

## Genital Tract Infection with *Chlamydia trachomatis* Fails To Induce Protective Immunity in Gamma Interferon Receptor-Deficient Mice despite a Strong Local Immunoglobulin A Response

MARTINA JOHANSSON,<sup>1</sup> KARIN SCHÖN,<sup>1</sup> MICHAEL WARD,<sup>2</sup> AND N. LYCKE<sup>1\*</sup>

*Department of Medical Microbiology and Immunology, University of Göteborg, S-413 46 Göteborg, Sweden,<sup>1</sup> and Chlamydial Research Laboratory, Molecular Microbiology Group, University of Southampton, Southampton SO16 4YD, United Kingdom<sup>2</sup>*

Received 12 July 1996/Returned for modification 14 August 1996/Accepted 10 December 1996

**CD4<sup>+</sup> T cells have been found to play a critical role in immune protection against *Chlamydia trachomatis* infection. Since both humoral and cell-mediated antichlamydial immunity have been implicated in host protection, the crucial effector functions provided by the CD4<sup>+</sup> T cells may rely on Th1 or Th2 functions or both. In the present study, we evaluated the development of natural immunity following vaginal infection with *C. trachomatis* serovar D in female gamma interferon receptor-deficient (IFN- $\gamma$ R<sup>-/-</sup>) mice with a disrupted Th1 effector system. We found that in comparison with wild-type mice, the IFN- $\gamma$ R<sup>-/-</sup> mice exhibited a severe ascending primary infection of prolonged duration which stimulated almost 10-fold-stronger specific local immunoglobulin A (IgA) and IgG responses in the genital tract. Following resolution of the primary infection and despite the augmented antibody responses to chlamydiae, the IFN- $\gamma$ R<sup>-/-</sup> mice were completely unprotected against reinfection, suggesting that local antibodies play a subordinate role in host protection against chlamydial infection. Immunohistochemical analysis of frozen sections of the genital tract revealed many CD4<sup>+</sup> T cells in the IFN- $\gamma$ R<sup>-/-</sup> mice, with a dominance of interleukin 4-containing cells in mice following resolution of the secondary infection. However, in contrast to the findings with wild-type mice, the typical clusters of CD4<sup>+</sup> T cells were not found in the IFN- $\gamma$ R<sup>-/-</sup> mice. Few and similarly distributed CD8<sup>+</sup> T cells were observed in IFN- $\gamma$ R<sup>-/-</sup> and wild-type mice. Whereas chlamydia-infected macrophages from wild-type mice had no inclusion bodies (IB) and produced significant amounts of nitric oxide (NO) in the presence of IFN- $\gamma$ , macrophages from IFN- $\gamma$ R<sup>-/-</sup> mice contained many IB but no NO. These results indicate that CD4<sup>+</sup> Th1 cells and IFN- $\gamma$ , rather than local antibodies, are critical elements in host immune protection stimulated by a natural ascending *C. trachomatis* infection in the female genital tract.**

*Chlamydia trachomatis* is an obligate intracellular pathogen of ocular and genital tract mucosal tissues. Trachoma is one of the world's major causes of preventable blindness, while severe sequelae of chlamydial genital tract infection, one of the world's most common sexually transmitted diseases (STD), include pelvic inflammatory disease, infertility, and ectopic pregnancy (49). Despite major research into chlamydial pathogenesis and host immune responses, vaccine development has been hampered by the incomplete understanding of the critical factors governing protective immunity in the genital tract (6, 32, 49).

Experimental studies suggest that both humoral and cell-mediated immunity are required for host resistance against chlamydial genital tract infection (8, 29, 37, 39). In particular, antibodies and T cells recognizing specific epitopes in the major outer membrane protein (MOMP) are strongly associated with immune protection, and their primary structures have been resolved to single-amino-acid resolution (1, 9, 11, 44, 45, 53, 55). An ongoing controversy is the relative contribution of humoral versus cell-mediated immunity in host resistance to chlamydiae (8, 11, 21, 29, 35, 37, 39, 44, 45, 53). For example, Cotter and coworkers, using the backpack syngeneic hybridoma tumor system, recently reported that passive delivery of anti-MOMP-specific monoclonal antibodies into serum and genital secretions had a marginal effect in preventing coloni-

zation and chlamydial shedding, whereas anti-MOMP immunoglobulin G (IgG), more than IgA, reduced the severity of inflammation and immunopathology of the ascending infection (11). Other studies have demonstrated that adoptive transfer of chlamydia-specific CD4<sup>+</sup> as well as CD8<sup>+</sup> T-cell clones alone can confer complete protection against a challenge infection with live chlamydiae (20, 34, 42, 43). Thus, the effector functions provided by protective antichlamydia T cells may include both regulatory cytokine production and chlamydia-specific cytotoxicity.

Although specific cytotoxic T-lymphocyte (CTL) activity has been demonstrated, recent studies in major histocompatibility complex (MHC) class I- or class II-deficient mice found that MHC class II-restricted T-cell responses were necessary for the development of protective immunity, whereas MHC class I-deficient mice, like normal mice, completely resolved genital tract infection with *C. trachomatis* (29). In fact, most reports have ascribed CD4<sup>+</sup>, rather than CD8<sup>+</sup>, T cells preeminent importance in chlamydia-specific immune protection (8, 26, 34, 43). It is unclear whether cytokine production itself or B-cell help provided by the CD4<sup>+</sup> T cells is the critical factor since, for example, gamma interferon (IFN- $\gamma$ ) has been found to reversibly inhibit chlamydial growth (5) while the appearance of antichlamydial IgA antibody in the genital tract has been associated with the resolution of the chlamydial infection (7, 29, 36, 37).

CD4<sup>+</sup> T cells may be divided into two functionally different subsets, the Th1 and Th2 subsets (30). In response to infections or immunizations, these subsets may develop from a common precursor into effector cells which produce distinctive cyto-

\* Corresponding author. Mailing address: Department of Medical Microbiology & Immunology, University of Göteborg, S-413 46 Göteborg, Sweden. Fax: 46 31 827647. E-mail: nils.lycke@microbio.gu.se.

kines: Th1 cells produce interleukin 2 (IL-2), IFN- $\gamma$ , and lymphotoxin, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 (30). There is most often a strong bias towards one or the other of the two subsets, and it is well documented that Th1 and Th2 subsets are reciprocally regulated and produce mutually inhibitory cytokines (30). Thus, the Th1 cells are involved in cell-mediated immunity, and their differentiation is augmented by IL-12 and IFN- $\gamma$  but suppressed by IL-4 (40). Conversely, IL-4 is required for Th2 differentiation, while IFN- $\gamma$  suppresses this development (30, 40). Whether protective immunity against chlamydial infection involves Th1 or Th2 or both CD4<sup>+</sup> effector T-cell subsets is presently unclear. Of two recent studies, one demonstrated a significantly higher frequency of IFN- $\gamma$ - than of IL-4-producing cells in the draining lymph nodes of the genital tract following chlamydial infection, whereas the other reported production of IFN- $\gamma$  as well as IL-6 from chlamydia-immune CD4<sup>+</sup> T cells (8, 43).

To delineate the possible role of Th1 and Th2 effector cells in host protection against genital tract infection with *C. trachomatis*, we undertook studies in mice made genetically deficient for the IFN- $\gamma$  receptor (IFN- $\gamma$ R<sup>-/-</sup>) (18). Mice with a targeted disruption of the IFN- $\gamma$  system have been found to exhibit increased susceptibility to facultatively intracellular bacterial infections, indicating a poor ability to control intracellular microbial infections (10, 18). Since chlamydiae are strictly intracellular pathogens, these mice should provide an excellent model for studies aiming at establishing whether cell-mediated, Th1-dependent or antibody, Th2-associated functions are best required for immune protection stimulated by a primary ascending genital tract infection.

#### MATERIALS AND METHODS

**Animals.** IFN- $\gamma$  receptor gene-targeted 129/Sv mice (i.e., IFN- $\gamma$ R<sup>-/-</sup> mice) and wild-type, control 129/Sv mice (i.e., IFN- $\gamma$ R<sup>+/+</sup> mice) were kindly provided by M. Auget, Genentech, San Francisco, Calif. (18). Female mice, 8 to 10 weeks old, were used at the onset of each experiment. Homozygous IFN- $\gamma$ R<sup>-/-</sup> mice were backcrossed for eight generations with 129/Sv mice and bred in ventilated cages under pathogen-free conditions at the animal facility of the Department of Medical Microbiology and Immunology in Göteborg, Sweden.

**Chlamydia bacteria.** A human genital tract isolate of *C. trachomatis* serovar D was propagated in buffalo green monkey kidney cells, gamma irradiated with 25 Gy prior to inoculation. The chlamydiae were bulk grown by conventional techniques, and aliquots containing  $2 \times 10^5$  elementary bodies (EB)/ $\mu$ l were frozen at -70°C. The purity and identity of the bulk-grown organisms were confirmed by PCR sequencing of the gene encoding the MOMP as described previously (17).

**Bacterial inoculation of the female genital tract.** We adapted a mouse model previously described by Tuffrey et al. (46). Briefly, the mice were given subcutaneous injections of 2.5 mg of medroxyprogesterone (DepoProvera; Upjohn, Puurs, Belgium) per dose, in 100  $\mu$ l of phosphate-buffered saline (PBS), 7 days prior to the intravaginal inoculation. At inoculation, mice were anesthetized by intraperitoneal injection with 200  $\mu$ l of a 1:10 dilution of Hypnorm (Janssen Pharmaceuticals, Beerse, Belgium) in PBS. For inoculations, 60  $\mu$ l of live chlamydia EB in PBS was injected intravaginally and a swab soaked with some of the inoculum was left in the vagina for about 20 min. The infectious dose of *C. trachomatis* serovar D, used throughout the study, was  $10^7$  EB, corresponding to 100 50% infectious doses (ID<sub>50</sub>). At reinoculation, occurring between days 35 and 45 after the primary infection, the mice were again treated with medroxyprogesterone subcutaneously and given a challenge dose of 100 ID<sub>50</sub>, i.e.,  $10^7$  EB of *C. trachomatis* serovar D. Of note, since some of the IFN- $\gamma$ R<sup>-/-</sup> mice were shedding bacteria (see below) for longer than 46 days, those mice were treated for 5 consecutive days with Doxyferm (Merckle, Hamburg, Germany) at 20 mg/ml in their drinking water prior to chlamydial challenge (the dose is approximately 120 to 140 mg/mouse/day). Only mice that tested negative for bacterial shedding were entered into the challenge study.

**Detection of genital tract infection.** Mice were screened for chlamydial shedding by use of the MikroTrak enzyme immunoassay (EIA) and immunofluorescence kits (Syva Company, San Jose, Calif.) as described in the manufacturer's instructions. Bacterial swabs of the vagina were performed on days 8, 20, 38, and 46 after inoculation. Chlamydial DNA in the upper genital tract was detected by PCR as described previously (16). Protection against reinfection was defined on the basis of chlamydial shedding, i.e., an absorbance of <0.8 in the EIA, and a negative immunofluorescence by the Syva MicroTrak test.

**Infertility testing.** As an indicator of an ascending genital tract infection, the extent to which a chlamydial infection had caused infertility was tested. Three to six weeks after the challenge infection, female IFN- $\gamma$ R<sup>-/-</sup> and wild-type mice were mated with fertile male mice and monitored for pregnancy for 7 to 9 weeks thereafter. Uninfected female mice of both strains were also mated with fertile male mice and used as controls. A total of 15 to 17 female mice from each group were included in the fertility testing.

**Serum and genital tract secretions.** Serum and genital tract secretions were collected immediately before the challenge with chlamydiae and 30 to 45 days later. Blood was collected from the lateral tail vein, and serum was aliquoted and frozen. Genital tract secretions were collected from anesthetized mice with a polywicks tampon (Lawton, Tuttingen, Germany) introduced into the vagina and left for 10 min (15). The tampon was then put in an Eppendorf tube containing 250  $\mu$ l of PBS, and the tube was placed on ice. Thereafter, the tube was vigorously vortexed and the tampon was fixed to the lid of the tube before centrifugation at 13,000 rpm at 4°C for 8 min. The serum and secretions were stored at -20 and -70°C, respectively, until further analysis.

