

## Dissemination of *Chlamydia trachomatis* Chronic Genital Tract Infection in Gamma Interferon Gene Knockout Mice

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Received 7 January 1997/Returned for modification 28 February 1997/Accepted 29 March 1997

Mice (C57BL/6), treated with progesterone and infected intravaginally with the mouse pneumonitis strain of *Chlamydia trachomatis* (MoPn), acquired genital tract disease that ascended from the endocervix to the uterine horns, oviducts, and ovaries in a temporal fashion before the occurrence of spontaneous microbiological resolution by about 28 days after infection. Surprisingly, dissemination of MoPn in small numbers to draining lymph nodes, the peritoneal cavity, spleen, liver, kidneys, and lungs occurred in normal mice during the early stages of disease (7 to 14 days) in a portion of infected animals but resolved from these tissues, by microbiological criteria, prior to resolution of genital tract involvement. In contrast, gamma interferon knockout (IFN- $\gamma$  KO) mice exhibited dissemination of infection to a greater extent and for longer periods in a variety of tissues, and a portion of infected IFN- $\gamma$  KO mice failed to microbiologically resolve their genital tract disease. By comparison, C57BL/6 SCID mice uniformly failed to resolve their genital tract disease and exhibited high levels of dissemination to all tissues tested for extended (50-day) periods of times. Interestingly, although IFN- $\gamma$  KO mice failed to completely clear organisms from their genital tracts, they exhibited an attenuated infection indistinguishable from that of heterozygous littermates when challenged 112 days after primary infection. These data support a role for IFN- $\gamma$  in containing dissemination of MoPn from the genital tract to extragenital sites and in the microbiological resolution of infection. Data also indicate that IFN- $\gamma$  is not required for modulating reinfections, which normally follow a shorter and less dramatic course.

*Chlamydia trachomatis* continues to be the most frequently reported human sexually transmitted pathogen (6) associated with severe adverse sequelae in women who develop complications of infection as a result of extension from the lower to the upper genital tract (3). The mouse pneumonitis (MoPn) model of *C. trachomatis* genital tract disease has been successfully used by several groups of investigators to provide information regarding ascending complications of chlamydial genital tract infection in females and development of infertility subsequent to acute disease (9, 30, 31).

The use of gene inactivation in murine embryonic stem cells by homologous recombination has been successfully applied to study a variety of developmental processes and genetic disorders in mice (20, 22, 23). Gene knockout strategies also have been used to investigate parameters associated with immune function (2, 19, 36), especially those related to the specific contributions of the immune system to the resolution of various infectious diseases (10, 16, 33).

Morrison et al. studied mice deficient in functional major histocompatibility complex (MHC) molecules or in CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes in the MoPn genital infection model to evaluate cell-mediated immunity (CMI) against *C. trachomatis* genital tract disease (21). This work documents the critical role for functional MHC class II molecules and CD4<sup>+</sup> T lymphocytes in recovery from chlamydial genital tract infection. Williams et al. used  $\gamma$ - $\delta$  T-cell knockout mice to show that this class of T cells plays a protective role early and a deleterious role late in a respiratory model of MoPn infection (35). The immune-regulated cytokine gamma interferon (IFN- $\gamma$ ) is a

T-cell and natural killer (NK) cell product that has been implicated in the control of acute stages of primary chlamydial genital tract disease (12–14, 18, 25, 28, 29) and in the development of chronic stages of infection (1). Gene knockout mice have been developed to study the role of IFN- $\gamma$  in immunity. Animals have been produced that lack either a functional IFN- $\gamma$  receptor gene (11) or the IFN- $\gamma$  gene itself (8). Studies were therefore initiated to examine the effects of IFN- $\gamma$  on the development and resolution of upper genital tract disease in the MoPn genital tract infection model. The results provide evidence suggesting that although IFN- $\gamma$  knockout (IFN- $\gamma$  KO) animals develop a normal degree of relative protection against second-challenge MoPn infections, IFN- $\gamma$  contributes demonstrably to the resolution of primary MoPn infection and containment of dissemination of this organism beyond the genital tract.

### MATERIALS AND METHODS

**Chlamydia.** *Chlamydia trachomatis* MoPn (Weiss) was grown in HeLa 229 cells, and elementary bodies were purified from infected cells by density gradient centrifugation as previously described (5).

**Mice.** IFN- $\gamma$  KO breeding pairs (8) provided to William Weidanz (University of Wisconsin) by D. Dalton (Genentech Inc., San Francisco, Calif.) were maintained and bred at the University of Wisconsin Gnotobiotic Laboratory. The mice used in these studies were backcrossed five times to the C57BL/6 background and therefore were not inbred. Homozygous IFN- $\gamma$  KO mice and heterozygous littermates were obtained from this colony. C57BL/6 SCID mice and their normal counterparts (C57BL/6J) were obtained from Jackson Laboratories, Bar Harbor, Maine. For experiments involving the characterization of MoPn dissemination in normal mice at 7, 10, and 14 days postinfection, C57BL/6NHsd mice were obtained from Harlan Sprague Dawley, Indianapolis, Ind.

