was sterilized by UV irradiation for 2–12 h. Cold extracellular matrix (ECM) (200 µL; BioTechnologies, Stoughton, MA) was dripped onto the screen mesh, and the chamber was allowed to sit for 1 h on ice to ensure that the pores were filled and both sides of the screen mesh were coated. Excess ECM was removed, and the chambers were warmed to 37°C to allow the ECM to polymerize into a thin gel. Following transfer of the chambers to 4-well multidish tissue culture plates (Nunc, Roskilde, Denmark) and equilibration with DMEM, HEC-1B cells (10^6/chamber) were seeded onto the ECM layer and incubated at 35°C until a polarized monolayer was obtained, which takes ~4–5 days.

A 50-µL inoculum of stock C. trachomatis serovar E EB, diluted in DMEM to a concentration sufficient to produce inclusions in 50% of the HEC-1B cells, was added to the apical surface of the monolayer and allowed to adsorb for 2 h at 35°C to effect attachment and entry of the chlamydiae. Nonadherent chlamydiae were removed by rinsing the apical surface twice, the chambers were replenished with fresh medium, and the cultures were reincubated at 35°C. At the indicated times, the medium was removed from both the apical and basal chambers, the chambers were inverted, and a layer of DMEM-agarose, containing normal human serum, was formed on (under) the ECM. A 3.0-mm (approximate diameter) well was cut in the center of the polymerized agarose bed and filled with 10 µL of PMNL (~10^6 PMNL), either unloaded or loaded with azithromycin. On occasion, a few red blood cells (RBC) were also added to the well as a marker for orientation of the center well in samples processed for microscopy. A drop of agarose was then added to the well to prevent leakage of the blood cells. The chambers were replenished with fresh medium and incubated for an additional 1.5, 3, 5, and 12 h to allow for PMNL migration. In preliminary experiments, it was determined that the orientation of the coculture chamber, that is, apical side up or basal side up, had no influence on PMNL chemotaxis. Thus, while most experiments were performed with the basal-to-apical orientation, certain manipulations or reagent additions required inversion of the chamber; the results were not affected by this maneuver.

Since endometrial epithelial cells cultured in a polarized orientation in vitro have a tendency to reform their in vivo glandular organization, resulting in the formation of scattered finger-like projections (organoids) arising from the monolayer, measurement of transepithelial resistance is precluded. One control to ensure there were no tiny gaps in the polarized epithelial monolayer from turgor pressure of organoids falling over included an inverted chamber to monitor escape of marker RBC from the PMNL well to the apical surface. If this phenomenon was seen, the sample was excluded from the study. Another control to confirm the intactness of the tight junctions in the polarized barrier was the addition of FMLP (10^-6 M) to the apical surface of the epithelial layer and the absence of PMNL migration over the subsequent 3 h.

Assessing PMNL migration by light microscopy. The coculture chambers were washed twice in 0.1 M Sorenson’s buffer (pH 7.2) and fixed in 2% paraformaldehyde–0.05% glutaraldehyde, prepared in Sorenson’s buffer, for 18 h at 4°C. Samples were processed, infiltrated, and embedded in glycol methacrylate resin (JB-4 Plus; Polysciences, Inc., Warrington, PA) according to standard procedures. Thick sections (2–5 µm) providing a sagittal view through the monolayer were cut with an ultramicrotome (Reichert UM-2; Leica, Deerfield, IL), and the sections were stained with toluidine blue. Because it was not possible to separate the agarose layer from the ECM layer for quantitation of PMNL by number or enzyme analyses, PMNL migration was assessed qualitatively by location in numerous histologic sections as follows: (1) no movement of PMNL into the ECM, (2) migration of PMNL through the ECM and to the basal domain of the epithelial layer; (3) infiltration of PMNL into the epithelial layer, and (4) transmigration of intraepithelial PMNL onto the apical (lumenal) surface. When quantitative assessment of PMNL chemotaxis was required, numerous histologic sections of each sample, from two separate experiments, were examined, and the number of intraepithelial or apical PMNL per 2-mm length of HEC-1B monolayer per section were counted and averaged.

