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Repeated *Chlamydia trachomatis* Infection of *Macaca nemestrina* Fallopian Tubes Produces a Th1-Like Cytokine Response Associated with Fibrosis and Scarring

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***Chlamydia trachomatis*-associated female infertility and ectopic pregnancy are caused by postinflammatory fibrosis and scarring of the upper genital tract. Scarring of the upper genital tract is associated with multiple infectious episodes with *C. trachomatis*. To study the immune response that occurs with multiple infections of *C. trachomatis* in the female upper genital tract, a *Macaca nemestrina* model was used. Subcutaneous pockets containing autologous salpingeal tissue implants were inoculated three times with *C. trachomatis*. The inflammation after three inoculations was associated with a mononuclear infiltrate dominated by CD8 T-cell lymphocytes. Perforin mRNA was induced in infected pockets, demonstrating that activated cytolytic lymphocytes were present in the lesions. Fibrosis, as evidenced by fibroblast proliferation and connective tissue deposition, was observed by the third infection. Cytokine mRNAs induced by repeated chlamydial infection included gamma interferon, interleukin-2 (IL-2), IL-6, and IL-10 mRNAs, but IL-4 mRNA was not induced. Nearly identical findings were found in macaque fallopian tubes infected in situ repeatedly with *C. trachomatis*, validating the subcutaneous pocket model of chlamydial salpingitis. However, it was not possible to evaluate if there was an induction of perforin mRNA in infected salpingeal tubes in situ, because there was a high basal level of perforin mRNA in these tissues. These results suggest that repeated chlamydial infection of the female upper genital tract leads to CD8 T-cell predominance, a Th1-like cytokine milieu, and these inflammatory changes are associated with progression to fibrosis associated with female infertility.**

Chlamydia trachomatis is one of the most frequent causes of sexually transmitted diseases in the United States, afflicting more than 4 million people annually and costing more than 1 billion dollars annually (32). The manifestations of *C. trachomatis* infection in women can range from asymptomatic cervicitis to pelvic inflammatory disease (PID), infertility, and ectopic pregnancy. Up to 20% of women studied at sexually transmitted disease clinics and 5 to 7% of all pregnant women are culture positive for *C. trachomatis* (reviewed in reference 26). Because of the high frequency of chlamydial infection, many women are believed to become infected with chlamydia multiple times. *C. trachomatis* infection has been associated with up to 20% of female infertility in the United States, as a high proportion of tubal infertility is believed to be caused by chlamydial infection (14). It has been hypothesized that multiple infections with *C. trachomatis* lead to increased tissue damage (8). Multiple episodes of PID are more likely to cause tubal scarring, infertility, and ectopic pregnancies than a single episode of PID (33). In the primate model of infection, a single inoculation with *C. trachomatis* leads to a self-limited infection of the lower reproductive tract, while repeated infection is required to cause distal tubal obstruction (16). Thus, repeated infections with *C. trachomatis* are more likely to cause infertility and ectopic pregnancy than single infections.

To study the immune response to *C. trachomatis* infection of the female genital tract, we have employed a primate model using female pig-tailed macaques (*Macaca nemestrina*) with subcutaneous autologous implants of salpingeal tissues inoculated with *C. trachomatis* (30). We have shown that after a single inoculation with *C. trachomatis*, mRNA synthesis is induced for interleukin-2 (IL-2), IL-6, IL-10, and gamma interferon (IFN-γ) but not IL-4, suggesting that a T helper cell type 1 (Th1)-like response predominates after a single chlamydial infection (30). In addition, CD8 T cells comprised over 60% of the total infiltrating lymphocytes and the infiltrating cells contained perforin mRNA, suggesting that activated cytolytic CD8 T cells predominated in the lesions (30).

These studies do not address whether the lymphocyte and cytokine phenotype is altered after multiple infections of chlamydia, which is more analogous to the actual process leading to tubal infertility of females. Furthermore, confirmation is needed that a similar histologic and cytokine milieu is found during chlamydial infection in salpingeal tissues in subcutaneous pockets and the salpingeal tissues in situ, i.e., in intact upper genital tract tissues.

In this report, we study multiple infections with *C. trachomatis*, and we show an outcome with multiple infections in an in situ model identical to that with the pocket model, validating the pocket model. The results show that (i) Th1-like cytokines (and not a switch to Th2) continue to dominate after multiple infections; (ii) fibrosis occurs after multiple infections; and (iii) CD8 T cells predominate over B cells, natural killer (NK) cells, and CD4 T cells.

MATERIALS AND METHODS

Animals and chlamydiae. Eighteen sexually mature female pig-tailed macaques (*M. nemestrina*) were enrolled in this study. All macaques were housed at the University of Washington's Regional Primate Research Center and were routinely screened for simian retroviruses. The protocol for this experiment was approved by the Animal Care Committee, University of Washington. Animals