**Genotyping of IFN- $\gamma$  KO mice.** When mice were 3 to 4 weeks of age, tail snips were obtained and total genomic DNA was purified by using the QIAamp Tissue Kit (Qiagen Inc., Chatsworth, Calif.) in accordance with the manufacturer's instructions. The resultant DNAs were tested simultaneously for the presence of the IFN- $\gamma$  gene and the gene coding for neomycin resistance by thermal amplification with specific primer pairs (32). Primers neo-204 (5' GCTATTGGCT ATGACTGGG 3') and neo-909 (5' GAAGCGATAGAAGGCGATG 3')

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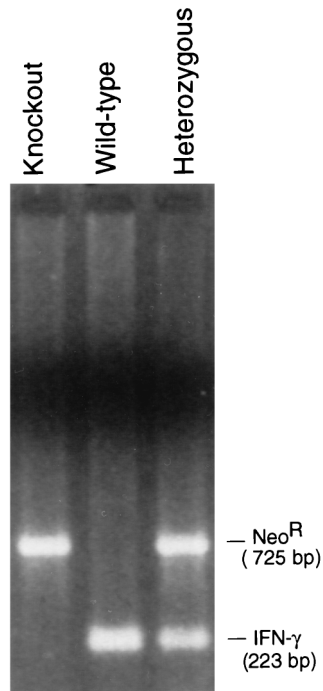


FIG. 1. Determination of IFN- $\gamma$  genotype. Shown is an ethidium bromide-stained 1.3% agarose gel on which were electrophoresed thermal-amplification products from genomic DNAs obtained from normal, IFN- $\gamma$  KO, and IFN- $\gamma$  heterozygous littermates. Neo<sup>R</sup>, neomycin resistance cassette.

were used to amplify a 725-bp product from the neomycin resistance gene, and primers GIFN 3 (5' AAGTGGCATAGATGTGGAAG 3') and GIFN 4 (5' GAATGCATCCTTTTTCGCCT 3') were used to amplify a 223-bp product from the normal IFN- $\gamma$  gene. Amplification reactions (reaction volume, 100  $\mu$ l; containing 10  $\mu$ l of a DNA sample) employed the Hot Start technique using paraffin beads (AmpliWax PCR Gems; Perkin-Elmer, Branchburg, N.J.) and were run in a PHC-3 thermocycler (Technic Inc., Princeton, N.J.) under the following cycling conditions: 40 cycles of 1 min at 94°C, 1 min at 60°C, and 1.5 min at 72°C. PCR amplification of DNA from mice homozygous for the wild-type or inactivated IFN- $\gamma$  gene yielded a single band migrating at either 223 or 725 bp, respectively, while DNA from heterozygous mice yielded both bands (Fig. 1). The gel image shown in Fig. 1 was scanned and cropped by using Adobe Photoshop 3.0.5 software, assembled with Canvas 3.5.4 software, and reproduced by dye sublimation printing.

**Infection of mice.** All mice were pretreated with progesterone and infected at 7 to 11 weeks of age. MoPn stocks were diluted in SPG (10 mM phosphate [pH 7.2], 0.25 M sucrose, and 5 mM L-glutamic acid) so that 4,800 inclusion-forming units (IFU) were contained in 5  $\mu$ l. This inoculum represents 100–50% infectious doses (ID<sub>50</sub>s) for progesterone-pretreated C57BL/6 mice (6a). This volume was inoculated into the vaginal vault with a micropipettor on day 0. Progesterone (Depo-Provera; Upjohn, Kalamazoo, Mich.) pretreatment consisted of two subcutaneous injections, each consisting of 2.5 mg of progesterone in 0.1 ml of saline, given 10 and 3 days prior to infection. Infection was confirmed and MoPn shedding was monitored by collection of vaginal swab specimens (Calgiswab, type 1; Spectrum Diagnostics, Houston, Tex.) in 0.5 ml of SPG (with two 4-mm-diameter glass beads) at the times indicated below; these specimens were frozen at –80°C until assayed for MoPn infectivity. Challenge infections of IFN- $\gamma$  KO and IFN- $\gamma$  heterozygous mice were given at 112 days postinfection following progesterone pretreatment as described for the primary infection.

**Characterization of MoPn genital tract infection and study of MoPn dissemination in IFN- $\gamma$  KO, IFN- $\gamma$  heterozygous, C57BL/6 SCID, and C57BL/6 wild-type mice.** Both strains of immunodeficient mice and their immunocompetent counterparts were inoculated with MoPn, and the course of primary infection was assessed by collection of vaginal swab specimens on days 4, 7, 10, and 14 postinfection and weekly thereafter. Dissemination of MoPn genital tract infection was investigated by sacrificing groups of animals (see Fig. 4 for group sizes) at 10, 23, 38, and 50 days postinfection and collecting various tissues for determination of MoPn infectivity. The lungs, liver, kidneys, and spleen were each placed in 1 ml of SPG and frozen at –80°C for future analysis. Genital tracts were removed and fixed in 10% buffered formalin for histopathological evaluation. C57BL/6 SCID and C57BL/6 wild-type mice were studied through day 50

postinfection, at which point all mice had been utilized. IFN- $\gamma$  KO and IFN- $\gamma$  heterozygous mice were followed through day 147 postinfection so that protection from challenge could be addressed.