Transmission electron microscopy. Samples were processed and embedded in epon-araldite for high contrast or in Lowicryl (Polysciences, Inc.) for immunoelectron microscopy, as described previously [12]. Prior to final embedding in resin, the samples were cut into strips (~1.5-mm wide) and oriented in the mold such that sectioning began with the periphery of the sample and proceeded inward to the PMNL well.
Antibodies. The primary antibodies used in this study were (1) rabbit polyclonal antibodies generated against purified Chlamydia trachomatis serovar E EB or reticulate bodies (RB), prepared in the authors' laboratory; (2) monoclonal antibodies directed against C. trachomatis lymphogranuloma venereum biovar (L2 serovar) lipopolysaccharide (LPS), provided by S. J. Richmond and S. Campbell (University of Manchester, Manchester, UK); and (3) rabbit polyclonal antibodies directed against C. trachomatis L2 EB, donated by D. Malinowski (Becton Dickinson, Research Triangle Park, NC); these latter antibodies, used at a 1:2000–1:4000 dilution, were monospecific for the chlamydial major outer membrane protein (MOMP). All primary antibodies were examined by Western blot analyses for their specificity and the strength of their reactivity with C. trachomatis serovar E EB and RB prior to their use in immunolabeling, and their titers for use in postembedding labeling with Lowicryl sections were also predetermined [12, 13].

ELISA for chemokines. Culture media from the basolateral compartment of infected cultured cells and cell lysates were analyzed by ELISA for tumor necrosis factor (TNF)-α, IL-1α (Quantikine; R&D Systems, Minneapolis), and IL-8 (Predicta; Genzyme, Cambridge, MA). Samples were collected immediately after inoculation and at 24, 36, 48, and 96 h postinoculation (hpi) and were stored frozen at −80°C until they could be analyzed as a complete set. Duplicate aliquots of supernatants were also analyzed separately for the L cell cytotoxicity assay [14].

RNA preparation and reverse transcriptase–polymerase chain reaction (RT-PCR). Polarized HEC-1B cells were cultured on Transwell inserts (4.5 cm²; Costar, Cambridge, MA) and infected with chlamydiae titered to yield a 30% infection in the host cells. Infected cultures were harvested at 1.5, 6, 12, 18, 24, and 36 hpi (4 inserts per harvest interval), and the samples were lysed with Trizol reagent (Life Technologies Gibco BRL); the lysates were pooled and aliquotted for storage at −70°C. Using this set of samples, total RNA was prepared in two separate instances, according to the manufacturer's instructions. Amounts of IL-8 mRNA and actin mRNA were measured by competitive RT-PCR, using the competitive plasmid pHCO1 [15], corresponding primers (Nucleic Acid Core Facility, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill [UNC-CH]), and amplification conditions essentially as previously described [15], except with substitution for the following reagents: Superscript II Reverse Transcriptase enzyme (Life Technologies Gibco BRL) with the addition of 20 U/reaction of RNasin ribonuclease inhibitor (Promega, Madison, WI); oligo(dT12-18) and dNTPs (Pharmacia, Piscataway, NJ); and AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA). Controls included samples that had not been reverse-transcribed and PCR in the presence of all reagents except for the cDNA. The PCR reaction products were fractionated on nondenaturing 5% polyacrylamide gels and analyzed using a PhosphorImager scanner and ImageQuant program (Molecular Dynamics, Sunnyvale, CA).

For the detection of other PMNL chemoattractants, the cDNA samples were normalized to yield identical amounts of actin and amplified by primers allowing the specific amplification of ENA-78, GCP-2, GRO-α, GRO-β, and GRO-γ. Primers and amplification conditions have been described by Froyen et al. [16] and Haskell et al. [17]. The 154-bp ENA-78 and 184-bp GCP-2 amplicons were gel purified (BIO-101, Vista, CA), reamplified with the primers used for the first amplification, and sequenced (UNC-CH Automated DNA Sequencing Facility on a model 373A DNA sequencer [Applied Biosystems, Foster City, CA]) using the Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The sequence obtained was compared with the published sequences of human ENA-78 and GCP-2 genes [16, 18] using the gap and best-fit sequence comparison computer programs (GCG, Madison, WI).

