Host Chemokine and Cytokine Response in the Endocervix within the First Developmental Cycle of *Chlamydia muridarum*[▽]

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The initial host response in a primary chlamydial infection is the onset of acute inflammation. However, we still know very little about the early temporal events in the induction of the acute inflammatory response and how these events relate to the initial chlamydial developmental cycle in an actual genital infection. Because it was critical to initiate a synchronous infection in the endocervix in the first 24 h to evaluate the sequential expression of the host response, we developed the surgical methodology of depositing *Chlamydia muridarum* directly on the endocervix. Cervical tissue was collected at 3, 12, and 24 h after inoculation and the expression array of chemokines, cytokines, and receptors was assessed to characterize the response during the initial developmental cycle. Polymorphonuclear leukocyte (PMN) infiltration was first observed at 12 h after inoculation, and a few PMNs could be seen in the epithelium at 24 h. Electron microscopic analysis at 24 h showed that virtually all inclusions were at the same stage of development, indicating a synchronous infection. Several chemokine and cytokine genes were expressed as early as 3 h after infection, but by 12 h, 41 genes were expressed. Thus, activation of the host response occurs both with the introduction of elementary bodies into the host and early replication of reticulate bodies. No significant response was observed when UV-inactivated organisms were inoculated into the cervix at any time interval. This model provides an ideal opportunity to investigate the mechanisms by which the early inflammatory response is induced in vivo.

In the early stages of infections with chlamydiae, whether genital, ocular, or respiratory, one typically finds a heavy acute inflammatory response at the site of infection. Thus, in these early stages, the pathological response is one of destruction of the superficial epithelium by polymorphonuclear leukocytes (PMNs) (25). As the adaptive response is initiated, one finds an influx of mononuclear cells, including CD4 and CD8 T cells, into the local site, although these cells tend to be present in the submucosa rather than directly in the epithelium (17, 23). Upon reinfection, the innate response is also activated, but there is a strong T-cell anamnestic response in repeated infection that likely also contributes to the pathological response (22, 24, 29).

Obviously, all of these cell types require the production of various chemokines and cytokines to attract them to the local site; however, there is relatively little known about all of the mechanisms which contribute to elicit the response. There has been a great deal of work published on the induction of various chemokines and cytokines using in vitro culture systems. Ingalls et al. first reported that chlamydial lipopolysaccharide (LPS) was able to elicit tumor necrosis factor alpha (TNF- α) production from peripheral blood leukocytes and from tissue culture cells (8). Rasmussen et al. later observed that chlamydial infection of epithelial cells in vitro resulted in the produc-

tion of interleukin 8 (IL-8), an important chemokine for PMNs, 20 to 24 h postinfection and required that the organisms be viable (26). Recently, it has been demonstrated that IL-8 production from epithelial cells requires active growth of the organism within the cell and activation of the ERK pathway independent of p38 and Jun N-terminal MAPK (2, 3). There have been numerous in vitro studies showing that chlamydiae can elicit various chemokines and cytokines from tissue culture cells (reviewed in reference 19). In general, IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α are elicited, and all require active infection of the target cell, strongly indicating that the intracellular growth of chlamydiae may be a key factor in stimulating the appropriate intracellular pathways for the production of these molecules. The obvious caveat with these studies is that they are all in vitro infection models in established cell lines, which do not represent the multitude of factors present in the natural infection or the natural target cell(s) at the tissue site.

There have also been a number of reports characterizing the chemokine and cytokine response in vivo using the mouse infected in the genital tract with *Chlamydia muridarum*, but the majority of these studies characterized the chemokine and cytokine response as beginning several days after inoculation (4, 5, 12, 28). Not surprisingly, a variety of chemokines and cytokines are produced as a result of infection, including TNF- α , IL-1 α , IL-1 β , IL-6, IL-10, IL-12p40, gamma interferon (IFN- γ), CCL2 (monocyte chemoattractant protein 1 [MCP-1]), CCL3 (macrophage inflammatory protein 1 α [MIP-1 α]), CCL5 (RANTES), CCL11 (eotaxin), CXCL2 (MIP-2 α), CXCL9 (monokine induced by IFN- γ [MIG]), and CXCL10

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(interferon-inducible protein 10 [IP-10]). However, it is not clear what mechanisms are responsible for eliciting these mediators, and none of the studies have looked at the response in the endocervix shortly after infection.