**Dissemination routes in wild-type (normal) mice.** To discriminate between internal dissemination from the genital tract to distal tissues and (i) horizontal spread from mouse to mouse, (ii) autoinfection, or (iii) environmental transmission and subsequent dissemination to extragenital organs, the following set of experiments was performed. Mice were separated into the following groups: group A, 24 animals housed individually without grooming collars; group B, 24 animals housed individually and fitted with Elizabethan collars (EJAY International, Glendora, Calif.) to prevent self-grooming; group C, 3 groups of 8 mice each housed in large rodent cages and rotated daily into cages containing dirty litter from cages containing infected mice; and groups D and E, 6 groups of 8 mice each housed in large rodent cages, with 4 mice of each set of 8 being infected (group D) and 4 being uninfected (group E). Infection was confirmed by collecting vaginal swabs on day 4 postinfection. At 7, 10, and 14 days postinfection, eight animals from each group were sacrificed. Vaginal swab, peritoneal lavage, lung, heart, liver, kidney, spleen, iliac lymph nodes, and upper genital tract tissue specimens were collected from animals in groups A, B, and C, while specimens of the same tissues plus mesenteric lymph nodes were collected from animals in groups D and E. Each tissue specimen was placed in 1 ml of SPG and frozen until it was assayed for MoPn infectivity.

**Measurement of MoPn infectivity from vaginal swabs and tissue samples.** Swab samples were thawed and vortexed vigorously, and recovery of infectivity was quantified by inoculating swab material onto DEAE-dextran (45  $\mu$ g per ml; Sigma, St. Louis, Mo.)-pretreated HeLa 229 cell monolayers in 96- and 24-well microtiter plates as described previously (7). Samples were refrozen at –80°C and stored for use in confirmatory cultures as needed. After 42 to 44 h of incubation at 37°C in an atmosphere of 5% CO<sub>2</sub>, the plates were washed with phosphate-buffered saline and fixed with methanol, and chlamydial inclusions were visualized by staining with the genus-specific monoclonal antibody 14M-3-B9 (generously provided by Harlan Caldwell, Rocky Mountain Laboratories, Hamilton, Mont.) and a fluorescein isothiocyanate-labeled anti-mouse immunoglobulin secondary antibody (Organon Teknica Corp., Durham, N.C.). Recovery was determined by counting inclusions viewed through an inverted fluorescence microscope.

Frozen tissue samples were thawed and maintained on an ice throughout processing. Each sample was homogenized for 30 to 40 s (Ultra-Turrax T25; Janke & Kunkel IKA-Labortechnik, Staufen, Germany) and sonicated for 20 s (Sonifier 250; Branson Ultrasonics Corp., Danbury, Conn.). Such suspensions were centrifuged for 10 min at 500  $\times$  g to clear the supernatants of large debris. Aliquots (0.4 ml) of supernatant were inoculated in duplicate onto DEAE-dextran-pretreated HeLa 229 cell monolayers in 24-well cell culture plates. Inoculum-containing trays were centrifuged at 500  $\times$  g for 1 h at 30°C; this was followed by a 30-min incubation at 37°C on a rocking platform. Samples were then removed by aspiration, and the monolayers were washed once with 0.5 ml of Hanks' balanced salts solution to remove adherent debris and then fed with 1 ml of minimum essential medium containing 10% fetal bovine serum, 10  $\mu$ g of gentamicin per ml, 50  $\mu$ g of vancomycin per ml, and 0.7  $\mu$ g of cycloheximide per ml. The trays of cells were fixed, and inclusions were visualized as described above for the swabs specimens.

**Histopathological evaluation of tissue samples.** Formalin-fixed genital tract tissues collected at 10, 23, 38, and 50 days postinfection were evaluated for histopathological changes by a veterinary pathologist at the University of Wisconsin Research Animal Resources Center. Two infected IFN- $\gamma$  KO mice and two and C57BL/6 SCID mice from each time point were compared to infected IFN- $\gamma$  heterozygous and normal C57BL/6 mice, respectively, with regard to alterations in the inflammatory response to MoPn genital tract infection. Longitudinal sections were stained with hematoxylin and eosin and graded for the type and intensity of inflammatory infiltrate in the vagina, uterus, oviducts, and ovaries.

## RESULTS

**Primary genital tract infection in C57BL/6 wild-type, C57BL/6 SCID, IFN- $\gamma$  KO, and heterozygous IFN- $\gamma$  KO littermates.** Progesterone-pretreated animals were infected with 100 ID<sub>50</sub>s of MoPn intravaginally as described in Materials and Methods. Although not required for genital tract infection by MoPn (26), progesterone pretreatment was used to ensure a 100% infection rate and to allow for use of a relatively low infecting dose. This route of infection is known to cause upper genital tract spread of the organism by 7 to 10 days after infection (7). The animals were monitored for the presence of MoPn by collection of vaginal swabs at specified intervals. The data presented in Fig. 2 show that both normal C57BL/6 and C57BL/6 SCID mice develop an active infection as evidenced by the increase in organisms shed over the initial inoculum size