Results

Polarized coculture model system for PMNL chemotaxis (figure 1A). On light microscopy examination of numerous histologic sections, it was clear that uninfected HEC-1B cells and HEC-1B cells infected for 6, 12, 18, or 24 h (figure 1B) failed to evoke PMNL chemotaxis. However, when HEC-1B cells had been infected for 36 h, PMNL chemotaxis did occur. By 1.5 h after addition, PMNL migrated out of the agarose well and through the ECM layer to the basal domains of the polarized epithelial cells (figure 1C). By 3 h, the PMNL had migrated in between the epithelial cells and were often found juxtaposed to infected epithelial cells. After 3–5 h, PMNL could be detected on the luminal or mucosal surface of the infected epithelia (figure 1D). Escape of marker RBC from the PMNL well to the apical surface (figure 1E), which would indicate a breach in the polarized monolayer, did not occur in these samples.

The same pattern of PMNL chemotaxis occurred to HEC-1B cells infected for 48 h. The only difference was a slightly increased number of PMNL responding at each interval examined (data not shown).

Chemotactic PMNL are biologically active. The biologic activity of the intraepithelial PMNL was confirmed by two methods: determining the infectious titers of chlamydiae harvested from infected monolayers unexposed or exposed to chemotactic PMNL and preloading the PMNL with the antibiotic azithromycin and analyzing for delivery of the bioreactive antibiotic to infected epithelial cells. This antibiotic has been documented to be concentrated in PMNL lysosomes in vivo and retained via protonation of the aglycone ring [19]. After chemotaxis, PMNL release azithromycin to targeted bacteria [20] or at sites of infection [19].

Following incubation of 36-h infected HEC-1B cells to allow for PMNL chemotaxis, the samples were fixed and processed for transmission electron microscopy. Chlamydiae inclusions in infected HEC-1B cells at the periphery of the culture chamber, where there was presumably little or no azithromycin, contained morphologically normal appearing metabolically active reticulate bodies (RB) and EB (figure 2A). A few millimeters inward, there was a remarkable change in the inclusions; the chlamydiae were enveloped in a mass of glycogen (figure 2B), as if sensing a change in their microenvironment that signaled a protective maneuver. In infected epithelial cells located half the distance to the center well, there was a noticeable increase in chlamydial outer membrane blebbing (figure 2C) that became more pro-
Polymorphonuclear leukocyte (PMNL) chemotaxis is triggered to polarized HEC-1B cells infected with *C. trachomatis* for 36 h. A. Schematic representation of cross-section of coculture model illustrating chlamydiae-infected HEC-1B cells polarized on extracellular matrix (ECM). B–D. Light microscopy photomicrographs of histologic sections of polarized HEC-1B cells infected with *C. trachomatis* for 24 or 36 h and then exposed to PMNL for 1.5–5 h. B. Epithelial cells infected for 24 h do not trigger PMNL migration. C. Epithelial cells infected for 36 h do trigger PMNL chemotaxis. After 1.5 h, PMNL migrate from agarose well through ECM and make contact with basal domain of polarized epithelial cells; D. by 5 h, there is extensive PMNL migration through the epithelial cell layer and out onto the apical cell surface. E. In some control experiments, escape of RBC from the PMNL well to the apical surface suggested a breach in the monolayer, and the monolayer was discarded. * Denotes inclusions; arrowheads indicate PMNL. Magnification: B = ×500, C = ×2200, D = ×1500, and E = ×1300.

nounced (figure 2D). Finally, inclusions in infected HEC-1B cells nearest the center well contained markedly damaged RB, large envelope ghosts, and few EB (figure 2E). The pattern of gradual destruction of metabolically active RB illustrated here was essentially identical to that described by our laboratory in a previous study [10] that examined, by transmission electron microscopy, the action of azithromycin added to the culture medium on chlamydiae in infected HEC-1B cells. Thus, the morphologic findings suggested that the migrating PMNL were delivering bioreactive azithromycin and releasing it in concen-
Figure 2. Transmission electron photomicrographs of effects of bioreactive azithromycin delivered by migrating polymorphonuclear leukocytes (PMNLs) on chlamydiae in HEC-1B cells infected for 36 h. A. Inclusion in infected epithelial cells at periphery of culture chamber containing normal elementary bodies (EB) and reticulate bodies (RB). B. Inclusion in infected epithelial cells 2 mm from periphery of chamber, showing chlamydiae enveloped in mass of glycogen. C, D. Inclusions in infected cells located half the distance to center well revealed an increase in chlamydial outer membrane blebbing (arrowheads). E, Inclusion in infected HEC-1B cells nearest center well contained damaged RB, large envelope ghosts (arrowheads), and few EB. Fluorescence photomicrographs of subpassage infectivity of chlamydiae obtained from infected HEC-1B cells (F) not exposed to PMNL versus (G) exposed to chemotactic PMNL loaded with azithromycin. Magnification: $A = \times 5000$, $B = \times 4300$, $C = \times 13,200$, $D = \times 13,400$, $E = \times 4600$, $F = \times 1200$, and $G = \times 1200$. 
trations sufficient to kill chlamydiae in infected polarized human endometrial epithelial cells.