**Antibody determinations.** Serum and secretions were assayed for antibodies to purified EB or recombinant three-quarter-length MOMP (rMOMP), derived from *C. trachomatis* serovar L1, by enzyme-linked immunosorbent assay (ELISA), as described previously (33, 41). Briefly, serum was assayed in flat-bottom 96-well plates (Nunc, Roskilde, Denmark), while secretions were tested in round-bottom 96-well polyvinyl chloride plates (InVivo, Stockholm, Sweden). The plates were coated with 50  $\mu$ l of L1 antigen or rMOMP per well at 5  $\mu$ g/ml in PBS and placed in a humidified chamber at 4°C overnight. After repeated washings with PBS and blocking with 0.1% (wt/vol) bovine serum albumin (BSA) in PBS, sera were diluted 1:200 and 1:100 (for IgG subclass detection) in 0.1% BSA-PBS in the first well followed by threefold dilutions in corresponding subwells. Genital tract secretions were initially diluted 1:4 in 0.1% BSA-PBS, and then threefold serial dilutions were performed in corresponding subwells. Samples were allowed to incubate at 4°C overnight. Secondary antibodies directed against mouse IgG, IgA, IgM, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Inc., Birmingham, Ala.) were conjugated to alkaline phosphatase and used at dilutions of 1:500 or 1:1,000 in 0.1% BSA-PBS. The plates were incubated for 2 h at room temperature. Bound antibodies were visualized by adding 100  $\mu$ l of *n*-nitrophenyl phosphatase substrate (Sigma Chemical Co., St. Louis, Mo.) in ethanolamine buffer to each microwell. The reaction was read at 405 nm with a Multiskan PLUS spectrophotometer (Labsystems, Hampshire, United Kingdom). Titers were determined with the linear slope of the curve and defined as the interpolated dilution of a sample giving rise to an absorbance 0.4 above background. The antibody titers were expressed as log<sub>10</sub>  $\pm$  standard deviation (SD) of four to seven mice per group.

**Cryosections and immunohistochemical analysis.** The cervix, uterus, tubes, and ovaries were removed, placed into Histocon (Histolab Products AB, Göteborg, Sweden), and kept on ice. The tissues were cut into appropriately sized pieces, placed, standing, in plastic forms (Cryomold; Miles Inc., Elkhart, Ind.) filled with O.C.T. Compound (Miles), subsequently snap frozen in isopentane in liquid N<sub>2</sub> for approximately 60 s, and then stored at -70°C until processed further. Frozen sections were prepared on microslides with a cryostat (Leitz, Wetzlar, Germany) and processed by fixing in 100% cold acetone for 5 min. To block endogenous peroxidase activity, the slides were treated with 0.3% H<sub>2</sub>O<sub>2</sub> for 5 min and then stained with the following labelled antibodies: biotin-conjugated anti-CD4 or biotin-conjugated anti-CD8 (Pharmingen, San Diego, Calif.). Staining for inclusion bodies (IB) was done on tissue slides fixed in 100% acetone for 5 min, washed in PBS, and subsequently incubated with a fluorescein isothiocyanate-labelled antichlamydia antibody (Syva MicroTrak kit) for 30 min as described in the manufacturer's instructions.

For detection of intracellular cytokines, we adapted a method described for human tissues (2). Briefly, cryostat sections, 5  $\mu$ m thick, were mounted and fixed for 15 min in 5% paraformaldehyde-PBS. After a thorough washing with Hank's balanced salt solution (HBSS), endogenous peroxidase was blocked by incubation in 1% H<sub>2</sub>O<sub>2</sub> in HBSS containing 0.1% saponin (Sigma) for 60 min. The slides were then washed three times, 5 min each, in 0.1% saponin-HBSS. To block nonspecific binding of antibodies, the slides were first incubated with normal horse serum, diluted 1:20 in 0.1% saponin-HBSS, for 15 min. Endogenous biotin was then blocked for 15 min with an avidin-biotin blocking kit (Vector, Burlingame, Calif.) as described in the manufacturer's instructions, and the slides were rinsed in 0.1% saponin-HBSS. The saponin treatment permeabilized the cell membrane to permit intracellular staining. The sections were then incubated with the primary antibody, rat anti-mouse IFN- $\gamma$  or rat anti-mouse IL-4 (Pharmingen), diluted 1:200 in 0.1% saponin-HBSS containing 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, overnight at room temperature in a humidified chamber. To detect bound primary antibodies, the slides were incubated for 1 h at room temperature with biotin-conjugated rabbit anti-rat IgG (Vector) diluted 1:100 in 0.1% saponin-HBSS. After careful washing in 0.1% saponin-HBSS, peroxidase-conjugated avidin (ABC complex; DAKO, Glostrup, Denmark) was added to each slide, and the slides were incubated for an additional hour at room temperature. Intracellular deposits of cytokines were then visualized by the addition of the peroxidase AEC substrate (Sigma). AEC is 10 mg of 3-amino-9-ethylcarbazole in 6 ml of dimethyl sulfoxide mixed with 50 ml of Na acetate and 4  $\mu$ l of H<sub>2</sub>O<sub>2</sub> and was allowed to react with the labelled antibodies for 15 min. Prior to inspection in the microscope, the tissue was washed, counterstained with HTX (Histolab Products

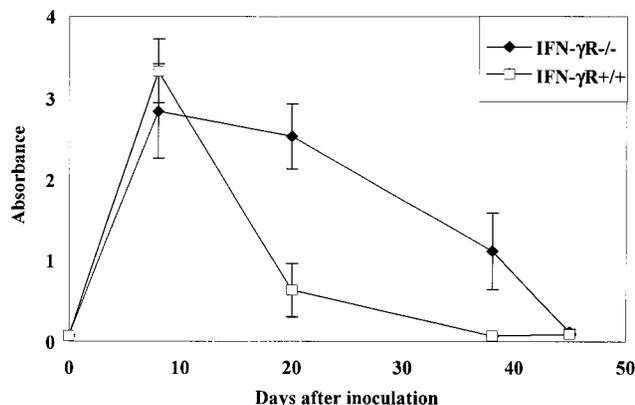


FIG. 1. Bacterial shedding from the genital tract following a primary inoculation with *C. trachomatis*. IFN- $\gamma$ R<sup>-/-</sup> mice exhibit more bacterial shedding with slower kinetics of bacterial clearance following a primary genital tract infection with chlamydiae than wild-type mice do. Chlamydial shedding was monitored in IFN- $\gamma$ R<sup>-/-</sup> and wild-type (IFN- $\gamma$ R<sup>+/+</sup>) mice following a primary intravaginal inoculation with *C. trachomatis* serovar D. The mice were tested for bacterial shedding on days 8, 20, 38, and 46 following inoculation by use of the Syva Mikrotrak EIA kit. The results are expressed as mean absorbance values  $\pm$  standard errors of the means of two experiments with four to eight mice per group.

AB), and mounted in Aquamount (BDH Laboratory Supplies, Poole, England). Sections were evaluated and photographed with an Axioskop microscope (Zeiss, Cambridge, United Kingdom). The relative distribution of labelled cells was calculated by enumeration of three light-microscopic sections with similar surface areas for each mouse and the mean number of labelled cells ( $\pm$ SD) of three mice per group. The labelling was highly specific: no staining was observed with an isotype-matched irrelevant control antibody as the primary antibody, and no staining was observed with the second-step anti-rat IgG (Vector) antibody used alone without a primary antibody.

**Control of chlamydial growth in cultured peritoneal macrophages.** Peritoneal macrophages (PM $\phi$ ) were isolated 3 days following an intraperitoneal injection with thioglycolate. The PM $\phi$  were washed in PBS, resuspended in Iscove's medium (Seromed, Biochrom KG, Berlin, Germany) containing 10% heat-inactivated fetal calf serum,  $5 \times 10^{-5}$  M 2-mercaptoethanol (Sigma), 1 mM L-glutamine, and 50  $\mu$ g of gentamicin per ml (hereafter referred to as Iscove's total medium), and adjusted to  $10^6$  cells/ml. After irradiation with 25 Gy,  $10^5$  PM $\phi$  were seeded in four-well glass slide chambers (Nunc) and allowed to adhere to the glass overnight in the CO<sub>2</sub> incubator at 37°C. The next day, nonadherent cells were removed by washing and the cultures were inoculated with  $10^7$  EB of *C. trachomatis* serovar D. To investigate the protective effect of IFN- $\gamma$  on chlamydial growth, the following additives were used alone or together as indicated: lipopolysaccharide (LPS; 10  $\mu$ g/ml), recombinant IFN- $\gamma$  (rIFN- $\gamma$ ; Genzyme, Cambridge, Mass.) at 100 or 500 U/ml, and the NO synthase inhibitor N<sup>G</sup>-monomethyl-L-arginine acetate (L-NMA; Calbiochem, San Diego, Calif.) at 1 mM. After 96 h of incubation, the PM $\phi$  were analyzed for presence of chlamydial IB with the Syva immunofluorescence staining kit. Parallel cultures were investigated for production of nitric oxide (NO). Briefly, irradiated PM $\phi$  were seeded at 100,000 cells/well in 96-well plates (Nunc) and infected with  $10^7$  EB of *C. trachomatis* serovar D. Infected or uninfected PM $\phi$  cultures were incubated with the following additives: LPS (1  $\mu$ g/ml), rIFN- $\gamma$  (100 U/ml), rIFN- $\gamma$ -LPS, and rIFN- $\gamma$ -LPS-L-NMA (1 mM). After 72 h of incubation, 50  $\mu$ l of the supernatants was added to 50  $\mu$ l of Greiss reagent as described previously (14) and the reaction was read at 550 nm in a Multiskan MS spectrophotometer. A standard curve was generated from serial dilutions of known concentrations of NaNO<sub>2</sub> in Iscove's medium and analyzed with a spectrophotometer after the addition of Greiss reagent as described previously (14). All samples were analyzed in triplicate.