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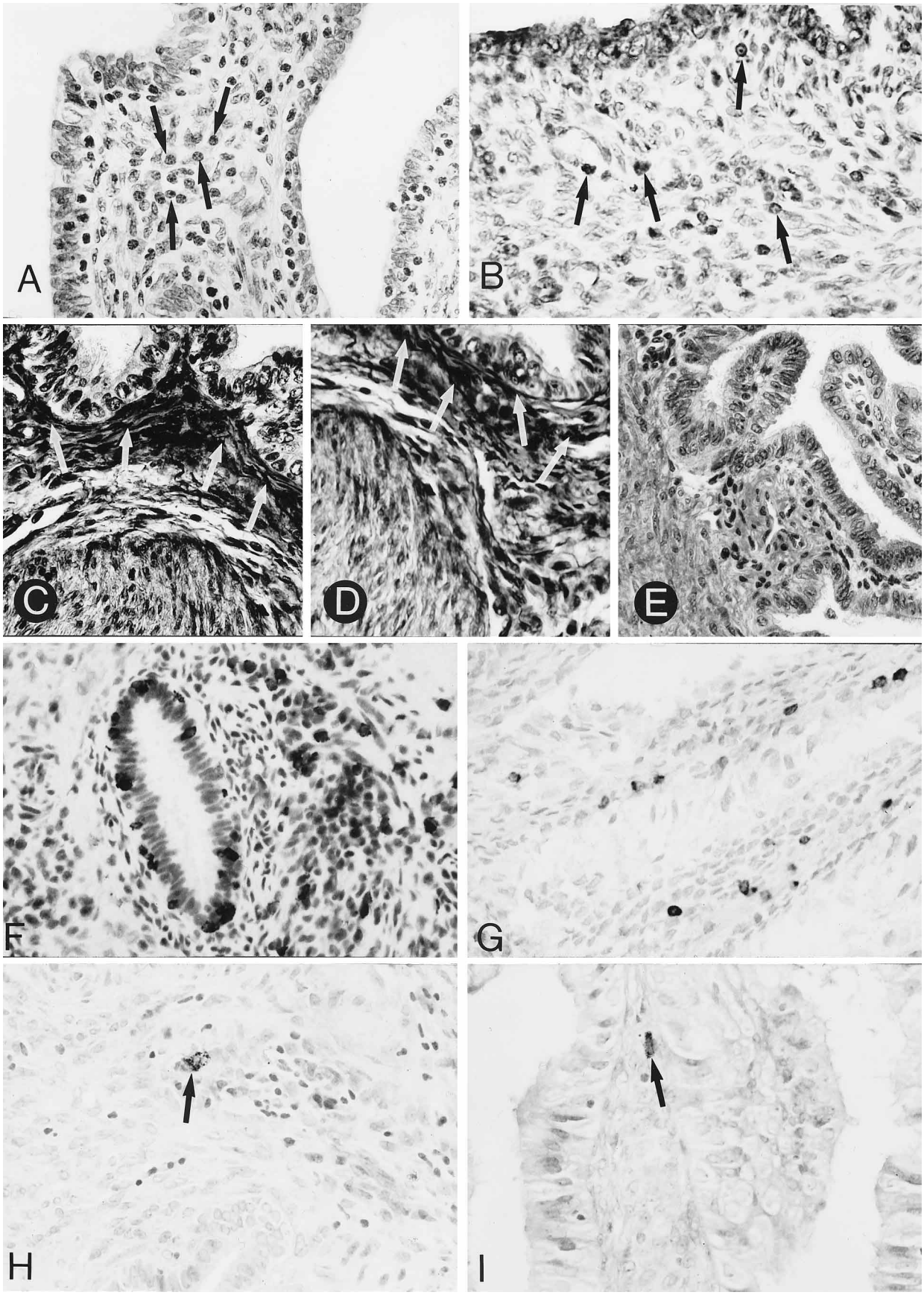
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were handled humanely, and experiments were carried out within NIH Animal Use Guidelines. *C. trachomatis*, serovar E (UW-5/Cx), was grown in HeLa cells. Organisms were purified by Hypaque (Hypaque-76; Sanofi, Winthrop Pharmaceuticals, New York, N.Y.) density gradient centrifugation. The chlamydia fraction was washed and diluted in sucrose-phosphate-glutamate buffer (219 mM sucrose, 12 mM phosphate buffer [pH 7.4], 5 mM glutamic acid) for inoculation.

Subcutaneous salpingeal pocket model. Nine macaques each had 24 to 30 subcutaneous pockets implanted with autologous salpingeal tissues (fallopian tubes and fimbriae) approximately 3 weeks prior to inoculation (17). Samples from three control (sham-inoculated with sucrose-phosphate-glutamate buffer) macaques and six infected (*C. trachomatis*-inoculated) macaques were studied. The pocket tissues were inoculated by three subcutaneous injections at weekly intervals. The dose delivered was 10^5 infection-forming units in 50 μl per pocket each time. Pocket tissues were explanted on days 3, 7, 14, and 21 after the final inoculation.

In situ model. Chlamydial PID was induced in six female macaques by inoculating the cervix and both fallopian tubes with 10^6 infection-forming units of *C. trachomatis* (18), followed in 2 weeks by a repeat inoculation at each site. Hysterectomy was performed 20 days following the final inoculation. Control fallopian tubes were obtained from three macaques that were not inoculated with chlamydia.

Histologic techniques. Routine histology was assessed from formalin- and/or Carnoy's fixative-fixed paraffin-embedded sections stained with hematoxylin and eosin or methyl green pyronine. Verhoeff-van Gieson staining was used to stain tissues for connective tissue elements. Immunocytochemistry (ICC) for detection of chlamydiae was performed on deparaffinized sections, using the avidin-biotin-peroxidase complex immunostaining system (Vector Laboratories Inc., Burlingame, Calif.) with the species-specific anti-*C. trachomatis* monoclonal antibody KK-12 (28). For CD8 immunolocalization, some pockets were fixed with 0.5% zinc acetate and 0.5% ZnCl was added (3). After paraffin embedding, sectioning, and deparaffinizing, the sections were exposed to monoclonal anti-human CD8 (clone SPV-T8; Zymed Inc., South San Francisco, Calif.) or monoclonal anti-human CD57 (HNK-1; Monosan, Caltag Laboratories, Burlingame, Calif.) for detection of NK cells, followed by avidin-biotin-peroxidase immunolocalization. Transmission electron microscopy was performed on uranyl acetate-lead citrate-stained thin sections as previously described (19).

Phenotype analysis of lymphocytes from salpingeal tissues. On days 3, 7, 14, and 21 after the last chlamydia inoculation, salpingeal tissues from three pockets were explanted and pooled, and the cells were dispersed by using a wire-mesh screen. The cells were reacted with phycoerythrin-conjugated antibodies to CD4, CD8, and CD20 (Becton Dickinson, Sunnyvale, Calif.), and the percentage of the lymphocytes (gated by forward and side scatter) staining with each monoclonal antibody was determined with a Coulter XL flow cytometer.