These data were confirmed by determining infectivity titers of chlamydia progeny on passage to fresh host cells. The infectivity of chlamydiae harvested from HEC-1B cells exposed to chemotactic PMNL loaded with azithromycin decreased 86% (figure 2G) relative to the infectivity of chlamydiae obtained from infected HEC-1B cells not exposed to chemotactic PMNL (figure 2F).

Secretion of chlamydial envelope antigens from chlamydiae-infected HEC-1B cells coincides with PMNL chemotaxis. LPS is a recognized potent PMNL chemoattractant [21, 22]. Richmond and Sterling [23] first demonstrated that chlamydiae-specific LPS could be detected on the surface of infected host cells concomitant with active RB replication at mid-development cycle, prior to rupture of the chlamydial inclusion at the end of the developmental cycle and subsequent release of chlamydial progeny. This finding has since been confirmed by other investigators [12, 13, 24]. High-magnification examination of chlamydial inclusions in infected cells exposed to chemotactic PMNL loaded with azithromycin revealed several vacuoles external to the inclusions (figure 3A). From high-contrast epon sections, these vacuoles frequently appeared to be filled with membrane blebs. Thin sections cut from duplicate samples embedded in Lowicryl resin yield images of less contrast, especially for membranous material, but offer far superior preservation of antigens for postembedding labeling with gold-conjugated antibodies. Using the latter method, the blebs within the extracellular vacuoles reacted specifically with primary monoclonal antibodies generated against chlamydial LPS (figure 3B and inset) and with primary polyclonal monospecific antibodies generated against chlamydial MOMP (figure 3C) but did not react with uninfected HEC-1B cells (figure 3D). Detection was accomplished with gold-conjugated second-affinity antibodies. The gold marker also denoted the presence of these chlamydial antigens on the surfaces of the infected cells. The timing of the appearance of chlamydial antigens on the host cell surfaces, ~36 hpi [12], coincided with PMNL chemotaxis.

It should be noted that midway through this study, complement was found by us to be sequestered in the commercially obtained ECM (data not shown). This finding was compatible with the finding of plasminogen [25] in a similar basement membrane extract (Matrigel; Collaborative Research, Bedford, MA). Both matrices are produced from the Engelbreth-Holm-Swarm murine sarcoma [26], incubated in subcutaneous pouches in mice for large-scale commercial production. Since both intact EB and MOMP have been reported to fix complement by the alternative pathway [27, 28], the involvement of C5a must also be implicated.

Persistence of chlamydial envelopes for 4 weeks in antibiotic-exposed infected epithelial cells. Whereas azithromycin effectively destroys metabolically active RB, chlamydial envelopes remain. The questions then became how long did the envelopes persist and was PMNL chemotaxis still stimulated to envelope-containing epithelial cells? Azithromycin was added to the medium bathing 36-h infected HEC-1B cells. By 72 hpi, primarily envelope ghosts and a few residual EB remained in the inclusions. Incubation of the polarized cultures was continued for 4 more weeks, with changes of the medium every 4 days, and the cultures were examined weekly by transmission electron microscopy. Chlamydial envelopes were easily detected at 7 (figure 4A) and 12 (figure 4B) days but were more difficult to find at 21 (figure 4C) and 28 days. Chlamydia-specific MOMP antigens (figure 4D–4F) and LPS (figure 4G–4I) were detected at 7, 12, and 21 days, respectively.

At 7, 12, 21, and 28 days, PMNL were added to the agarose wells and the coculture chambers were reincubated for 3 h to monitor for PMNL migration. By light and fluorescence microscopic analyses of stained histologic sections cut from JB4 resin–embedded samples, it was clear that the monolayers were still intact (figure 5A, 5B, 5E, 5F) and that inclusions were devoid of intact chlamydiae. In all test samples, PMNL migration did occur and numerous intraepithelial PMNL were visible by 3 h (figure 5B, 5E, 5F). Of equal importance, LPS antigen in 28 day HEC-1B cells was clearly being transferred to intraepithelial PMNL (figure 5G).

