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Susceptibility of Mice to Vaginal Infection with *Chlamydia trachomatis* Mouse Pneumonitis Is Dependent on the Age of the Animal

SUKUMAR PAL, ELLENA M. PETERSON, AND LUIS M. DE LA MAZA*

Department of Pathology, Medical Sciences, University of California, Irvine, Irvine, California 92697-4800

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Mice from three strains, BALB/c (H-2^d), C3H (H-2^k), and C57BL/6 (H-2^b), ranging from 5 to 14 weeks of age, were inoculated intravaginally with different doses of the *Chlamydia trachomatis* mouse pneumonitis serovar. Vaginal swabs taken at weekly intervals showed that the percentage of animals with positive cultures and the number of inclusion-forming units recovered per mouse were higher in the younger animals. Furthermore, vaginal shedding lasted longer in the young mice than in the older mice. In addition, following mating higher rates of infertility and a decrease in the number of embryos were observed in the infected young mice. In conclusion, susceptibility to a chlamydial vaginal infection is dependent on the age of the mice, with the older animals being more resistant.

Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen in humans (3, 11, 20). It is estimated that in the United States 3 to 4 million new cases of *C. trachomatis* infection occur each year (12). Most published studies report that the highest rates of infection occur in adolescent sexually active women, with a reduced rate of infection with increasing age (12). Different reasons for the apparent decrease in susceptibility to *C. trachomatis* in older individuals have been proposed, including physiological changes occurring with aging, reduced exposure, and immunity following exposure (3, 11, 12). In an attempt to start ascertaining the role that each one of these factors may play in the pathogenesis of the disease, we inoculated mice to determine if age at time of exposure is a factor in susceptibility to a *C. trachomatis* vaginal infection.

The *C. trachomatis* mouse pneumonitis (MoPn) biovar (strain Nigg II) was purchased from the American Type Culture Collection (Manassas, Va.) and grown in HeLa-229 cells (6). Elementary bodies were purified as described by Caldwell et al. (1) and frozen in SPG (0.2 M sucrose, 20 mM sodium phosphate [pH 7.4], and 5 mM glutamic acid). The number of inclusion forming units (IFU) was determined using McCoy cells (7). BALB/c, C3H/HeN, and C57BL/6 mice were purchased from Simonsen Laboratories (Gilroy, Calif.) and from Charles River (Wilmington, Mass.). The animal protocol was approved by the University of California, Irvine, Animal Care and Use Committee. Mice were infected intravaginally with different infectious doses of *C. trachomatis* MoPn in SPG, and vaginal cultures were taken at weekly intervals (2). Vaginal swabs were inoculated in McCoy cells seeded on 48-well plates and centrifuged at 1,000 × *g* for 1 h at 24°C. At 30 h postinfection the monolayers were fixed with methanol and stained as previously described (2). Six weeks after the intravaginal challenge groups of four female mice were housed with a proven breeder male mouse, and pregnancy was determined by weighing the animals. Mice that did not become pregnant after 1 month were mated with a second male mouse (7). All pregnant mice were euthanized on days 14 to 17 of gestation, and the embryos in each uterine horn were counted. The mean number of embryos per mouse was determined by dividing the total number of embryos present in both uterine horns in a group of animals by the number of female mice in that group. Nonpregnant mice were euthanized 42 days after the last mating.

As shown in Table 1, of the three strains tested, C3H/HeN mice were the most susceptible to a chlamydial vaginal infection. Overall the younger animals had a more severe infection as shown by the percentage of mice with positive vaginal cultures and the length of time that the animals had the positive cultures and by the number of IFU recovered per mouse. Almost all the C3H/HeN mice, of four different ages, that were infected intravaginally with 10^7 or 10^6 IFU of *C. trachomatis* MoPn had positive vaginal cultures. Furthermore, a significant decrease in fertility, as shown by the number of infertile animals and by a decrease in the number of embryos per mouse, occurred in all the four age groups inoculated with 10^7 *C. trachomatis* IFU. However, only the 5- to 6- and 7- to 8-week-old mice inoculated with 10^6 or 10^5 IFU had a significant decrease in fertility. As expected, the number of mice with hydrosalpinx closely paralleled the infertility results. All of the 5- to 6-week-old BALB/c mice inoculated with doses of *C. trachomatis* MoPn ranging from 10^4 to 10^7 IFU/mouse had positive cultures some time during the first 4 weeks of the 6-week observation period. In general, the length of time that these animals had positive genital cultures and the number of IFU recovered were greater than corresponding values for the 14-week-old BALB/c mice. All animals had negative vaginal cultures during the 5th and 6th weeks of observation. Also, significant decreases in fertility and the number of embryos per