While we know a reasonable amount about the array of chemokines and cytokines that are produced following chlamydial infection and are able to associate their production with certain pathways, we know very little about the early temporal events in the induction of the acute inflammatory response and how these events relate to the developmental cycle of chlamydiae in the actual mammalian host. Even though the *in vitro* studies would suggest that the induction of chemokines and cytokines occurs very quickly, there has been only one *in vivo* study thus far that has assessed the cytokine response in the first 24 h of *C. muridarum* genital infection in the mouse (28). In that study, Tseng and Rank reported increased TNF- α and CCL3 (MIP-1 α) gene expression in the cervix as early as 6 h after infection, IFN- γ expression by 18 h, and NK cell activity at 12 h after infection, all clearly within the time frame of the first developmental cycle. Thus, it was the goal of this study to characterize the host response in greater detail, particularly as related to when key chemokine receptor, chemokine, and cytokine genes are expressed during the first developmental cycle in the mouse cervix. This information will serve as a baseline for future studies of the mechanisms by which production of these mediators are elicited.

Because the target tissue of chlamydial infection is the endocervix, it is problematic to inoculate chlamydiae vaginally and then evaluate the response in the endocervix in the first 24 h. Undoubtedly, many organisms are lost by leakage of the inoculum to the exterior, and most likely, only a few organisms pass through the cervical os and through the mucous layer to infect the endocervix within the first few hours, so that the number of infected cells is comparatively low with respect to the inoculum, or they enter the endocervix in stages, so that the resulting infection is most likely asynchronous. Thus, the subsequent events in the initiation of the inflammatory response are difficult to discern because they represent a continuum rather than a stepwise process. Consequently, we developed an inoculation method that would allow the deposition of a large number of organisms directly on the endocervical tissue so that we could then remove tissues at early times during the first developmental cycle, with the assumption that the infection within the first developmental cycle would be relatively synchronous. This should allow us to determine exactly when, during the developmental cycle, specific chemokine and cytokine expression occurs. Based on the mediators produced at each time interval, we could then begin to determine when specific pathways are initiated and how they are temporally regulated. Therefore, in this study, we describe the intracervical inoculation method and characterize the chemokine and cytokine response within the first 24 h following endocervical infection.

MATERIALS AND METHODS

Experimental animals. Six-week-old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were housed in a barrier facility with a 12:12 light/dark cycle and food and water *ad libitum*.

Intracervical inoculation of chlamydiae. The Nigg strain of *C. muridarum* was utilized in these experiments. The culture was originally obtained from ATCC as

a yolk sac preparation in 1977 and has been subsequently passaged in this laboratory first in yolk sacs and then in cell culture.

Seven days prior to infection, mice are injected subcutaneously with 25 mg each of Depo-Provera to force the mice into a state of anestrus, thus ensuring that all mice are inoculated at the same stage of the estrous cycle to eliminate that variable. Mice are anesthetized with 5 mg/kg of Nembutal intraperitoneally. The lower abdomen is shaved and swabbed with Betadine and 70% ethanol. A 1-cm incision is made ventrally in the skin, and the peritoneum is bluntly dissected directly over the cervix. The uterine horns are exposed, and the left horn is ligated directly distal of the uterine horn branching point by use of a medium LigaClip (Ethicon Endo-surgery, Inc., Cincinnati, OH). The right horn is ligated approximately 1 cm distal of the branching point. The chlamydial inoculum containing 10^7 inclusion-forming units (IFU) in 20 μ l of sucrose-phosphate-glutamate buffer (SPG), pH 7.2, is injected into the right uterine horn immediately proximal to the ligation point in the direction of the endocervix by use of an insulin syringe fitted with a 30-g, one-half-inch needle bent at a 45° angle. The skin is then closed with a standard surgical staple. This method permits the deposition and containment of the chlamydial suspension directly at the endocervix, which is the primary target tissue of chlamydial genital infection. Moreover, the site of injection in the uterine horn was designed to preclude any damage to the endocervix itself by mechanical insertion of the needle.

Control groups are inoculated intracervically with SPG buffer (sham inoculated) or UV-inactivated chlamydiae (21). All groups inoculated with viable chlamydiae or UV-inactivated bacteria were always compared to the sham-inoculated group. All animal experiments were preapproved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences.