**Statistical analysis.** We used Student's *t* test for unmatched data for analysis of significance.

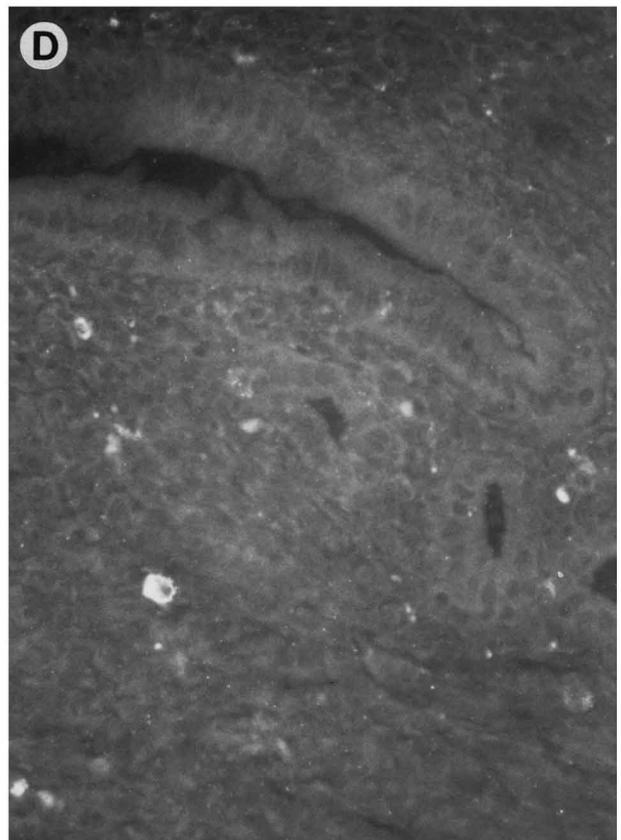
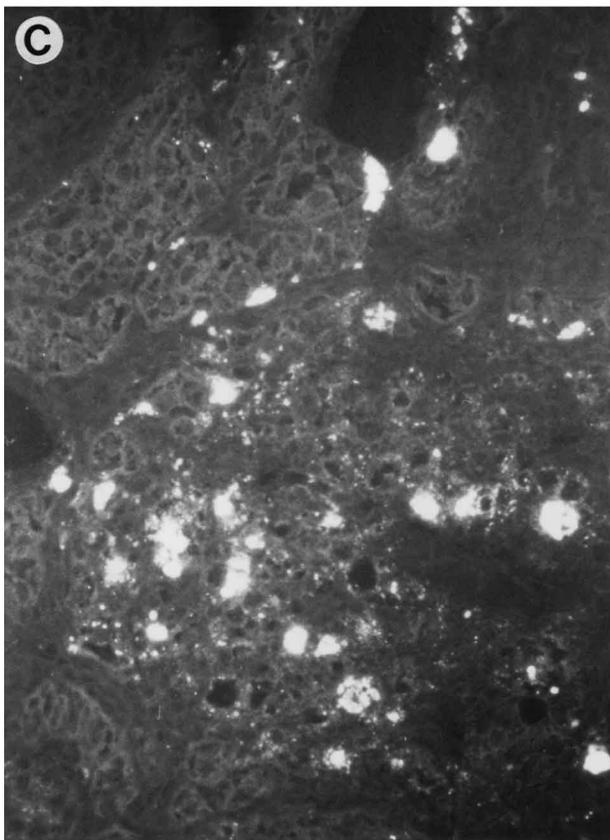
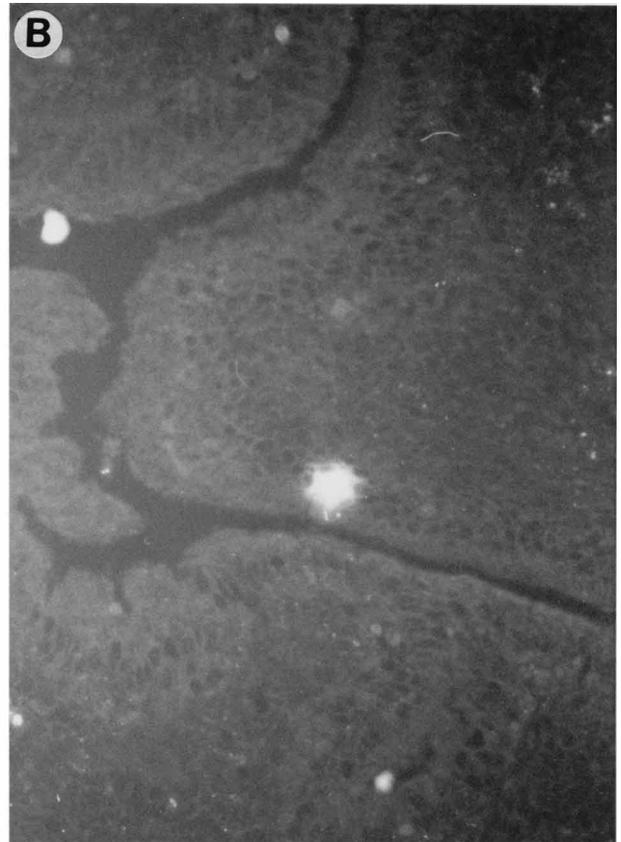
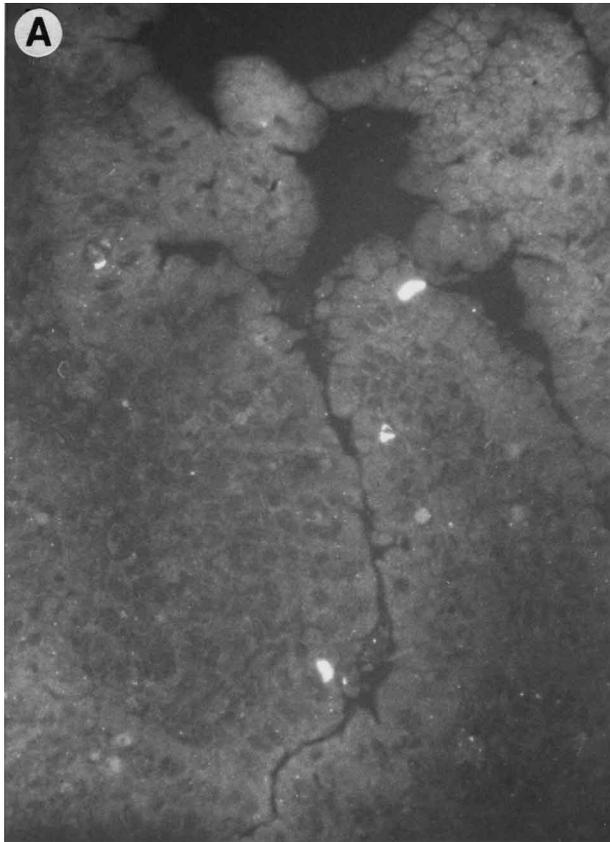
## RESULTS

**Failure to develop immune protection against chlamydial infection in IFN- $\gamma$ R<sup>-/-</sup> mice.** CD4<sup>+</sup> T cells have been ascribed a critical role in protective immunity against infection with *C. trachomatis* (8, 26, 29, 43). The two functionally different CD4<sup>+</sup> T-cell effector populations, the Th1 subset involved in cell-mediated immunity and the Th2 subset associated with antibody and allergic responses, produce distinct cytokines and are reciprocally regulated in response to microbial infections (30, 40). Since chlamydia-immune individuals have been found to exhibit both humoral and cell-mediated immunity, both or one of these CD4<sup>+</sup> T-cell effector systems could be critically required for host resistance against infection with *C. trachomatis* (8, 26, 29, 43). To shed light on this issue, we studied the development of protective immunity in mice with a defect in their Th1 effector system, i.e., the IFN- $\gamma$ R<sup>-/-</sup> mice (18). The mice were inoculated intravaginally with a human isolate of *C. trachomatis* serovar D and monitored for shedding (47) of bacteria at regular intervals beginning on day 8. We found that IFN- $\gamma$ R<sup>-/-</sup> and wild-type (IFN- $\gamma$ R<sup>+/+</sup>) mice were equally susceptible to a primary intravaginal inoculation with live bacteria, with more than 85% of the mice developing a primary infection as assessed by bacterial shedding. All of the wild-type and most of the IFN- $\gamma$ R<sup>-/-</sup> mice cleared their infection by 46 days following inoculation (Fig. 1). However, the IFN- $\gamma$ R<sup>-/-</sup> mice exhibited augmented bacterial shedding and the kinetics of bacterial clearance was slower than that of wild-type mice (Fig. 1). In both wild-type and IFN- $\gamma$ R<sup>-/-</sup> mice, the secondary infection resulted in infertility in 60 to 70% of the infected mice, indicating that intravaginal inoculation caused an ascending infection that involved the upper genital tract.

This notion was further confirmed by immunohistochemical analysis of frozen tissue sections for chlamydial inclusions from the upper genital tracts of infected and control mice. At 8 days following inoculation, both wild-type and IFN- $\gamma$ R<sup>-/-</sup> mice demonstrated similar numbers and appearances of chlamydial IB in the uterus, but at 13 days, IFN- $\gamma$ R<sup>-/-</sup> mice exhibited significantly more and larger IB than wild-type mice did (Fig. 2). No staining with antichlamydial monoclonal antibody was found in uninfected control mice from either strain (data not shown). In a separate set of mice, chlamydial DNA was also demonstrated in the ovarian bursa and uterus by PCR amplification of the *omp1* gene (16, 17) (data not shown).

We then asked whether a primary infection in IFN- $\gamma$ R<sup>-/-</sup> mice would stimulate protective immunity against a second challenge with *C. trachomatis*. We elected to use the overwhelming dose of 100 ID<sub>50</sub> of *C. trachomatis* to ensure that only major protective events were assessed (11). Only mice that had recovered from the primary infection and that were negative for chlamydial shedding were rechallenged. However, since the recovery from the primary infection was slower in IFN- $\gamma$ R<sup>-/-</sup> mice than in wild-type mice, with shedding of chlamydiae in some animals occurring more than 50 days after the inoculation, we treated some of the mice with tetracycline (120 to 140 mg/day), an antichlamydial drug, prior to reinoculation. Following reinoculation, shedding of bacterial antigen was again monitored at regular intervals. We found that almost all

FIG. 2. Chlamydial IB in the genital tract of infected mice. IFN- $\gamma$ R<sup>-/-</sup> and wild-type mice were inoculated intravaginally with a human isolate of *C. trachomatis* serovar D. Mice were killed 8 and 13 days postinoculation, and frozen tissue sections were prepared from mice that tested positive for chlamydial shedding. The sections were stained with fluorescein-labelled antichlamydial antibody by use of the Syva kit and analyzed for the presence of IB in a microscope at  $\times 40$  magnification. The number of IB in the uterus of IFN- $\gamma$ R<sup>-/-</sup> (A) and wild-type (B) mice did not differ after 8 days, whereas on day 13, the IFN- $\gamma$ R<sup>-/-</sup> mice (C) had significantly more IB than the wild-type mice (D) did.



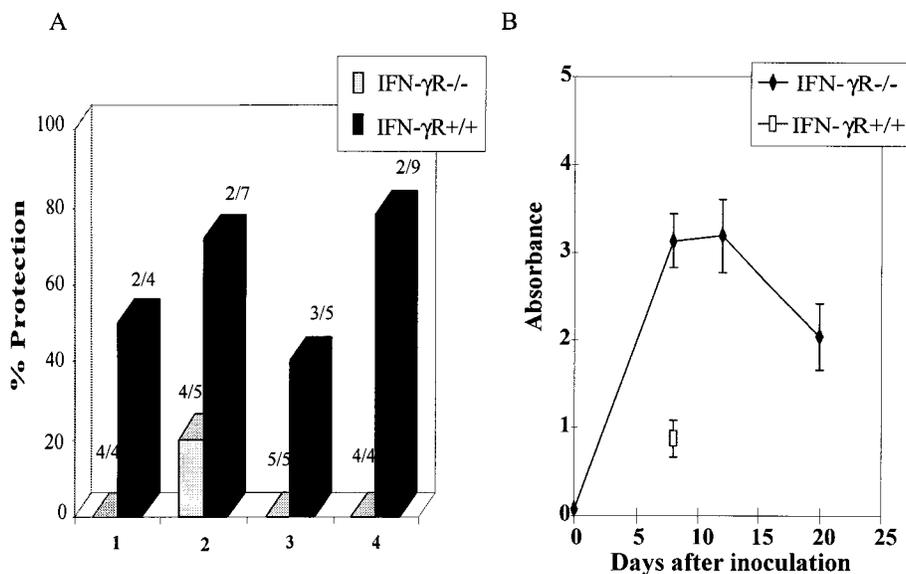


FIG. 3. Lack of protective immunity following resolution of a primary genital tract infection with *C. trachomatis* in IFN- $\gamma$ R<sup>-/-</sup> mice. (A) The percent level of protection is given for four separate experiments, and the results demonstrate no or poor protection in IFN- $\gamma$ R<sup>-/-</sup> mice (shaded bars) and good protection in wild-type mice (solid bars). Protection was defined as lack of bacterial shedding on day 8 after challenge. The numbers represent individual unprotected mice per total mice in each experimental group. (B) Chlamydial shedding was monitored in IFN- $\gamma$ R<sup>-/-</sup> and wild-type (IFN- $\gamma$ R<sup>+/+</sup>) mice following a second intravaginal inoculation with *C. trachomatis*. The results are expressed as mean absorbance values  $\pm$  standard errors of the means of four to five experiments with four to nine mice per group. Protected as well as unprotected mice were included in this analysis. Only mice that had developed a primary infection and subsequently cleared that infection, i.e., exhibited no bacterial shedding by day 46, were included in the challenge experiments.

