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Analysis of the Immune Response in Mice following Intrauterine Infection with the *Chlamydia trachomatis* Mouse Pneumonitis Biovar

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A Swiss Webster white mouse model of salpingitis was used to characterize the immune response following an intrauterine infection with the *Chlamydia trachomatis* mouse pneumonitis biovar. Western blot (immunoblot) analyses of the serum samples showed that the immunodominant bands corresponded to molecular masses of 72, 60, 42, and 28 kDa and to the lipopolysaccharide. Antibodies to the 60-kDa heat shock protein and to the 60-kDa cysteine-rich protein were detected at 2 and 3 weeks postinfection, respectively. Neutralization was observed in an in vitro assay with serum samples as early as the 3rd day postinfection and remained high for the 7 weeks of observation. The mice were mated in the 7th week following infection. Of the infected experimental mice, 71.4% were found to be either unilaterally or bilaterally infertile, whereas only 27.4% of the noninfected control mice were found to be infertile.

Several studies have looked at the role of *Chlamydia trachomatis* in human infertility. Seroepidemiological analyses in Western countries have found an increased prevalence of high antibody titers to chlamydiae in women with tubal infertility and ectopic pregnancy (7, 9, 29, 31). Recently, Soong et al. (22) found chlamydial DNA in the endocervical cells of 26.3% of infertile patients but in only 12.5% of the fertile control individuals. Thus, infection with *C. trachomatis* appears to be a significant problem that may cause permanent tubal damage.

The sequelae to a chlamydial infection are thought to be mainly the results of the immune reaction mounted by the host and not a direct effect of the chlamydial infection (4, 10–14, 25, 26, 30, 31). For example, Westrom and Mardh (31) monitored a cohort of women with pelvic inflammatory disease proven by laparoscopy and found that those individuals with a single episode of pelvic inflammatory disease had an infertility rate of 6.1% while those who had three or more episodes had an infertility rate of 54%. In the case of trachoma, the 60-kDa heat shock protein (hsp) has been shown to elicit an ocular delayed-type hypersensitivity reaction in both monkeys and guinea pigs (10, 11, 25, 26, 30). This delayed-type hypersensitivity reaction is now thought to be due to an autoimmune reaction occurring as a result of the sequence homology between the chlamydial 60-kDa hsp and human hsp60 (10, 21).

Experimental models of salpingitis have been established by the inoculation of human and mouse *C. trachomatis* serovars into the upper reproductive tract of female mice (23, 24, 27). In these models, following the acute infectious episode a significant number of mice developed oviductal pathology that resulted in unilateral or bilateral infertility. There are, however, still many unanswered questions regarding the role of the host immune response in infertility. In an attempt to gain a better understanding of the immunopathological changes that lead to infertility, we have further characterized the immune responses in mice following an intrauterine infection with the *C. trachomatis* mouse pneumonitis (MoPn) biovar.

The *C. trachomatis* MoPn strain Nigg II was grown and purified as previously described (5, 23). Six- to eight-week-old Swiss Webster white female mice were obtained from Simonsen Laboratories (Gilroy, Calif.). For inoculation the animals were anesthesized with methoxyflurane and a lateral abdominal incision was made. In the experimental group, 35 animals were injected in the left uterine horn with 10^5 inclusion-forming units of *C. trachomatis* MoPn in 20 μl of sugar phosphate glutamate (SPG), while the 35 control mice were inoculated with mock-infected HeLa cell material processed by the same protocol as the MoPn elementary bodies (EBs). Groups of two experimental and two control mice were sacrificed at several intervals up to 7 weeks following the initial inoculation. Seven weeks following the initial inoculation, groups of two female control and two experimental mice were independently paired with a single proven breeder male mouse. Eighteen days after mating, the female mice were sacrificed, tissues and blood were collected, and the number of embryos in each uterine horn was counted. The Student *t* test was used for statistical analyses.