Assessment of chemokine/cytokine transcription and quantification of chlamydial 16S RNA. Upon dissection, care was taken to remove only the cervix so that there was only minimal contaminating tissue from the vagina and from the endometrium. Care was also taken to allow sufficient distance from the ligation point so that any inflammatory response elicited by the ligation was not included in the RNA preparation. RNA was prepared from mouse cervix immediately following excision using the RNeasy (Qiagen) method following homogenization. The RNA samples were DNase I (Roche Laboratories) treated and further purified on a column from an RNeasy kit (Qiagen). Reverse transcription was carried out using an RT2 first-strand kit (SABiosciences) using 1 μ g of RNA. The inflammatory cytokine-chemokine "RT2 profiler array" from SABiosciences was used to measure the transcript levels of various cytokines. The array contains primer sets for 80 cytokines/chemokines and appropriate housekeeping controls. Real-time PCR was carried out as described by the manufacturer, using a Bio-Rad I cycler. Threshold cycles for all PCR runs were set at the same level, arbitrarily chosen as 150, with minimum background signal, for comparison between different samples. The data analysis software from SABiosciences was used to perform all threshold cycle-based change calculations from the raw threshold cycle data. Data represented for each transcript in the infected group are expressed relative to the transcript levels from mock-infected groups. At least three samples were processed for each time point for the infected or mock-infected group, and significance was assessed by a *t* test, with a *P* value of <0.05 being considered significant. Data are presented only if a minimum of a twofold increase was reported compared to sham-inoculated animals, as is standard for gene array experiments (15).

To assess the levels of chlamydial 16S rRNA, reverse transcription was carried out as described earlier (16) using Superscript III and quantitative PCR carried out using primers for *C. muridarum* rs16, AGACAACAAGGACGCAAGAACC (sense) and GGATCATACCACCCTAACAACTCG (antisense), and host β -actin, GGCTATGCTCTCCCTCAG and CGCTCGGTGACGGATCTTCAT. The data for chlamydial rRNA were normalized to host actin expression and expressed as the relative change over the mock-infected group.

Histopathology. Tissues were fixed directly in buffered formalin and were then prepared and stained with hematoxylin and eosin according to standard methodology. Chlamydial inclusions were directly visualized on tissue sections by immunohistochemistry. Briefly, sections were incubated with a monoclonal mouse antichlamydial LPS antibody prepared from the clone EVI H1 (a kind gift of You-xun Zhang, Boston University) followed by reagents from a horseradish peroxidase-diaminobenzidine tetrahydrochloride kit (R&D).

High-contrast transmission electron microscopy. The genital tract from one animal euthanized at 24 h after infection was placed in 2% glutaraldehyde-0.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) for subsequent standard processing, infiltration, and embedding in Epon 812 resin (30). Sequential semithin sections of sagittal views of the cervical tissue were first cut; placed on a glass slide, stained with epoxy tissue stain (EM Sciences, Hatfield, PA), which is a toluidine blue O-basic fuchsin in a water-alcohol solution; and examined by

bright-field microscopy for orientation. When the section depth revealed areas of early inflammation, indicating the likely presence of chlamydial infection, the area was isolated and retrimmed, and thin silver-gold sections were cut with a diamond knife on a Reichert ultratrac S ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), collected on gold grids, and examined in a Philips Tecnai-10 electron microscope (FEI) at 80 kV. Each section spanned the entire circumference of the epithelial lining and subepithelial layers, and the entire epithelial lining was examined, not just preselected areas of inflammation. This process was repeated at four different depths.

Flow cytometric analysis of mouse cervical cells. For analysis of cell populations in infected mice, the cervix was excised from the genital tract of an individual mouse, rinsed twice, and incubated separately with 1 ml of 0.3% collagenase I (Worthington Biochemical) for 20 min at 37°C. After the enzymes were neutralized with EDTA (10 mM), cells were washed and resuspended in fluorescence-activated cell sorter buffer (phosphate-buffered saline, 1% bovine serum albumin, and 1 mM EDTA). The cells (2×10^4 to 5×10^4 cells/25 μ l) were incubated in Fc Block (5 μ g/ml) for 10 min and stained for individual cell surface markers or isotype controls (5 μ g/ml) for 20 min on ice. A mixture of the following monoclonal antibodies (BD Biosciences) was used: CD45 PerCP5.5, NK1.1-PE (NK cells), Ly6G-FITC (PMNs), and CD11c-APC (dendritic cells [DC]). Cells were washed with fluorescence-activated cell sorter buffer and treated with DAPI (4',6-diamidino-2-phenylindole) (2 μ g/ml) to stain for dead cells. Data were acquired in a FACSaria (BD Biosciences) and analyzed using FloJo software (Tree Star Inc.).