IFN- $\gamma$ R<sup>-/-</sup> mice were reinfected; in four separate experiments, less than 4% of the mice were protected (Fig. 3A). Even inoculation with live chlamydiae on a third occasion caused further genital tract infection and undiminished chlamydial shedding in IFN- $\gamma$ R<sup>-/-</sup> mice (data not shown). By contrast, in wild-type mice, the degree of protection varied between 40 and 80% in the different experiments, with a mean of more than 55% of the mice being protected (Fig. 3A). Moreover, when the mean levels of bacterial shedding in the reinoculated IFN- $\gamma$ R<sup>-/-</sup> and wild-type mice in all experiments were compared, wild-type mice demonstrated much lower mean EIA detection

of chlamydiae than IFN- $\gamma$ R<sup>-/-</sup> mice did, indicating significantly ( $P < 0.001$ ) stronger protection (Fig. 3). Thus, these results clearly demonstrate that IFN- $\gamma$ R<sup>-/-</sup> mice are impaired in their ability to develop protective immunity in the genital tract following primary infection with *C. trachomatis*.

**Presence of strong local antichlamydial IgA and IgG antibody production in IFN- $\gamma$ R<sup>-/-</sup> mice following infection.** Host protection against many pathogenic microorganisms at mucosal surfaces is thought to depend on the presence of local antibodies, particularly secretory IgA (28). In the clinic as well as in experimental models, antichlamydial IgA and IgG in the

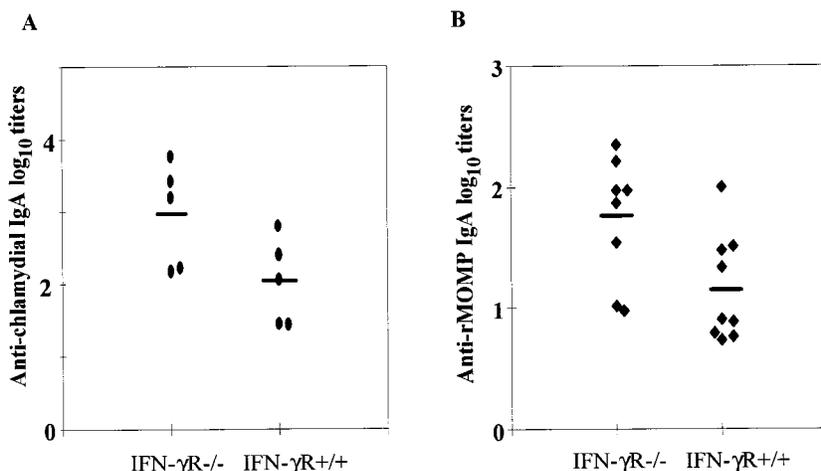


FIG. 4. Strong local antichlamydial IgA antibody responses stimulated by a primary genital tract infection. Genital tract secretions were collected 35 to 45 days after a primary vaginal inoculation of *C. trachomatis* in IFN- $\gamma$ R<sup>-/-</sup> and wild-type (IFN- $\gamma$ R<sup>+/+</sup>) mice. The samples were analyzed for specific IgA antibodies against whole chlamydial EB (A) or rMOMP (B) by an ELISA (see Materials and Methods). Antibody responses are given as mean (bars) and individual log<sub>10</sub> titers of five experiments with four to seven mice per group in each experiment (A) or mean log<sub>10</sub> titers (bars) from individual mice (diamonds) in two experiments (B). The IFN- $\gamma$ R<sup>+/+</sup> mice have significantly ( $P < 0.01$  for EB,  $P < 0.05$  for rMOMP) lower antibody titers than the IFN- $\gamma$ R<sup>-/-</sup> mice do.

TABLE 1. Specific antibody responses in genital tract secretions and serum to primary and secondary infections with *C. trachomatis*<sup>a</sup>

Sample	Isotype	Antibody response <sup>b</sup> to:			
		Primary infection		Secondary infection	
		IFN- $\gamma$ R <sup>-/-</sup> mice	IFN- $\gamma$ R <sup>+/+</sup> mice	IFN- $\gamma$ R <sup>-/-</sup> mice	IFN- $\gamma$ R <sup>+/+</sup> mice
Secretions	IgG	2.43 ± 0.73	1.92 ± 0.81	2.43 ± 0.71	2.11 ± 0.73
	IgA	2.79 ± 0.83	1.97 ± 0.73	2.88 ± 0.58	1.94 ± 0.77
	IgM	0.69 ± 0.09*	0.82 ± 0.18†	1.08 ± 0.38‡	0.94 ± 0.50§
Serum	IgG	4.39 ± 0.64	4.19 ± 0.89	4.70 ± 0.61	4.67 ± 0.67
	IgA	2.50 ± 1.08	2.13 ± 1.16	3.63 ± 0.51	2.36 ± 1.20
	IgM	NT <sup>c</sup>	NT	NT	NT

<sup>a</sup> Genital tract secretions and serum were collected 35 to 45 days after a primary vaginal inoculation of chlamydiae and 35 to 45 days after a challenge reinoculation. The samples were analyzed for specific antibodies in various isotypes against whole EB by an ELISA (see Materials and Methods).

<sup>b</sup> Antibody responses are given as mean log<sub>10</sub> titers ± SD of five experiments with four to seven mice per group in each experiment. \*, †, ‡, and §, 15 of 23, 22 of 29, 18 of 23, and 24 of 29 mice, respectively, did not demonstrate detectable antichlamydial IgM titers.

<sup>c</sup> NT, not tested.

genital tract have been associated with host protection against infection. Recently, we found that IFN- $\gamma$ R<sup>-/-</sup> mice were impaired in their ability to respond with gut mucosal IgA to oral immunizations with soluble protein antigens (23). Therefore, we asked whether the absence of local antibodies to chlamydiae could explain the lack of protection against reinfection observed in the IFN- $\gamma$ R<sup>-/-</sup> mice. Genital tract secretions, either from uninfected mice or collected from infected mice on days 34 to 46 after the primary inoculation, were analyzed by ELISA for their content of IgA, IgG, and IgM antibodies to chlamydiae. To our surprise, we found significantly ( $P < 0.01$  and  $P < 0.05$ , respectively) higher titers of specific IgA antibodies against purified whole EB as well as rMOMP in uninfected IFN- $\gamma$ R<sup>-/-</sup> than in well-protected wild-type mice (Fig. 4). The mean antichlamydial IgA titer in IFN- $\gamma$ R<sup>-/-</sup> mice was roughly 10-fold higher than that in wild-type mice, suggesting, paradoxically, that host protection against reinfection poorly correlated (correlation coefficient [ $r$ ], 0.39) with the presence of local IgA antibodies. Specific IgG antibodies in the genital tract secretions similarly correlated ( $r = 0.16$ ) poorly with immune protection as the IFN- $\gamma$ R<sup>-/-</sup> mice also exhibited stronger IgG titers than the wild-type mice (Table 1). Interestingly, whereas antibody titers of both the IgA and the IgG classes to chlamydiae were significantly ( $P < 0.01$  and  $P < 0.05$ , respectively) higher in secretions from IFN- $\gamma$ R<sup>-/-</sup> mice than in those found in wild-type mice, no difference was observed at the systemic, serum antibody level between the two mouse strains (Table 1). Moreover, IgG titers to chlamydiae in the sera of both strains were roughly 100-fold higher than those in genital tract secretions while, by contrast, the IgA titers were comparable in secretions and sera of IFN- $\gamma$ R<sup>-/-</sup> mice but were lower in secretions than in serum in wild-type mice (Table 1). IgM titers to chlamydiae were low or undetectable in both strains of mice (Table 1), while uninfected mice of both strains exhibited no specific antibodies of any isotype against chlamydiae (data not shown).

A proxy measure of Th1 and Th2 activity in immune responses may be discerned from the IgG subclass distribution, with the Th1 cytokine IFN- $\gamma$  regulating IgG2a responses and the Th2 cytokine IL-4 promoting IgG1 responses (30). As expected, the serum antichlamydial IgG subclass analysis revealed significantly lower ( $P < 0.05$ ) IgG2a-specific antibody titers in IFN- $\gamma$ R<sup>-/-</sup> than in wild-type mice (Fig. 5). Moreover, few wild-type mice responded with IgG1 antibodies to the primary infection, suggesting that this intracellular bacterial infection promoted Th1 rather than Th2 responses (Fig. 5). However, in the absence of a functioning IFN- $\gamma$  system, the

IFN- $\gamma$ R<sup>-/-</sup> mice exhibited significantly ( $P < 0.01$ ) stronger IgG1 responses to chlamydiae than the wild-type mice did (Fig. 5). It is important to note, though, that compared to the other IgG subclass responses, even that of IgG2a, the IgG1 response was weak, suggesting a strong Th1 influence on antichlamydial antibody responses in the IFN- $\gamma$ R<sup>-/-</sup> mice as well (Fig. 5).

Rechallenge with chlamydiae did not alter the local antichlamydial IgA or IgG responses in IFN- $\gamma$ R<sup>-/-</sup> or wild-type mice, indicating that in fact, peak antibody responses in secretions had been stimulated by the primary infection in both strains of mice (Table 1). By contrast, in IFN- $\gamma$ R<sup>-/-</sup> but not in wild-type mice, serum IgA and IgG antibody responses increased even further upon rechallenge, with the serum antichlamydial IgA titers increasing by almost fivefold (Table 1). Thus, despite Th1 activity and a strong local mucosal and serum IgA and IgG response to chlamydiae, poor protection against reinfection was observed in IFN- $\gamma$ R<sup>-/-</sup> mice. These results suggest that there is no direct correlation between high

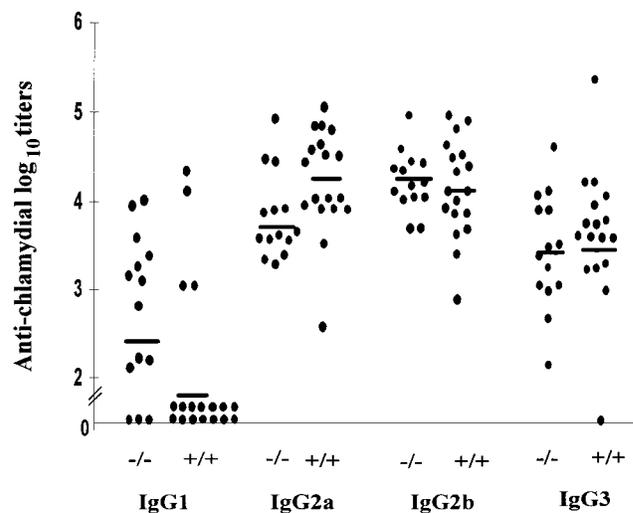


FIG. 5. Serum antichlamydial IgG subclass distribution following a primary genital tract infection in IFN- $\gamma$ R<sup>-/-</sup> mice. Serum samples from IFN- $\gamma$ R<sup>-/-</sup> and from wild-type (IFN- $\gamma$ R<sup>+/+</sup>) mice were collected and analyzed by an ELISA for IgG1-, IgG2a-, IgG2b-, and IgG3-specific antibodies. The symbols represent the mean (bars) and individual log<sub>10</sub> titers of animals of three experiments. The IFN- $\gamma$ R<sup>-/-</sup> mice have significantly lower ( $P < 0.05$ ) antichlamydial IgG2a titers but significantly higher ( $P < 0.01$ ) IgG1 titers than the IFN- $\gamma$ R<sup>+/+</sup> mice.

