

Intranasal Immunization Induces Long-Term Protection in Mice against a *Chlamydia trachomatis* Genital Challenge

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In an attempt to confer long-term protective immunity, BALB/c female mice were immunized intranasally with 10^4 inclusion-forming units (IFU) of the *Chlamydia trachomatis* mouse pneumonitis biovar (MoPn). Animals were subsequently challenged in the ovarian bursa with 10^5 *C. trachomatis* MoPn IFU at 60, 120, or 180 days post-intranasal immunization. Two control groups were included in the study. One control was sham immunized and mock challenged, and another group was sham immunized and challenged with 10^5 *C. trachomatis* MoPn IFU. Vaginal cultures were collected at regular intervals following the intrabursal challenge. In comparison with the sham-immunized mice, the animals that were intranasally immunized with *C. trachomatis* had significant protection, as shown by a reduction in the number of animals that had positive vaginal cultures and by a decrease in the intensity and length of the shedding. Furthermore, histopathological characterization of the genital tract following challenge, in the three groups of mice, showed a minimal inflammatory infiltrate in the *C. trachomatis*-immunized animals, when compared with the sham-immunized control group. Subsequently, the three groups of female mice that were challenged at 60, 120 and 180 days postimmunization were mated at 6 weeks following the challenge. Overall, in the mice intranasally immunized with *C. trachomatis* the fertility rates and the number of embryos were similar to those in the sham-immunized and mock-challenged group. In contrast, there was a significant increase in infertility in the groups of mice that were sham immunized and *C. trachomatis* challenged. In conclusion, intranasal immunization with *C. trachomatis* induces long-term protection against a genital challenge as shown by a decrease in the infection and infertility rates when compared with sham-immunized animals. Thus, this model may help to characterize the parameters of the immune response that are important in maintaining long-term protection and may aid in identifying the antigenic determinants involved in eliciting protection.

Chlamydia trachomatis has for several decades been recognized as the etiological agent of trachoma, the leading cause of preventable blindness in the world (13, 39). Recently, however, more emphasis has been placed on characterizing the role that this intracellular bacteria plays in genital and perinatal infections. As a result, this organism has now been found to be one of the leading sexually transmitted pathogens (13, 39). Among the sexually transmitted diseases caused by *C. trachomatis*, endometritis and salpingitis can result in long-term sequelae, including chronic abdominal pain, ectopic pregnancy, and infertility (1, 3, 5, 16, 33, 42, 47, 48). Although *C. trachomatis* is susceptible to antibiotic treatment, over 50% of the genital infections in females are asymptomatic and thus the only effective approach against this pathogen is to use immunoprophylactic measures (10, 14, 15, 46).

Over the last four decades, a significant amount of effort has been focused on developing vaccines for *C. trachomatis* infections. Several vaccination trials were performed in humans in an attempt to protect individuals at high risk for trachoma (14, 15, 46). These trials were for the most part quite disappointing. For example, by using viable or inactivated *C. trachomatis* elementary bodies (EB), it was observed that the protection was short-lived and serovar specific. In addition, some vaccinated individuals developed a worse disease than the nonvaccinated controls when they subsequently became reexposed. Furthermore, long-term follow-up studies indicated that there

were no significant differences between the clinical status of eyes of the vaccinated individuals and those of the control groups. Similar results were obtained in animal models such as the Taiwan monkey *Macaca cyclopis* (14). For example, it was shown that monkeys immunized with 10^9 EB were protected for 1 to 2 years, while those receiving 10^7 EB had an increased susceptibility to the infection and more severe disease upon reexposure.