* Corresponding author. Mailing address: Department of Pathology, Medical Sciences, Room D440, University of California, Irvine, Irvine, CA 92697-4800. Phone: (949) 824-7450. Fax: (949) 824-2160. E-mail: lmdelama@uci.edu.

TABLE 1. Intravaginal infection of C3H/HeN, BALB/c, and C57BL/6 mice with *C. trachomatis* MoPn

Mouse strain	Inoculum (IFU/mouse)	No. of mice	Age (wk)	Mean vaginal shedding score[a] (% of mice with + culture[f]) at wk:				% Mice with + culture[b]	% Mice infertile[c]	Mean no. of embryos/ mouse ± 1 SD	% Mice with hydrosalpinx
				1	2	3	4				
C3H/HeN	10^7	10	5–6	4+ (100)	4+ (100)	2+ (100)	1+ (20)	100[d]	100[d]	1.5 ± 2.1[e]	70[d]
	10^6	10	5–6	4+ (100)	4+ (90)	3+ (90)	1+ (40)	100[d]	80[d]	1.9 ± 2.9[e]	30
	10^5	10	5–6	4+ (100)	4+ (80)	3+ (80)	1+ (30)	100[d]	60	4.1 ± 3.6	60[d]
	10^4	10	5–6	0 (0)	3+ (10)	2 + (20)	1+ (10)	20	10	6.2 ± 2.6	10
	SPG	10	5–6	0 (0)	0 (0)	0 (0)	0 (0)	0	10	6.0 ± 2.7	0
	10^7	10	7–8	4+ (100)	4+ (100)	1+ (60)	2 + (10)	100[d]	100[d]	0[e]	80[d]
	10^6	10	7–8	4+ (100)	4+ (90)	2+ (40)	1 + (10)	100[d]	80[d]	1.5 ± 3.4[e]	60[d]
	10^5	10	7–8	4+ (90)	3+ (90)	3+ (80)	1 + (10)	90[d]	70[d]	2.9 ± 3.8[e]	30
	10^4	10	7–8	3+ (40)	3+ (40)	3+ (50)	1+ (10)	60[d]	30	7.6 ± 4.4	30
	SPG	10	7–8	0 (0)	0 (0)	0 (0)	0 (0)	0	10	9.2 ± 1.9	0
	10^7	10	10	4+ (90)	3+ (80)	1+ (30)	0 (0)	90[d]	80[d]	3.1 ± 4.0	60[d]
	10^6	11	10	3+ (100)	4+ (82)	4+ (46)	0 (0)	100[d]	36	4.9 ± 4.1	0
	10^5	11	10	3+ (46)	4+ (46)	1+ (55)	1+ (9)	64[d]	36	5.7 ± 4.5	18
	SPG	11	10	0 (0)	0 (0)	0 (0)	0 (0)	0	18	5.9 ± 3.4	0
	10^7	10	14	3+ (100)	2+ (60)	1+ (10)	4 + (30)	100[d]	70[d]	2.5 ± 4.1	50
	10^6	10	14	3+ (90)	4+ (70)	0 (0)	0 (0)	100[d]	30	6.0 ± 3.0	10
	10^5	10	14	2+ (50)	3+ (30)	2+ (60)	0 (0)	70[d]	10	6.8 ± 1.9	0
	SPG	9	14	0 (0)	0 (0)	0 (0)	0 (0)	0	10	6.1 ± 2.2	0
BALB/c	10^7	10	5–6	4+ (100)	4+ (100)	4+ (90)	2 + (20)	100[d]	90[d]	0.8 ± 1.9[e]	90[d]
	10^6	10	5–6	4+ (100)	4+ (100)	4+ (80)	2+ (10)	100[d]	100[d]	1.1 ± 2.6[e]	80[d]
	10^5	11	5–6	4+ (100)	4+ (100)	4+ (82)	3+ (27)	100[d]	82[d]	2.2 ± 2.0[e]	55[d]
	10^4	10	5–6	4+ (100)	4+ (100)	4+ (100)	3+ (20)	100[d]	80[d]	2.5 ± 2.6[e]	60[d]
	SPG	11	5–6	0 (0)	0 (0)	0 (0)	0 (0)	0	9	5.6 ± 2.5	0
	10^7	10	7–8	4+ (80)	3+ (70)	1+ (10)	0 (0)	80[d]	60	3.0 ± 3.0[e]	50[d]
	10^6	10	7–8	4+ (90)	3+ (60)	2+ (20)	0 (0)	90[d]	60	3.5 ± 3.4	40
	10^5	10	7–8	4+ (30)	3+ (30)	0 (0)	0 (0)	20	20	6.0 ± 2.5	0
	SPG	11	7–8	0 (0)	0 (0)	0 (0)	0 (0)	0	20	6.0 ± 2.8	0
	10^7	12	14	4+ (83)	1+ (42)	4+ (33)	0 (0)	83[d]	58[d]	4.8 ± 3.4	42[d]
	10^6	12	14	2+ (75)	1+ (42)	2+ (25)	0 (0)	75[d]	50[d]	5.0 ± 3.5	33[d]
	10^5	12	14	3+ (25)	1+ (8)	4+ (33)	2+ (8)	58[d]	25	7.5 ± 2.9	17
	SPG	12	14	0 (0)	0 (0)	0 (0)	0 (0)	0	8	6.8 ± 2.6	0
C57BL/6	10^7	10	5–6	4+ (100)	3+ (90)	2+ (50)	0 (0)	100[d]	60	5.5 ± 3.5	20
	10^6	9	5–6	4+ (100)	4+ (100)	2+ (44)	0 (0)	100[d]	44	6.1 ± 3.8	11
	SPG	9	5–6	0 (0)	0 (0)	0 (0)	0 (0)	0	22	7.7 ± 3.3	0