Experimental design. Because we were interested only in the host response to chlamydiae within the time span of the first developmental cycle, which for *C. muridarum* is approximately 36 h, tissues were collected at either 3, 12, or 24 h after intracervical inoculation. This enabled us to evaluate the response at early, mid-, and late stages to determine whether there were any differences in the responses associated with the developmental cycle. Control mice included animals that were inoculated with an equivalent amount of UV-inactivated chlamydiae or were sham inoculated with phosphate-buffered saline. All chemokine and cytokine levels in the infected animals were compared to those of sham-infected animals at the comparable time period.

RESULTS

In order to determine whether an inflammatory response could be detected within the initial chlamydial developmental cycle of *C. muridarum*, which is about 36 h, we removed tissues from three mice each at 3, 12, and 24 h after infection. Upon histopathologic examination, no evidence of an inflammatory response could be detected in the cervix 3 h after inoculation with any of the mice. However, at 12 h after inoculation, an increased number of PMNs were observed apparently attached to the walls of the venules or in the immediate perivascular areas in the submucosa of the endocervix (Fig. 1A). PMNs were not seen in or near the luminal epithelium. By 24 h, however, a substantial increase in the number of PMNs in the submucosa was observed (Fig. 1B). The venules appeared dilated, and many PMNs could be seen attached to the endothelium. Occasionally, PMNs were found in the superficial epithelial layer, and there were numerous PMNs in the area between the venules and the epithelium. When 24-h tissues were stained with antibody to chlamydial LPS, areas of staining could be visualized in the epithelium (Fig. 1C), although the staining did not appear to be obvious inclusions as anticipated based on the number of IFU inoculated and the time after infection. Instead, rather smaller granular areas of cytoplasmic staining were observed.

In addition to the histopathologic examination, five mice were inoculated intracervically, and the cervixes were collected at 24 h and processed for flow cytometrical analysis of PMNs, NK cells, DC, and CD45⁺ cells. Three animals were sham inoculated with SPG as controls. Because we were careful to obtain only the cervixes of the mice, the number of viable cells

FIG. 1. Histopathologic response in the mouse endocervix following intracervical inoculation of chlamydiae. (A) Hematoxylin-and-eosin-stained section of the endocervix at 12 h after inoculation, showing three PMNs in a venule (circle and arrow). Magnification, $\times 25$. (B) Hematoxylin-and-eosin-stained section of the endocervix at 24 h after inoculation. Many PMNs are now visible in venules and capillaries as well as the submucosa. Magnification, $\times 25$. (C) Section of endocervix at 24 h after inoculation stained with antibody to chlamydial LPS and visualized by immunohistochemistry, showing several chlamydial inclusions (arrows). PMNs are also visible in the epithelium and submucosa. Magnification, $\times 25$.

FIG. 2. Flow cytometrical analysis of the cervixes of mice at 24 h after intracervical inoculation. PMNs were significantly greater in the cervixes of infected animals than in those of sham-inoculated animals (one-tailed *t* test). The bars represent the mean results for amounts in four animals (infected) and three animals (sham) $\pm$ standard deviation.

obtained from a single animal was relatively low, so we had to restrict our analysis to only four surface markers. Nevertheless, we did observe a significant increase in PMNs compared to the sham-inoculated animals, supporting the histopathologic observations ($P < 0.025$, according to a one-tailed *t* test) (Fig. 2). There was also an increase in CD45⁺ cells, albeit not significant. Similarly, a slight but insignificant increase in NK cells and DC in the infected animals was noted. Because of the low number of PMNs at 24 h and the very low number of PMNs observed with histopathologic samples at 12 h, we did not perform flow cytometry at that time.

When cervical tissue was examined at 24 h after infection by transmission electron microscopy, numerous infected superficial epithelial cells were observed (Fig. 3). Interestingly, the inclusions appeared to be very early in the developmental cycle, with only a few reticulate bodies (RB) observed with the inclusions. In some cases, only a single RB was observed with a vacuole, with multiple inclusions/vacuoles present in individual cells. It appears that the cells were infected with multiple organisms and that the inclusions had not yet fused. Importantly, virtually all inclusions were at the same stage, strongly indicating that the intracervical inoculation procedure resulted in a synchronous infection. Moreover, there was a high percentage of cells infected in the epithelium. The presence of PMNs in the epithelium and in the submucosa (Fig. 3B) with only very small inclusions clearly demonstrated that a chemokine gradient develops relatively quickly during the initial developmental cycle.