Although efforts are still devoted to preventing trachoma, lately the focus of attention in Western countries has shifted to the production of vaccines against the sexually transmitted diseases produced by *C. trachomatis*. In general, to date, the results from the animal studies support the overall conclusions reached with the trachoma vaccines; e.g., the protection is weak and short-lived (7, 25, 34, 37). In an attempt to learn more about this problem, we have recently reported that in a BALB/c mouse model intranasal immunization with the *C. trachomatis* mouse pneumonitis biovar (MoPn) protected the animals against a genital tract challenge (29). In those studies we challenged the mice at 2 months following intranasal immunization. Thus, the question of the length of protection remains to be answered. Here, we report on the results obtained when mice were challenged up to 6 months postimmunization.

MATERIALS AND METHODS

Organisms. The *C. trachomatis* MoPn strain Nigg II, obtained from the American Type Culture Collection (Rockville, Md.), was grown in HeLa 229 cells, and the EB were purified as previously described (4, 32). For the isolation of *C. trachomatis* MoPn, vaginal swabs were collected at different intervals following intrabursal inoculation and cultured into McCoy cell monolayers (28).

Immunization and challenge protocols. Seven- to eight-week-old BALB/c

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(*H-2^d*) female mice obtained from Simonsen Laboratories (Gilroy, Calif.) were used in this study. Mice received a standard mouse diet at the University of California, Irvine, vivarium. Three groups of mice were entered in this study: intranasally immunized with *C. trachomatis* MoPn and *C. trachomatis* MoPn challenged (immunized, challenged); sham immunized and then challenged with *C. trachomatis* MoPn (sham immunized, challenged); sham immunized but not challenged (sham immunized, nonchallenged). Mice were immunized by intranasal inoculation with 10⁴ inclusion-forming units (IFU) of *C. trachomatis* MoPn per mouse, and the control mice received mock-infected HeLa 229 cell extracts (29).

To determine the length of protection, each group of mice was divided into three subgroups that were challenged at 60, 120, or 180 days postimmunization. Each mouse was inoculated in the left ovarian bursa with 10⁵ IFU of *C. trachomatis* MoPn. The right ovarian bursa received mock-infected HeLa cell extract processed by using the same protocol employed for purification of EB. The experiment was repeated twice.

Immunoassays for humoral immunity. Blood and vaginal secretions were collected by using the procedures described elsewhere (28). The inclusion immunofluorescence assay (IFA) was performed by using McCoy cell monolayers infected with *C. trachomatis* MoPn (31). Fluorescein-labeled goat anti-mouse immunoglobulin G (IgG), IgM, and or IgA diluted 1:50 in phosphate-buffered saline (PBS) containing 0.02% Evans blue served as the secondary antibody.

The enzyme-linked immunosorbent assay (ELISA) was performed as described by Pal et al. (28). Briefly, 96-well flat-bottom plates (Corning Glass Works, Corning, N.Y.) were coated overnight with 1 µg of viable, purified *C. trachomatis* MoPn EB per well. A 100-µl aliquot of a twofold serial dilution of a serum sample was added to each well, and the wells were incubated 1.5 h at 37°C. The EB-antibody complexes were detected by adding horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies. The following class/subclass-specific antibodies were tested: IgG, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM (Southern Biotechnology Associates, Inc., Birmingham, Ala.), and the binding was measured at 405 nm by using an ELISA reader (Bio-Rad Corp., Hercules, Calif.). For color development, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was used as the substrate.

Western blots (immunoblots) were performed as previously described using nitrocellulose membranes (28). *C. trachomatis* MoPn EB, 250 µg on a 7.5-cm-wide mini slab gel, were resolved by 10% tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (40). Serum samples diluted 1:100 with PBS-0.05% Tween 20 (PBS-T) (Fisher Chemical, Fair Lawn, N.J.) were incubated 1.5 h at room temperature. The strips were then incubated 1 h with a 1:500 dilution of HRP-conjugated goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology Associates, Inc.) and the bands were developed with a 4-chloro-1-naphthol solution.