[a] Mean vaginal shedding score per mouse of each group. 4+, $>10^4$ IFU; 3+, 10^4 to 10^3 IFU; 2+, $<10^3$ to 10^2 IFU; 1+, $<10^2$ IFU; 0, negative.
[b] Percentage of mice that had at least one positive vaginal culture for *C. trachomatis* during the 6 weeks of observation.
[c] Percentage of mice that had no embryos in one or both uterine horns.
[d] $P < 0.05$ by Fisher's exact test.
[e] $P < 0.05$ by the Mann-Whitney U test.
[f] + culture, positive culture.

mouse for the four groups of 5- to 6-week-old BALB/c mice were observed, while in the 14-week-old mice a decrease in fertility was noted only in groups inoculated with 10^7 and 10^6 IFU. Even in these two groups the number of embryos per mouse was not statistically significantly different from the number for the controls. As expected, the C57BL/6 mice were the most resistant strain of the three tested to an intravaginal *C. trachomatis* infection. Positive vaginal cultures were obtained from all the 5- to 6-week-old mice inoculated with 10^6 or 10^7 *C. trachomatis* IFU. However, no statistically significant differences in fertility, number of embryos per mouse, and hydrosalpinx formation between any of the C57BL/6 groups and the control animals inoculated with SPG were observed. We have previously reported that 7- to 8-week-old C57BL/6 mice infected intravaginally with 3×10^7 IFU of *C. trachomatis* MoPn had fertility rates that were very similar to those for the control noninfected mice (2). It was for that reason that here we only tested 5- to 6-week-old C57BL/6 mice.

