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Immune Response in Mice Infected in the Genital Tract with Mouse Pneumonitis Agent (*Chlamydia trachomatis* biovar)

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Female Swiss-Webster mice were inoculated intravaginally with mouse pneumonitis agent (MoPn), a *Chlamydia trachomatis* biovar. Inoculation with 3.5×10^5 egg lethal doses per mouse resulted in shedding of the agent from the genital tract for as long as 21 days. Immunoglobulin M antibodies to MoPn were detected in plasma by day 7 post-inoculation, and immunoglobulin G antibodies were detected by day 14. Antibodies were detected in genital secretions by day 20, and titers in plasma and secretions were still considerable on day 56. Delayed-type hypersensitivity tests, determined by footpad swelling, were not positive in appreciable numbers of animals until after day 25. Delayed-type hypersensitivity reactions were maximal 24 h after testing and were preceded by an Arthus-like reaction, which appeared within 3 h and declined by 12 h. Convalescent animals were rechallenged by intravaginal inoculation and were found to be solidly immune.

The importance of human genital infections caused by *Chlamydia trachomatis* is now well established. Because the organism isolated is essentially a human agent, studies in animals have been somewhat restricted. The use of various animal models for the study of chlamydial infections has been recently reviewed (1).

A number of investigators have employed the agent of mouse pneumonitis (MoPn). This organism, a mouse agent, is a distinct *C. trachomatis* biovar, producing iodine-positive inclusions and exhibiting sensitivity to sulfadiazine, like the human agent (J. W. Moulder, T. P. Hatch, C.-C. Kuo. J. Schachter, and J. Storz, *In* N. Krieg (ed.), Bergey's manual of systematic bacteriology, 9th ed., vol. 1, The Williams & Wilkins Co., Baltimore, in press). Williams et al. (14, 15) have studied chlamydial pneumonitis in nude mice and heterozygous litter mates by using MoPn. Antibiotic therapy for treatment of chlamydial pneumonitis was evaluated by Kramer et al. (7) in mice infected with MoPn.

In 1981, we reported on genital tract infection of female mice with MoPn (2). We have extended our studies to investigate immune response and resistance to reinfection. The results are reported here.

MATERIALS AND METHODS

Mice. Six-week-old female Swiss-Webster mice were purchased from Harlan Sprague-Dawley, Indianapolis, Ind. Mice were housed in cages covered with fiber glass filter bonnets and supplied food and water ad libitum in an environmentally controlled room at 24°C with a 12:12 light-dark cycle.

MoPn. MoPn was maintained by passage in chicken embryos via the yolk sac route as described previously (2). Fifty percent homogenates were prepared in sucrose-potassium-glutamate (pH 7.0) containing 50 μg of gentamicin and 100 μg of vancomycin per ml. The 50% egg lethal dose (ELD_{50}) was determined by the method of Reed and Muench (10).

A particle suspension of chlamydiae was prepared for inoculation as follows. The 50% homogenate was centrifuged at 192 × *g* at 5°C for 10 min. The middle layer between sediment and fatty material was carefully removed, diluted with sucrose-potassium-glutamate, and recentrifuged at 30,900 × *g* at 5°C for 30 min. The supernatant was discarded and the pellet was evenly suspended in sucrose-potassium-glutamate to one-tenth the original volume centrifuged. This particle suspension was considered to be 10× concentrated. Fresh particle suspensions were prepared for each experiment.

Inoculation of mice. Mice were inoculated intravaginally under pentobarbital sodium anesthesia with 0.03 ml of MoPn particle suspension, using a syringe-needle assembly modified for this purpose (2).