Chlamydial antigens secreted to the surfaces of infected epithelial cells influence PMNL chemotaxis. To begin to explore whether or not the appearance of chlamydial antigens on the infected host plasma membrane and initiation of PMNL chemotaxis were related, polyclonal antibodies generated against purified EB (in which antibodies to LPS and MOMP are predominant) and separately against purified RB (in which antibodies to LPS and MOMP are less predominant) were added individually to the tissue culture medium bathing polarized infected HEC-1B cell monolayers at 12 hpi. The samples were reincubated for 24 h until a 36-h infection was attained. PMNL were subsequently added, and incubation was continued for 5 h to allow for chemotaxis. Advanced transepithelial PMNL migration was reduced 65% in samples containing anti–EB antibodies relative to control samples where no antibodies were present, whereas transepithelial PMNL migration was reduced only 12% in samples containing added anti-RB antibodies (data not shown).

Do chemokines secreted from chlamydiae-infected epithelial cells trigger PMNL chemotaxis? It was suspected that proinflammatory chemokines released from chlamydiae-infected endometrial epithelial cell lines would be one of the early signals to initiate PMNL chemotaxis. Therefore, supernatants and cell lysates from the chambers containing HEC-1B cells infected for 24, 36, 48, and 96 h were collected and analyzed by ELISA for IL-8, IL-1α, and TNF-α. Whereas all the control samples were positive, the results for all test samples were negative for cytokine product. These results were in apparent conflict with data recently reported by Rasmussen et al. [29], who reported...
Figure 3. Transmission electron photomicrographs of secretion of chlamydial envelope antigens from chlamydiae-infected HEC-1B cells; 36-h infected HEC-1B cells exposed to polymorphonuclear leukocytes (PMNLs) loaded with azithromycin (A) revealed several vacuoles (arrowheads), external to inclusions, that appeared to be filled with chlamydial outer membrane blebs. By immunoelectron microscopy (B–D), blebs within the extrainclusion vacuoles reacted with (B, inset) primary monoclonal antibodies generated against chlamydial lipopolysaccharide (LPS), and (C) primary polyclonal monospecific antibodies generated against chlamydial major outer membrane protein (MOMP); both primary antibodies were visualized with 30-nm gold-conjugated second-affinity antibodies. Note presence of chlamydial LPS and MOMP on surfaces of host cells. D, Uninfected epithelial cells did not react with anti-LPS monoclonal antibody, thereby confirming specificity of this antibody for chlamydial LPS. Magnification: $A = \times 12,250$, $B = \times 13,500$, (inset) $= \times 19,400$, $C = \times 9000$, and $D = \times 4500$. 
Figure 4. Transmission electron photomicrographs of persistence of chlamydial envelopes in azithromycin-exposed infected epithelial cells. Following azithromycin action on chlamydiae, cultures were processed weekly thereafter and embedded in epon (A–C) or Lowicryl (D–I) resin. Residual chlamydial envelopes were easily detected at (A) 7 days and (B) 12 days but were difficult to find at (C) 21 days. Chlamydial (D–F) MOMP antigens and (G–I) lipopolysaccharide antigen were detected at 7, 12, and 21 days, respectively, based on reaction with specific primary antibodies and visualization with 30-nm gold-conjugated second-affinity antibodies. Magnification: A = ×7700, B = ×6400, C = ×20,800, D = ×11,100, E = ×13,500, F = ×15,000, G = ×17,500, H = ×14,400, and I = ×8400.
Figure 5. Polymorphonuclear leukocyte (PMNL) chemotaxis is triggered to persistent chlamydial envelopes in azithromycin-exposed infected epithelial cells. At 7 and 28 days after azithromycin action, PMNL chemotaxis was allowed to proceed for 3 h; samples were then harvested and processed for (A, B, E, F) light, (C, D) fluorescence, and (G) transmission electron microscopy analyses. A, B, Azithromycin-exposed infected cells reincubated for 7 days revealed intact monolayers and migration of PMNL (arrowheads) through extracellular matrix, epithelial monolayers, and out onto the apical surface; C, D, envelope-containing inclusions were easily detected by staining cell monolayers with a pool of fluorescein-labeled anti-major outer membrane protein monoclonal antibodies (MAbs). E, F, Azithromycin-exposed infected cells reincubated for 28 days continued to show intact monolayers and extensive migration of PMNL (arrowheads) through the monolayer. G, Transmission electron photomicrograph of a culture at 28 days reacted with primary MAbs generated against chlamydial lipopolysaccharide (LPS). Note transfer of chlamydial LPS antigen (arrowheads) from the epithelial cell (HEC-1B) to 2 intraepithelial PMNL. * Denotes inclusions. Magnification: A = ×2700, B = ×2700, C = ×1000, D = ×1400, E = ×3200, F = ×2880, and G = ×8250.
that infection of nonpolarized HeLa cells (an endocervical cell line) with the invasive LGV biovar of *C. trachomatis* (serovar L2) induced IL-8, IL-6, IL-1α, GRO-α, and granulocyte-macrophage colony-stimulating factor by 48 hpi, as determined by both ELISA and RT-PCR. Therefore, we compared *C. trachomatis* serovar E and serovar L2 infection of both HeLa and HEC-1B cells for secretion of IL-8 product by ELISA. Whereas serovar L2 and serovar E infection of HeLa cells did induce IL-8 secretion, there was no up-regulation of IL-8 secretion in infected HEC-1B cells by either serovar (table 1). However, if nontransformed human lung fibroblast cells (WI-38; ATCC CC175) were added to the agarose layer (see figure 1A) underneath the ECM layer, to mimic stromal cell presence, and polarized HEC-1B cells were infected with serovar E for 48 h, considerable secretion of IL-8 was easily detected. These data suggested that signals from serovar E–infected endometrial cells were, indeed, being produced, which, in turn, triggered IL-8 production in WI-38 cells.