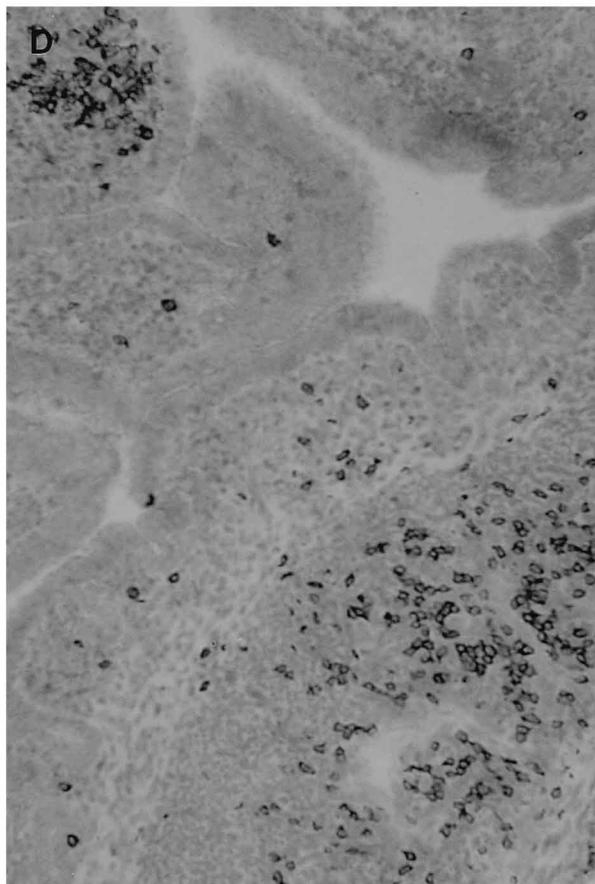
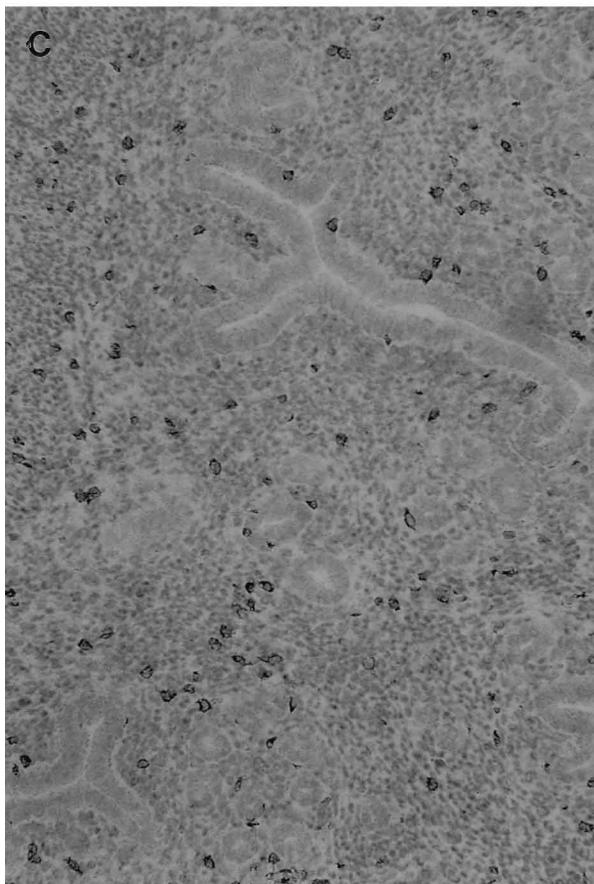
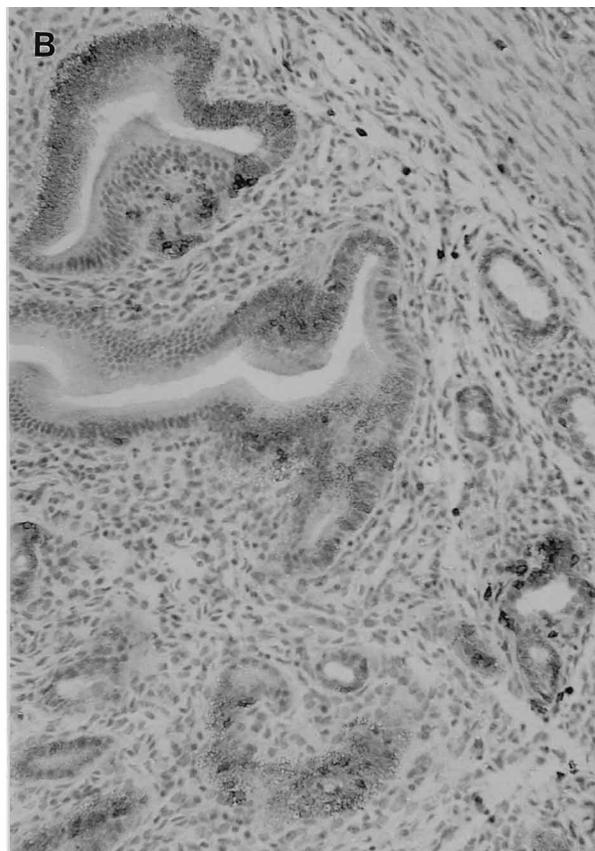
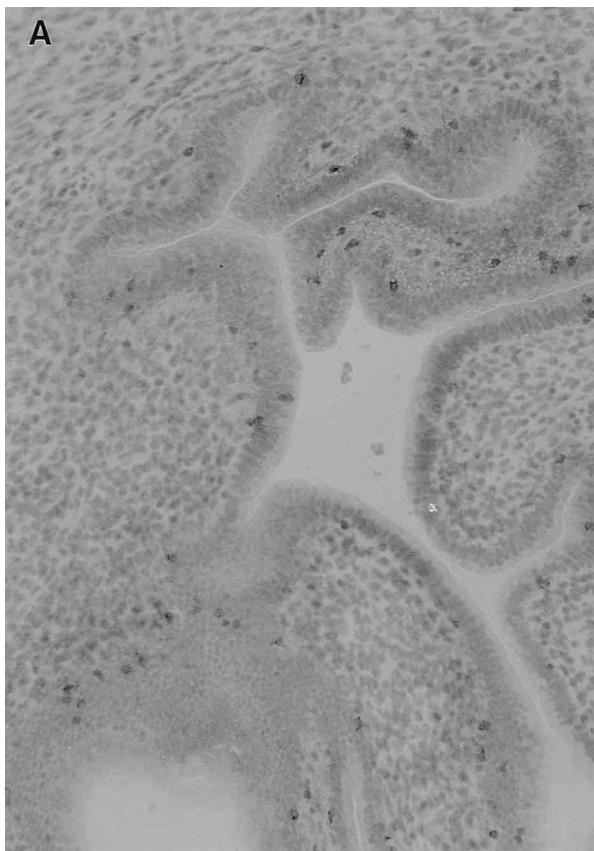


FIG. 6. Distribution of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in genital tract tissues following a challenge infection with *C. trachomatis*. Frozen tissue sections of the uterus taken 30 days after a secondary infection were labelled with anti-CD4<sup>+</sup> or anti-CD8<sup>+</sup> biotinylated antibodies followed by horseradish peroxidase-conjugated avidin-biotin complexes. Both IFN- $\gamma$ R<sup>-/-</sup> (A) and wild-type (IFN- $\gamma$ R<sup>+/+</sup>) (B) mice had few but similarly distributed CD8<sup>+</sup> T cells. In contrast, although CD4<sup>+</sup> T cells were frequent in both IFN- $\gamma$ R<sup>-/-</sup> (C) and wild-type (D) mice, the distribution differed in that clusters of CD4<sup>+</sup> T cells were found only in wild-type mice. The tissues were examined by light microscopy at ca.  $\times 20$  magnification.

local antibody titers against chlamydial antigens in the genital tract and resistance to chlamydial infection.

**Presence of Th1 and Th2 cells in the genital tract of chlamydia-infected mice.** Since Th1 activity dominated serum responses in both IFN- $\gamma$ R<sup>-/-</sup> and wild-type mice and local IgA and IgG antibodies to chlamydiae were not sufficient for host protection in IFN- $\gamma$ R<sup>-/-</sup> mice, we investigated to what extent the inability to respond to IFN- $\gamma$  may have affected the presence and distribution of T-cell subsets in the genital tract. Immunohistochemical analyses were performed on frozen sections taken from mice rechallenged by a second inoculation with chlamydiae. The sections were labelled with anti-CD4- or anti-CD8-specific antibodies. We found low frequencies and similar distributions of CD8<sup>+</sup> T cells in IFN- $\gamma$ R<sup>-/-</sup> and wild-type mice (Fig. 6). Most of the CD8<sup>+</sup> T cells appeared to be intraepithelial lymphocytes (Fig. 6). However, CD4<sup>+</sup> T cells occurred much more frequently than CD8<sup>+</sup> T cells in the genital tract and localized in distinct clusters in submucosal tissues in the wild-type mice (Fig. 6). In IFN- $\gamma$ R<sup>-/-</sup> mice, the CD4<sup>+</sup> T cells occurred as frequently as they did in wild-type mice but did not localize in clusters (Fig. 6). Naive, uninfected mice of both strains exhibited very few or no CD8<sup>+</sup> or CD4<sup>+</sup> T cells in tissue sections (data not shown), indicating that the immune and inflammatory reaction towards the chlamydial infection had attracted these T cells to the genital tract. Sections were then incubated with anti-IFN- $\gamma$  or anti-IL-4 to assess the relative distribution of Th1 and Th2 cytokine-producing cells in the chlamydia-infected genital tract. We found similar frequencies and distributions of IFN- $\gamma$ -containing cells in both IFN- $\gamma$ R<sup>-/-</sup> and wild-type mice, whereas cells containing IL-4 occurred three times more frequently in IFN- $\gamma$ R<sup>-/-</sup> than in wild-type mice (Fig. 7). The immunohistochemical findings are summarized in Table 2. Similar findings with regard to distribution of T-cell subsets and frequencies of cytokine-containing cells were made with sections from the cervix or oviduct.

**Cells from IFN- $\gamma$ R<sup>-/-</sup> mice do not produce NO in response to IFN- $\gamma$  and fail to control chlamydial infection.** Chlamydia-infected mice of both IFN- $\gamma$ R<sup>-/-</sup> and wild-type strains developed local CD8<sup>+</sup> and CD4<sup>+</sup> T-cell responses in the genital tract, with similar frequencies of IFN- $\gamma$ -producing cells; however, there were more IL-4-producing cells in the IFN- $\gamma$ R<sup>-/-</sup> mice than in the wild-type mice. The increased IL-4 or Th2 activity could explain the comparatively stronger local IgA and serum IgG1 responses to chlamydiae in IFN- $\gamma$ R<sup>-/-</sup> mice (40, 48). However, despite the stronger specific IgA or IgG response and the similar frequency of IFN- $\gamma$ -producing cells in the IFN- $\gamma$ R<sup>-/-</sup> mice, they were poorly protected against a second or third challenge with chlamydiae. Thus, in agreement with previous studies, it appeared that the ability to respond to IFN- $\gamma$  played a central role in host resistance against chlamydial infection (8, 19, 38, 43, 54). It is well known that IFN- $\gamma$  may mediate antimicrobial activity as an inducer of the intracellular enzymes NO synthase and indoleamine-2,3-dioxygenase (5, 19, 27, 50). Therefore, we investigated in vitro the extent to which PM $\phi$  from naive IFN- $\gamma$ R<sup>-/-</sup> or wild-type mice could control chlamydial infection in the presence or absence of rIFN- $\gamma$ . The formation of NO was correlated with the frequencies of IB in the cultured cells. The PM $\phi$  were cultured for 3 days with LPS,

rIFN- $\gamma$ , or L-NMA, an inhibitor of NO synthase, added alone or in different combinations. In the absence of additives, PM $\phi$  from both IFN- $\gamma$ R<sup>-/-</sup> and wild-type mice demonstrated similar levels of IB (Fig. 8). However, in PM $\phi$  from wild-type mice treated with rIFN- $\gamma$ , no IB were seen but scattered EB were found inside some of the cells (Fig. 8). On the contrary, PM $\phi$  from IFN- $\gamma$ R<sup>-/-</sup> mice cultured with rIFN- $\gamma$  contained large IB (Fig. 8). This result correlated well with the inability to form NO in macrophages from IFN- $\gamma$ R<sup>-/-</sup> mice, whereas high levels of NO were detected in cultures with chlamydia-infected macrophages from wild-type mice treated with rIFN- $\gamma$  or rIFN- $\gamma$ -LPS (Table 3). The production of NO in wild-type macrophages could be abrogated by the addition of the NO inhibitor L-NMA to the cultures (Table 3). Thus, the inability to respond to IFN- $\gamma$  was associated with the failure to form NO in response to an intracellular infection with live *C. trachomatis* in IFN- $\gamma$ R<sup>-/-</sup> mice.

