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In Situ Analysis of the Evolution of the Primary Immune Response in Murine *Chlamydia trachomatis* Genital Tract Infection

SANDRA G. MORRISON AND RICHARD P. MORRISON*

Department of Microbiology, Montana State University, Bozeman, Montana 59717

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Adaptive immune responses contribute to the resolution of *Chlamydia trachomatis* genital tract infection and protect against reinfection, but our understanding of the mechanisms of those protective responses is incomplete. In this study, we analyzed by in situ immunohistochemistry the progression of the inflammatory and cytokine responses in the genital tracts of mice vaginally infected with *C. trachomatis* strain mouse pneumonitis. The cellular inflammatory response was characterized by an initial elevation in myeloid cells in the vagina (day 3) and uterine horns (day 7), followed by a marked rise in the number of T cells, predominantly $CD4^+$ cells. $CD8^+$ T cells and $CD45R^+$ B cells were also detected but were much less numerous. Perivascular clusters of $CD4^+$ T cells, which resembled clusters of T cells seen in delayed-type hypersensitivity responses, were evident by 2 weeks postinfection. Following the resolution of infection, few $CD8^+$ T cells and $CD45R^+$ B cells remained, whereas numerous $CD4^+$ T cells and perivascular clusters of $CD4^+$ T cells persisted in genital tract tissues. Interleukin-12 (IL-12)- and tumor necrosis factor alpha (TNF-α)-producing cells were observed in vaginal tissue by day 3 of infection and in uterine tissues by day 7. Cells producing IL-4 or IL-10 were absent from vaginal tissues at day 3 of infection but were present in uterine tissues by day 7 and were consistently more numerous than IL-12- and TNF-α-producing cells. Thus, the evolution of the local inflammatory response was characterized by the accumulation of $CD4^+$ T cells into perivascular clusters and the presence of cells secreting both Th1- and Th2-type cytokines. The persistence of $CD4^+$-T-cell clusters long after infection had resolved (day 70) may provide for a readily mobilizable T-cell response by which previously infected animals can quickly respond to and control a secondary infectious challenge.

In the murine model of *Chlamydia trachomatis* genital tract infection, intravaginal inoculation of *C. trachomatis* strain mouse pneumonitis (MoPn) produces an initial infection of vaginal and cervical epithelial cells, which progresses to involve the uterine horns and oviducts (1, 22, 24, 40). Animals generally resolve the infection and are culture negative by 4 weeks (22). The resolution of primary *C. trachomatis* genital tract infection in mice is dependent on T-cell-mediated immune responses. Genital infection of major histocompatibility complex class II-deficient gene knockout mice results in a chronic course of infection compared to that in normal immunocompetent mice (22). Furthermore, depletion of $CD4^+$ T cells prior to infection (20) delays the resolution of infection, and the transfer of immune T cells or T-cell clones or lines confers a moderate level of protective immunity to naive recipients (3, 12, 31, 36). Conversely, chlamydia-specific antibodies are not needed to bring about the resolution of primary infection (14, 37) but may contribute to the protection of mice from reinfection (37).

Th1-type cytokines, such as interleukin-12 (IL-12) and gamma interferon (IFN-γ), are needed for the resolution of chlamydial infection. Mice treated with anti-IL-12 resolve primary infection more slowly than nontreated mice (25). Mice deficient in IFN-γ are unable to completely resolve genital tract infection, and chlamydial infection in those mice disseminates to systemic sites (5, 25). The importance of other cytokines and immunological mediators in protective immunity to primary chlamydial genital tract infection has been studied, but none are known to be as critical as IL-12 and IFN-γ in controlling and resolving primary chlamydial infection (25–27).

Our understanding of the systemic immune responses that contribute to the resolution of primary chlamydial genital tract infection has been broadened through the use of specific gene knockout mice. However, our knowledge of the evolution of the local immune response during the course of infection has not been thoroughly documented. Previous studies have examined to some extent the cellular and cytokine compositions of the local inflammatory response to chlamydial genital tract infection (13, 15, 24, 25, 27, 28, 41), and others have analyzed the systemic immune responses (16, 26, 27, 37). Various methodologies have been used in those studies to detect the presence or absence of cytokines, including detection of cytokine mRNA by reverse transcription-PCR in homogenates of genital tract tissue from infected mice or detection of cytokines in culture supernatants from antigen-stimulated splenic lymphocytes by enzyme-linked immunosorbent assay. However, to understand the antimicrobial properties of the adaptive immune response to chlamydial infection, it is important to gain an understanding of the local inflammatory responses elicited during the course of an infection that resolves without therapeutic intervention.

The purpose of the present study was to characterize the evolution of the local inflammatory response to chlamydial genital tract infection. In situ immunohistochemistry was used to depict changes in lineage-specific cell populations and the pattern of cytokine production in the murine genital tract during the course of chlamydial infection. Our results provide a foundation from which we can study the effects of experimen-

* Corresponding author. Mailing address: Department of Microbiology, Lewis Hall Room 109, Montana State University, Bozeman, MT 59717. Phone: (406) 994-7959. Fax: (406) 994-4926. E-mail: morrison@montana.edu.

tally induced perturbations in the systemic immune response on the development of local (genital tract) immunity.

MATERIALS AND METHODS

Mice. Female C57BL/6J mice were purchased from the National Cancer Institute (Bethesda, Md.) and maintained in the animal facilities at Montana State University. Mice 8 to 12 weeks of age were used throughout the study.

Growth, purification, and enumeration of *C. trachomatis*. The MoPn strain of *C. trachomatis* was grown in HeLa 229 cells. Elementary bodies were purified and inclusion-forming units (IFU) were determined as described previously (4).

Antibodies. The following reagents were purchased from Pharmingen (San Diego, Calif.) and used at the dilutions indicated: monoclonal antibodies to CD3e (clone 145-2C11) (1/200), CD4 (clone RM4-5) (1/500), CD8a (clone 53-6.7) (1/500), CD11b (clone M1/70) (1/500), CD45R/B220 (clone RA3-6B2) (1/5000), Ly-6G (clone RB6-8C5) (1/500), IL-4 (clone BVD4-1D11) (1/200), and IL-10 (clone JES5-16E3) (1/200); and isotype control monoclonal antibodies for hamster immunoglobulin G (IgG) (clone G235-2356) (1/200), rat IgG2a (clone R35-95) (1/500), rat IgG2b (clone R35-38) (1/500), and biotinylated goat anti-rat Ig (1/200). Monospecific biotinylated goat anti-mouse IL-12 and tumor necrosis factor alpha (TNF-α) were purchased from R&D Systems (Minneapolis, Minn.) and used at a 1/20 dilution. Biotinylated goat anti-hamster IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) was diluted 1/500 for use.