To further assess chlamydial replication following intracervical injection, mice were injected intracervically with *C. muridarum* and groups of three mice each were euthanized at 3, 12, and 24 h after inoculation. The cervical tissue was removed, RNA was extracted, and the relative levels of chlamydial 16S RNA were determined. There were no detectable 16S rRNA transcripts at 3 h (Fig. 4), but by 12 h, there was a definite increase in 16S rRNA levels, which increased substantially by 24 h. Coupled with the electron microscopy data, it was apparent that at the 3-h period, presumably the elementary bodies

FIG. 3. Ultrastructure of *C. muridarum*-infected mouse cervix 24 h postinoculation. (A) Multiple early inclusions, containing one or a few RB (arrows), are visible in superficial epithelial cells; no PMNs are seen in this area of the tissue. (B) In other tissue sites, a few PMNs (stars) can be seen migrating to the epithelial layers, eventually (C) targeting chlamydia-infected epithelial cells. Magnification, $\times 3,900$; bars, 1 μ m.

(EB) were still traversing the mucous layer and seeking the susceptible epithelial layers for attachment in vivo in contrast to easier direct deposition of EB onto the surface of nonmucus-coated epithelial cells in vitro. Subsequently, EB had evidently attached to and entered the mouse cervical epithelial cells and

FIG. 4. Change in chlamydial 16S rRNA at 3, 12, and 24 h after intracervical inoculation.

FIG. 5. Increase in transcripts of chemokine receptor genes at 3, 12, and 24 h after intracervical inoculation. Only chemokine receptor genes with a twofold or greater increase are shown. Significant differences ($P < 0.05$ or less) compared to sham-inoculated animals are designated by *.

begun transition into RB, and RB replication had been initiated, as evidenced by the now detectable 16S RNA transcript levels at 12 h. The dramatic increase in chlamydial transcripts by 24 h coincides with chlamydial vesicle fusion to form ultrastructurally visible, albeit small, inclusions containing a few RB.

To obtain a profile of which chemokine, cytokine, and receptor genes are transcribed 3, 12, and 24 h following inoculation of chlamydiae in the cervix, the transcription profile for 80 cytokines, chemokines, receptors, and housekeeping controls was assessed using the inflammatory cytokine-chemokine RT2 profiler array. As controls, tissue was obtained from three sham-inoculated mice at each time. Tissues were also obtained from three mice, each inoculated with UV-inactivated chlamydiae at 3 and 12 h after inoculation. The results from each time were compared to those for the sham-inoculated mice at the same time, and the increase over results for the sham-inoculated mice was determined.

By 3 h after inoculation, expression of 11 genes was induced, and the number increased to 41 genes at 12 h but decreased to

28 genes expressed at 24 h after inoculation (Fig. 5 to 8). The response at 3 h after inoculation included chemokine receptors, CCR2 and CCR6; and chemokines, CCL3 (MIP-1 α), CCL20 (MIP-3 α), CCL24, CCL25, and CXCL15. Only three cytokines were transcribed at 3 h: IL-1F8, IL-13, and TNF- α . Complement factor 3 was also transcribed at this time. Interestingly, only two genes, CCR6 (5.34-fold increase) and IFN- γ (2.27-fold increase), were transcribed at 12 h after inoculation. Neither gene was significantly different from those for the sham-inoculated animals. These data strongly suggest that viable chlamydiae are required to initiate the chemokine and cytokine cascade essential for induction of the inflammatory response. Moreover, the fact that only EB are present, or, at most, the very early conversion of EB to RB, would indicate that this would be sufficient to initiate the transcription process for these chemokines, cytokines, and receptors.

FIG. 6. Increase in transcripts of CC chemokine genes at 3, 12, and 24 h after intracervical inoculation. Only genes with a twofold or greater increase are shown. Significant differences ($P < 0.05$ or less) compared to sham-inoculated animals are designated by *.

FIG. 7. Increase in transcripts of CXC chemokine genes and complement factor 3 at 3, 12, and 24 h after intracervical inoculation. Only genes with a twofold or greater increase are shown. Significant differences ($P < 0.05$ or less) compared to sham-inoculated animals are designated by *.