Isolation of MoPn. Unless indicated otherwise, infection was monitored by isolation of MoPn from cervical-vaginal swabs. A calcium alginate swab on a flexible aluminum shaft (Calgiswab, type 1, Spectrum Diagnostics, Glenwood, Ill.) was inserted into the vagina and rotated. The swab was placed in 1.0 ml of 2SP (16), containing 50 μg of gentamicin, 100 μg of vancomycin, and 2.5 μg of amphotericin B per ml. Specimens were stored at −70°C before inoculation. In later experiments, freezing was avoided and the specimens were inoculated into cell cultures immediately after collection. The swabs were chilled in ice water until processed.

MoPn was isolated by inoculation of McCoy cell cultures grown on cover slips in shell vials, using centrifugation essentially as described by Evans and Taylor-Robinson (4). The medium consisted of Eagle minimal essential medium, 10% fetal calf serum, 5.0% glucose, 0.5 μg of cycloheximide per ml, and 50 μg of gentamicin per ml. Two vials per specimen were inoculated with 1.0 ml of swab fluid each; one cover slip was fixed with methanol and stained with Giemsa after 48 h of incubation at 36°C, and the other was blind passaged. Microscopic examination of the first-passage cover slips for chlamydial inclusions was frequently hampered because of toxicity. Reliance was placed on results of the second passage.

Determination of antibodies to MoPn in plasma and secretions. Micro-immunofluorescence was used, employing previously described procedures (2). Antigen slides were prepared by placing small drops of a 2.5% yolk sac homogenate from MoPn-infected embryos on glass microscope slides.

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TABLE 1. Development of delayed hypersensitivity and Arthus-like reactions in mice during genital infection with MoPn

Reaction (time post-inoculation)	No. positive/no. challenged on day post-inoculation with MoPn:									
	5	10	15	20	25	30	32	35	40	54
Delayed hypersensitivity (24 h)	0/5	1/10	1/10	1/10	3/10	4/5	1/4	3/5	4/5	2/4
Arthus (3 h)	0/5	3/10	1/10	2/10	4/10	4/5	4/4	5/5	5/5	3/4

Antigen slides were fixed in acetone and stored at −70°C. The assay was performed by incubating serial two-fold dilutions of plasma on the antigen slides for 45 min, one dilution per antigen well. Immunoglobulin (IgG) antibody to MoPn was measured by adding fluorescein-labeled rabbit anti-mouse IgG (H and L chain specific) (Miles Laboratories, Inc., Elkhart, Ind.) diluted 1:20 to the washed slides and incubating for 30 min. The slides were again washed and were counterstained with 0.1% Evans blue. IgM was determined by using fluorescein-labeled rabbit anti-mouse IgM (μ chain specific) diluted 1:10 (Cappel Laboratories, West Chester, Pa.). Slides were examined under a Zeiss microscope with a vertical fluorescence illuminator (Carl Zeiss, West Germany).

Genital secretions were collected from mice by inserting a section (2 by 4 mm) of a surgical sponge (Weck-Cel, Edward Weck and Co., Inc. Durham, N.C.) into the vagina of the mouse under anesthesia with pentobarbital sodium and collecting it approximately 4 h later. Sponges were stored at −20°C. The sponges from each group collected on the same day were pooled, and 0.1 ml of phosphate-buffered saline (pH 7.2) containing 50 μg of gentamicin and 100 μg of vancomycin per ml was added to 0.04 g of secretion. The eluted secretions were arbitrarily considered undiluted, and serial twofold dilutions were performed. Indirect immunofluorescence was performed by using rabbit anti-mouse IgA (α chain specific) (Miles Laboratories) and then fluorescein-labeled goat anti-rabbit IgG. There was some question as to the specificity of this procedure for mouse IgA since the labeled goat anti-rabbit IgG reagent apparently cross-reacted with mouse immunoglobulin. Thus, specific antibody in secretions will be considered as total antibody rather than IgA.

Determination of delayed-type hypersensitivity reaction by footpad swelling. Delayed-type hypersensitivity (DTH) to MoPn was determined by measuring the increase in footpad thickness after injection of MoPn antigen. Antigen was prepared by heat killing (100°C, 30 min) MoPn propagated in McCoy cells and then concentrating the antigen 2× by centrifugation at 30,900 × *g* for 30 min. Uninfected McCoy cells were prepared similarly to serve as control antigen.