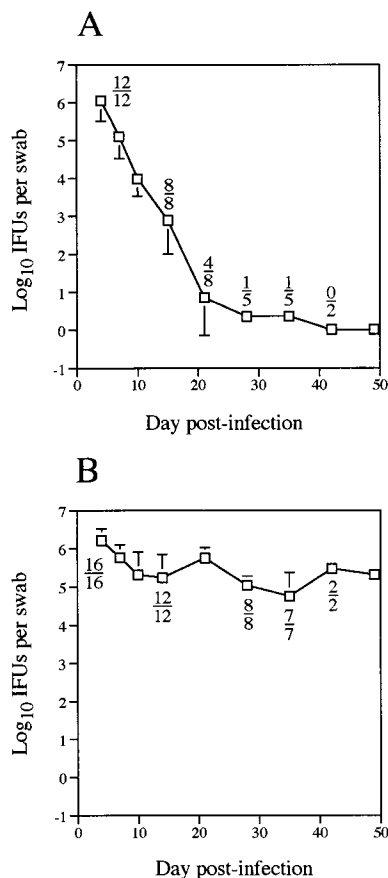


FIG. 2. Course of MoPn genital tract infection in C57BL/6J mice (A) and C57BL/6 SCID mice (B). The data represent the recovery of infectivity from vaginal swab specimens collected at various times postinfection. The ratios indicate the number of culture-positive mice over the total number of mice swabbed at a given time point. The total number of mice (denominators) decreased over time because animals were intermittently sacrificed to study disseminated infection. When no fraction is indicated, it should be assumed that the value for that time point is the same as that in the preceding ratio. The bars represent standard deviations.

of  $4.8 \times 10^3$  organisms to more than  $10^6$  organisms shed per swab by 4 days after infection. C57BL/6 SCID animals, however, were incapable of clearing infection during the entire 50 days of observation, whereas strain-matched wild-type mice showed little shedding by 28 days after infection and were chlamydia negative by 42 days postinfection. The group sizes decreased with time due to the fact that animals were removed from the study to evaluate the tissue distribution of MoPn at specified times after intravaginal infection.

The data presented in Fig. 3 show that littermates heterozygous for the inactivated IFN- $\gamma$  gene exhibit shedding patterns similar to those observed in wild-type C57BL/6 animals. In contrast, IFN- $\gamma$  KO animals showed continuous shedding throughout the study period, although the proportion of shedding animals decreased with time, as did the amount of MoPn shed per swab. A few of the IFN- $\gamma$  KO mice cleared the infection within 28 days, but this capacity could not be correlated with any other parameter, including breeding history. Shedding levels were low for the IFN- $\gamma$  KO mice remaining after 50 days, and some animals were alternately culture positive and culture negative. From days 15 to 45 postinfection, 37% of the IFN- $\gamma$  KO mice developed systemic illness as evidenced by their ruffled fur, hunched posture, and elevated

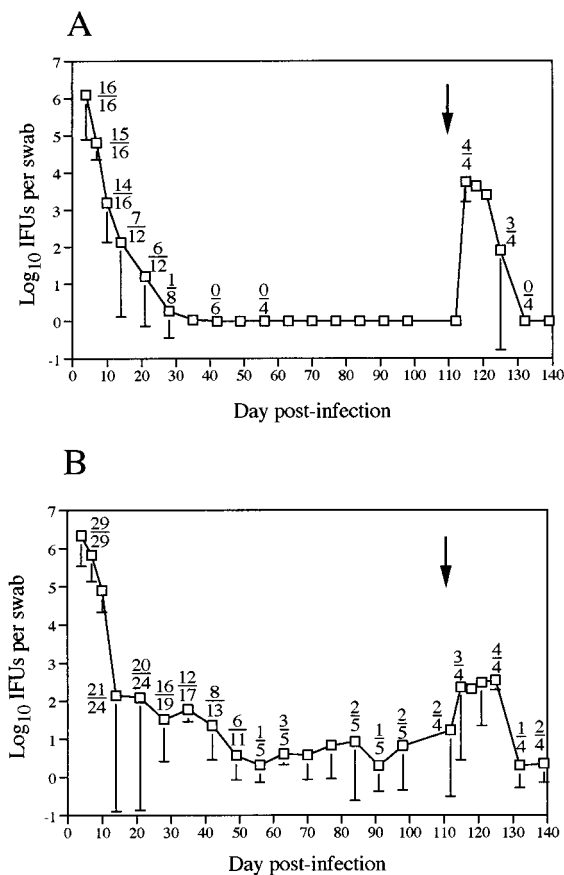


FIG. 3. Course of MoPn genital tract infection in IFN- $\gamma$  heterozygous mice (A) and IFN- $\gamma$  KO mice (B). The data are presented as described in the legend to Fig. 2. The time at which challenge inoculations were given (112 days postinfection) is indicated by vertical arrows. The bars represent standard deviations.

breathing rate. A few of these animals died as a result of this illness, but the exact death rate could not be determined because numerous animals were sacrificed during this period for evaluation of disseminated infection. Those IFN- $\gamma$  KO mice that suffered illness but did not die recovered spontaneously. In such mice, the occurrence of illness did not correlate with clearance of genital tract infection or any other parameter. Such illness was never observed in any other mouse strain following MoPn infection, nor was it seen in eight uninfected IFN- $\gamma$  KO mice used as sentinels (data not shown). This fact indicates that the illness was not due to the presence of a pathogen in the cage environment and that it was most likely a result of chlamydial infection, although we did not test for other pathogens, such as coronavirus. Furthermore, the control IFN- $\gamma$  mice were not mock infected, so the presence of other pathogens in the MoPn inoculum cannot be ruled out. Interestingly, when all four remaining IFN- $\gamma$  KO mice (two culture positive and two culture negative) were challenged with a second intravaginal inoculation of MoPn, as indicated by the vertical arrows in Fig. 3, a relative degree of protection similar to that observed in heterozygous animals was seen. This relative protection included a reduced peak shedding level of MoPn organisms and a protracted shedding time, although IFN- $\gamma$  KO mice were unable to completely eradicate the infection in a way similar to that observed subsequent to primary infection.