Genital tract infection. Mice were treated with Depo-Provera (medroxy-progesterone acetate) and infected with 1,500 IFU (100 50% infectious doses) of *C. trachomatis* MoPn as previously described (22). The course of infection in a group of nine mice was monitored by enumerating the number of IFU recovered from cervicovaginal swabs (Calgiswab; Spectrum Medical Industries, Los Angeles, Calif.) taken at various time points following infection (22). Inclusions were visualized by indirect immunofluorescence using the MoPn-specific anti-major outer membrane protein monoclonal antibody Mo-33b and fluorescein isothiocyanate-labeled goat anti-mouse IgG (22). Because swabbing disrupted the vaginal and cervical mucosal epithelium, tissues from these mice were not used for immunohistological analysis. A separate group of 45 mice were infected concurrently, and their genital tracts were harvested and used for immunohistochemistry. At days 3, 7, 10, 14, 21, 28, 35, 42, and 70 following infection, five mice at each time point were sacrificed; the entire genital tract was removed, placed in embedding medium (OCT) (Tissue-Tek; Sakura Finetek, Torrance, Calif.), snap frozen in dry ice-cooled 2-methylbutane, and stored at −85°C until sectioned and processed for immunohistochemistry. Because chlamydial cultures were not performed on mice used for histological analysis, infection was confirmed either by staining genital tract tissues for chlamydial inclusions (days 3, 7, and 10 postinfection) or by analyzing sera for antichlamydial IgG and IgA (days 14, 21, 28, 35, 42, and 70 postinfection) (22).

Immunohistochemistry. Cryostat sections of the entire genital tract, 5 μm thick, were placed onto Superfrost slides (Fisher Scientific, Santa Clara, Calif.) and air dried at room temperature (~21°C). All staining procedures were performed in a humidified chamber at room temperature.

(i) Staining of cell surface antigens. Air-dried sections were fixed in acetone for 5 min, air dried, and then rehydrated in phosphate-buffered saline (PBS) for 15 min. The endogenous peroxidase activity of genital tract tissue was blocked by incubating the sections in Peroxo-Block (Zymed Laboratories, San Francisco, Calif.) for 40 s. Sections were washed in PBS (10 mM phosphate, 0.13 M NaCl, pH 7.4) for 5 min and blocked with avidin-biotin-containing 5% normal serum (Vector Laboratories, Burlingame, Calif.) according to the manufacturer's protocol. Following a 5 min rinse with PBS, sections were incubated for 1 h with primary antibody (anti-CD3, -CD4, -CD8, -CD11b, -CD45R, or -Ly6G), diluted in Hanks balanced salt solution containing 1% normal serum, which corresponded to the species from which the secondary antibody was derived. Tissues were rinsed in PBS for 5 min and then incubated for 30 min with biotinylated secondary antibody diluted in Hanks balanced salt solution with 1% normal serum. Tissues were rinsed with PBS, incubated with Vectastain ABC complex (Vector Laboratories) for 30 min, and washed with PBS, and color was developed by adding 3,3′-diaminobenzidine (Vector Laboratories) substrate. Sections were then counterstained with hematoxylin, rinsed with distilled water, cleared with xylene, and mounted with Permount (Fisher Scientific). Isotype-matched negative control antibodies and antisera were used to stain normal and infected tissues to ensure the specificity of positive staining reactions. Neither normal nor chlamydia-infected tissues stained with the negative control monoclonal antibody or sera (data not shown).

(ii) Intracellular cytokine staining. Air-dried sections were fixed for 15 min in PBS containing 4% paraformaldehyde and washed in PBS, and endogenous peroxidase activity was blocked by incubating sections for 1 h in a solution of PBS containing 1% H_2O_2 and 0.1% saponin (Sigma Chemical Company, St. Louis, Mo.). Tissues were then washed for 15 min in PBS–0.1% saponin and blocked with avidin-biotin-containing 5% normal serum (Vector Laboratories) according to the manufacturer's protocol, except that 0.1% saponin was added to the blocking solution. When monoclonal primary antibodies (i.e., anti-IL-4 and -IL-10) were used, tissues were incubated overnight at room temperature with primary antibody diluted in PBS containing 1% normal serum, 0.1% saponin, and 0.02% azide. Sections were washed for 15 min in PBS–0.1% saponin and then incubated for 1 h with secondary biotinylated antibody diluted in PBS–1% normal serum–0.1% saponin. Tissues were incubated with the Vectastain ABC reagent and developed as described above, except that all washes and incubation mixtures contained 0.1% saponin and the incubation with the ABC reagent was for 1 h.

Biotinylated, monospecific polyclonal antibodies (anti-IL-12 and anti-TNF-α) were used for the detection of some cytokines. In those assays tissues were treated as described for the detection of cytokines with monoclonal antibodies, except that tissues were incubated for 2 h at room temperature in a humidified chamber with biotinylated primary antibody and no secondary antibody was used.

To insure that all anticytokine antibodies would stain their respective cytokines under the conditions described above, positive control cells (MiCK-1, -2, and -3) (Pharmingen) were spread onto Superfrost slides, fixed, and stained by the procedures described above for cytokine staining. Positive control cell populations for TNF-α, and IFN-γ were MiCK-1 cells, those for IL-4 and IL-10 were MiCK-2 cells, and those for IL-12 were MiCK-3 cells.

Qualitative evaluation of genital tract inflammation. Tissue sections from four or five mice at each indicated time point postinfection were stained for cell surface antigens and cytokines as described above. Cell populations and subpopulations were enumerated by counting positive-staining cells in 10 high-power (40×) microscopic fields, an area of approximately 2.3 mm^2. Tissues were then assigned an inflammatory score for each cell surface phenotype, as follows: 0, <1 positive cell/mm^2; 1, 1 to 50 positive cells/mm^2; 2, 51 to 250 positive cells/mm^2; 3, 251 to 500 positive cells/mm^2; 4, >500 positive cells/mm^2 without evidence of cell clusters; and 5, >500 positive cells/mm^2 with cell clusters present. Cytokine-producing cells were enumerated by counting the number of positive-staining cells in 10 20× fields, an area of approximately 9.5 mm^2.

RESULTS

In situ analysis of lineage-specific cells in genital tract tissue during the course of *C. trachomatis* infection. At various times during the course of chlamydial genital tract infection, animals were sacrificed and the entire genital tract (vagina, uterine horns, and oviducts) was removed and analyzed by immunohistochemistry for cell surface markers that define specific cell lineages. Cells of the myeloid lineage express cell surface molecules recognized by anti-CD11b, a phenotype shared by macrophages/monocytes and polymorphonuclear neutrophils (PMN) (21). Anti-Ly6G reacts with a surface molecule on mature granulocytes (8). Anti-CD3e and anti-CD45R (B220) were used to identify T cells and B cells, respectively.

The course of chlamydial genital tract infection is shown in Fig. 1. Tissues were harvested from infected mice at various times following infection and correspond to different stages of infection. For example, tissues harvested at days 3 and 7 represent a time when infection is progressing and specific immune responses are developing (22), whereas tissues harvested at days 21 and 28 are representative of a resolving infection and those harvested at days 42 and 70 are representative of a resolved infection.