By 12 h after inoculation, there was a significant burst of activity with numerous receptor, chemokine, and cytokine genes being expressed (Fig. 5 to 8). CCR2 and CCR6 continued to increase, with the latter increasing to very high levels. In addition, CCR1, CCR3, CCR5, IL-2rb, IL-6ra, IL-8rb, and IL-10ra were expressed. A large number of additional chemokines were now expressed in this time frame, including CCL2 (MCP-1), CCL4 (MIP-1 β), CCL5 (RANTES), CCL6, CCL7 (MCP-3), CCL8, CCL9, CCL11 (eotaxin), CCL12, CCL17 (TARC), CCL22 (MDC), CCL24, CXCL1 (KC), CXCL4, CXCL10 (IP-10), and CXCL11 (I-TAC). With respect to cytokines, TNF- α continued to increase, and expression of IFN- γ , IL-1 α , IL-1 β , IL-1F6, IL-11, IL-17 β , and transforming growth factor β genes was also observed.

At 24 h after inoculation, still well within the time frame of the first developmental cycle, there was a decrease in expression of several receptors and chemokines (Fig. 5 to 8). However, other chemokines continued to increase, including CCL2 (MCP-1), CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES),

CCL20 (MIP-3 α), CXCL1 (KC), CXCL9 (MIG), CXCL10 (IP-10), and CXCL15. CXCL5 was the only chemokine to be newly expressed at 24 h. TNF- α continued to increase, and there were marked increases in IFN- γ , IL-1 α , and IL-1 β at 24 h. That 41 genes were transcribed between 3 and 12 h, including 30 genes not transcribed at 3 h, suggests that a second wave of gene transcription is caused by activation of pathways resulting from the interaction of the intracellular replicating chlamydiae with the host cell and/or the influx of PMNs or other inflammatory cells, like macrophages and NK cells, which are also producing chemokines and cytokines. It is important to note that there were several chemokines and cytokines that were not induced by chlamydial infection of the cervix within the first 24 h of infection, including CCL1, CCL19 (MIP-3 β), CXCL12, CXCL13, IL-3, IL-4, IL-10, IL-16, IL-18, and IL-20 (data not shown). The decrease in the level of other chemokines suggests a waning of the response as the inclusion becomes mature, prior to cell death. Likewise, increases in cytokines such as TNF- α , CXCL10, and IL-1 β suggest that

FIG. 8. Increase in transcripts of cytokine genes at 3, 12, and 24 h after intracervical inoculation. Only cytokine genes with a twofold or greater increase are shown. Significant differences ($P < 0.05$ or less) compared to sham-inoculated animals are designated by *.

they could be made by PMNs that are infiltrating the infection site.

DISCUSSION

This study marks the first comprehensive assessment of the chemokine and cytokine response within the first 24 h of chlamydial infection of the cervix. This is a critical time in the host response to the infection because the array of chemokines and cytokines produced will determine the nature of the host inflammatory response, with respect to the speed of the response and the types of cells recruited. It is also the only time frame in vivo in which one is able to associate the expression of particular chemokine and cytokine genes with stages in the chlamydial developmental cycle. For instance, at 3 h after inoculation, there cannot possibly be any advanced stage inclusions, so any response must be associated with the presence of EBs in the tissue or the earliest infection stage of the host cells. By definition, the threshold for the induction of receptor, chemokine, and cytokine gene expression must be the later stage of the developmental cycle attained. The electron microscopy data reveal that at 24 h after the inoculation, the infection is very synchronous, with regard to the early stage in the chlamydial developmental cycle, so one can make some approximations of when during the developmental cycle specific chemokine and cytokine expression is initiated. However, after approximately 36 to 48 h for *C. muridarum*, a new round of replication will occur, and the infection is likely to become asynchronous. Moreover, as is evident in this study, by 12 h after inoculation, PMNs have begun to enter the local site and could be contributing to the array of chemokines and cytokines produced. Therefore, when observing cervical tissue after the initial developmental cycle, not only will one have both EBs and inclusions at various stages, but there will also be a very intense inflammatory response. Consequently, any assessment of the expression profile in relation to the mechanism that elicits the particular response would be difficult at best to interpret, other than stating which chemokine and cytokine genes are being expressed.

In order to address the time frame in which receptor, chemokine, and cytokine genes are expressed in relationship to the chlamydial developmental cycle, we developed a new technique which enabled us to deposit a large number of chlamydiae at the cervix, with the goal of infecting sufficient cells to increase our sensitivity in the quantification of the gene transcription by quantitative PCR. The primary reason for doing this was prompted by our concern that vaginal inoculation would not result in sufficient organisms passing through the cervical os and mucous layer to come into contact with endocervical cells in a short period of time and in a synchronous fashion, thereby diminishing the sensitivity of the assay. While we recognize that this method is very artificial and that the number of organisms deposited is far above the likely infectious dose in nature, the signals, pathways, and sequences of events should be the same at the level of a single cell, whether the inoculum contains 10 IFU or 10⁷ IFU. Also, this method gives us a greater level of consistency with regard to the number of organisms deposited because intravaginal inoculation routinely results in the loss of some of the inoculum exiting back through the vagina.