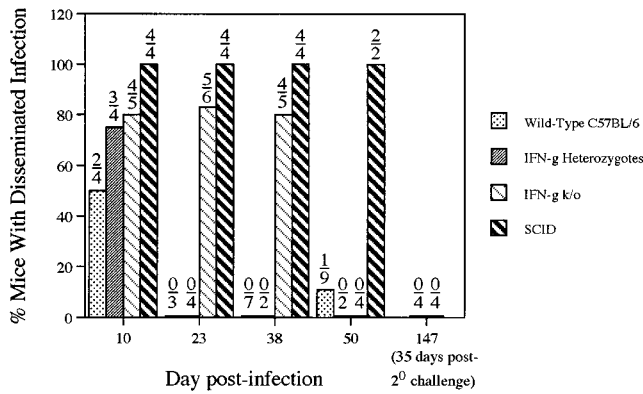


FIG. 4. Time course of disseminated MoPn infection in C57BL/6 SCID and IFN- $\gamma$  KO mice, with normal C57BL/6 and IFN- $\gamma$  heterozygous mice included as controls. Only IFN- $\gamma$  KO and heterozygous mice were tested at 147 days postinfection (35 days post-secondary infection). The results were calculated from the ratios of the numbers of mice showing dissemination to the different sites tested (lungs, liver, kidneys, and spleen) to the total numbers of animals examined (shown above the bars).

**Dissemination of MoPn to extragenital tissues following intravaginal infection.** Some animals from infected groups were sacrificed at various times after infection, and selected tissues were collected and processed to evaluate for the presence of MoPn. Evidence of disseminated infection was obtained for IFN- $\gamma$  KO and C57BL/6 SCID mice extending to 38 and 50 days postinfection (the full term of the study), respectively (Fig. 4). Dissemination of MoPn genital tract infection in IFN- $\gamma$  KO mice has recently been independently documented by Perry and Caldwell (22a). Surprisingly, disseminated infection was also documented for IFN- $\gamma$  heterozygotes and wild-type C57BL/6 mice at the earliest time point tested (10 days postinfection). To more precisely define dissemination of MoPn following intravaginal infection, the distribution of infectious organisms was measured in the lungs, liver, kidneys, and spleen at various times after infection (Fig. 5). Small numbers of MoPn IFU were found in the lungs and liver at 10 days postinfection for all groups of animals tested. Chlamydiae were isolated from all organs tested for knockout and SCID mice for the first 38 days of infection, whereas, with one exception,