Qualitative and quantitative RT-PCR confirmed there was no up-regulation of IL-8 mRNA in serovar E–infected polarized HEC-1B cells; the amount of IL-8 RNA, quantitated against a competitive standard, never reached 1 or 2 molecules per uninfected or infected HEC-1B cell (data not shown). In addition, there was no evidence for increased presence of GRO-α, GRO-β, or GRO-γ in infected cell lysates standardized to actin cDNA and using an amplified plasmid containing GRO-α cDNA sequence as a positive control (data not shown). However, a clear difference was found between uninfected and infected samples in the production of ENA-78 and GCP-2 messages. While neither of these chemokine mRNAs was detected in uninfected control host cells, ENA-78 mRNA was detected 6 h after infection and GCP-2 mRNA was produced 12 h after infection (figure 6). Of interest, the latter was very short-lived: No message was detected 24 h after infection. The identity of these two chemokine messages was confirmed by the presence of a unique *Ptrl* site in the GCP-2 DNA fragment (data not shown) and by sequencing the amplified amplicons after agarose gel purification. The sequence obtained for the ENA-78–specific amplicon was identical to the known sequence of the human ENA-78 gene [18], except for one ambiguous base. The 184-bp GCP-2–specific fragment yielded 151 bases of sequences of which 7 were ambiguous. Alignment with the published sequence of the human GCP-2 gene [18] gave a 97% identity ratio. Thus, the length, restriction pattern, and sequence of both DNA fragments were consistent with their presumed identities.

**Discussion**

The role of neutrophils in *C. trachomatis* infections is complex. The complexities, elucidated in animal models, span beneficial effects in the early stages of chlamydial infection to detrimental effects in the later stages. For example, intravaginal inoculation of BALB/c mice with the indigenous *C. trachomatis* mouse pneumonitis strain (MoPn) results in a vigorous neutrophil response and a significant reduction in vaginally shed chlamydiae. If the mice received the granulocyte-depleting monoclonal antibody RB6-8C5 intravaginally 1 day prior to inoculation, a more intense infection ensued, implicating an important role for neutrophils in controlling the early stages of *C. trachomatis* infection [30]. On the other hand, chlamydiae-infected C57Bl/6 knockout mice (β2m<sup>−/−</sup>, class II<sup>−/−</sup>, and CD4<sup>−/−</sup>) experienced a marked acute inflammatory response that persisted and was accompanied by ascending infection, tubal blockage, and hydrosalpinx [31]. In an elegant study by Darville et al. [32], the variations in outcomes of manifestations of *C. trachomatis* infection in humans was replicated in different inbred mouse strains infected with MoPn. C57 mice had the