Our results show that, in addition to differences in strain susceptibility, differences in susceptibility to an intravaginal infection with the *C. trachomatis* MoPn serovar are contingent on the age of the animal. Differences in susceptibility to a *C. trachomatis* infection among strains of mice have been known for several years (2, 16, 17). More specifically, for an intravaginal challenge with 3×10^7 IFU/mouse, 6- to 8-week-old C3H/HeN mice have been shown to be the most susceptible, with 30% (6 of 20) fertility, while the C57BL/6

mice had 75% (15 of 20) fertility and the BALB/c mice had 40% (8 of 20) fertility (2).

The high prevalence of *C. trachomatis* genital infections in young adults, in comparison with older individuals, has been considered to be the result of several factors (3, 11, 12). On the one hand, physiological changes resulting from sexual and/or immunological maturation have been cited as possible causes. An overall relative decrease in sexual activity and a decrease in the number of different sexual partners with aging are components that have also been considered. The contribution of adaptive immunity following several exposures has also been evaluated. This last component has been given significant emphasis since the ability of an individual to mount protective immunity following a natural infection suggests the possibility of developing an efficacious vaccine (14, 19). Based on the results obtained with this murine model it appears that physiological changes resulting from sexual and/or immunological maturation play a significant, although not necessarily exclusive, role in the increased resistance to a chlamydial infection observed with aging. Newborn mice are considered to be immunologically mature at the time of birth, but on the other hand sexual maturation does not occur in most strains of mice until the animals are 6 to 10 weeks old (15). In this respect, progesterone and estradiol can have an effect on the local immune responses, and this could affect susceptibility to a chlamydial infection (5). Our results with the C3H/HeN and BALB/c mice suggest that sexual maturation plays a significant role in the decrease in susceptibility to a chlamydial upper genital tract infection. On the other hand, C57BL/6 mice seem to be quite resistant to a vaginal challenge even before the start of the estrus cycle, clearly indicating the importance of the genetic background in susceptibility to a chlamydial infection.

An increase in susceptibility to a *C. trachomatis* infection during the luteal phase of the estrus cycle has been reported in several studies (4, 8). This increase in susceptibility may be due to the local effects of progesterone in the genital tract, such as thinning of the mucosa, or to a more systemic phenomenon, including a shift toward a Th2 immune response (5, 10). In women the menarche and the initiation of sexual activity frequently occur in parallel during the teenage years. It is possible that before the establishment of the menstrual cycle the epithelium of the upper genital tract is highly susceptible to a chlamydial infection and subsequently becomes relatively more resistant depending on the hormonal balance. Furthermore, changes in the composition of the cervical plug are known to occur as a result of the estrus cycle, and they may influence the ability of pathogens to travel to the upper genital tract (12).

In addition to shedding some light on the susceptibility to a chlamydial infection our data have also some practical implications for the utilization of these murine models for testing candidate vaccines to prevent infertility. Of the three long-term sequelae from a genital chlamydial infection occurring in humans, chronic abdominal pain, ectopic pregnancy, and infertility, only the last one can be evaluated in a murine model. Based on our results it appears that, when an intravaginal challenge is used, this parameter can only be assessed in certain strains of mice and preferentially in young animals. This age limitation significantly narrows the window period in which animals can be immunized, challenged intravaginally, and subsequently mated to evaluate their fertility status. To overcome this limitation, some investigators treat mice with progesterone before the intravaginal challenge (18). As discussed above a concern about this approach is that the immunomodulation induced by the progesterone may make it difficult to assess the protective response elicited by the candidate vaccine (5, 10). An alternative approach is to challenge the mice directly in the upper genital tract since this type of inoculation more consistently induces infertility independently of the age of the mice (7, 13, 17). The obvious concern with this model is that it bypasses the natural route of infection. The optimal model for characterizing the pathogenesis of a chlamydial infection and testing candidate vaccines is the monkey (9). For obvious reasons working with monkeys is not a feasible alternative when large numbers of animals are required. Thus, at least for now, the murine model appears to be the best choice to characterize chlamydial genital infections. The lack of an ideal murine model, however, should not discourage research in this field. The information that we gain with the various murine models should be valuable for developing strategies to control these diseases in humans.

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