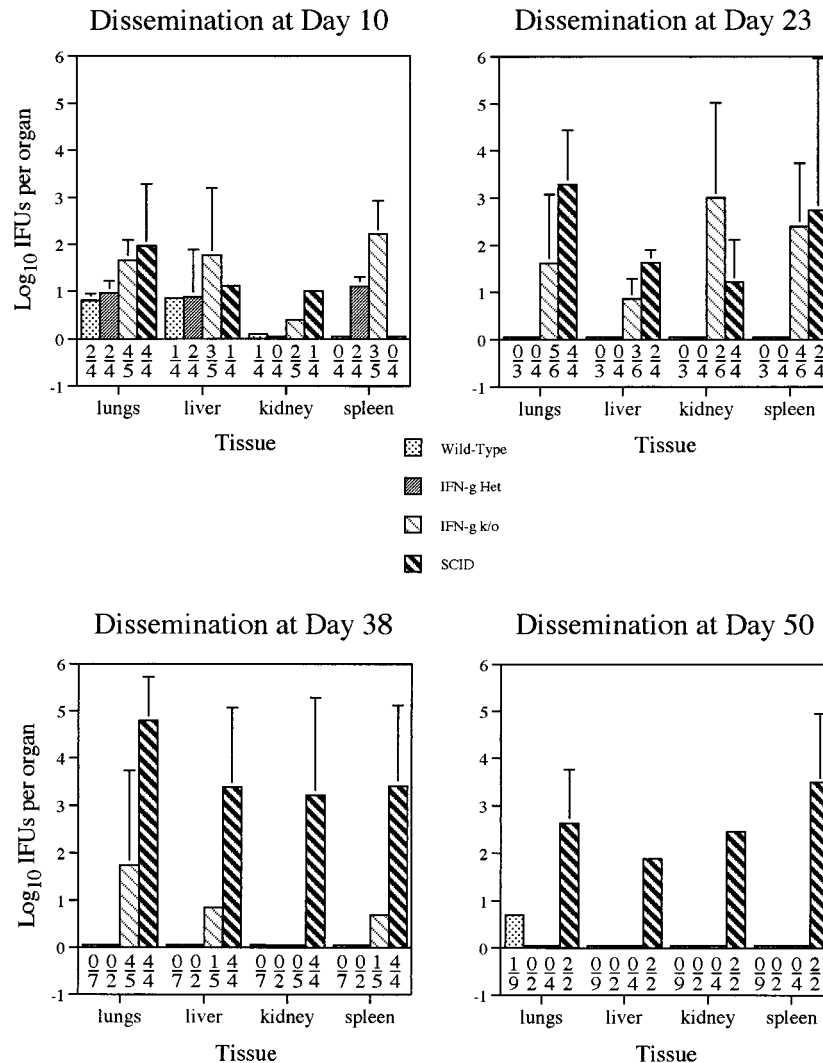


FIG. 5. Quantitative recovery of MoPn from extragenital sites at 10, 23, 38, and 50 days postinfection in normal C57BL/6, IFN- $\gamma$  heterozygous, C57BL/6 SCID, and IFN- $\gamma$  KO mice. The ratio below each bar represents the number of culture-positive tissues over the total number of tissues analyzed from that group at that time. Zero values are not included in the means. The bars represent standard deviations.



TABLE 1. Dissemination of MoPn genital tract infection in C57BL/6 mice

Group	Condition	% of mice with disseminated infection and tissues affected as of day					
		7		10		14	
		% of mice with disseminated infection <sup>a</sup>	Tissue(s) affected <sup>b</sup>	% of mice with disseminated infection <sup>a</sup>	Tissue(s) affected <sup>b</sup>	% of mice with disseminated infection <sup>a</sup>	Tissue(s) affected <sup>b</sup>
A	Infected mice, caged individually without grooming collars	27 (2/7)	ILN (2/2)	67 (4/6)	ILN (2/4), lung (1/4), PL (2/4)	0 (0/8)	None
B	Infected mice, caged individually with grooming collars	57 (4/7)	ILN (4/4), kidney (1/4), lung (2/4)	63 (5/8)	ILN (3/5), PL (3/5)	14 (1/7)	PL (1/1)
C	Uninfected mice, caged in groups with soiled bedding	0 (0/8)	None	0 (0/8)	None	0 (0/8)	None
D	Infected mice, caged in groups (cocaged with groups of uninfected mice)	100 (7/7)	ILN (7/7), MLN (3/7), PL (1/7), spleen (1/7)	100 (8/8)	ILN (8/8), liver (1/8), lung (1/8), MLN (4/8), PL (3/8)	67 (6/9)	ILN (4/6), liver (2/6), MLN (2/6), PL (1/6)
E	Uninfected mice, caged in groups (cocaged with groups of infected mice)	0 (0/8)	None	63 (5/8)	MLN (5/5)	78 (7/9)	MLN (7/7)

<sup>a</sup> Percentages were calculated from the ratios of the numbers of mice showing dissemination to the sites to the total numbers of mice tested per group (raw numbers are in parentheses).

<sup>b</sup> Abbreviations: ILN, iliac lymph nodes; MLN, mesenteric lymph nodes; PL, peritoneal lavage. Indicated in parentheses are the ratios of the numbers of mice showing dissemination at the indicated sites to the total numbers showing dissemination at any site in that group.

immunologically competent animals cleared disseminated MoPn after the 10-day time point. Dissemination in individual wild-type and IFN- $\gamma$  heterozygous animals, if detected, was usually seen in only one extragenital site. Dissemination was often detected at multiple sites in individual IFN- $\gamma$  KO mice at 10, 23, and 38 days postinfection and in all tissues tested in individual C57BL/6 SCID mice at 38 and 50 days postinfection. Interestingly, IFN- $\gamma$  KO animals were capable of controlling dissemination before 50 days postinfection, but severely immunocompromised animals (C57BL/6 SCID) were incapable of resolving disseminated chlamydial disease. Although extensive dissemination was observed in C57BL/6 SCID mice, those animals did not exhibit the illness seen in some IFN- $\gamma$  KO mice (described above). These results indicate that disseminated infection alone was not responsible for the illness observed in some IFN- $\gamma$  KO mice.

**Pathology.** Severe peritoneal disease was observed in one of five IFN- $\gamma$  KO mice examined at day 23, in one of five of these mice at day 38, and in two of five of these mice at day 50 postinfection. In these cases, the peritoneal contents were found to have adhered into one large mass. This was likely due to an excess of peritoneal adhesions, although histopathological analysis was not performed on these tissues. This pathology did not correlate with the occurrence of illness, and it was not observed in any other mouse strain. The absence of peritoneal pathology in SCID mice with disseminated infection indicated that factors in addition to disseminated MoPn contributed to this pathology. Compensatory cytokine production resulting from the absence of IFN- $\gamma$  may have contributed to peritoneal disease; however, cytokine levels were not measured, and therefore this possibility cannot be addressed.

Histopathological evaluation of genital tissues from each group on days 10 and 23 postinfection revealed the presence of profound inflammatory infiltrates throughout the genital tract with numerous polymorphonuclear leukocytes in the ovarian bursa. This inflammation had cleared by day 50 postinfection in all groups except C57BL/6 SCID mice, in which large numbers of polymorphonuclear leukocytes were still present in the uterine horns.

The results of gross pathological evaluation of the organs

taken for MoPn recovery analyses were unremarkable and did not reveal a characteristic pathology for MoPn-positive tissues.