The cellular profiles of vaginal tissues from control mice and from mice on day 3 of infection are shown in Fig. 2. Cells with the Ly6G and CD11b cell surface phenotype predominate and correspond to the PMN infiltrate that has been reported previously (22). Numerous CD11b- and Ly6G-positive cells were found in the luminal exudate, the epithelial layer, and the lamina propria (Fig. 2E and F). Cells of the B-cell lineage (CD45R) were rare (Fig. 2H), and a few T cells ($CD3e^+$ cells) (Fig. 2G) were found in the mucosal epithelium and submucosa. As infection progressed, cells began to infiltrate and accumulate in the uterine horns (Fig. 3). Ly6G- and CD11b-positive cells were numerous by day 7 postinfection (Fig. 3E and F) and localized to the uterine lumen, mucosal epithelium, and lamina propria. As the infection resolved (3 to 4 weeks postinfection), the PMN population decreased (Fig. 3Q and R). T cells ($CD3e^+$ cells) and B cells ($CD45R^+$ cells) were rarely observed in uterine tissues until about day 7 postinfection, at which time they contributed significantly to the inflammatory infiltrate (Fig. 3G and H). T cells and B cells localized primarily to the lamina propria but were also occasionally

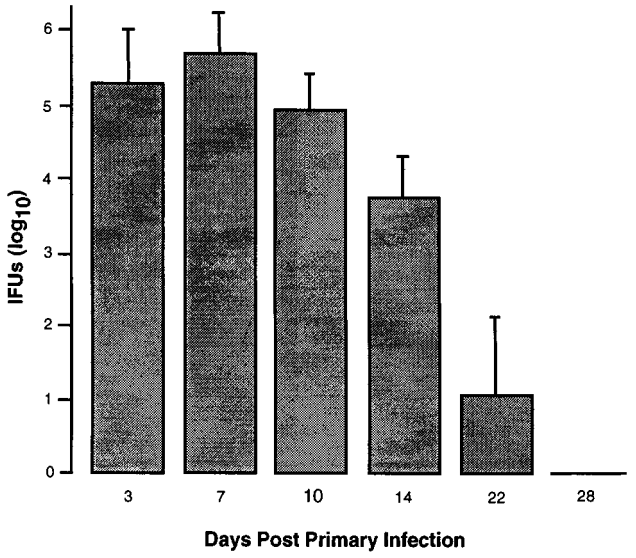


FIG. 1. Time course of chlamydial genital tract infection. Mice were infected with 100 50% infectious doses of *C. trachomatis* strain MoPn. The course of infection was monitored by swabbing the vaginal vault at selected times and enumerating IFU by isolation onto HeLa cell monolayers. Inclusions were visualized by indirect immunofluorescence using monoclonal antibody Mo-33b and fluorescein-labeled goat anti-mouse IgG. Data are presented as mean IFU ± standard error of the mean for nine mice.

observed within the mucosal epithelium. $CD3^+$ T cells were the predominant cell type during and following the resolution of infection (days 14 to 70).

An interesting feature of the T-cell inflammatory response in the uterus, and to some extent in the vagina, was the development of perivascular T-cell clusters. Clusters became apparent by day 21 postinfection (Fig. 3O) and were observed as late as 70 days postinfection (data not shown). B-cell clusters were less evident, but anti-CD11b also stained clusters of cells. Low-power magnification of immunostained tissues (Fig. 3U to X) clearly demonstrated the clustering of T cells throughout the uterine horns.

In the murine model of chlamydial genital tract infection, the oviducts are the primary site for inflammatory damage, which subsequently results in infertility. The cellular infiltrate of the oviducts following chlamydial infection was evaluated and found to be similar to that of uterine tissues (data not shown). Briefly, as infection ascended into the oviducts (approximately day 7 postinfection), PMN predominated in the luminal exudate and in mucosal and submucosal tissues. T cells and B cells accumulated as the course of infection progressed. Unlike that of uterine tissues, however, the cellular infiltrate of oviducts diminished greatly, and only a few scattered T cells were observed following the resolution of infection. Thus, few inflammatory cells remained in oviductal tissue, but as reported previously (22), tubal ectasia, loss of ciliated columnar epithelial cells, and hydrosalpinx were frequently observed.

In situ analysis of CD4 and CD8 T-cell subsets. Both $CD4^+$ and $CD8^+$ T cells have been implicated in the protective immune response to *C. trachomatis* genital tract infection (11, 22, 34–36). At 3 days following infection, $CD4^+$ and $CD8^+$ T cells were observed in the vaginal mucosal epithelium and lamina propria (Fig. 4C and D). By 7 days postinfection, the uterine mucosal epithelium and lamina propria contained $CD4^+$ and $CD8^+$ T cells (Fig. 5). $CD4^+$-T-cell clusters were evident by 14 to 21 days postinfection (Fig. 5C and D). Clusters were observed throughout the uterine horns, and, in general, clusters of $CD4^+$ T cells were more numerous and were comprised of more cells than $CD8^+$-T-cell clusters (Fig. 5F and L).

The frequencies of cell populations in genital tract tissue during the course of infection are depicted in Fig. 6. Myeloid-lineage cells (CD11b) and $CD3^+$ T cells were the predominant cell types in early infection (day 7), but T cells, and particularly $CD4^+$ T cells, were the predominant cell type during the resolution (days 14 to 21) and following the resolution (days 28 to 70) of infection. The kinetics of B-cell infiltration into infected genital tract tissue was similar to that of T cells, but the number

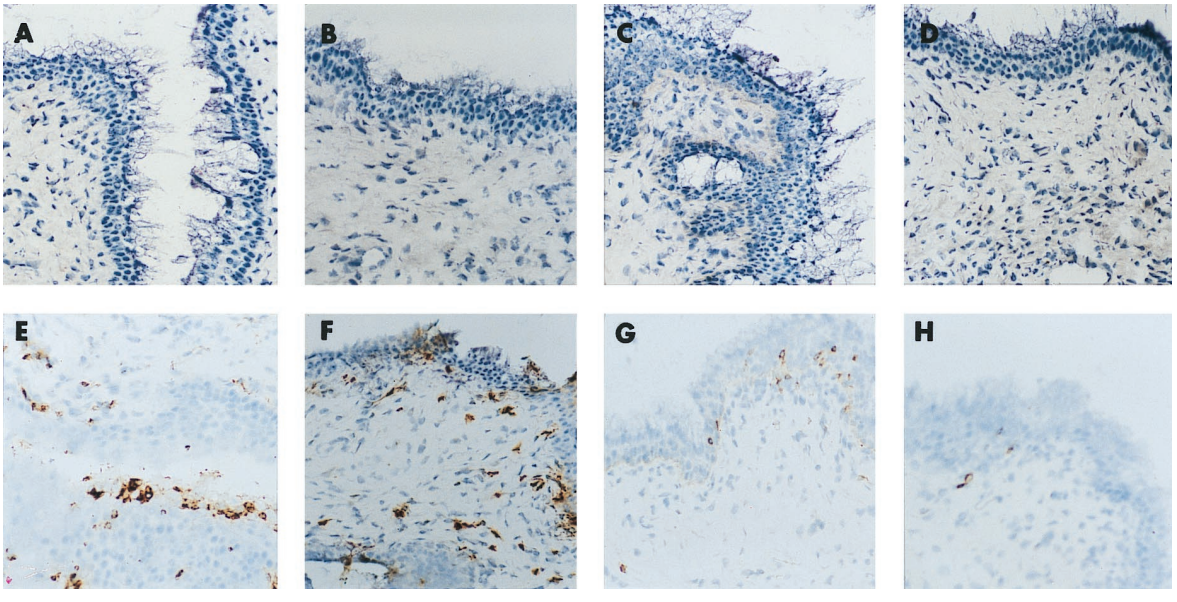


FIG. 2. Immunohistochemical staining of CD11b, Ly6G, CD3e, and CD45R in vaginal tissue. Vaginal tissues were collected from uninfected mice (A to D) and from chlamydia-infected mice at 3 days post-infectious challenge (E to H) and stained with anti-CD11b (A and E), anti-Ly6G (B and F), anti-CD3e (C and G), or anti-CD45R (D and H). Magnification, ×300. Representative tissues from four or five mice at each time point are shown.