For the isolation of *C. trachomatis* MoPn, vaginal swabs were obtained at different intervals following inoculation and the specimens were cultured as previously described (16). Tissues for histological analysis were fixed in buffered formalin and processed by standard techniques. Blood was collected by periorbital or heart puncture, and vaginal secretions were collected by rinsing the vaginal cavity with 40 μl of sterile phosphate-buffered saline (PBS) (10 mM, pH 7.4). The inclusion immunofluorescence assay (IFA) and the in vitro neutralization assays were performed as previously described (15, 16). For Western blots (immunoblots), chlamydia EBs were resolved by 10% tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20). Approximately 250 μg of purified EBs were loaded on a 7.5-cm-wide slab gel. In addition to EBs, serum samples were also probed with the affinity-purified *C. trachomatis* serovar A 60-kDa

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TABLE 1. Culture and immunological results for mice infected with the *C. trachomatis* MoPn biovar

Days post-infection	MoPn isolation		Serum antibody titer[a]		Vaginal antibody titer[a]		% Inhibition of IFUs[b]
	Positive total	% Positive	IgG	IgM	IgG	IgA	
3	ND[c]	0	0	40	0	0	62
5	13/27	48	0	320	0	0	80
13	9/21	43	80	640	0	4	83
15	ND	ND	160	320	0	0	87
20	3/19	16	ND	ND	ND	ND	ND
24	ND	ND	640	160	8	8	86
27	1/18	6	ND	ND	ND	ND	ND
31	0/8	0	2,560	80	8	16	94
38	0/8	0	1,280	80	16	4	85
44	ND	ND	2,560	40	8	4	86

[a] Sera and vaginal secretions were collected for each time point from two mice and were pooled for testing.

[b] Percent inhibition of chlamydial inclusion-forming units (IFUs) relative to control mock-infected-mouse serum.

[c] ND, not done.

hsp (kindly supplied by Richard Morrison, Rocky Mountain Laboratory, Hamilton, Mont.) or with Sarkosyl-extracted EBs prepared as described previously (5, 28). Serum samples were diluted 1:100 with PBS containing 0.05% Tween 20, incubated with a nitrocellulose membrane for 2 h at room temperature, and processed as previously described (5). A 1:500 dilution of rabbit antiserum to hsp60/63 of the moth *Heliophis virescens* (a generous gift from StressGen Biotechnologies Corp. Victoria, British Columbia, Canada), a 1:200 dilution of a mouse monoclonal antibody (MAb) to the MoPn 60-kDa cysteine-rich protein (crp) isolated in our laboratory (unpublished results), and a 1:10 dilution of a MAb to the *C. trachomatis* serovar A 60-kDa hsp (provided by R. Morrison) were used as positive controls.

C. trachomatis MoPn was isolated from vaginal swabs during the first 4 weeks postinfection (Table 1). All chlamydia cultures from the control animals were negative. Control and experimental animals were sacrificed, and their genital tracts were inspected for gross and microscopic morphological changes. In the control groups no abnormalities were found throughout the 7 weeks of observation (Fig. 1A). As determined by gross examination of the six infected mice sacrificed between the 4th and 6th weeks, three had a unilateral hydrosalpinx and one had a bilateral hydrosalpinx. In the experimental group, by 3 and 7 days postinfection, on microscopic examination there was a significant acute inflammatory infiltrate permeating all the layers of the uterine horn and oviduct. Areas with a marked acute inflammatory infiltrate with necrosis and abscess formation were found by 11 days postinoculation (Fig. 1B). The animals with hydrosalpinx showed signs of dilation of the oviduct, with flattening or disappearance of the cells in the epithelial layer (Fig. 1C). By this time the inflammatory infiltrate was mainly composed of lymphocytes and plasma cells.

As shown in Table 1, a serum IFA immunoglobulin M (IgM) antibody titer of 320 was detected at 5 days and peaked at 640 by the 2nd week following inoculation, while the IgG titers continued to rise to 2,560 during the 7 weeks of observation. Significant IgA and IgG titers were detected in the vaginal washes by 3 weeks and peaked approximately on the 5th week postinoculation (Table 1). The IFA titers in the control animals were negative throughout the experiment.