## DISCUSSION

The local immune system in the genital tract has attracted much interest in recent years (6, 32, 49). This is partly because of the growing problem with human immunodeficiency virus and other STD and the wish to find effective prophylactic vaccines against these diseases (49). However, vaccine development has been slow because of the complexity of host immune responses against STD and the inability to recreate appropriate models in vitro of the local immune system in the genital tract. Therefore, in spite of many years of intense investigations, we still lack basic information on the complex regulatory mechanisms governing local immune responses in the genital tract (49). The recent development of genetically deficient mice through homologous gene recombination techniques has provided a new and powerful tool for studies of host-parasite relationships that could be used to better understand STD-specific immunity and facilitate the development of effective vaccines (22, 24).

In the present study, we provide evidence suggesting that the most important protective factor against a genital tract infection with *C. trachomatis* is IFN- $\gamma$  (5, 8, 19, 38, 43, 54). Using IFN- $\gamma$ R<sup>-/-</sup> mice, we found that a primary genital tract infection failed to stimulate protection against reinfection with live chlamydiae. However, despite the almost complete lack of resistance against reinfection, the mice exhibited stronger specific local IgA and IgG responses in the genital tract than the well-protected wild-type mice did. The direct demonstration of IgA-containing cells in infected genital tract tissue, but not in uninfected control tissue (21a), as well as the comparatively high titers of IgA relative to those of IgG in secretions as opposed to the reverse situation in serum indicates that strong local antibody responses develop in response to a primary infection with *C. trachomatis*. In an extended analysis of these antibodies, no apparent qualitative difference with regard to epitope recognition, including reactivity with rMOMP, was observed. Although it was not possible to test the neutralizing ability of the antibodies, the fact that B-cell reactivity against MOMP epitopes constitutes a major part of the humoral immune response and that such antibodies, in particular, those of the IgA class, have been associated with protection and found

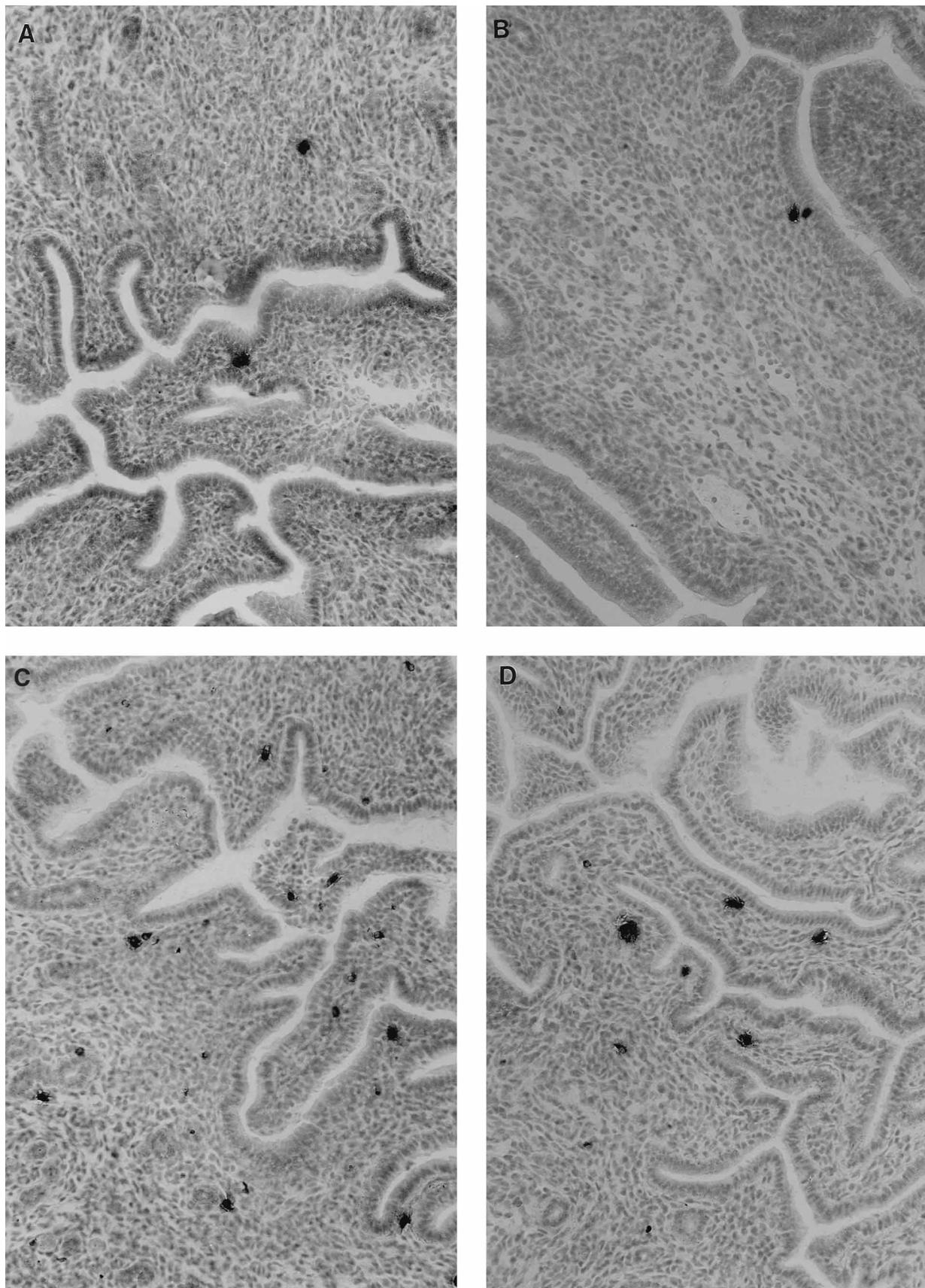


FIG. 7. Distribution of IFN- $\gamma$ - and IL-4-containing cells in genital tract tissues following a challenge infection with *C. trachomatis*. Frozen tissue sections of the uterus taken 30 days after a secondary infection were labelled with rat anti-IFN- $\gamma$  or anti-IL-4 antibodies followed by biotin-conjugated rabbit anti-rat IgG. Both IFN- $\gamma$ R<sup>-/-</sup> mice (A) and wild-type (IFN- $\gamma$ R<sup>+/+</sup>) mice (B) demonstrated similar frequencies of IFN- $\gamma$ -containing cells, whereas more IL-4-containing cells were found in the IFN- $\gamma$ R<sup>-/-</sup> mice (C) than in the wild-type mice (D). The tissues were examined by light microscopy at ca.  $\times 20$  magnification.

TABLE 2. Distribution of CD4<sup>+</sup>, CD8<sup>+</sup>, IL-4, or IFN- $\gamma$ -containing cells in the genital tract of *C. trachomatis*-infected IFN- $\gamma$ R<sup>-/-</sup> and wild-type mice<sup>a</sup>

Mice	No. of labelled cells (mean $\pm$ SD)			
	CD4 <sup>+</sup>	CD8 <sup>+</sup>	IFN- $\gamma$	IL-4
IFN- $\gamma$ R <sup>-/-</sup>	464 $\pm$ 137	77 $\pm$ 30	34 $\pm$ 23	223 $\pm$ 76
IFN- $\gamma$ R <sup>+/+</sup>	432 $\pm$ 86	94 $\pm$ 45	27 $\pm$ 13	74 $\pm$ 20

<sup>a</sup> The relative distribution of T-cell subsets in the uterus following resolution of an ascending *C. trachomatis* infection in the genital tract is shown. IFN- $\gamma$ R<sup>-/-</sup> and wild-type mice were killed 35 to 45 days after a challenge reinoculation with live chlamydiae. Immunohistochemical analysis was performed on frozen tissue sections with labelled antibodies directed against CD4<sup>+</sup>, CD8<sup>+</sup>, IL-4, or IFN- $\gamma$  (see Materials and Methods). The tissues were examined by light microscopy at  $\times 20$  magnification. Light-microscopic cross sections were evaluated, and three sections were counted per mouse. The values represent the mean number of labelled cells  $\pm$  SD of three mice per group. These are results from one representative experiment of three giving similar results. Uninfected mice had fewer than five labelled cells of any kind per cross section.

to neutralize infectivity supports the assumption that no functional difference existed between local IgA and IgG antibodies in IFN- $\gamma$ R<sup>-/-</sup> and wild-type mice (1, 9, 11, 25, 44, 55). Moreover, information as to whether epitope-specific IgA antibodies generated in the absence or presence of IFN- $\gamma$  would be functionally different is lacking, and no report to date has demonstrated that mice deficient for IFN- $\gamma$  develop antibody responses characterized by poor neutralizing ability. On the contrary, we recently found in cholera toxin-immune IFN- $\gamma$ R<sup>-/-</sup> mice strong specific intestinal IgA responses, which were associated with antitoxic protection in ligated loops of a magnitude comparable to that seen in wild-type mice (23). The issue of neutralization by antibodies to chlamydiae also remains controversial since some previous studies have failed to find a correlation between neutralization and host protection against genital tract infection (11).

Our results are at apparent variance with earlier studies showing that increased levels of specific antibodies in secretions, especially antichlamydial IgA, were associated with the resolution of a primary infection or resistance against reinfection (7, 12, 29, 36, 37), and in the guinea pig model, passive transfer of antibodies has been found to significantly reduce chlamydial shedding in the genital tract (37). By contrast, despite high antichlamydial antibody levels, no reduction of chlamydial shedding in reinoculated IFN- $\gamma$ R<sup>-/-</sup> mice was found. A similar observation was reported by Cotter and coworkers by use of the hybridoma backpack model (11). They found that anti-MOMP IgA or IgG had only marginal effects on colonization and shedding while the antibodies appeared to protect against the immunopathology of an ascending infection (11). We did not specifically evaluate the inflammatory response to a second infection with chlamydiae in IFN- $\gamma$ R<sup>-/-</sup> mice. Nevertheless, none of the parameters we studied, i.e., the presence of T cells or IgA- and cytokine-containing cells or the extent of acquired infertility, would indicate that the local antichlamydial antibodies significantly protected against the immunopathology caused by an ascending infection. The most likely explanation for the discrepancy between the present results and those of earlier studies is the fact that immune protection against a challenge infection in previous studies was investigated in animals with a functioning IFN- $\gamma$  system, whereas we evaluated the protective activities of local antibodies in the complete absence of this important system. Furthermore, it may be that local IgA antibodies would have shown some modest protective effect had we chosen to use a less severe challenge. Thus, Cotter et al. showed that IgA antibody had a

small effect on chlamydial colonization following infection with 5 ID<sub>50</sub> of *C. trachomatis* that was not observed at 50 or 500 ID<sub>50</sub> (11). Notwithstanding this, in the context of these experiments and the severe challenge used, it is clear that IFN- $\gamma$ , and not local antibody, was the critical factor. Also, in the normal animal, the release of IFN- $\gamma$  into the infected tissue may occur early on in colonization as part of the inflammatory response. The source for this protective cytokine might be natural killer (NK) cells rather than chlamydia-specific T cells (52). Such a sequence of events has been found to be critical in host protection against facultatively intracellular bacteria such as *Listeria monocytogenes*, where production of the proinflammatory cytokine tumor necrosis factor alpha and IL-12 triggers secretion of IFN- $\gamma$  by NK cells (3). Whether NK cells are involved in host resistance against chlamydial infection, as has been suggested, needs further investigations (52).