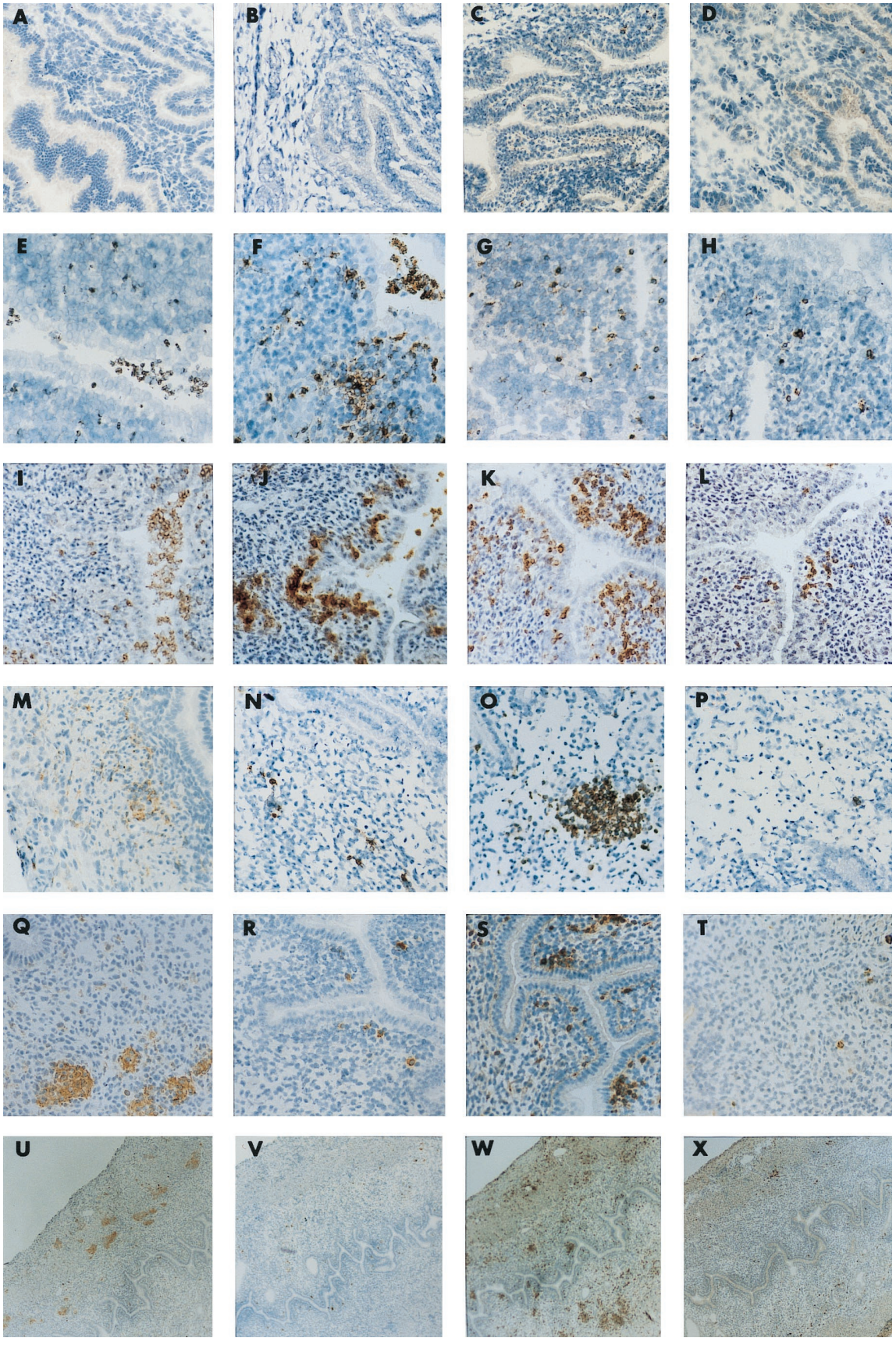


FIG. 3. Immunohistochemical staining of myeloid and lymphoid cell populations in uterine tissue. At weekly intervals uterine horns were harvested from chlamydia-infected mice and stained for CD11b, Ly6G, CD3e, or CD45R cell surface antigens. (A to D) Noninfected mice; (E to H) 7 days postinfection; (I to L) 14 days postinfection; (M to P) 21 days postinfection; (Q to T and U to X) 28 days postinfection. Anti-CD11b (A, E, I, M, Q, and U), anti-Ly6G (B, F, J, N, R, and V), anti-CD3e (C, G, K, O, S, and W), and anti-CD45R (D, H, L, P, T, and X) were used. Magnifications, ×300 (A to T) and ×60 (U to X). Representative tissues from four or five mice at each time point are shown.

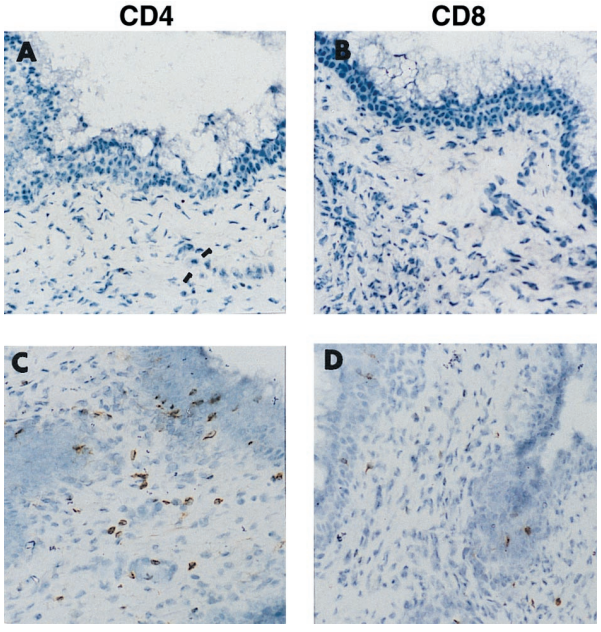


FIG. 4. Immunohistochemical staining of $CD4^+$- or $CD8^+$-T-cell subsets in vaginal tissues from noninfected mice (A and B) and chlamydia-infected mice (C and D) at 3 days postinfection, stained with either anti-CD4 (A and C) or anti-CD8 (B and D). Magnification, ×300. Representative tissues from four or five mice at each time point are shown.

of B cells never approached the level of T cells. The inflammatory infiltrate of the genital tract tissues diminished as infection resolved, but appreciable numbers of T cells remained localized to uterine tissue for at least 70 days postinfection.