Cytokine mRNA analysis. In the pocket model, three salpingeal tissues were explanted on days 3, 7, and 14 after the last chlamydia inoculation. Tissues from three pockets were pooled and ground with a disposable pestle in the presence of 0.5 ml of RNAzol B (Tel-Test Inc., Friendswood, Tex.). RNA was isolated by the RNAzol B procedure as recommended by the manufacturer. In the in situ model, a 0.5-cm section of fallopian tube, acquired 20 days after the last chlamydia inoculation, was similarly extracted for RNA. The RNA was reverse transcribed by using random hexamer primers, and the total cDNA for hypoxanthine-guanine phosphoribosyltransferase (HPRT) was normalized by quantitative competitive PCR as previously described (30). Primers, PCR conditions, negative and positive controls, and precautions to avoid cross-contamination were as previously described (30) except that the 5′ IL-4 primer was 5′-TTAA TGGGTCTCACCTCCCA-3′ and the 3′ IL-4 primer was 5′-CAGAAGGTTTC CTTCTCAGTT-3′, and conditions for IL-4 cycling were 45 cycles of 94°C for 1 min, 58°C for 2 min, and 72°C for 1 min. The number of cycles was varied between 30 and 45 to obtain PCR products that were less than maximal in intensity so that their intensities could be compared. Under these conditions, the efficiencies of the primers for cytokines and perforin to yield a PCR product were compared by using serial dilutions of cDNA from *M. nemestrina* mitogen (phytohemagglutinin)-stimulated blood mononuclear cells (30). Each of the sets of primers gave a detectable PCR product within a threefold dilution of the others. This demonstrates that the primers had similar efficiencies in detecting cytokine and perforin mRNAs in a mitogen-stimulated cDNA sample. Samples were assayed more than once for each cytokine mRNA, and the intensities of the cytokine PCR bands were reproducible. PCR products were electrophoresed (Ellard Instrumentation, Seattle, Wash.) with ethidium bromide and photographed. For comparison of the bands from the pocket series, photographs of PCR products were scanned and the intensities of the images of the PCR products were quantitated with the Imagequant program (National Institutes of Health, Bethesda, Md.), subtracting the background fluorescence for the gel from the fluorescence of each band. For comparison of the bands in the in situ series, the fluorescence of the gels was digitized (GDS 7500; UVP Inc., Upland, Calif.), and the intensities of the PCR products were quantitated with the Imagequant program as described above.

Statistical analysis. The intensity values of chlamydia-inoculated samples and the control samples were compared by the two-tailed Mann-Whitney (nonparametric analysis) test to compare the means.

RESULTS

The effects of multiple infections with *C. trachomatis* on the female upper genital tract were examined by using two experimental models. The first involved three inoculations of subcutaneous pockets containing salpingeal tissues and studying the infected tissues explanted from the pockets at various time points after infection. The second involved two inoculations of the cervix and the fallopian tubes in situ and studying the fallopian tissues at hysterectomy.

Pocket model. The salpingeal tissues from the pocket model were found to be infected with chlamydiae as assessed by ICC staining for chlamydial inclusions, using pockets explanted on days 2 through 14 after inoculation with chlamydia (not shown). No infection was detected in the pockets explanted from sham-inoculated controls (not shown).

A widespread mononuclear cell infiltrate was observed on days 3 through 21 in the chlamydia-inoculated pocket tissues (Fig. 1A). Clusters of lymphocytes formed lymphoid follicle-like structures within the deep stroma of infected salpingeal tissues (not shown). A mononuclear exudate was seen in the lumen of the infected salpingeal tissues. Lymphocytes predominated in the lesions at all times, but some neutrophils were observed on day 3 after reinoculation (not shown). Plasma cell infiltration was evident in the infected tissues (Fig. 1A). There was clear evidence of fibrosis, as evidenced by fibroblast proliferation and collagen and elastin deposition in the stroma of infected salpingeal tissues (Fig. 1C and 2A). These changes were not observed in the sham-inoculated controls (Fig. 1E).

ICC analysis showed that CD8 T cells dominated the mononuclear infiltrates of the chlamydia-infected salpingeal tissues (Fig. 1F). We were unable to assess CD4 T cells on fixed macaque tissues with ICC. Only trace numbers of NK cells (<1 CD57 cell per five high-powered fields), as shown by ICC (Fig. 1H), were observed in the infected pocket tissue.

Dispersing cells from the infected salpingeal tissues explanted from pockets allowed quantitative assessment of lymphocyte phenotypes (Fig. 3). As early as 3 days after the final inoculation, the predominant phenotype of lymphocytes was

FIG. 1. Histology of chlamydia-inoculated tissues from the pocket and the in situ macaque models. (A) Light micrograph of tubal tissue inoculated with *C. trachomatis* in the pocket model. The cellular infiltrate within the submucosa at day 7 after tertiary inoculation reveals densely stained mononuclear cells and numerous plasma cells (arrows). Hematoxylin and eosin stain; magnification, ×400. (B) Light micrograph of a tubal biopsy obtained at hysterectomy day 20 after secondary inoculation from the in situ macaque model. A widespread inflammatory cellular infiltrate of mononuclear and plasma cells (arrows) are present. Methyl green pyronine stain; ×400. (C and D) Light micrographs of chlamydia-inoculated tubal tissues from the pocket model day 21 after tertiary inoculation (C; ×630) and the in situ model day 20 after secondary inoculation (D; ×400) demonstrating fibrosis following chronic chlamydial infection. Tissues were stained by Verhoeff-van Gieson stain to indicate increased deposition of elastin and collagen after chronic infection. Both tissues show heavy deposits of elastin and collagen in the basement membrane (arrows) and within the submucosal compartment. These changes were not seen in uninfected salpinx tissues (E). (F and G) Immunocytochemistry of chlamydia-inoculated tubal tissues obtained from the pocket model on day 7 after tertiary inoculation (F; ×400) and the in situ model on day 20 after secondary inoculation (G; ×400). The CD8 T cells were visualized with anti-CD8 monoclonal antibody and immunoperoxidase. Numerous CD8 T cells are within the submucosal compartment, and several have migrated into the epithelial layer. Individual lymphocytes are circumscribed by the antibody. (H and I) Immunocytochemistry of chlamydia-inoculated tubal tissues obtained from the pocket model on day 7 after tertiary inoculation (H; ×400) and from the in situ model day 20 after secondary inoculation (I; ×400). The NK cells, reacting with anti-CD57, were infrequent (mean count, <1 per five high-powered fields) in the infected tissues, and a field with a single staining cell is shown.