Taken together, our findings showed that local antichlamydial antibody in the absence of a functioning IFN- $\gamma$  system provided no or poor protection against chlamydial infection. With regard to seemingly conflicting findings in other studies, we are aware that data obtained with mice may not apply completely to other species; therefore, e.g., protective immunity against chlamydiae in guinea pigs may still rely more heavily on local antibody production than such immunity in mice does (37). Moreover, it should be noted that a majority of the IFN- $\gamma$ R<sup>-/-</sup> mice resolved the genital tract infection, implying that factors other than IFN- $\gamma$  can salvage host resistance against chlamydiae. Thus, we believe that IFN- $\gamma$  plays a key role in protective immunity, i.e., in preventing infection in the immune animal, but clearly, other factors influence clearance of an ongoing infection. One such factor may be tumor necrosis factor alpha (13, 51), which was also found locally in the infected genital tract of the IFN- $\gamma$ R<sup>-/-</sup> mice (unpublished observation). Importantly, our study does not exclude a protective role for local antibodies. Rather, our results with IFN- $\gamma$ R<sup>-/-</sup> mice argue that local antibodies play a subordinate role to T cells and IFN- $\gamma$  in host immune protection against an ascending genital tract infection with *C. trachomatis*. This notion is further corroborated by studies showing that mice made B-cell deficient by anti-IgM treatment, with little antibody response but with a functioning IFN- $\gamma$  system, can still develop protection against genital tract infection with chlamydiae (35).

Adoptive transfer experiments in *nu/nu* and normal mice have unequivocally demonstrated that T cells, in particular, CD4<sup>+</sup> T cells, are essential for the development of protective immunity against *C. trachomatis* (34, 43). However, apart from two studies by Cain and Rank and Su and Caldwell, no previous study has addressed the relationship between immune protection and the degree of Th1 and Th2 activities in the chlamydia-infected genital tract tissue (8, 43). After a secondary genital tract infection, up-regulated CD4<sup>+</sup> T-cell numbers were found, with an increase in IL-4-containing cells in IFN- $\gamma$ R<sup>-/-</sup> mice, while unaltered frequencies of IFN- $\gamma$ -containing cells were found in IFN- $\gamma$ R<sup>-/-</sup> mice compared to that in wild-type mice. Therefore, we may conclude that even strong Th2 activity in the genital tract immune response, in the absence of an IFN- $\gamma$  system, cannot confer protection against reinfection with chlamydiae. By contrast, chlamydia-protective Th1 CD4<sup>+</sup> T cells produce IFN- $\gamma$ , and several previous studies have documented a strong correlation between IFN- $\gamma$  production and host resistance against infection (27, 38, 50, 54). Indeed, treatment of chlamydia-infected *nu/nu* mice, which lack  $\alpha\beta$  T-cell-receptor-positive CD4<sup>+</sup> T cells, with IFN- $\gamma$  resolved the genital tract infection in some of the mice (38). The present study extends this information by demonstrating in vivo that irrespective of the presence of both CD8<sup>+</sup> and CD4<sup>+</sup> T cells in the genital

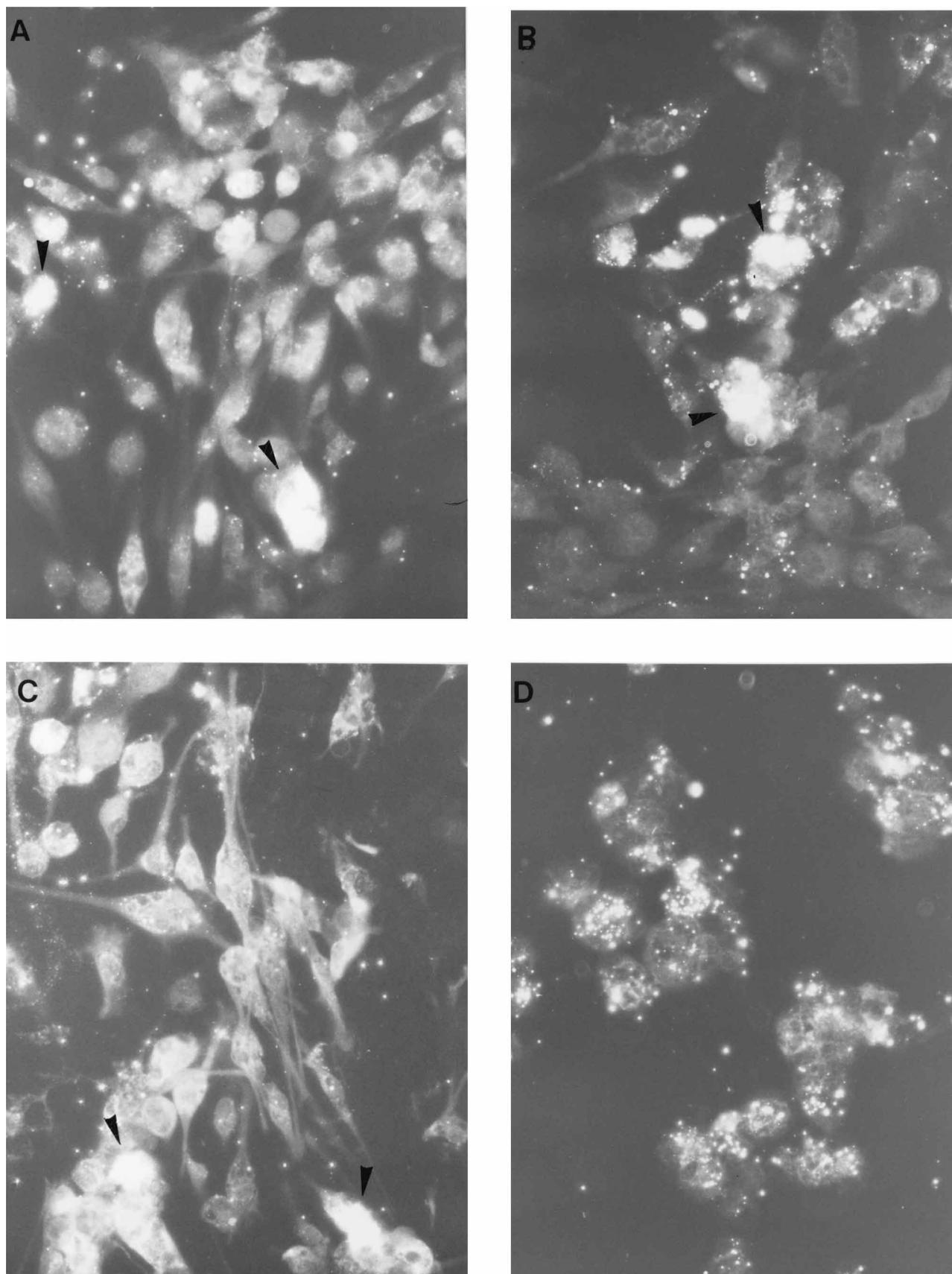


FIG. 8. Macrophages from IFN- $\gamma$ <sup>-/-</sup> mice fail to control chlamydial growth in vitro. PM $\phi$  were cultured with or without addition of rIFN- $\gamma$  for 3 days. Thereafter, the cells were stained with fluorescein-labelled antichlamydial antibody with the Syva kit and examined for the presence of IB (arrowheads) by fluorescence microscopy at  $\times 40$  magnification. The presence of IB in macrophages from IFN- $\gamma$ <sup>-/-</sup> mice treated with IFN- $\gamma$  (A) was unaltered compared with macrophages cultured in plain medium (B), whereas wild-type cells demonstrated no IB in the presence of IFN- $\gamma$  (D), in contrast to the untreated macrophages containing multiple IB (C).

TABLE 3. Macrophages isolated from IFN- $\gamma$ R<sup>-/-</sup> mice fail to respond with NO production to a chlamydial infection<sup>a</sup>

Mouse IFN- $\gamma$ R status	Infected	NO concn produced ( $\mu$ M) <sup>b</sup> in presence of:					
		No additive	rIFN- $\gamma$	rIFN- $\gamma$ -L-NMA	LPS	LPS-rIFN- $\gamma$	LPS-rIFN- $\gamma$ -L-NMA
+/+	Yes	0	62.7 $\pm$ 3.5	7.8 $\pm$ 0.3	0	64.5 $\pm$ 14.3	16.5 $\pm$ 2.2
+/+	No	0	21.8 $\pm$ 3.7	NT <sup>c</sup>	0	77.0 $\pm$ 16.4	13.7 $\pm$ 5.1
-/-	Yes	0	0	0	0	0	0
-/-	No	0	0	0	0	0	0

<sup>a</sup> The production of NO by PM $\phi$  in the presence or absence of *C. trachomatis* was assessed after 72 h in culture with or without the following additives: 10  $\mu$ g of LPS per ml, 100 U of rIFN- $\gamma$  per ml, or 1 mM of the NO synthase inhibitor L-NMA added alone or in combination as indicated. The production of NO was measured with a spectrophotometer as the concentration of NO<sub>2</sub> in supernatants after the addition of Greiss reagent (14).

<sup>b</sup> The micromolar concentrations of NO<sub>2</sub> are expressed as means  $\pm$  SD of triplicate cultures and calculated from a standard curve generated with absorbance values from known concentrations of NaNO<sub>2</sub>. These results are from one representative experiment of three giving similar results.

<sup>c</sup> NT, not tested.

tract, IFN- $\gamma$  appears to be the principal protective factor in host resistance against chlamydiae. Although IFN- $\gamma$  was shown to affect chlamydia-specific CTL activity (42), previous studies found that CTL activity against other intracellular pathogens was unaffected in IFN- $\gamma$ -deficient mice (10, 18). Since MHC class I-restricted CD8<sup>+</sup> T cells occurred as frequently in the chlamydia-infected tissue of IFN- $\gamma$ R<sup>-/-</sup> mice as they did in wild-type mice, we believe it is unlikely that cytotoxicity plays a major role in immune protection against chlamydiae (4, 20, 42). This is further supported by the demonstration of intact immune protection against reinfection with chlamydia in MHC class I-deficient mice (29). Rather, IFN- $\gamma$  probably has a direct effect on epithelial cells and other infected cells. This notion was also corroborated by our findings in vitro showing that peritoneal macrophages from IFN- $\gamma$ R<sup>-/-</sup> mice did not produce NO and failed to control chlamydial growth in the presence of IFN- $\gamma$ . The central role of IFN- $\gamma$  in the control of chlamydial growth in infected cells in vitro has been documented by several other groups (27, 38, 50, 54). Whether the role is to stimulate inducible NO synthase or to affect tryptophan metabolism via indoleamine-2,3-dioxygenase or both cannot be discerned from the present study since both events are regulated by IFN- $\gamma$  (5, 19, 31). Additional studies in mice made genetically deficient for inducible NO synthase may help clarify this point (50).

#### ACKNOWLEDGMENTS

We are grateful to Sylvia Everson and Marie Bengtsson for skilled technical assistance. We also thank the staff in the Diagnostic Bacteriology Section of the Sahlgrenska Hospital in Göteborg, Sweden, for continuous support in screening for chlamydial shedding.

The study was supported by grants from the WHO GPV-Transdisease-programme, the Swedish Medical Research Council, SAREC, and in Southampton, United Kingdom, by the UK Medical Research Council.