Cellular characteristics of the vagina and uterus following the resolution of genital tract infection. Previous studies have shown that the adaptive immune responses which develop during the course of primary chlamydial infection confer a considerable degree of protection from a secondary infectious challenge (22). The protective response is characterized by the shedding of fewer chlamydiae and a much shortened course of infection. To determine the characteristics of the inflammatory cell response in genital tract tissue at a time when infection had resolved and animals demonstrated a level of immunity to reinfection, we examined vaginal and uterine tissues for various cell populations at 42 days following primary infection (2 weeks past the first culture-negative time point).

Considerable numbers of inflammatory cells remain localized to the vaginal and uterine tissues following the resolution of primary infection (Fig. 7). At 42 days following infection, moderate numbers of $CD11b^+$ cells and $CD3^+$ T cells were detected in the vagina. $CD45R^+$ B cells were also detected but were less numerous than T cells. B cells and T cells were found to localize to both the lamina propria and mucosal epithelium. $CD4^+$-T-cell clusters were evident, and $CD8^+$ T cells were often observed to be localized to the mucosal epithelium. The uterus displayed a similar inflammatory picture; T cells pre-

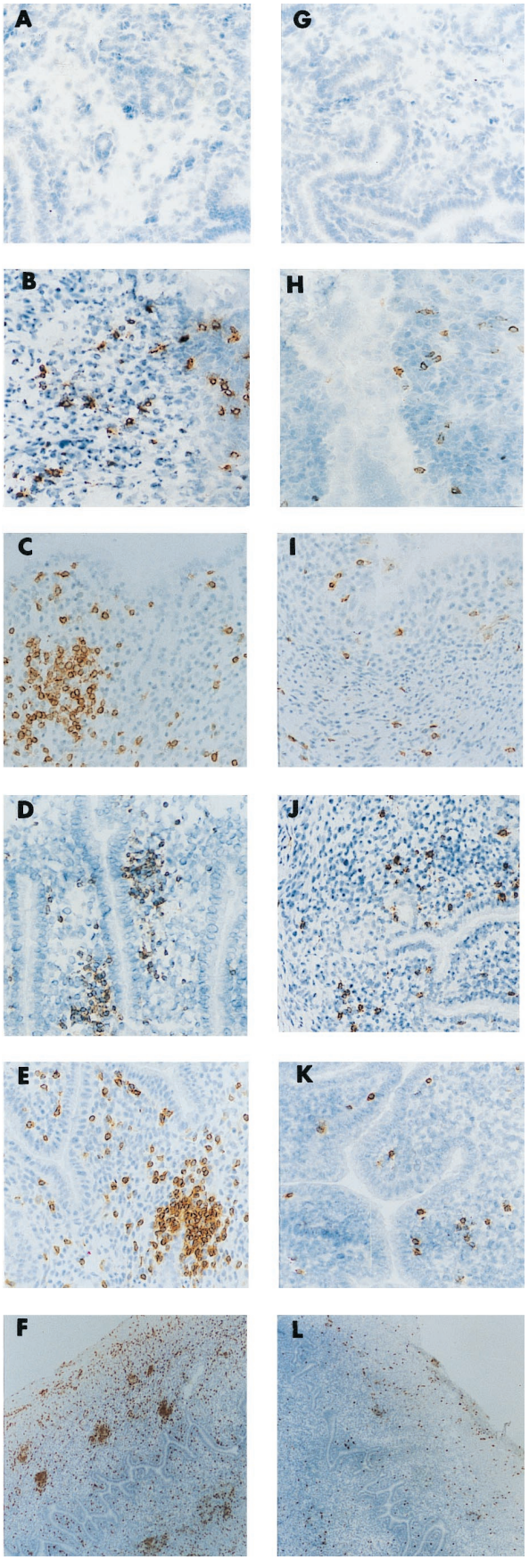


FIG. 5. Immunohistochemical staining of either $CD4^+$- or $CD8^+$-T-cell subsets in uterine tissue from noninfected or chlamydia-infected mice. (A to F) Staining with anti-CD4; (G to L) staining with anti-CD8. (A and G) Noninfected; (B and H) 7 days postinfection; (C and I) 14 days postinfection; (D and J) 21 days postinfection; (E, K, F, and L) 28 days postinfection. Magnifications, ×300 (A to K) and ×60 (F and L). Representative tissues from four or five mice at each time point are shown.

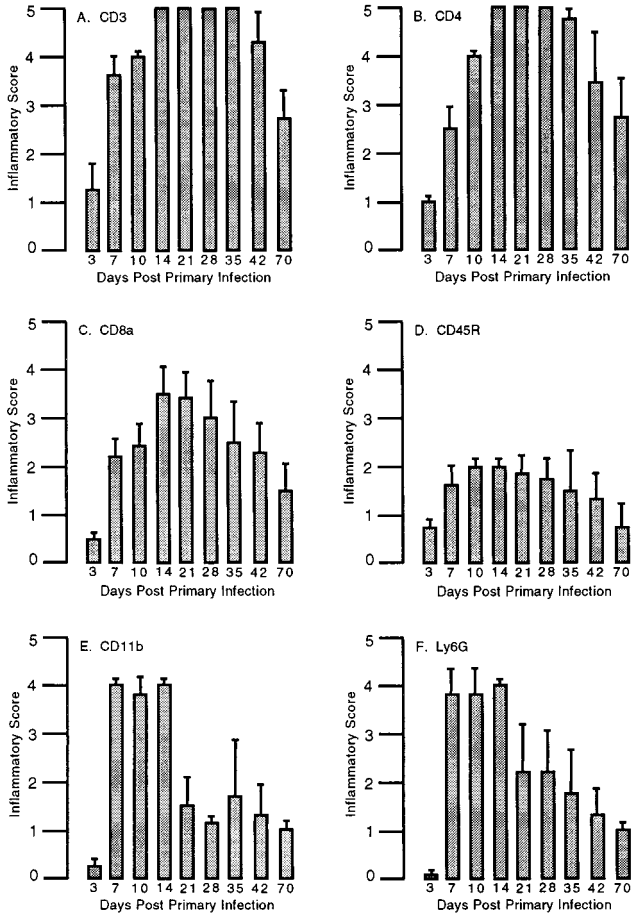


FIG. 6. Characterization of the local cellular inflammatory response following genital tract infection with *C. trachomatis*. Uterine horns were harvested at various times postinfection, processed, stained, and enumerated as described in Materials and Methods. Bars represent the mean inflammatory score ± standard deviation in uterine tissues from groups of four or five mice at each time point.

dominated, and only scattered $CD45R^+$ B cells and $Ly6G^+$ PMN were observed. $CD4^+$ T cells were scattered throughout the lamina propria and within clusters, whereas the less numerous $CD8^+$ T cells were observed within the mucosal epithelium and sparsely scattered throughout the lamina propria. Although single $CD11b^+$ cells were present in the uterine tissue at this time, a more dominant feature was the diffuse CD11b staining of regions that appeared to be near perivascular clusters of lymphocytes.