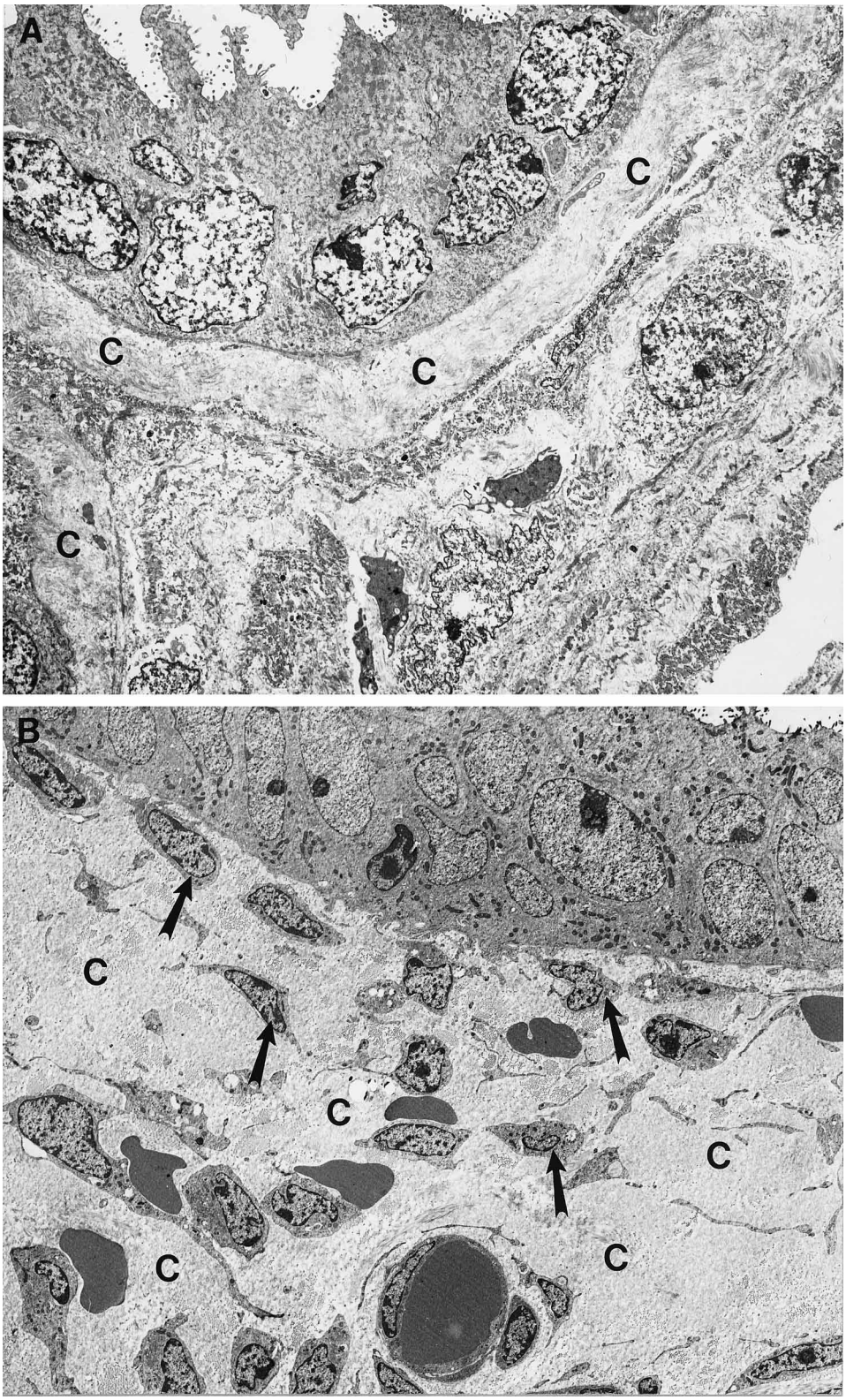

FIG. 2. Transmission electron micrographs of chlamydia-inoculated tubal tissues from the pocket and the in situ models. In the chronically infected salpingeal pocket tissues (A), note the extensive collagen (C) depositions throughout the submucosal compartment (magnification, ×2,000). Numerous fibroblasts (arrows) can be seen in these sections. In the salpingeal tissues from the in situ model (B), a similar distribution of collagen (C) is observed throughout the submucosa (×3,200).

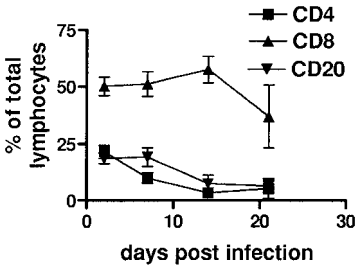

FIG. 3. Phenotype of lymphocytes in chlamydia-inoculated pockets. Shown are the means and standard errors of the percentage of total lymphocytes that were CD4, CD8, and CD20 (B-cell) positive in flow cytometry. Data are expressed as a function of days after the third inoculation with *C. trachomatis*. Each data point represents three to six independent measurements.

CD8 T cells, followed by CD4 T cells and CD20 B cells. Too few lymphocytes were found in uninfected salpingeal tissues to accurately assess lymphocyte phenotypes from sham-inoculated pocket samples, as expected from the lack of inflammation in these specimens.

Using reverse transcription-PCR (RT-PCR), we studied the cytokine responses in salpingeal pocket tissues. In pilot experiments, cytokine mRNA in chlamydia-inoculated salpingeal pockets was induced over the background levels in sham-inoculated control pockets on days 2 through 7 after the final inoculation, with the response trailing off on day 14 and absent by day 21. Based on this observation, cytokine mRNA levels were studied in detail on days 3, 7, and 14.

To normalize for variability in the explanted pockets, the RNA was extracted from three replicate pockets and pooled. cDNA was made from the RNA pool, and quantitative competitive PCR was performed to determine HPRT cDNA levels. Finally, the concentration of each sample was adjusted such that equal concentrations of HPRT cDNA were present in all samples. The cDNA samples required a maximum threefold adjustment to normalize them to the cDNA HPRT levels of other samples. In Fig. 4, the HPRT row shows the HPRT PCR, demonstrating the equivalence points of the competitor and adjusted-pocket cDNAs.