![Figure 6](https://academic.oup.com/jid/article-abstract/179/4/954/2907727)

Figure 6. Expression of GCP-2 and ENA-78 chemokine mRNA in *C. trachomatis*–infected HEC-1B cells examined by reverse transcriptase–polymerase chain reaction. RNA was prepared from samples harvested at postinfection h (hpi) indicated. Amplification of cDNA with GCP-2 (lanes 2–8)– or ENA-78 (lanes 9–15)–specific primers. Lane 1, 100-bp standard DNA ladder; lanes 3, 4, 6–8, 10, 11, 13–15, infected cultures; lanes 2, 5, 9, 12, uninfected cultures. Arrows indicate specific amplicons.
shortest course of infection and the lowest incidence of hydrosalpinx; BALB/c mice had an intermediate course of infection, but all developed hydrosalpinx; and C3H mice had the longest course of infection and the most oviductal pathology. Significant increases in neutrophils were noted early in infection in mice (C57) with the shortest course of infection; these mice also had increased transforming growth factor-β, which may have damped the inflammatory response once the organisms had been eradicated, thereby protecting the oviduct from severe destruction [33].

In this study, using a coculture model system consisting of genital C. trachomatis-infected polarized epithelial cells and human blood neutrophils, at least two homing signals were identified that triggered an initial neutrophil response from the submucosa: chlamydial outer membrane components (LPS and, possibly, MOMP) and two chemokines (ENA-78 and GCP-2). In addition, the involvement of complement, most likely in the form of C5a, was also implicated.

An excellent study by Ingalls et al. [34] confirmed the important role of chlamydial LPS in the proinflammatory response. They demonstrated that purified LPS from C. trachomatis serovar F EB could induce TNF-α production from whole blood ex vivo and translocation of nuclear factor κB in CHO cells expressing the CD14 (LPS) receptor, albeit the responses were ~100-fold less potent than Salmonella minnesota Re LPS and Neisseria gonorrhoeae lipooligosaccharide. The accumulation of chlamydial LPS in the eukaryotic membrane has been shown by spin-probe electron-spin resonance spectroscopy to decrease plasma membrane fluidity [35], which could compromise the efficiency of immune recognition and, perhaps, immune cytosis. Of interest, the chemotype of LPS harvested from chlamydiae grown in vitro is rough (Re) and the lipid A is pentaacyl, perhaps accounting for its reduced biologic activity. Ingalls et al. [34] proposed that a chlamydial LPS-induced less-brisk inflammatory response may be correlated clinically with the predominant asymptomatic nature of chlamydial genital infection.

While it is clear that chlamydial LPS specifically can induce a proinflammatory cytokine response via the CD14 signaling pathway, the role of chlamydial outer membrane proteins in influencing a PMN influx is not clear. Even though chlamydial MOMP has been shown to bind C3b in the absence of antibody and generate C5a [27], there is some debate as to whether MOMP is free from contaminating LPS. Perhaps extracellular MOMP can be acquired by surveillant dendritic cells and then processed and presented to T cells [36, 37], explaining the early and predominant antibody response to MOMP in chlamydiae-infected individuals. In a study of 33 women in the San Francisco Bay area stratified by symptoms, clinical findings, and histopathology and evaluated for C. trachomatis MOMP gene (omp1) polymorphism in isolates, serovar E MOMP genotypes were stably associated with asymptomatic infection, nonvariant serovar F genotypes were correlated with lower genital tract infection, and omp1 F variant genotypes were associated with pelvic inflammatory disease [38].

An important cellular microbiology question is how do LPS and MOMP get to the surface of the infected host cell? Even though these chlamydial envelope antigens have been shown to be selectively secreted to the surfaces of infected host epithelial cells in the absence of azithromycin [12, 13], during a stage in the antibiotic killing process of metabolically active chlamydiae by azithromycin, excessive RB outer membrane blebbing occurred. Subsequently, numerous vacuoles containing such blebs appeared external to the chlamydial inclusion, and chlamydial LPS and MOMP then began to be detected on the infected epithelial cell plasma membrane and extracellularly. It is tempting to speculate that inhibition of chlamydial protein synthesis prevented incorporation of chlamydial proteins [39] into the inclusion membrane; as a result of the altered inclusion membrane fluidity, the vacuoles arose from pinching-off or eversion of inclusion membrane, were trafficked to the epithelial surface via the exocytic pathway and, on fusion with plasma membrane, delivered the chlamydial envelope antigens to the epithelial surface. While it has been demonstrated that trans-Golgi exocytic vesicles containing spherogomyelin fuse with the chlamydial inclusion and the spherogomyelin becomes incorporated in the chlamydial envelope [40], there is no evidence this flow is bidirectional. There is, however, precedence for the concept of eversion or budding of LPS-containing vesicles from bacterial-containing vacuoles, even in the absence of antibiotic [41].