**Dissemination of MoPn genital tract infection in normal mice.** It was somewhat surprising to find that transient dissemination of MoPn to extragenital sites had occurred in immunocompetent animals (Fig. 4). To determine if this observation was a result of true dissemination, as opposed to reinfection via the airborne or oral route, groups of control animals whose members were infected intravaginally and housed individually (Table 1, group A), infected vaginally and housed individually while wearing grooming collars (to prevent autoinoculation by the oral route) (Table 1, group B), or housed in groups with soiled bedding from cages containing infected mice (environmental exposure) (Table 1, group C) were studied. Dissemination to extragenital sites was observed in groups A and B, but evidence of genital or extragenital infection was not detected in group C. The occurrence of dissemination in mice wearing grooming collars indicated that spread occurred internally and did not require autoinoculation through self-grooming. The levels of recovery of infectious MoPn from major organs (kidneys and lungs) and peritoneal washes in groups A and B were low (5 to 100 IFU per sample), while the iliac lymph nodes yielded larger numbers (100 to 500 per sample). The absence of infection in group C indicated that the mice involved in these studies were free of detectable MoPn and that MoPn was not transmitted via the environment under the conditions tested. The bulk of dissemination was seen on days 7 and 10 postinfection, while dissemination was rare at 14 days postinfection. These results, combined with the lack of dissemination in previous experiments in immunocompetent animals on day 23 postinfection (Fig. 4), indicated that although commonly seen in normal mice, disseminated infection is largely controlled by 2 to 3 weeks postinfection.

We next tested for transmission and subsequent dissemination in mixed groups of infected and uninfected mice (Table 1, groups D and E). In this experiment, mesenteric lymph nodes were also tested for MoPn to further study dissemination through the lymphatic system. Dissemination was again somewhat common among infected animals (group D), with the bulk being seen in the lymph nodes draining the genital tract

(the iliac nodes). Surprisingly, infectious MoPn was frequently recovered from the mesenteric lymph nodes of uninfected mice (group E) on days 10 and 14 postinfection. No other tissue from group E yielded infectious MoPn, indicating that these infections probably occurred via the oral route because the mesenteric lymph nodes are the major draining nodes of the gastrointestinal tract. The absence in group E of MoPn at other extragenital sites also indicates that oral transmission does not result in disseminated infection, at least through 14 days. Together, these results indicate that MoPn genital tract infection can disseminate to extragenital sites and that transmission of MoPn from animal to animal can occur, probably by the oral route. These findings represent unexpected sequelae of chlamydial genital tract infection in mice.

## DISCUSSION

The development of murine models has enabled researchers to experimentally address both pathological sequelae and naturally occurring protective immunity following chlamydial genital tract infection. Vaginal inoculation of MoPn causes an ascending infection that reaches the oviduct epithelium (7, 21). Such infections frequently result in infertility, and the comparison of different inbred mouse strains suggests that the H-2 haplotype influences infertility development (9). *C. trachomatis* serovar E also causes infertility in female mice following upper genital tract inoculation, but under these conditions the genetic factor(s) affecting infertility is not H-2 linked (31). To date, two different studies have reported the reduction of upper genital tract pathology following immune intervention. Modest protection from salpingitis was achieved via parenteral immunization with recombinant major outer-membrane protein (30), and passive administration of major outer-membrane protein-specific immunoglobulin G (IgG) and IgA monoclonal antibodies significantly reduced the intensity of oviductal inflammation at 8 days postinfection (7).

Significant progress has been made toward understanding the basis for naturally occurring immune protection following murine genital tract infection. The central role of T cells in protection was demonstrated by the inability of athymic nude mice to resolve MoPn genital tract infections (26). Furthermore, the capacity of B-cell-deficient mice to resolve MoPn genital tract infection and develop protective immunity in a manner similar to normal mice establishes that the requirement for T-cell help is related to the development of CMI and not antibody production (24). A dominant role for CD4 T cells was indicated by adoptive transfer studies in which the transfer of freshly isolated CD4 T cells obtained from genitally infected mice significantly enhanced the rate of clearance of genital tract infection in naive mice whereas CD8 T-cell transfer did not (29). Gene knockout mice have also been used to explore the role of T cells in protection from chlamydial infection. MHC class I-deficient (MHC class I KO) mice are indistinguishable from normal mice in clearance of an MoPn genital tract infection and protection from challenge, confirming results from adoptive immunity experiments showing that CD8 T cells do not play a major role (21). Other groups have shown that there are protective effects following adoptive transfer of *Chlamydia*-specific CD8 T-cell lines and clones (13, 28), although the observed *in vivo* protection is related to IFN- $\gamma$  production and therefore may not involve cytolytic mechanisms (28). MHC class II KO mice behave similarly to nude mice in that they are incapable of resolving a primary MoPn infection. This defect is likely related to the deficiency in antigen presentation by MHC class II molecules and the resulting CD4 T-cell response (21). CD4-deficient (CD4 KO) mice dis-

play an intermediate capacity to eradicate MoPn in that resolution of infection is delayed by about 2 weeks when compared to normal mice (21). There is evidence to suggest that MoPn specific antibody, although subordinate to the role of CMI, also contributes to protection. Resolution of MoPn infection in CD4 KO mice corresponds temporally with the appearance of MoPn-specific IgA in secretions (21), and passive transfer of both IgG and IgA MoPn-specific monoclonal antibodies confers protection from low-dose inoculation (ID<sub>50</sub>s [4]) and significantly reduces ascending infection (7).