Then semiquantitative (nonplateau) PCR was performed for each cytokine, comparing the normalized cDNA samples from sham-inoculated pockets with those from matched chlamydia-inoculated pockets for each time point. For the cytokines IFN-γ, IL-2, IL-6, and IL-10, bands corresponding to cytokine mRNA were generally absent or weak from sham-inoculated pockets, but strong bands were present from chlamydia-inoculated pockets. The intensity of the PCR products for these cytokines was generally brighter on days 3 and 7 than on day 14 after infection. A representative group of PCR products from one sham-inoculated macaque and two chlamydia-inoculated macaques is present in Fig. 4. Similar results were obtained for two additional groups of the same size (data not shown). To assess the significance of the differences in intensity of the bands from the sham-inoculated and the chlamydia-inoculated pockets in all macaques (nine macaques in all, three with sham-inoculated pockets and six with chlamydia-inoculated pockets), the band intensities were digitized and compared. The mean intensity of the PCR products from the sham-inoculated control pockets was significantly ($P < 0.04$) less than the mean intensity of the PCR products from the chlamydia-inoculated pockets for IFN-γ, IL-2, IL-6, and IL-10, demonstrating that mRNA for each of these cytokines was induced by chlamydia infection (Fig. 5). The mRNA for IL-4 was not generally detected in any of the pockets assayed, yet IL-4 mRNA was detected from mitogen-stimulated macaque blood mononuclear cells (Fig. 4), even when diluted 10-fold (not shown). The expected quantity of IL-4 PCR product was obtained when a limiting quantity of mitogen-stimulated cDNA was added to cDNA obtained from chlamydia-inoculated samples. This finding demonstrates that an inhibitor of the IL-4 PCR was not present in these samples. At high numbers of PCR cycles, weak bands for IL-4 were detected in a few of the chlamydia-inoculated and the sham-inoculated samples, but the differences between the infected and control samples were not significant (Fig. 4 and data not shown). Thus, IL-4 was not induced by repeated chlamydial infection.

Because of the predominance of CD8 T lymphocytes in the chlamydia-infected salpingeal pockets, the induction of perforin mRNA synthesis was studied as a marker for cytolytic lymphocyte function. The signal for perforin mRNA was significantly greater in chlamydia-inoculated pockets than in sham-inoculated controls (Fig. 4 and 5). The presence of perforin mRNA suggests that activated cytolytic lymphocytes were present in the chlamydia-inoculated tissues.

In situ model. In the in situ model, fallopian tubes that were inoculated with chlamydia had evidence of chlamydial inclusions at hysterectomy by ICC (not shown). The submucosal compartment of fallopian tubes harvested at day 20 after the second inoculation of chlamydia showed a mixed infiltration with lymphocytes and plasma cells (Fig. 1B) and evidence of fibrosis in the deep stroma (Fig. 1D and 2B), similar to that seen with pocket salpingeal tissues after the last inoculation of chlamydiae (Fig. 1A, 1C, and 2A). Lymphoid follicle-like formations were also observed in the deep stroma (not shown). CD8 T lymphocytes predominated in the mononuclear infiltrates (Fig. 1G). NK cells were rarely observed in the inflammatory infiltrate (Fig. 1I).

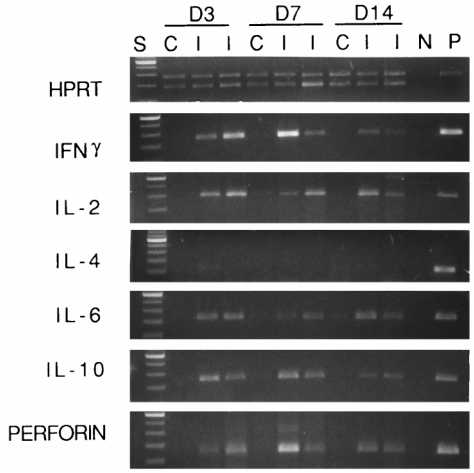

FIG. 4. mRNA expressed in chlamydia-inoculated pockets and sham-inoculated controls. Shown are the PCR products from RT-PCR representing the mRNA levels of *C. trachomatis*-inoculated pockets (I) of two animals and pockets from a sham-inoculated control (C), explanted on day 3 (D3), day 7 (D7), and day 14 (D14) after the third inoculation. The cDNA pools were first adjusted to equal concentrations of HPRT cDNA. Shown in the HPRT row are the equivalence points of the cDNAs with a competitor plasmid, pDC10, which gives a higher-molecular-weight band than the native HPRT cDNA (lane P, in the HPRT row, shows the PCR product obtained with only the competitor plasmid as a template). The next rows represent mRNAs from the various samples for IFN-γ, IL-2, IL-4, IL-6, IL-10, and perforin. Also shown are 100-bp standards (BRL-GIBCO, Gaithersburg, Md.) (S column) and control PCR products from no cDNA (N column) and phytohemagglutinin-stimulated macaque blood mononuclear cells (P column, except HPRT row). Two other groups of equal size gave similar results.