Addition of purified Shigella flexneri LPS to the apical surface of polarized colonic intestinal epithelial cells resulted in transcytosis of LPS to epithelial basolateral domains [42]. In the epithelial cells, this event triggered induction and secretion of IL-8 at the basolateral poles and resulted in increased PMNL adherence to the epithelia. Given that IL-8 has been connected with the response of numerous cell types to bacterial infection [15], has been shown to be produced by a cervical epithelial cell line in response to C. trachomatis infection [29], and has also been identified as one of the cytokines expressed by the endometrium at specific stages of gestation and menstruation [43], it seemed the obvious choice when beginning to look at chemokine production. To our surprise, although low levels of IL-8 message could be detected in both infected and uninfected samples, a connection between IL-8 message or protein product could not be made with the infection process. Instead, two chemokines of the same family, ENA-78 and GCP-2, were shown to be expressed in response to chlamydial infection at the RNA level. Very little data are available concerning the production of these two chemokines in the endometrium, but both chemokines have clearly been connected to various inflammatory situations, mostly chronic types such as arthritis and inflammatory bowel syndrome, and ENA-78 has been shown to be secreted by hepatocytes infected with Salmonella and Yersinia species [44]. It is likely that in vivo infection in

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the lower genital tract induces an ascending cascade of signals that programs endometrial epithelial cells to produce a different chemokine response to deal with invasive infection versus primary infection. Perhaps because the endometrium is programmed to undergo normal physiologic changes that are apparently very similar to an inflammatory process, chlamydial infection in the endometrium results in a cytokine “signature” that is subtly different, so that the immune system is, in fact, able to distinguish between these events in order to respond appropriately. In any event, our results suggest a dynamic interaction between the specific host epithelial cell and chlamydiae, and they illustrate the usefulness for the host to possess a battery of chemical messengers with apparently redundant functions.

A particularly significant finding in this study was that following azithromycin exposure of infected cells, residual chlamydial envelopes and MOMP and LPS antigens could persist in inclusions for up to 4 weeks and that PMNL migration was still stimulated. Our data, albeit generated in vitro, provide one plausible explanation for the theory that a prolonged inflammatory response to persistent chlamydial antigens is believed to be responsible for the damage and sequelae in chlamydial infections [45]. Clearly, the ability to extrapolate from the in vitro tissue culture situation to the in vivo situation is compromised because many more physiologic processes are underway in vivo, including more rapid cell turnover, repair responses, and humoral mechanisms that allow for clearance of microbial antigens. However, in a study of 25 women with postinfectious tubal infertility who had been treated previously with antibiotics regarded as effective against C. trachomatis, Patton et al. [45] detected evidence of chlamydiae in tubal biopsy specimens from 17 of these women by culture, immunocytochemistry using an anti-MOMP monoclonal antibody, or in situ hybridization for detection of C. trachomatis plasmid. In a follow-up study model of experimentally induced salpingitis–pelvic inflammatory disease in female macaques [3], monkeys were given doxycycline, doxycycline plus ibuprofen, doxycycline plus triamcinolone, or placebo to assess the effects of antimicrobial and antiinflammatory drugs on oviductal pathology. At hysterectomy, there was no evidence that treatment regimens had any effect on gross or histologic pathology. Despite negative posttreatment cervical cultures, the persistence of chlamydial MOMP and plasmid DNA was found in cervix, endometrium, and oviducts in the majority of treated macaques.

In summary, an in vitro model system has been developed for examining in more depth the mechanisms generating neutrophil migration signals and how nuances of the mechanisms modulate neutrophil migration to effect a mild or acute response to chlamydial infection of epithelial cells. Current studies in our laboratory include a comparison of neutrophil migration patterns to endocervical cells (HeLa) versus endometrial epithelial cells (HEC-1B) infected with C. trachomatis serovar E versus serovar F and serovar F variant.

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References