To perform the assay, 0.05 ml of MoPn antigen and control were injected into rear footpads with a 30-gauge needle. The thickness of the footpad was measured before injection and at 3, 6, 12, 24, and 48 h after injection with a Schnelltaster dial micrometer (H. C. Kroplin, Hessen, West Germany), and the increase in footpad thickness was recorded.

A positive DTH reaction was considered to be an increase of 3.0 footpad units (1 unit = 0.1 mm) at 24 h. A positive Arthus reaction was considered to be an increase of 3.5 footpad units at 3 h. DTH and Arthus reaction values were derived by determining one standard deviation above the mean of 29 readings of footpads injected with control antigen at 24 or 3 h after challenge, respectively. These values were arbitrarily considered to be the minimum increases in footpad thickness for positive reactions. Mice were only tested once for a DTH reaction.

Challenge experiments. Mice that had recovered from genital tract infection, as monitored by consistent failure to isolate MoPn, were challenged for resistance (immunity) to reinfection. The doses used were equivalent to primary infection doses: 10^3 to 10^4 ELD_{50} per mouse. Isolation of MoPn was attempted on days 5 and 10 postchallenge. Normal animals were also inoculated at the time of challenge to confirm infectivity of challenge inoculum.

RESULTS

Duration of shedding of MoPn. The results of two experiments in which mice were inoculated with MoPn and individually followed for shedding of the agent are shown in Fig. 1. Recovery of MoPn on day 3 was attributed to survival of residual inoculum and was discounted as evidence for infection. A total of 12 of 15 animals were successfully infected. In experiment A, with 3.5×10^5 ELD_{50} per mouse, infected animals continued to shed the agent at least until day 21 (three of four), whereas in experiment B, with 3.6×10^4 ELD_{50} per mouse, only one of eight was positive for MoPn about this time. Variability in the results may be accounted for by the numbers of animals and the doses. In experiment B, an unexpected finding was obtained for two animals in that they were apparently not successfully infected after inoculation yet yielded positive results on day 26. Since five animals were caged together, it is possible that these mice were infected by exposure to secretions of infected animals. Other than these two exceptions, MoPn was not recovered after day 20 to 21, and intermittent shedding was not observed; in experiment B, the animals were monitored through day 42.

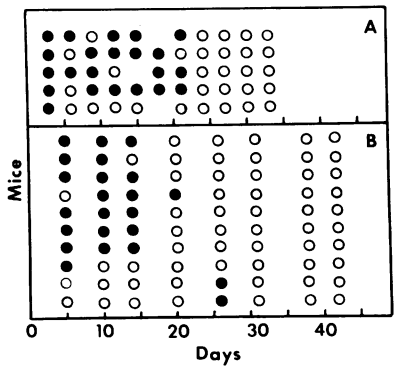

FIG. 1. Course of MoPn infection in individual mice. Each row of circles represents isolation attempts from an individual mouse. Symbols: ●, MoPn isolated; ○, MoPn not isolated.

Antibody response in plasma and secretions. Antibody response was determined in mice infected in parallel with those in infection experiments. IgM antibodies to MoPn in plasma were detected by day 7, and IgG antibodies were detected by day 14 (Fig. 2). IgM titers increased until day 29 and were still strongly positive on the last day of testing (day 56). IgG titers continued to increase after day 14 and were quite high ($\bar{x}$ = 794) on day 56. Antibodies to MoPn were detected in secretions by day 14, and peak titers were obtained by day 20. The titers were still appreciable in secretions on day 56. The presence of high antibody titers in plasma and genital secretions corresponded temporally with the absence of shedding of MoPn.