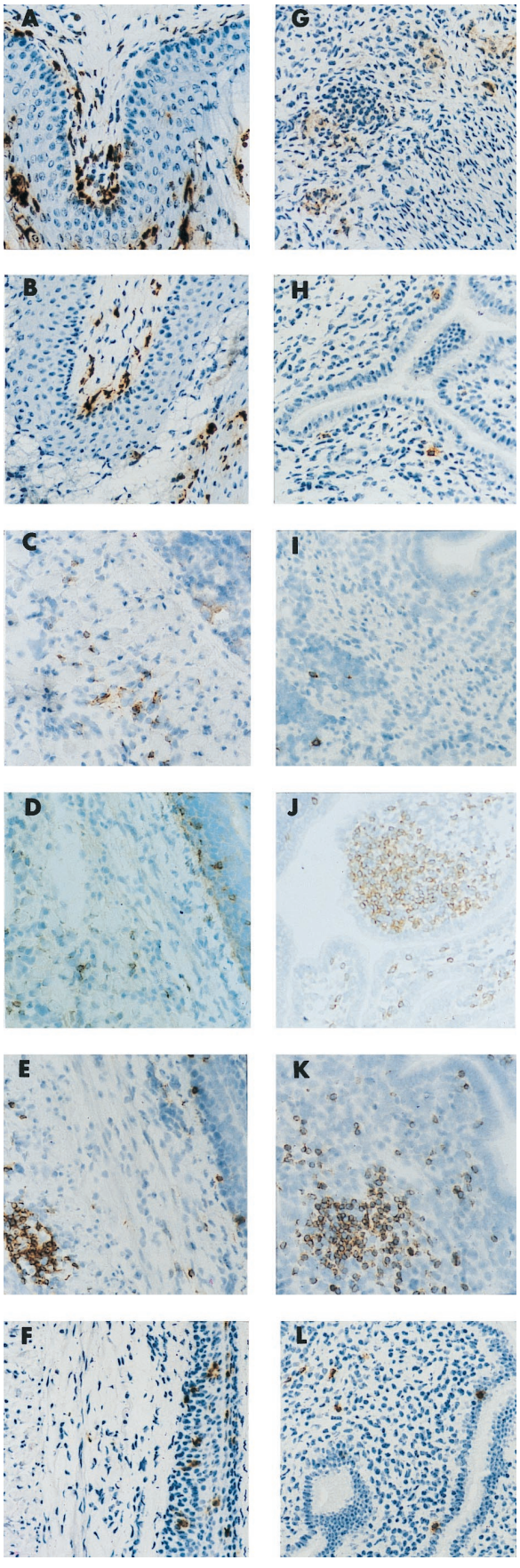
Detection of cytokine-producing cells in the genital tracts of infected mice. To determine the kinetics of cytokine production and the location of cytokine-producing cells during genital tract infection, vaginal and uterine tissues were analyzed for cells producing specific cytokines at various times throughout the course of infection (Fig. 8; Table 1). Cells producing IL-12 or TNF-α were detected in vaginal tissues by day 3 postinfection (Fig. 8); however, neither IL-4- nor IL-10-producing cells were detected at that time. At 7 days postinfection, cytokine-producing cells were evident in uterine tissue (Table 1). The individual variation in cytokine-expressing cells was large, but as a whole IL-4- and IL-10-producing cells outnumbered IL-12- and TNF-α-producing cells throughout the course of infection. Considerable variability in the number of IL-12- and TNF-α-producing cells also occurred during the course of infection and was not significantly different at any time point analyzed. Similar variability in the TNF-α response has been reported previously (6). In contrast, the number of IL-4- and IL-10-producing cells was greatest at 3 weeks postinfection, a time when infection is nearing resolution, and declined thereafter. However, even at 70 days postinfection the number of cytokine-producing cells in genital tract tissues was greater than that in naive mice.

Tissues were also probed for IL-1β, IL-2, IL-5, IL-6, and IFN-γ, but we were unable to convincingly demonstrate the production of those cytokines using immunohistochemical staining. Those data do not necessarily imply that the cytokines were not produced, but may instead simply reflect the limitations of the detection methology. Our enumeration of IFN-γ-producing cells in genital tract tissues of infected mice was also confounded by the observation that immunoaffinity-purified anti-IFN-γ stained chlamydial inclusions quite intensely. The reason for the cross-reactivity of anti-IFN-γ with chlamydial inclusions is not understood, but because of that reactivity we were unable to confidently enumerate IFN-γ-producing cells.

DISCUSSION

Previous studies of the primary immune response to *C. trachomatis* genital tract infection have utilized methods that disrupt the genital tract tissue to enumerate infiltrating cell populations or to determine the pattern of cytokine production by infiltrating cells (25, 27, 41). The in vitro production of cytokines by antigen-stimulated splenic lymphocytes has also been used to define the systemic cellular immune response following primary chlamydial genital tract infection (27, 37–39). In recent studies, the molecules that traffic lymphocytes to genital tract tissue following chlamydial infection have been defined using in situ immunohistochemical analysis (15, 28). In the present study, we have extended those findings by analyzing the development of the local cellular inflammatory response using in situ immunohistochemical staining for cell surface antigens and for the production of intracellular cytokines. Our intent was to identify and characterize changes in the local inflammatory and cytokine responses induced during the course of chlamydial genital tract infection.

The influx of hematopoietic cells into the vagina at day 3 of infection, and into the uterus at day 7 of infection, correlated with a large increase in cells of the myeloid lineage ($CD11b^+$ cells). Although we did not distinguish unequivocally between PMN and macrophages, Ly6G is a cell surface antigen found predominantly on PMN (8), and previous studies using hematoxylin and eosin staining of infected genital tract tissue have indicated that the early inflammatory cellular infiltrate is comprised primarily of PMN (22). The mere presence of PMN at the site of infection is not sufficient to resolve infection, however. For example, mice deficient in T-cell responses do not resolve infection even though a marked PMN inflammatory response is evident (22), and in vivo depletion of PMN with anti-Ly6G only slightly prolongs the course of chlamydial infection (2). It is not known if that modest effect on chlamydial shedding induced by PMN depletion results from decreased killing of chlamydiae by PMN or from the loss of mediators produced by PMN that direct lymphoid cells to the site of infection. PMN also comprised the predominant cell type in the vaginal and uterine luminal exudates, and cells of the lymphoid lineage were rarely observed in the lumens. Thus, the analysis of vaginal washes for specific cytokines might not necessarily reflect the predominant cytokines produced during infection but instead may provide information only on those



cytokines released by cells of the exudate (PMN) or on mediators released by epithelial cells.