Although CMI is the dominant factor in immune protection against acute MoPn genital tract disease in mice, it remains unclear what specific cytokines and effector mechanisms are responsible. IFN- $\gamma$  has long been known to inhibit chlamydial growth (4, 27), and an important role for this cytokine in murine genital tract chlamydial infections was first indicated by passive protection experiments (25). Protective T-cell populations produce IFN- $\gamma$  (13, 14, 28, 29), and in some cases IFN- $\gamma$  production has been shown to be responsible for their protective effects. The importance of IFN- $\gamma$  in chlamydial immunity has also been explored by using IFN- $\gamma$  receptor knockout (IFN- $\gamma$  R KO) mice in a model of *C. trachomatis* serovar D genital tract infection (15, 32, 34). In this system, IFN- $\gamma$  R KO mice suffered a more severe and prolonged infection than normal mice, providing further evidence that IFN- $\gamma$  is important for control of murine genital tract infection.

Gene knockout mice were used in studies reported here to explore the role of IFN- $\gamma$  in immunity to chlamydial infection. Our results showed that the lack of a functional IFN- $\gamma$  gene resulted in a prolonged infection that was largely controlled by 49 days postinfection ( $\leq 10$  IFU recovered per swab), compared to 28 days for heterozygous littermates. Low-level shedding continued in some IFN- $\gamma$  KO mice through 112 days postinfection. In spite of the delay in resolution, IFN- $\gamma$  KO mice were protected from reinfection to the same degree as heterozygous animals, indicating that if IFN- $\gamma$  was important to protection from challenge, compensatory immune activity developed that obviated the need for this cytokine. Similarly, vaccination of IFN- $\gamma$  KO mice overcomes their susceptibility to *Listeria monocytogenes* infection (10). The lack of acquired immunity in C57BL/6 SCID mice rendered them incapable of resolving infection through 50 days of observation. Not surprisingly, this is similar to the course of MoPn genital tract infection observed in athymic nude mice and MHC class II KO mice, both of which are also substantially impaired with respect to acquired immunity and cannot resolve MoPn genital infections.

Common, low-level dissemination of chlamydiae and the importance of acquired immunity for the control of dissemination of mucosal chlamydial infection have not been documented previously. The lack of IFN- $\gamma$  production resulted in more severe dissemination, but as with resolution of MoPn genital tract shedding, IFN- $\gamma$  KO mice were able to control dissemination by 50 days postinfection. The courses of genital tract and disseminated infections in SCID mice were similar, with neither being controlled by 50 days postinfection. It may be that effector mechanisms important for control of local infection are also important for control of dissemination to extragenital sites.

Although dissemination of chlamydial genital tract infection has long been assumed, the somewhat high incidence of dissemination in normal, immunocompetent mice was surprising. Dissemination was shown to occur via the draining lymph system as well as through the oviduct into the peritoneal cavity, although it is unknown which route carried MoPn to the major internal organs. Due to technical difficulties in culturing blood

samples, we were unable to determine if spread also occurred via the bloodstream. In addition, we found that progesterone treatment prior to infection was not required for movement of MoPn from the genital tract, since dissemination was observed in untreated IFN- $\gamma$  KO mice (data not shown). The high frequency of dissemination seen in this system may or may not reflect the human situation, and it is unclear if dissemination in the murine system plays a central role in the development of extragenital complications similar to human reactive arthritis and Fitz-Hugh-Curtis syndrome.

The discovery of animal-to-animal transmission, apparently by the oral route, was also an unexpected finding. Since mesenteric lymph nodes from infected mice that were housed alone (Table 1, groups A and B) were not assayed for chlamydial infectivity, it is not known if infection at this site can occur by autoinoculation. Infection of the gastrointestinal tract by MoPn has been observed following gastric inoculation, as determined by recovery of infectious MoPn on rectal swabs for up to 3 weeks postinoculation (6b), and oral immunization with viable MoPn does confer a degree of protection against MoPn genital tract challenge (17).

Although disseminated infection occurred in both SCID and IFN- $\gamma$  KO mice, illness and peritoneal pathology were seen only in the IFN- $\gamma$  KO animals. This difference makes it unlikely that multiplication of MoPn at extragenital sites was solely responsible for these conditions; however, the underlying basis for the pathology in IFN- $\gamma$  KO mice is not known.

It is clear that IFN- $\gamma$  is important to the development of protection in mice with chlamydial infections. It remains to be determined if IFN- $\gamma$  acts primarily by stimulating the appropriate T-cell populations and/or by directly inhibiting chlamydial growth in infected cells. If low-level dissemination is a common feature of chlamydial infection, then the murine model will be an excellent system for determining whether dissemination affects the protective or pathological immune responses and leads to extragenital complications similar to those observed in humans.

#### ACKNOWLEDGMENTS

We thank Anette Gendron for performing the histopathological evaluations, Edward Balish and William Weidanz for providing IFN- $\gamma$  KO mice, and Jon Woods for facilitating the reproduction of digital images.

This work was supported by U.S. Public Health Service grant AI-19782.

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Editor: R. E. McCallum