Not surprisingly, a large number of receptor, chemokine, and cytokine genes were expressed following intracervical inoculation. However, there appear to be two phases of expression, the first associated with the initial inoculum in the first 3 h and the second appearing between 3 and 12 h. It has been shown in vitro that by 2 h after infection of the host cell, EB have already begun to progress in the developmental cycle and are beginning the transformation into RB (18). Also, at this time period, before the EB have visibly converted into RB, fusion with exocytic vesicles begins (7). Thus, at 3 h after infection, there is clearly active gene transcription and protein synthesis by the organism, but bacterial replication has not yet been initiated. The initial host response at 3 h included expression of CCR2, a receptor for CCL2 (MCP-1), CCL7 (MCP-3), CCL8, and CCL12; and CCR6, a receptor for CCL20 (MIP-3 α). CCR6 was very strongly expressed, which was noteworthy in that, of the chemokines transcribed at 3 h, CCL20 (MIP-3 α) was the most strongly expressed. CCL20 (MIP-3 α) is chemotactic for immature DC and memory T cells and B cells, particularly in mucosal tissues (27); therefore, this would be an important chemokine for the initiation of the adaptive response and the recruitment of memory T cells in the case of reinfection. CCL3 (MIP-1 α) and CCL24 (eotaxin-2), which are chemotactic for immature DC, were also expressed at 3 h. CXCL15 was the only chemokine for PMNs expressed at 3 h after inoculation.

Not unexpectedly, TNF- α was expressed early as well and continued to increase at 12 h and 24 h. TNF- α is a key proinflammatory cytokine and an important mediator of the acute inflammatory response through an increase of vascular permeability and the expression of VCAM-1 on endothelial cells. Its production is probably initiated through the activation of the TLR2 pathway (6), but there may be other pathways involved as well. The transcription of C3 suggests that the alternative complement pathway may be activated. If this is the case, then the chemotactic factor C5a could be implicated. It has been shown previously that chlamydial EB could activate the alternative complement pathway (13), but stimulation of the biosynthesis of C3 by TNF- α has also been demonstrated (10). It is important to note that at this early stage after inoculation that UV-inactivated organisms did not elicit a chemokine or cytokine response, even though it was probable that there was free LPS or other products in the inoculum which one might suspect to be capable of eliciting a response. Therefore, these data would indicate that actual initiation of infection of the host cell was critical in the activation of the appropriate pathways. Moreover, it is interesting that in this first phase of the response, the primary chemokines that are produced are those which attract immature DC to the local site, an essential step in the activation of the adaptive response. The stage is also set for eliciting the inflammatory response through the activation of complement and production of TNF- α .

By 12 h after infection, there was a large number of chemokines and cytokines expressed, such that there were chemotactic factors for most cell types. By histopathology, it was apparent that PMNs had begun to localize at the site of infection, although most were perivascular and not yet associated with the epithelium. This would suggest that the chemokine gradients had developed, addressins were expressed on endothelial cells, and initial recognition of those addressins by PMNs had

occurred between 3 and 12 h. Based on our electron microscopic studies and the marked increase in chlamydial 16S RNA expression at 12 h, we would predict that EB were converting into RB during the period from 3 to 12 h and beginning to initiate metabolic activity. Nevertheless, one cannot clearly determine the source of the chemokines because of the influx of NK cells, DC, macrophages, and PMNs, all of which are capable of producing a variety of chemokines and cytokines.

Of the chemokines expressed in this time frame, it is interesting that CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC) were all transcribed at relatively high levels. Each of these, as well as CCL2 (MCP-1), CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL7 (MCP-3), CCL8, and CCL12, all of which were transcribed, are chemotactic for NK cells. Indeed, we previously reported that NK cell activity was present in the genital tract as early as 12 h after infection (28), so it is apparent that NK cells are recruited to the site of infection very early and probably have a major role in controlling the infection until the adaptive response develops, most likely through the production of IFN- γ . Previously, we detected IFN- γ expression in the genital tract by 18 h, and in the current study, we observed IFN- γ expression at 12 h. Not only are NK cells potentially a major factor in controlling the early course of infection, but they also participate in upregulating the Th1 response to chlamydial infection (28).