Delayed hypersensitivity. The nature of the footpad swelling response is shown in Fig. 3. Three hours after inoculation, an increase in swelling (footpad units) was observed in footpads of convalescent animals challenged with MoPn antigen. This was statistically higher than the change detected in other footpads of the same animals inoculated with control antigen ($P < 0.05$). Within 12 h, this response had declined. This early swelling was considered to be an Arthus-like reaction. A true DTH reaction followed and was at a maximum at 24 h; the reaction declined after 48 h. It was noted that response to McCoy cell antigen in infected animals was stronger than the response of uninfected to MoPn antigen. However, the response of uninfected to McCoy cell antigen was equivalent to that of infected animals (data not shown). This could be accounted for by the fact that the amount of cell material in MoPn antigen was probably less as a result of cell destruction due to infection.

Table 1 summarizes the results of two experiments in which the time course of appearance of DTH was followed. Arthus-like reactions are also shown for comparative purposes. Only 1 of 10 animals yielded a positive DTH reaction when the test was performed on days 10, 15, and 20. Beginning on day 25, more consistent positive reactions were obtained which lasted up to day 54, the last day of testing. Positive Arthus-like reactions were also observed in the majority of the animals tested after day 25.

Resistance to reinfection. Mice that had recovered from genital tract infection as measured by consistent failure to isolate MoPn were tested for immunity (resistance) to reexposure. Infection was detected in none of six mice challenged 42 days post-primary inoculation with a dose equivalent to the primary dose. In a second experiment, mice were challenged 42 (five mice), 51 (four mice), and 60 days (five mice) post-primary inoculation. No infection was detected in any of the animals challenged. Thus, solid immunity to reexposure was a consequence of primary genital infection with MoPn. Based on data obtained for shedding of the agent, the time period tested would be comparable to 20 to 50 days after primary infection had resolved.

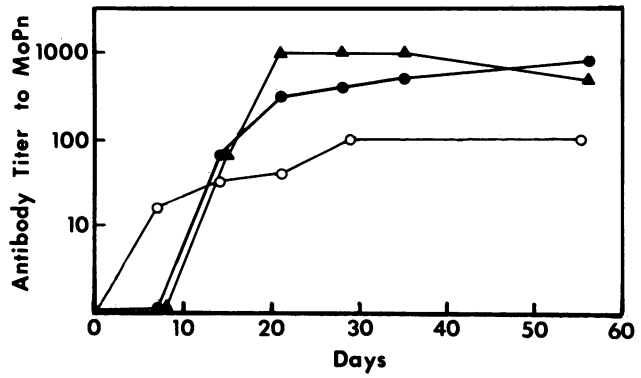

FIG. 2. Antibody response in mice infected with MoPn. Symbols: ●, plasma IgG response to MoPn; ○, plasma IgM response (obtained from a different experiment); ▲, immunoglobulin response in genital secretions. Each point represents the geometric mean antibody titer of four to five animals.

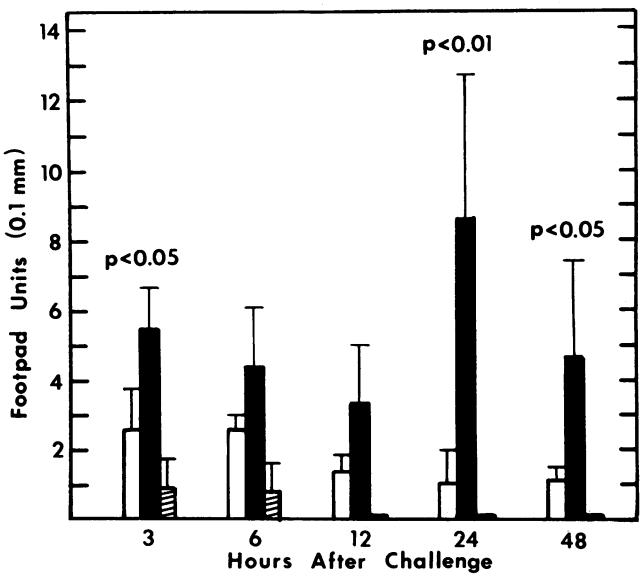

FIG. 3. Footpad response to infection with MoPn antigen in mice 30 days after genital inoculation with MoPn. Each bar represents the mean ± standard deviation of five mice. Solid bar: MoPn antigen in McCoy cells; open bar: McCoy cells; hatched bar: MoPn antigen injected into uninfected mice. A one-tailed *t* test was performed between MoPn antigen and McCoy cells alone in the infected animals. *P* values are given where significant differences were found.