#### REFERENCES

- Allen, J. E., R. M. Locksley, and R. S. Stephens. 1991. A single peptide from the major outer membrane protein of *Chlamydia trachomatis* elicits T cell help for the production of antibodies to protective determinants. *J. Immunol.* **147**:674-679.
- Andersson, J., J. Abrams, L. Björk, K. Funä, M. Litton, and K. Ågren. 1994. Concomitant *in vivo* production of 19 different cytokines in human tonsils. *Immunology* **83**:16.
- Bancroft, G. J. 1993. The role of natural killer cells in innate resistance to infection. *Curr. Opin. Immunol.* **5**:503-510.
- Beatty, P. R., and R. Stephens. 1994. CD8<sup>+</sup> T lymphocyte-mediated lysis of *Chlamydia*-infected L cells using an endogenous antigen pathway. *J. Immunol.* **153**:4588.
- Beatty, W. L., T. A. Belanger, A. A. Desai, R. P. Morrison, and G. I. Byrne. 1994. Tryptophan depletion as a mechanism of gamma interferon-mediated chlamydial persistence. *Infect. Immun.* **62**:3701-3711.
- Brandtzaeg, P., E. Christiansen, F. Muller, and K. Purvis. 1993. Humoral immune response patterns of human mucosae, including the reproductive tracts, p. 97-130. *In* P. D. Griffin and P. M. Johnson (ed.), *Local immunity in reproductive tract tissues*. Oxford University Press, Oxford, United Kingdom.
- Brunham, R. C., C.-C. Kuo, L. Cles, and K. K. Holmes. 1983. Correlation of host immune response with quantitative recovery of *Chlamydia trachomatis* from the human endocervix. *Infect. Immun.* **39**:1491-1494.
- 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**:1784-1789.
- Conlan, J. W., I. N. Clarke, and M. E. Ward. 1988. Epitope mapping with solid phase peptides: identification of type-reactive, subspecies-reactive, species-reactive and genus-reactive antibody binding domains on the major outer membrane protein of *Chlamydia trachomatis*. *Mol. Microbiol.* **2**:673-679.
- Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme. 1993. Disseminated tuberculosis in interferon  $\gamma$  gene-disrupted mice. *J. Exp. Med.* **178**:2243-2247.
- Cotter, T. W., Q. Meng, Z.-L. Shen, Y.-X. Zhang, H. Su, and H. D. Caldwell. 1995. Protective efficacy of major outer membrane protein-specific immunoglobulin A (IgA) and IgG monoclonal antibodies in a murine model of *Chlamydia trachomatis* genital tract infection. *Infect. Immun.* **63**:4704-4714.
- Cui, Z.-D., D. Tristram, L. J. LaScola, T. Kwiatkowski, Jr., S. Kopti, and P. L. Ogra. 1991. Induction of antibody response to *Chlamydia trachomatis* in the genital tract by oral immunization. *Infect. Immun.* **59**:1465-1469.
- Darville, T., K. K. Laffoon, L. R. Kishen, and R. Rank. 1995. Tumor necrosis factor alpha activity in genital tract secretions of guinea pigs infected with chlamydiae. *Infect. Immun.* **63**:4675-4681.
- Green, S. J., J. Anigolou, and J. J. Raney. 1994. Oxidative metabolism of murine macrophages, p. 14.5.1-14.5.11. *In* J. E. Coligan (ed.), *Current protocols in immunology*. John Wiley & Sons, Inc., New York, N.Y.
- Haneberg, B., D. Kendall, H. M. Amerongen, F. M. Apter, J.-P. Kraehenbuhl, and M. R. Neutra. 1994. Induction of specific immunoglobulin A in the small intestine, colon-rectum, and vagina measured by a new method for collection of secretions from local mucosal surfaces. *Infect. Immun.* **62**:15-23.
- Hayes, L. J., R. L. Bailey, D. C. W. Mabey, I. N. Clarke, M. A. Pickett, P. J. Watt, and M. E. Ward. 1992. Genotyping of *Chlamydia trachomatis* from trachoma-endemic village in the Gambia by a nested polymerase chain reaction: identification of strain variants. *J. Infect. Dis.* **166**:1173-1177.
- Hayes, L. J., S. Pecharatana, R. L. Bailey, T. J. Hampton, M. A. Pickett, D. C. W. Mabey, P. J. Watt, and M. E. Ward. 1995. Extent and kinetics of genetic change in the *omp1* gene of *Chlamydia trachomatis* in 2 villages with endemic trachoma. *J. Infect. Dis.* **172**:268-272.
- Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R. M. Zinkernagel, and M. Auger. 1993. Immune response in mice that lack the interferon- $\gamma$  receptor. *Science* **259**:1739.
- Igietseme, J. U. 1996. The molecular mechanism of T-cell control of *Chlamydia* in mice: role of nitric oxide. *Immunology* **87**:1-8.
- Igietseme, J. U., D. M. Magee, D. M. Williams, and R. G. Rank. 1994. Role for CD8<sup>+</sup> T cells in antichlamydial immunity defined by chlamydia-specific T-lymphocyte clones. *Infect. Immun.* **62**:5195-5197.
- Igietseme, J. U., and R. G. Rank. 1991. Susceptibility to reinfection after a primary chlamydial genital infection is associated with a decrease of antigen-specific T cells in the genital tract. *Infect. Immun.* **59**:1346-1351.
- Johansson, M. Unpublished observation.
- Kaufmann, S. H. 1994. Bacterial and protozoal infections in genetically disrupted mice. *Curr. Opin. Immunol.* **6**:518.
- Kjerrulf, M., D. Grdic, M. Vajdy, and N. Lycke. Interferon- $\gamma$  deficient mice exhibit impaired gut mucosal IgA responses but intact oral tolerance. Submitted for publication.
- Lycke, N., A.-K. Bromander, L. Ekman, D. Grdic, E. Hörnquist, M. Kjerrulf, M. Kopf, M. Kosco-Vilbois, K. Schön, and M. Vajdy. 1995. The use of

- knock-out mice in studies of induction and regulation of gut mucosal immunity. *Mucosal Immunol.* Update **3**:1.
25. **MacDonald, A. B., D. McComb, and L. Howard.** 1984. Immune-response of owl monkeys to topical vaccination with irradiated chlamydia-trachomatis. *J. Infect. Dis.* **149**:439–442.
  26. **Magee, D. M., D. M. 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.
  27. **Mayer, J., M. L. Woods, Z. Vavrin, and J. B. Hibbs, Jr.** 1993. Gamma interferon-induced nitric oxide production reduces *Chlamydia trachomatis* infectivity in McCoy cells. *Infect. Immun.* **61**:491–497.
  28. **Mestecky, J., R. Abraham, and P. L. Ogra.** 1994. Common mucosal immune system and strategies for the development of vaccines effective at the mucosal surfaces, p. 357–372. *In* P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee, and J. Bienenstock (ed.), *Handbook of mucosal immunology*. Academic Press, Inc., San Diego, Calif.
  29. **Morrison, R. P., K. Feilzer, and D. B. 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.
  30. **Mosmann, T. R., and S. Sad.** 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today* **17**:138–146.
  31. **Park, Y. C., C. D. Jun, H. S. Kang, H. M. Kim, and H. T. Chung.** 1996. Role of intracellular calcium as a priming signal for the induction of nitric oxide synthesis in murine peritoneal macrophages. *Immunology* **87**:296–302.
  32. **Parr, M. B., and E. L. Parr.** 1994. Mucosal immunity in the female and male reproductive tracts, p. 677–685. *In* P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee, and J. Bienenstock (ed.), *Handbook of mucosal immunology*. Academic Press, Inc., San Diego, Calif.
  33. **Pickett, M. A., M. E. Ward, and I. N. Clarke.** 1988. High-level expression and epitope localization of the major outer-membrane protein of chlamydia-trachomatis serovar-11. *Mol. Microbiol.* **2**:681–685.
  34. **Ramsey, K. H., and R. G. Rank.** 1991. Resolution of chlamydial genital infection with antigen-specific T-lymphocyte lines. *Infect. Immun.* **59**:925–931.
  35. **Ramsey, K. H., L. S. F. Soderberg, and R. G. Rank.** 1988. Resolution of chlamydial genital infection in B-cell-deficient mice and immunity to reinfection. *Infect. Immun.* **56**:1320–1325.
  36. **Rank, R. G., and A. L. Barron.** 1983. Humoral immune response in acquired immunity to chlamydial genital infection of female guinea pigs. *Infect. Immun.* **39**:463–465.
  37. **Rank, R. G., and B. E. Batteiger.** 1989. Protective role of serum antibody in immunity to chlamydial genital infection. *Infect. Immun.* **57**:299–301.
  38. **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.
  39. **Rank, R. G., L. S. F. Soderberg, M. M. Sanders, and B. E. Batteiger.** 1989. Role of cell-mediated immunity in the resolution of secondary chlamydial genital infection in guinea pigs infected with the agent of guinea pig inclusion conjunctivitis. *Infect. Immun.* **57**:706–710.
  40. **Reiner, S. L., and R. A. Seder.** 1995. T helper cell differentiation in immune response. *Curr. Opin. Immunol.* **7**:360–366.
  41. **Robertson, J. N., M. E. Ward, D. Conway, and E. O. Caul.** 1987. Chlamydial and gonococcal antibodies in sera of infertile women with tubal obstruction. *J. Clin. Pathol.* **40**:377–383.
  42. **Starnbach, M. N., M. J. Bevan, and M. Lampe.** 1994. Protective cytotoxic T lymphocytes are induced during murine infection with *Chlamydia trachomatis*. *J. Immunol.* **153**:5183.
  43. **Su, H., and H. D. Caldwell.** 1995. CD4<sup>+</sup> T cells play a significant role in adoptive immunity to *Chlamydia trachomatis* infection of the mouse genital tract. *Infect. Immun.* **63**:3302–3308.
  44. **Su, H., and H. D. Caldwell.** 1992. Immunogenicity of a chimeric peptide corresponding to T helper and B cell epitopes of the *Chlamydia trachomatis* major outer membrane protein. *J. Exp. Med.* **175**:227–235.
  45. **Su, H., R. P. Morrison, N. G. Watkins, and H. D. Caldwell.** 1990. Identification and characterization of T-helper cell epitopes of the major outer-membrane protein of chlamydia-trachomatis. *J. Exp. Med.* **172**:203–212.
  46. **Tuffrey, M., F. Alexander, C. Inman, and M. E. Ward.** 1990. Correlation of infertility with altered tubal morphology and function in mice with salpingitis induced by a human genital-tract isolate of *Chlamydia trachomatis*. *J. Reprod. Fertil.* **88**:295.
  47. **van der Pol, B., J. A. Williams, and R. B. Jones.** 1995. Rapid antigen detection assay for identification of *Chlamydia trachomatis* infection. *J. Clin. Microbiol.* **33**:1920–1921.
  48. **Wakatsuki, Y., and S. Warren.** 1993. Effect of downregulation of germline transcripts on immunoglobulin A isotype differentiation. *J. Exp. Med.* **178**:129–138.
  49. **Ward, M. E.** 1995. The immunobiology and immunopathology of chlamydial infections. *APMIS* **103**:769–796.
  50. **Wei, X.-Q., I. G. Charles, A. Smith, J. Ure, G.-J. Feng, F.-P. Huang, D. Xu, W. Muller, S. Moncada, and F. Y. Liew.** 1995. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* **375**:408.
  51. **Williams, D. M., D. M. Magee, L. F. Bonewald, J. G. Smith, C. A. Bleicker, G. I. Byrne, and J. Schachter.** 1990. A role in vivo for tumor necrosis factor alpha in host defense against *Chlamydia trachomatis*. *Infect. Immun.* **58**:1572–1576.
  52. **Williams, D. M., J. Schachter, and B. Grubbs.** 1987. Role of natural killer cells in infection with the mouse pneumonitis agent (murine *Chlamydia trachomatis*). *Infect. Immun.* **55**:223–226.
  53. **Zhang, Y. X., S. Stewart, T. Joseph, H. R. Taylor, and H. D. Caldwell.** 1987. Protective monoclonal antibodies recognize epitopes located on the major outer membrane protein of *Chlamydia trachomatis*. *J. Immunol.* **138**:575–581.
  54. **Zhong, G., E. M. Peterson, C. W. Czarniecki, R. D. Schreiber, and L. M. de la Maza.** 1989. Role of endogenous gamma interferon in host defense against *Chlamydia trachomatis* infections. *Infect. Immun.* **57**:152–157.
  55. **Zhong, G., and R. C. Brunham.** 1991. Antigenic determinants of the chlamydial major outer membrane protein resolved at a single amino acid level. *Infect. Immun.* **59**:1141–1147.

Editor: J. R. McGhee