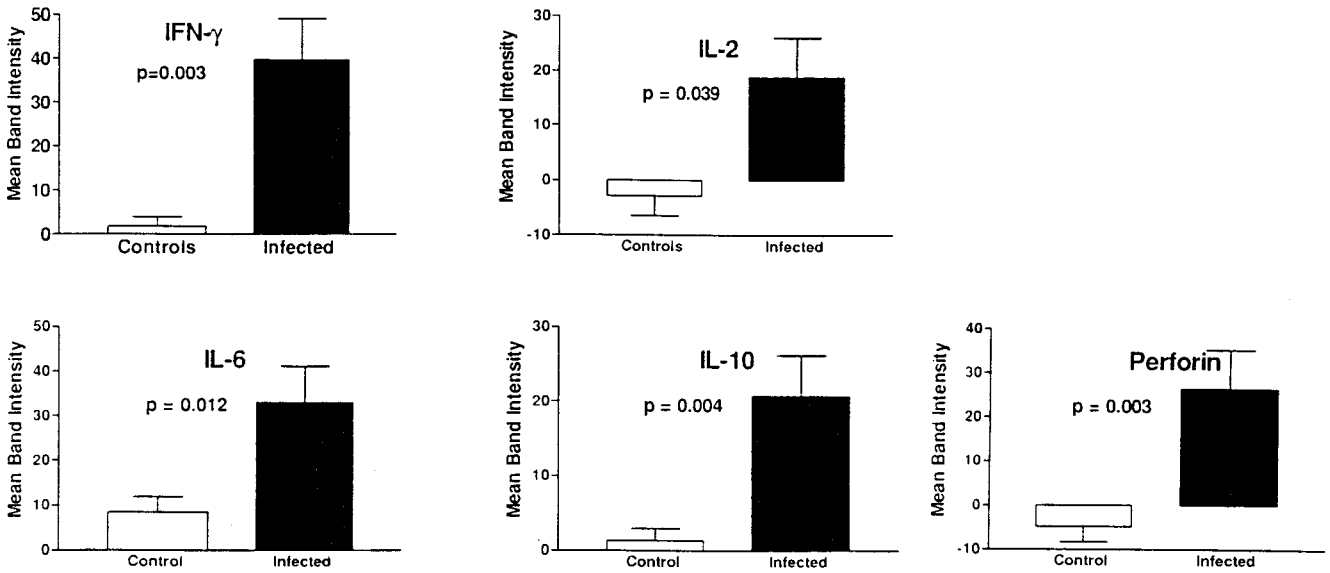

FIG. 5. Differences in intensity of cytokine PCR bands for chlamydia-inoculated and control pockets. The filled columns are the mean intensities of the PCR products from the salpingeal pockets taken on days 3, 7, and 14 after tertiary chlamydia inoculation from six macaques (infected), and the open columns are the mean intensities of the PCR products from the pockets taken on days 3, 7, and 14 after tertiary sham inoculation from three macaques (controls). The error bars represent the standard errors of the means. The *P* values are given for the differences in intensity between the chlamydia-inoculated and sham-inoculated pockets.

Cytokine mRNA induction in fallopian tubes infected in situ was analyzed by RT-PCR. Figure 6 shows that infected tubes had evidence of mRNA induction for IFN-γ, IL-2, IL-6, and IL-10, but control tubes did not contain detectable mRNA for any of these cytokines. Image analysis of the intensity of the bands for each of these cytokines showed that the signal for each was significantly ($P < 0.03$) higher in chlamydia-infected tubes than the signal for control uninfected tubes (Fig. 7). None of the infected fallopian tubes had detectable IL-4 mRNA (not shown). Perforin mRNA levels were high in uninfected tissues, such that no specific induction of perforin mRNA could be demonstrated.

DISCUSSION

The inflammatory response of salpingeal tissues to repeated chlamydial infections was studied, because repeated infections are more likely than single infections to lead to upper genital tract scarring and complications (8, 33). The mRNAs for IFN-γ, IL-2, IL-6, and IL-10, but not IL-4, were induced by repeated chlamydial infection of salpingeal tissues. IFN-γ and IL-2 are made by Th1 CD4 T cells, whereas IL-4 is made by Th2 cells. IL-6 and IL-10 are made by both Th1 and Th2 cells in humans (6, 22, 24) and presumably macaques. This finding suggests that Th1-like cytokines (IFN-γ and IL-2), but not Th2-like (IL-4) cytokines, are made by repeated infection with *C. trachomatis*. Since a variety of cell types were present in the infected tissues, and the RT-PCR technique does not identify which cell types are producing the mRNA, it is not possible to conclude that the Th1-like cytokines originated from Th1 CD4 T cells. Indeed, IL-2 and IFN-γ may be made by CD8 T cells; IL-6 can be produced by CD8 T cells, macrophages, fibroblasts, and endothelial cells; and IL-10 can be from macrophages and B cells (11, 24).

The same pattern of cytokine induction was found after a single infection with *C. trachomatis* in the pocket model; i.e., Th1-like cytokines but not Th2-like cytokines were induced (30). This finding demonstrates that repeated infection does not lead to a shift from Th1-like to Th2-like (IL-4) cytokines. Similar results were found in murine models of chlamydial infection. In the mouse pneumonitis strain of *C. trachomatis*, splenic CD4 cells from infected mice stimulated with chlamydial antigen produced Th1-like (IFN-γ, IL-2, and IL-6) but not Th-2-like (IL-4) cytokines (29). When mice were infected in the genital tract with the mouse pneumonitis strain of *C. trachomatis*, lymphocytes in the genital tract and draining lymph nodes produced IFN-γ and IL-2 but not IL-4 (5). The data reported here confirm the murine findings in a study using a primate system with a strain of *C. trachomatis* frequently found in human genital infections and using a multiple-infection model that leads to fibrosis of the salpingeal tissues. Though the primate model may be similar to human chlamydial salpingitis because of similarities in the immune system, anatomy, and strains of *C. trachomatis*, it should be noted that the route of inoculation is different from that for humans and that the

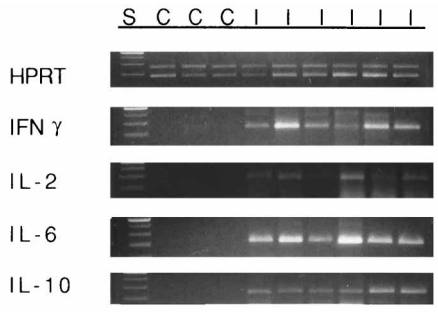

FIG. 6. mRNA expressed in chlamydia-inoculated fallopian tubes in situ and in control uninfected tubes. Shown are the PCR products from RT-PCR representing the mRNA levels of fallopian tubes from the in situ model, 20 days after inoculation with *C. trachomatis* for six animals (I), and in fallopian tubes from three uninfected control animals (C). The equivalence points of the cDNAs with competitor template for HPRT are shown in the first row, demonstrating that the cDNAs were normalized for the amount of HPRT cDNA. The next rows represent mRNAs for IFN-γ, IL-2, IL-6, and IL-10. Also shown are 100-bp standards (S). None of the chlamydia-inoculated tubes had detectable mRNA for IL-4 (not shown).

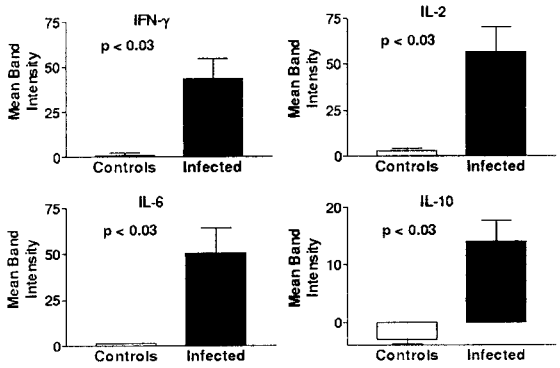

FIG. 7. Differences in intensity in PCR products representing cytokine mRNAs from chlamydia-inoculated and uninfected fallopian tubes. Shown are means and the standard errors of the means of the intensities (see Materials and Methods) of ethidium bromide-stained PCR products representing cytokine mRNA obtained from uninfected fallopian tubes (Controls, $n = 3$) or chlamydia-inoculated fallopian tubes (Infected, $n = 6$) in the in situ model. The P values are given for the differences in intensities of the PCR products of the chlamydia-inoculated tissues and the uninfected controls.

inoculum size to produce disease in humans is unknown. Nonetheless, the inoculation protocols were chosen to best mimic the histopathologic changes observed after repeated human chlamydial infections in a timely manner.