DISCUSSION

Immune response during human genital infection with *C. trachomatis* has been a subject of considerable interest. More information is currently available concerning infection of females than of males. Antibody response to chlamydiae has been documented in the IgM, IgG (12), and IgA (11) classes in serum and cervical secretions (12). Anti-chlamydial sIgA has also been detected in secretions (12). Cell-mediated immune response has been demonstrated by leukocyte migration inhibition (5) and lymphocyte transformation (3). Despite this information, the role of the immune response in recovery from genital infection and resistance to reinfection requires further definition.

Considerable knowledge has been obtained from animal model studies (1). In the system reported here, mice were inoculated intravaginally with MoPn. A high incidence of infection was obtained by this route, which simulates infection by sexual transmission. Mice ceased to shed MoPn in the genital tract between days 15 and 21, at a time when near-peak antibody titers in plasma and genital secretions had been achieved. In contrast, Tuffrey and Taylor-Robinson (13) were relatively unsuccessful in producing infection in mice by the intravaginal route with a genital strain of *C. trachomatis* or SA_2(f) (LGV) strain. Infection was only

successful by the intrauterine route in progesterone-treated animals with the "fast" strain. Under these conditions, the agent could be isolated from 33 to 43 days post-inoculation.

Animals challenged in our studies between days 42 and 68 post-primary inoculation were solidly resistant (immune) to reinfection. These results provide base-line information concerning immunity to chlamydial genital infection in mice. Williams et al. (15) did show protection to intranasal challenge in heterozygous littermates (nu/+) of nude mice after intrauterine immunization with MoPn.

The early appearance of IgM antibodies to MoPn in plasma is not surprising, but persistence until day 56, the last day tested, might be unexpected. In pneumonia studies of mice with *C. trachomatis* (6) and MoPn (15), IgM antibodies in serum were not detected. The results obtained to date in our study should be interpreted with caution. They may reflect the sensitivity and specificity of the reagents used or an important feature of genital tract infection. Patton et al. (9) found persistence of IgM antibodies through 12 weeks after inoculation of pig-tailed macaque monkeys with *C. trachomatis* into fallopian tubes.

The incidence of cell-mediated immune response, as detected by footpad swelling (DTH), was not appreciable until day 25; by this time the animals had ceased to shed the agent. An interesting contrast was reported by Kuo and Chen (8), who studied pneumonitis in mice produced by an ocular strain of *C. trachomatis*. They found that DTH, also determined by footpad swelling, could be detected as early as day 5 and peaked at day 7, preceding peak antibody titers. Since growth of the agent in lungs was apparently maximal on day 2 and declined rapidly, emphasis was placed on the role of cell-mediated immunity in regulating shedding of *C. trachomatis*. In investigating pneumonia in nude mice caused by MoPn, Williams et al. (15) found a significant role for humoral antibody in recovery from infection.

In our studies of footpad swelling, an Arthus-like reaction was observed. To our knowledge, this phenomenon has not been previously reported regarding chlamydial infections. However, it is not surprising since high levels of IgM and IgG are routinely observed in animals infected with chlamydiae. The development of the Arthus-like reactions reported here occurred concomitantly with the appearance of serum antibodies to MoPn.

The data reported in this study define the immunological parameters of the mouse-MoPn system and provide a basis for immune manipulation to further analyze immune mechanisms in chlamydial genital infections.

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