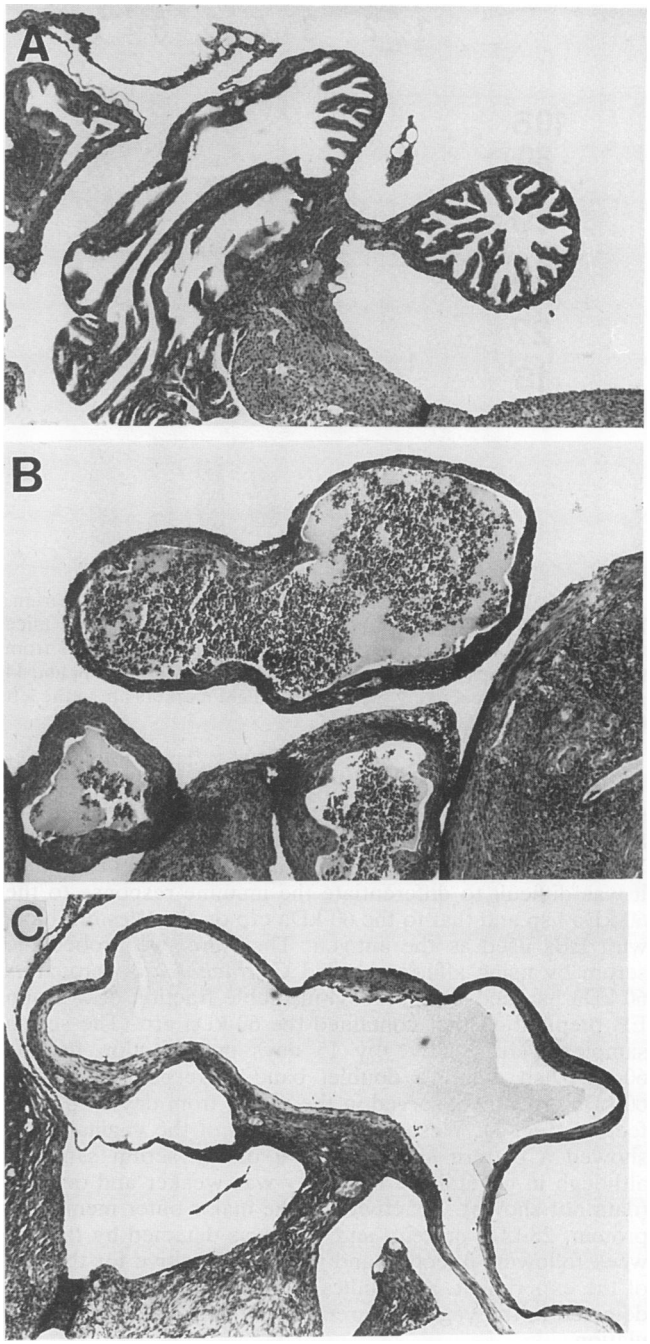

FIG. 1. Microscopic sections of mouse oviducts. (A) Normal control at 11 days following a mock inoculation. (B) Experimental animal at 11 days postinfection. Note heavy acute inflammatory infiltrate with abscess formation. (C) Experimental mouse oviduct 31 days postinfection. The wall of the oviduct is significantly dilated as a result of the hydrosalpinx. The mucosal lining has disappeared from most of the wall of the oviduct. Magnification, ×16.

By immunoblot analyses the control samples were negative in all instances while the experimental animals showed reactive bands by 2 weeks following infection (Fig. 2). The first bands detected corresponded to the 72-, 60-, 42- (major outer membrane protein), and 28-kDa proteins and the lipopolysaccharide (LPS). Reactivity to these bands re-

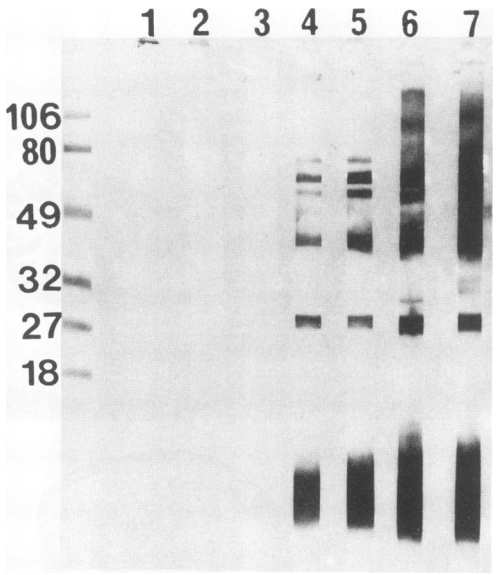

FIG. 2. Western blot analysis of the total Ig response in serum. Each antigen strip was probed with a pool of sera from mice sacrificed on the indicated day. Lanes 1 to 7, serum samples from the infected groups corresponding to days 0, 5, 13, 15, 24, 38, and 44 postinfection, respectively. Molecular weight markers are in the left lane of the blot.