Though the precise role of these cytokines in chlamydial infections has not been defined, Th1-like cytokines facilitate killing of intracellular organisms, whereas Th2-like cytokines can promote persistence of intracellular organisms (21). The presence of the Th1-like cytokine IFN-γ has been associated with enhanced killing and elimination of chlamydiae in a variety of rodent and in vitro models of chlamydial infection (2, 4, 20, 23, 27). Thus, the continued production of Th1-type cytokines, particularly IFN-γ, may help to contain the chlamydial infection. IFN-γ has been detected in vaginal secretions of women infected with *C. trachomatis* (1), suggesting its role during human infection. However, IFN-γ, IL-2, and IL-6 cytokines promote inflammatory damage and fibrosis (10), and the induction of these cytokines may lead to the fibrosis that complicates repeated chlamydial infections.

In contrast, IL-10 has been shown to reduce inflammatory damage (7) but also reduces killing of intracellular microbes, such as *Leishmania major* and *Trypanosoma cruzi* (25, 31). Thus, the induction of IL-10 in repeated infections may help limit the inflammatory and fibrotic damage but may enhance the persistence of chlamydial infection. Experimental manipulation of levels of these cytokines in the pocket model may help to define their role in infection and inflammatory damage during repeated infection of salpingeal tissues.

The results of immunostaining in both models and flow cytometry phenotype analysis of lymphocytes eluted from infected pockets demonstrate that CD8 T cells predominate after multiple chlamydial infections of salpingeal tissues. A marker for NK cells (CD57) revealed that very few NK cells were present in the infiltrates (Fig. 1H and I). In the in situ model, high levels of perforin mRNA in normal fallopian tube tissue made it difficult to determine whether there was further induction in perforin mRNA. Indeed, perforin-expressing cells have been demonstrated in normal human uterine tissue (9). However, unlike human uterine tissues, macaque fallopian tubes had few NK cells (Fig. 1I). However, perforin mRNA was absent in salpingeal tissues in the pocket model and was induced after repeated chlamydial infection. Perforin is a marker for activated cytolytic lymphocytes (12). Since CD8 cells predominate in the infiltrate after repeated infection, and NK cells appear to be absent, it seems likely that activated cytolytic CD8 cells are present. However, this conclusion should be tempered by the fact that CD4 cells may sometimes make perforin and have cytolytic function (34).

In the murine system, there is conflicting evidence about the relative roles of CD4 and CD8 T cells in protective immunity to *C. trachomatis*. One group found that adoptive transfer of CD4 T cells, but not CD8 cells, led to more rapid resolution of infection (29). Experiments using gene knockout mice suggest that CD4 T cells, and not CD8 T cells, are important for establishing primary protection to *C. trachomatis* genital infection (15). However, two groups have shown that CD8 T cells were important in containing chlamydial infection in rodent models and that CD8 T cells may function primarily through production of IFN-γ rather than cytolytic capacity (13, 27). By extrapolation, it seems likely that the CD8 cells in this primate system produce some of the IFN-γ, and the IFN-γ contributes in containing the infection. Because of the genetic differences between primates and mice and between the *C. trachomatis* strains used in the murine (mouse biovar) and primate (human biovar) systems, there may be differences in the relative roles and functions of T-cell subsets in containing *C. trachomatis* infection in primates.

We compared the histopathology and immunopathology of the pocket model to those of the salpingeal tissues in situ. The advantage of the pocket model is that samples from a single macaque can be taken at multiple time points, increasing the yield of information from each macaque. In both models, we found evidence of efficient chlamydial infection by ICC. The two models were similar with respect to histopathology, with a predominantly mononuclear infiltration composed of predominantly CD8 T cells and with lymphoid follicle formation. Both models showed evidence of progression to fibrosis. Both models showed an induction of mRNAs for IFN-γ, IL-2, IL-6, and IL-10 but not IL-4. The similarity of the results validates the salpingeal pocket model for the study of the inflammatory and subsequent fibrotic response of salpingeal tissues to chlamydial infection.

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REFERENCES

1. **Arno, J. N., V. A. Ricker, B. E. Batteiger, B. P. Katz, V. A. Caine, and R. B. Jones.** 1990. Interferon-γ in endocervical secretions of women infected with *Chlamydia trachomatis*. J. Infect. Dis. **162:**1385–1389.
2. **Beatty, W. L., G. I. Byrne, and R. P. Morrison.** 1993. Morphologic and antigenic characterization of interferon gamma-mediated persistent *Chlamydia trachomatis* infection *in vitro*. Proc. Natl. Acad. Sci. USA **90:**3998–4002.
3. **Beckstead, J. H.** 1994. A simple technique for the preservation of fixation-sensitive antigens in paraffin-embedded tissues. J. Histochem. Cytochem. **42:** 1127–1134.
4. **Byrne, G. I., and D. A. Krueger.** 1983. Lymphokine-mediated inhibition of