Changes in the cellular composition of infected genital tract tissues were determined throughout the course of infection. In addition to measuring the myeloid cells, the influx of T and B cells was monitored. By 7 days postinfection, cells of the lymphoid lineage had increased significantly in vaginal and uterine tissues. The delay in lymphoid cell expansion is not surprising, since signals from myeloid cells are necessary for their activation. The accumulation of $CD3e^+$ cells (T cells) and $CD45R/B220^+$ cells (B cells) into genital tract tissue was very rapid and thus was unlikely to result from the in situ expansion of antigen-specific cells. Instead, the increased number of lymphocytes probably resulted from the influx of non-antigen-specific cells. T cells outnumbered B cells throughout the course of infection, and $CD4^+$ T cells were more numerous than $CD8^+$ cells at all times evaluated. Consistent with the findings of others (28), T cells were predominantly T-cell receptor αβ positive, and T-cell receptor γδ-positive cells were only rarely observed (data not shown).

CD45R/B220 and CD19 are B-cell lineage differentiation antigens expressed on the surface of B lymphocytes from the pro-B-cell through the mature B-cell stage (10, 19). Both CD45R/B220 and CD19 have been used as pan-B-cell markers, although CD19 expression is reported to be more restricted to the B-cell lineage (18). Neither CD45R nor CD19 is found on plasma cells. In our initial studies we attempted to use anti-CD19 antibody to identify cells of the B-lymphocyte lineage. Although we found that splenic B cells were visualized using anti-CD19 antibody, only a few weakly staining cells were observed in genital tract tissues from infected mice (data not shown). However, numerous cells were easily visualized when anti-CD45R/B220 was used as a marker for B cells. We have interpreted the $CD45R/B220^+$ cells as representing cells of the B-lymphocyte lineage, even though we were unable to confirm those results using anti-CD19. Those disparate results may simply reflect differences in the utility of the antibodies in immunohistochemistry and may not have occurred if a different detection methodology, such as fluorescence-activated cell sorting was used for these studies. However, because splenic B cells stained with anti-CD19 antibody under identical experimental conditions, our results may indicate the presence of a $CD45R^+$ $CD19^-$ cell population in chlamydia-infected genital tract tissues. Natural killer (NK) cells do not express CD19 or other B-cell markers but have been reported to express CD45R (33). The CD45R-positive cells in genital tract tissue may therefore represent a population of NK cells. Our attempts to stain genital tract tissues for NK cells using other NK cell markers (anti-NK1.1 or anti-asialo-GM1) were unsuccessful. Alternatively, the CD45R/B220-staining cells may represent a population of activated T cells (42). At this time we know only that a population of $CD45R/B220^+$ cells infiltrate genital tract tissue following chlamydial infection and that few cells expressing the B-cell lineage CD19 cell surface antigen are present. If this $CD45R^+$ population of cells does represent an NK cell or activated T-cell population, then quite possibly those cells might contribute to the resolution of intracellular chlamydial infection. Additional studies using other methods

FIG. 7. Immunohistochemical staining of inflammatory cells in the vagina (A to F) and uterine horns (G to L) following the resolution of chlamydial genital tract infection (day 42 postinfection). Anti-CD11b (A and G), anti-Ly6G (B and H), anti-CD45R (C and I), anti-CD3e (D and J), anti-CD4 (E and K), and anti-CD8 (F and L) were used. Magnification, ×300. Representative tissues from four or five mice are shown.

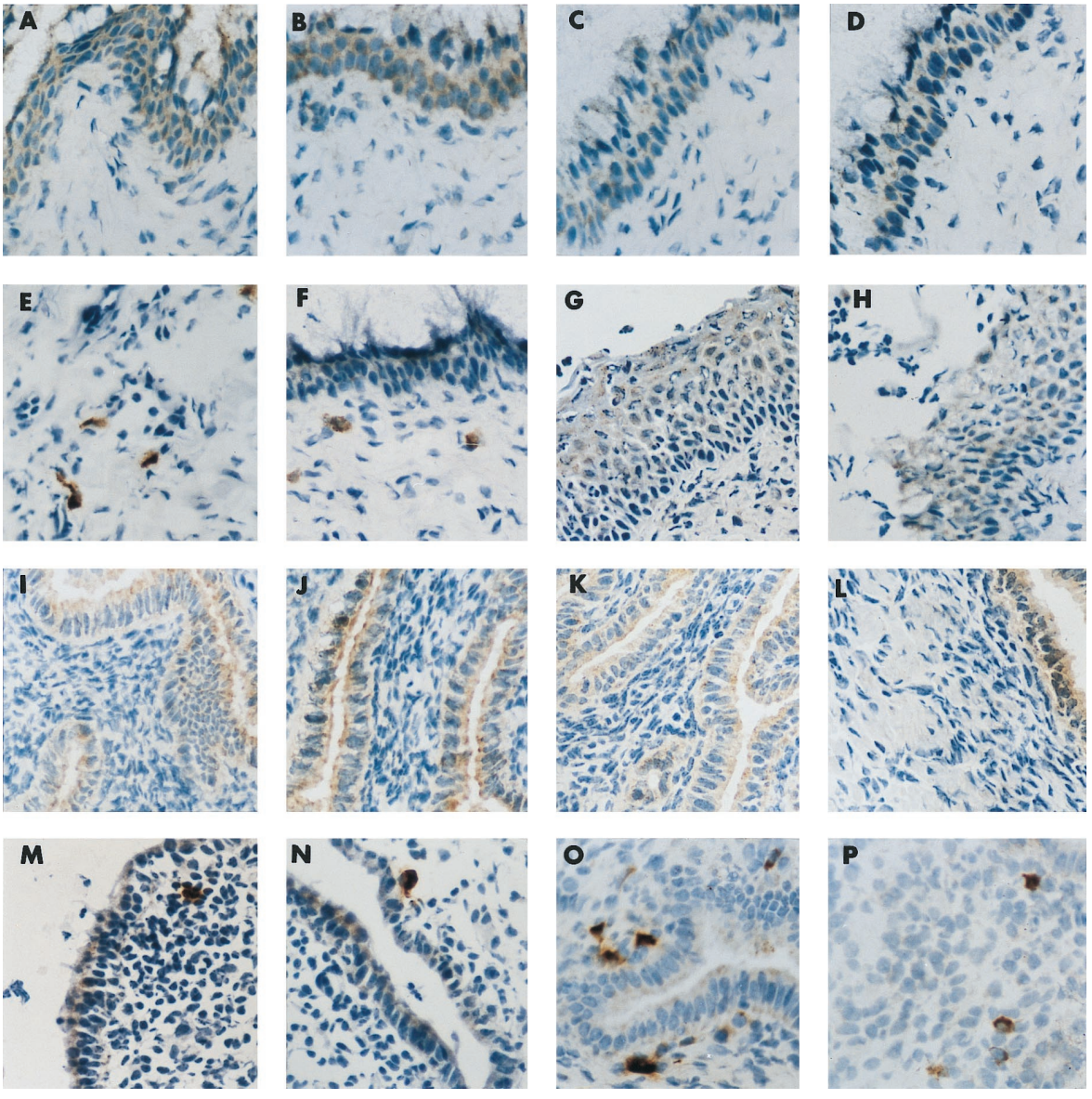


FIG. 8. Immunohistochemical staining of cytokine-producing cells in the vaginal (A to H) and uterine (I to P) tissues of chlamydia-infected mice. Tissues were harvested, processed and stained as described in Materials and Methods. (A to D) Vagina, noninfected; (E to H) vagina 3 days postinfection; (I to L) uterine horn, noninfected; (M to P) uterine horn 28 days postinfection. Anti-TNF-α (A, E, I, and M), anti-IL-12 (B, F, J, and N), anti-IL-4 (C, G, K, and O), and anti-IL-10 (D, H, L, and P) were used. Representative tissues from four or five mice at each time point are shown. Magnification, ×600.

to separate cell populations (e.g., fluorescence-activated cell sorting) will be needed to address that question.