mained strong for the rest of the experiment. By day 38, antibodies to high-molecular-mass proteins (approximately 100 kDa) were observed. Because of the overlap of the bands it was difficult to differentiate the immune response to the 60-kDa hsp and that to the 60-kDa crp on the Western blots with EBs used as the antigen. Therefore, we probed the serum by using affinity-purified *C. trachomatis* serovar A 60-kDa hsp and the Sarkosyl-insoluble fraction of a MoPn EB preparation that contained the 60-kDa crp. The serum samples were positive by 15 days postinfection for the 60-kDa hsp, while a doublet band corresponding to the 60-kDa crp was observed in the serum from day 24 postinfection (Fig. 3). Western blot analysis of the vaginal wash showed a pattern similar to that of the serum samples, although in general the reactivity was weaker and delayed (data not shown). Reactivity to the major outer membrane protein, 28-kDa protein, and LPS was detected by the 3rd week following infection and remained positive for the rest of the experiment. Antibodies to the 60-kDa proteins were detected in the vaginal secretions at day 27 following inoculation.

To determine the presence of neutralizing antibodies in serum and vaginal secretions, the specimens were incubated with *C. trachomatis* MoPn EBs and assayed to determine their infectivity in HeLa cell monolayers. At a 1:100 dilution the serum samples from the 1st through the 7th week of observation neutralized in vitro 80 to 90% of the infectivity (Table 1). On the other hand, we failed to detect any neutralizing antibody in vaginal washes even at low dilutions of the sample (1:2).

In two separate experiments a total of 14 mice in the experimental group and 11 mice in the control group were mated at 7 weeks following the initial inoculation. In the experimental group 10 of 14 (71.4%) of the mice had unilateral or bilateral infertility, while in the control group only 3 of 11 (27.4%) of the mice were infertile ($P < 0.05$). Furthermore, the mean number of embryos per mouse was 4.9 in the experimental group and 9.7 in the control group ($P < 0.05$).

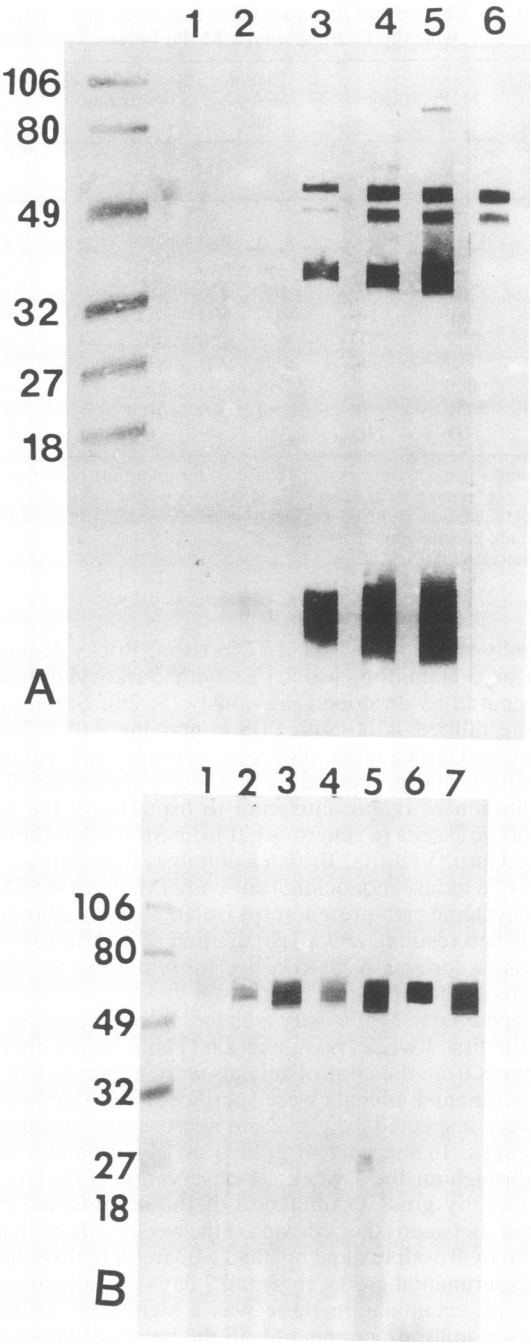

FIG. 3. Western blot analysis of total Ig response in serum samples to the Sarkosyl-insoluble fraction, containing the 60-kDa crp (A) and to the recombinant *C. trachomatis* serovar A 60-kDa hsp (B). Lanes 1 to 5, serum samples corresponding to days 0, 15, 24, 38, and 44 postinfection. Positive control antibodies for both antigens are shown in lanes 6 (anti-60-kDa crp MAb, panel A; anti-60-kDa hsp MAb, panel B) and 7 (rabbit polyclonal antibody to hsp60/63 of the moth *H. virescens*, panel B). Molecular weight markers are in the left lane.