Chlamydia replication in mouse fibroblasts is neutralized by anti-gamma interferon immunoglobulin. Infect. Immun. **42:**1152–1158.
5. **Cain, T. K., and R. G. Rank.** 1995. Local Th1-like responses are induced by intravaginal infection of mice with the mouse pneumonitis biovar of *Chlamydia trachomatis*. Infect. Immun. **63:**3302–3308.
6. **Del Prete, G., M. DeCarli, F. Almerigogna, M. G. Guidizi, R. Biagotti, and S. Romagnani.** 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. J. Immunol. **150:**353–360.
7. **de Vries, J. E.** 1995. Immunosuppressive and anti-inflammatory properties of interleukin 10. Ann. Med. **27:**537–541.
8. **Grayston, J. T., S.-P. Wang, L.-J. Yeh, and C.-C. Kuo.** 1985. Importance of reinfection in the pathogenesis of trachoma. Rev. Infect. Dis. **7:**717–725.
9. **Hameed, A., W. M. Fox, R. J. Kurman, R. H. Hruban, and E. R. Podack.** 1995. Perforin expression in endometrium during the menstrual cycle. Int. J. Gynecol. Pathol. **14:**143–150.
10. **Kovacs, E. J.** 1991. Fibrogenic cytokines: the role of immune mediators in the development of scar tissue. Immunol. Today **12:**17–23.
11. **Liles, W. C., and W. C. Van Voorhis.** 1995. Review: nomenclature and biological significance of cytokines involved in inflammation and the host immune response. J. Infect. Dis. **172:**1573–1580.
12. **Liu, C. C., C. M. Walsh, and J. D. E. Young.** 1995. Perforin: structure and function. Immunol. Today **16:**194–201.
13. **Magee, D. M., D. W. Williams, J. G. Smith, C. A. Bleicker, B. G. Grubbs, J. Schachter, and R. G. Rank.** 1995. Role of CD8 T cells in primary *Chlamydia* infection. Infect. Immun. **63:**516–521.
14. **Moore, D. E., and W. Cates, Jr.** 1990. Sexually transmitted diseases and infertility, p. 763–769. *In* K. K. Holmes, P.-A. Mardh, P. F. Sparling, and P. J. Wiesner (ed.), Sexually transmitted diseases, 2nd ed. McGraw-Hill, New York, N.Y.
15. **Morrison, R. P., K. Feilzer, and D. P. Tumas.** 1995. Gene knockout mice establish a primary protective role for major histocompatibility complex class II-restricted responses in *Chlamydia trachomatis* genital tract infection. Infect. Immun. **63:**4661–4668.
16. **Patton, D. L., C.-C. Kuo, S.-P. Wang, and S. A. Halbert.** 1987. Distal tubal obstruction induced by repeated *C. trachomatis* salpingeal infections in pig-tailed macaques. J. Infect. Dis. **155:**1292–1299.
17. **Patton, D. L., C.-C. Kuo, S.-P. Wang, R. M. Brenner, M. D. Sternfeld, S. A. Morse, and R. C. Barnes.** 1987. Chlamydial infection of subcutaneous fimbrial transplants in cynomolgus and rhesus monkeys. J. Infect. Dis. **155:**229–235.
18. **Patton, D. L., P. Wolner-Hanssen, S. J. Cosgrove, and K. K. Holmes.** 1990. The effects of *Chlamydia trachomatis* on the female reproductive tract of the *Macaca nemestrina* after a single tubal challenge following repeated cervical inoculations. Obstet. Gynecol. **76:**643–650.
19. **Patton, D. L., and C.-C. Kuo.** 1989. Histopathology of *Chlamydia trachomatis* salpingitis after primary and repeated reinfections in the monkey subcutaneous pocket model. J. Reprod. Fertil. **85:**647–656.
20. **Rank, R. G., K. H. Ramsey, E. A. Pack, and D. M. Williams.** 1992. Effect of gamma interferon on resolution of murine chlamydial genital infection. Infect. Immun. **60:**4427–4429.
21. **Reiner, S. L., and R. M. Locksley.** 1992. Lessons from *Leishmania*: a model for investigation of $CD4^+$ subset differentiation. Infect. Agents Dis. **1:**33–42.
22. **Romagnani, S.** 1995. Biology of human Th1 and Th2 cells. J. Clin. Immunol. **15:**121–129.
23. **Rothelmel, C. D., B. Y. Rubin, and H. W. Murray.** 1983. γ-Interferon is the factor in lymphokine that activates human macrophages to inhibit intracellular *Chlamydia psittaci* replication. J. Immunol. **131:**2542–2544.
24. **Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom.** 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. Science **254:**279–282.
25. **Silva, J. S., P. J. Morrissey, K. H. Grabstein, K. M. Mohler, D. Anderson, and S. G. Reed.** 1992. Interleukin 10 and interferon gamma regulation of experimental *Trypanosoma cruzi* infection. J. Exp. Med. **175:**169–174.
26. **Stamm, W. E., and K. K. Holmes.** 1990. *Chlamydia trachomatis* infection of the adult, p. 917–925. *In* K. K. Holmes, P.-A. Mardh, P. F. Sparling, and P. J. Wiesner (ed.), Sexually transmitted diseases, 2nd ed. McGraw-Hill, New York, N.Y.
27. **Starnbach, M. N., M. J. Bevan, and M. F. Lampe.** 1994. Protective cytotoxic T lymphocytes are induced during murine infection with *Chlamydia trachomatis*. J. Immunol. **153:**5183–5189.
28. **Stephens, R. S., C.-C. Kuo, and M. R. Tam.** 1982. Sensitivity of immunofluorescence with monoclonal antibodies for detection of *Chlamydia trachomatis* inclusions in cell culture. J. Clin. Microbiol. **1:**250–255.
29. **Su, H., and H. D. Caldwell.** 1995. $CD4^+$ T cells play a significant role in adoptive immunity to *Chlamydia trachomatis* infection of the mouse genital tract. Infect. Immun. **63:**516–521.
30. **Van Voorhis, W. C., L. K. Barrett, Y. T. Cosgrove Sweeney, C.-C. Kuo, and D. L. Patton.** 1996. Analysis of lymphocyte phenotype and cytokine activity in the inflammatory infiltrates of the upper genital tract of female macaques infected with *Chlamydia trachomatis*. J. Infect. Dis. **174:**647–650.
31. **Vieth, M., A. Will, K. Schroppel, M. Rollinghoff, and A. Gessner.** 1994. Interleukin-10 inhibits antimicrobial activity against *Leishmania major* in murine macrophages. Scand. J. Immunol. **40:**403–409.
32. **Washington, A. E., R. E. Johnson, and L. L. Sanders.** 1987. *Chlamydia trachomatis* infections in the United States: what are they costing us? JAMA **27:**2070–2072.
33. **Westrom, L., R. Joesoef, G. Reynolds, A. Hagdu, and S. E. Thompson.** 1992. Pelvic inflammatory disease and fertility. A cohort study of 1,844 women with laparoscopically verified disease and 657 control women with normal laparoscopic results. Sex. Transm. Dis. **19:**185–192.
34. **Williams, N. S., and V. H. Englehard.** 1996. Identification of a population of CD4+ CTL that utilizes a perforin- rather than a Fas ligand-dependent cytotoxic mechanism. J. Immunol. **156:**153–159.

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