While presumably not yet critical in the first 24 h of a primary infection, it should be noted that several chemokines expressed at 12 h, including CCL4 (MIP-1 β), CCL5 (RANTES), CCL7 (MCP-3), CCL8, CCL9, CCL12, CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC), are chemotactic for activated or memory T cells. Therefore, in the case of reinfection, by 12 h, chemokine gradients would be in the process of being established to recruit memory T cells to the local site to help bring about resolution of infection. It is somewhat surprising that CCL11 (eotaxin), CCL17 (TARC), and CCL24 (eotaxin-2) were also expressed since these are chemokines for Th2 cells, and the response to chlamydiae is predominantly a Th1 response. Nevertheless, these chemokines have other activities as well.

Obviously, PMNs are entering the local site at 12 h, and there are a number of proinflammatory cytokines expressed, including IL-1 α , IL-1 β , IL-1F6, and IL-1F8, that are important in the inflammatory process. In addition, CXCL1 (KC), CXCL5, and CXCL15, which are chemokines for PMNs, are expressed at 12 h, concomitant with the appearance of PMNs in the tissue.

By 24 h after infection, expression of the chemokines for T cells and NK cells, CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC), continued to increase to higher levels. The higher levels of NK chemokines is supportive of our previous observations that NK cell activity markedly increases in the genital tract by 24 h after infection and reaches peak levels by 48 h (28). As expected, IFN- γ has also attained high levels concomitant with the increase in NK cell activity in the genital tract. Nevertheless, it is clearly possible that there may be differences since the method of inoculation in each study is different. It is also apparent that the chemokine gradient for T cells is already established in order to recruit the specific T cells when they become available about 7 days after infection (11).

When the tissues were examined by histopathology at 24 h, there was a dramatic increase in PMNs in the endocervix, both in the venules and the submucosa, with some cells entering the epithelium and even the lumen at that time. Chlamydiae could easily be visualized in epithelial cells by immunohistochemistry. Moreover, by electron microscopy, PMNs could be observed migrating to the epithelium, and an occasional PMN was actually in the epithelium in the vicinity of infected cells. It is indeed remarkable that even though the inclusions at this point were quite small, there were apparently sufficient levels of chemokines already produced to attract the PMNs to the local site. Not surprisingly, proinflammatory cytokines, IL-1 α , IL-1 β , and TNF- α , continued to increase at 24 h. The marked increase in IL-1 β was expected at 24 h, as *in vitro* studies indicate activation of caspase-1 at that time via the inflammasome pathway (4).

This study demonstrates that virtually all of the important chemokines and cytokines essential for induction of both the innate response and the adaptive response are elicited within the time frame of the very early stages of the first developmental cycle of chlamydiae. Nevertheless, while the gene transcription data give a tremendous amount of information, with regard to possible pathways and mechanisms that are activated, we cannot overlook that there are most likely a number of proteins, constitutive and preformed in cells of the endocervix, which can be released immediately upon the inoculation of an infectious agent yet are not quantified by the gene array that was used in this study.

An important finding in this study, although not unexpected, is that the acute inflammatory response as well as the NK response occurs literally within hours of the introduction of the organism into the endocervix. Both are potentially important controls on the course of the infection until the adaptive response can be deployed and in the case of the NK cells may also be important in governing the upregulation of the Th1 response (1, 28). The speed and intensity of the chemokine and cytokine response at the tissue level are undoubtedly regulated by the inoculating dose and the replicative ability of the particular serovar or variant. In this study, we used a high inoculating dose to increase the level of the response so that our sensitivity was enhanced; however, one would predict that if lower inoculating doses were used, the sequence of events would be the same, but the overall level of each chemokine and cytokine would be reduced until the infection has progressed. Consequently, the chemokine gradients would be initially lower, and the recruitment of PMNs would be slower until the chemokine levels had increased. Thus, a critical factor in determining the "virulence" of chlamydiae, with respect to inducing an inflammatory response, is the size of the inoculating dose and the speed of replication of the variants as well as the yield of EB (9, 14, 20).

An important message from this study is that the host response is determined by interaction of chlamydiae and chlamydial products with components of the innate host response. Thus, to a great extent, it is the organism itself that determines the nature of the innate and adaptive response and, as a result, its fate within the host. This is certainly a complex process, best studied *in vivo* if we are to learn how the organism engineers the host response, and the advantages of the model we have presented, with the characterization of the early response, pro-

vide a firm foundation upon which to explore the interaction of the organism and the host response.

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