A hallmark of both ocular (trachoma) and urogenital chlamydial infections is the development of lymphoid follicles (7, 9, 17, 23, 32, 43). The follicles present in children with active trachoma appear to have germinal centers composed of B cells (7), but in adults with conjuctival scarring, the follicles lack germinal centers and T cells ($CD4^+$) are much more numerous than B cells (32). The composition of follicles that develop during chlamydial genital tract infection is less well characterized (9, 13, 17, 23, 43). The mechanism(s) of follicular hyperplasia has not been elucidated, but it may hold important clues to the adaptive immune responses that confer immune protection and/or the pathogenetic immune responses that are thought to contribute to the severe sequelae of chlamydial infection. Although typical lymphoid follicles (i.e., with the architecture of follicles found in primary or secondary lymphoid tissues) were not observed in the genital tract tissues of infected mice, perivascular lymphocyte clusters were easily discernable (Fig. 3, 5, and 7). Clusters were composed predominantly of $CD3e^+$ cells and $CD4^+$ cells, but much smaller clusters of $CD45R^+$ B cells and $CD8^+$ T cells were also present. The genitourinary tract, as well as the skin and the pulmonary and gastrointestinal tracts, are the most immunologically active tertiary lymphoid sites. The clusters of T cells that develop during chlamydial genital tract infection are reminiscent of clusters of T cells that accumulate in the dermis following a delayed-type hypersensitivity reaction (30). T-cell clusters were not apparent until about 10 to 14 days following genital tract infection (Fig. 3 and 5), and some clusters remained for as long as 70 days postinfection (data not shown). In a recent study we demonstrated that the level of protective immunity that devel-

TABLE 1. Enumeration of cytokine-producing cells following primary *C. trachomatis* genital tract infection[a]

Days postinfection[c]	No. of cytokine-producing cells/10 fields[b]			
	IL-4	IL-10	IL-12	TNF-α
3	0	0	0	0
7	6.5 (0–19)	4.2 (0–15)	2 (0–4)	16 (1–31)
10	12 (7–16)	19 (11–41)	3 (2–4)	2 (0–3)
14	130 (104–213)	254 (159–320)	0.5 (0–1)	3 (1–5)
21	242 (102–388)	340 (106–619)	7 (2–13)	21 (10–40)
28	168 (128–247)	303 (182–468)	6 (3–10)	18 (4–42)
35	122 (114–133)	234 (179–342)	18 (5–36)	29 (2–57)
42	182 (84–270)	257 (134–338)	8 (6–16)	38 (21–49)
70	65 (41–85)	69 (45–103)	12 (2–16)	42 (7–90)

[a] Uterine horns were removed from infected mice at various times following primary infection, and the tissue was processed as described in Materials and Methods for the detection of cytokine-producing cells.

[b] Cytokine-producing cells were enumerated by counting positive-staining cells in 10 microscopic fields using a 20× objective (total area evaluated, 9.5 mm^2). Data are presented as the mean number of cytokine-producing cells per 10 fields from four or five mice per time point; the range is given in parentheses.

[c] Days following primary genital tract infection.

ops following primary genital tract infection was significantly impaired if animals were treated with doxycycline before the infection resolved (39). The most significant effects were found when mice were treated prior to 10 days postinfection. Perhaps early antibiotic treatment of infected mice ameliorates the development of T-cell clusters. If the clusters consist of memory and/or effector populations of T cells that contribute to the protective immune response in animals that resolve primary infection, then curtailing the formation of T-cell clusters with antichlamydial chemotherapy may significantly impair the development of the long-term protective immune response.

Recently, Su et al. demonstrated that passive immunization of naive mice with chlamydia-pulsed dendritic cells confers a very significant level of protective immunity (38). Protection was equivalent to that of mice which had resolved a primary chlamydial genital tract infection and was greater than that conferred by the adoptive administration of immune T cells (36). Perhaps the activation of postcapillary venules by genital infection enables mice to mobilize chlamydia-pulsed dendritic cells from the circulation and to initiate the perivascular clustering of T cells more readily than mice receiving only immune T cells. Further studies are needed, though, to determine the importance, or lack thereof, of the perivascular T-cell clusters in protective immunity.

Cytokines have been the focus of many recent studies on the adaptive immune response to chlamydial genital tract infection. IL-12 and IFN-γ play crucial roles in antichlamydial immunity, since mice deficient in those cytokines fail to resolve genital tract infection (5, 25). Less is known about local cytokine production during the course of genital infection. However, using reverse transcription-PCR methodologies, IFN-γ, IL-12, IL-10, and TNF-α transcripts have been detected in homogenates of infected genital tract tissues (25, 29, 41). In our present study, IL-12- and TNF-α-producing cells were detected in vaginal tissues by day 3 following infection and in uterine tissues by day 7. The vagina was negative for IL-4- or IL-10-producing cells at 3 days postinfection, but positive cells were numerous in uterine tissues by 14 days following infection and remained elevated even after the infection had resolved. Our results confirm those of other investigators regarding the presence of IL-12, IL-10, and TNF-α and extend the findings by demonstrating the kinetics of appearance and the duration of the responses. Noteworthy is our demonstration of IL-4-producing cells in genital tract tissue of infected mice, compared to results of other investigators who have failed to detect IL-4 transcripts from infected genital tract tissue or IL-4 protein by enzyme-linked immunosorbent assay of supernatants of chlamydia-stimulated T cells (25, 29, 38, 41). Our staining appears to be specific, because we used monoclonal antibodies and we did not experience false-positive reactions due to incompletely blocked endogenous peroxidase activity. Thus, the discrepancy may be due to differences in detection methodologies or in the tissue preparation. Nevertheless, by immunohistochemistry, considerable numbers of IL-4-producing cells contribute to the cellular infiltrate in genital tract tissue of chlamydia-infected mice.

Clearly, the Th1-type T-cell response contributes importantly to the resolution of intracellular infection, and studies generally do not support an essential role for a Th2-type T-cell response. However, we previously reported that antibody-deficient gene knockout mice were more susceptible to reinfection, and we attributed that susceptibility to the lack of antichlamydial antibody (37). Thus, it may be of interest to further investigate the role of IL-4 and IL-10 in the development of Th2-type antichlamydial immune responses and of specific antibody in acquired resistance to chlamydial genital tract infection.

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