In this study we characterized the immune response in Swiss Webster white mice infected in the uterine horn with

the *C. trachomatis* MoPn biovar in an attempt to better understand the mechanisms that lead to infertility. In the serum we could detect an IgM chlamydia-specific immune response as early as 3 days postinfection that peaked at 2 weeks and subsequently declined. The IgM response corresponded to the appearance of neutralizing antibodies to chlamydiae in the serum. This is the first time that neutralizing antibodies have been detected so early after infection, suggesting that a very rapid protective humoral immune response is elicited in this model. However, to determine the role that these neutralizing antibodies play in the progression of the disease will require additional studies. The results for the serum IgG are similar to those reported by Barron et al. (1, 2) and Ramsey et al. (17–19) for mice challenged intravaginally with *C. trachomatis* MoPn. The IgG response was first detected at 2 weeks postinoculation and continued for the 7 weeks of observation. In the vaginal wash we detected an immune response similar to the one in serum although at much lower antibody levels. These titers however should be considered significant, particularly if we take into account the dilution factor resulting from washing the vagina with 40 μl of PBS. On the other hand, it is difficult to ascertain the validity of these titers as absolute values because of the variability introduced by the washing step, a problem already reported by Ramsey et al. (17). An inverse correlation between the level of IgA in cervical secretions and the recovery of the organism from the cervix in humans was described by Brunham et al. (3), suggesting that this immunoglobulin may regulate the shedding of chlamydiae. So far, no such a correlation has been described for a mouse model, so characterization of this parameter will require further studies.

Cross-reactivity between the chlamydial 60-kDa hsp and the host 60-kDa hsp has been postulated as a possible mechanism for the pathogenesis of the sequelae to a chlamydial infection (6, 10, 11, 21, 25, 26, 30). In this respect it is interesting that in the cervicitis mouse model a strong immune response to the 60-kDa crp but not to the 60-kDa hsp was noticed (17). In patients with ectopic pregnancies a high prevalence of antibodies to these two proteins has been reported, and for monkeys and guinea pigs the 60-kDa hsp appears to be involved in hypersensitive reactions in the ocular models of chlamydial infection (6, 10, 11, 25, 26, 28, 30). Here, we describe for the first time a strong immune response to both the 60-kDa crp and the 60-kDa hsp in the mouse salpingitis model. Thus, the possible role of the chlamydial 60-kDa hsp as a causative factor in the pathogenesis of infertility and ectopic pregnancy can be analyzed in this mouse model since this protein has significant sequence homology to the mouse 60-kDa hsp (8). However, it probably will be more appropriate to characterize this phenomenon in an inbred line of mice in order to eliminate the individual variability in the immune response expected from outbred mice such as Swiss Webster.

As of now, it is not clear whether the infertility in this model is due to the acute infectious episode, the result of an autoimmune phenomenon in response to the 60-kDa hsp or other chlamydial component(s), or the consequence of other pathogenic mechanisms. The acute lesions could directly lead to severe tissue damage with destruction of the normal architecture of the oviduct. However, the acute damage could be repaired if the local mucosa and supporting structures have good regenerating capacity and no long-term chronic damage occurs as a result of the infection. Thus, at this point it is not known if in this animal model normal fertility rates could be restored if a period of several months was allowed to lapse between the time of the acute infection and the time the mice are mated. The female upper genital tract is a privileged immune site that has to tolerate paternal antigens during pregnancy, the immune insults resulting from the reproductive cycle, and most likely continuous antigenic challenges by sperm and microorganisms ascending from the lower genital tract. If the long-term damage resulting from a *C. trachomatis* infection is due to an autoimmune mechanism, that would indicate that this organism is able to trigger some very unique immunological responses.

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