To further characterize the specificities of the B-cell epitopes of the major outer membrane protein (MOMP) a 1:500 dilution of sera was used to probe octameric peptides representing MOMP. Overlapping octameric peptides of MOMP were synthesized with a commercially available kit (Cambridge Research Biochemicals Inc., Wilmington, Del.) (11, 12). By using goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Inc.) conjugated to HRP as the second antibody and ABTS as the substrate, reactions were read as indicated above. The assay was repeated twice, using sonication in hot SDS and 2-β-mercaptoethanol to remove the antibodies between each assay (30).

The in vitro neutralization assay was performed as previously described, using centrifugation to infect the HeLa cell monolayers (32). The neutralization titer of a serum sample was the dilution which yielded 50% neutralization over the control serum.

Detection of ASC. Spleen cells isolated from mice were used in an ELISPOT assay to enumerate chlamydia-specific antibody-secreting cells (ASC) (29, 41). Sterile flat-bottom 96-well ELISA plates were coated overnight with 50 µl of *C. trachomatis* MoPn EB (1 mg/ml) in 10 mM PBS (pH 7.4) at 4°C. Each well then received 10⁵ unseparated spleen cells in 0.1 ml of RPMI 1640 containing 2 mM L-glutamine, 10% inactivated fetal calf serum, 2-β-mercaptoethanol (0.05 mM), and gentamicin (50 µg/ml), and the wells were incubated overnight at 37°C with 5% CO₂. A 1:500 dilution in PBS-T of alkaline phosphatase conjugated with goat anti-mouse IgG, IgM, or IgA antibody was added to each well. Finally, the antigen-antibody spot was developed by adding 5-bromo-3-chloro-3-indolyl phosphate (BCIP).

Assays for cell-mediated immunity. The lymphoproliferative assay was performed as previously described (29). Briefly, the spleens from two mice from each group were harvested, and the cells were teased into a single-cell suspension and enriched for T cells in a nylon wool column. Using a fluorescence-labeled monoclonal antibody to CD3⁺ (GIBCO-BRL, Grand Island, N.Y.) 85 to 90% of the suspension was found to be T cells. Antigen-presenting cells were prepared by irradiating (3,300 rad of ¹³⁷Cs) unseparated spleen cells and incubating them with various concentrations of MoPn EB. The enriched T cells were cultured in 96-well plates at a concentration of 8 × 10⁴ cells per well in 0.2 ml of RPMI 1640 supplemented with 10% fetal bovine serum. Lymphocytes were cocultured for 5 days with 1.2 × 10⁵ antigen-presenting cells with or without antigen and stimulated with MoPn EB. Concanavalin A was added to the wells as a positive stimulant. At the end of the fourth day of incubation, 1.0 µCi of (methyl-³H) thymidine (47 Ci/mmol; Amersham, Arlington Heights, Ill.) in 25 µl of RPMI 1640 was added to each well and the uptake of (³H)thymidine was measured.

For the detection of gamma interferon (IFN-γ) and interleukin-4 (IL-4), splenic T cells were stimulated as described above and the tissue culture supernatants were collected at 48 h of incubation. The instructions provided by the manufacturer with the enzyme immunoassay kits for the quantitation of IFN-γ and IL-4 were followed (Endogen, Cambridge, Mass.).

Histology. Animals were killed at different days following intrabursal inoculation. Tissues were fixed in buffered formalin and processed by standard techniques. Histological sections were stained with hematoxylin and eosin (29).

Fertility studies. Six weeks following intrabursal challenge, groups of four or five female mice were housed together with a proven breeder male mouse (Simonsen Laboratories) for 18 days and the pregnancies were monitored following the previously described protocol (29). Briefly, pregnancy was assessed by measuring the weight gained following mating. The mice that gained 5 to 10 g of weight starting at 18 days postmating were considered to be pregnant and were euthanized. After the first mating, the female mice that did not gain weight were mated again with male mice that had been successfully mated with another group of female mice and monitored as described above. Finally, all animals that had not gained weight were euthanized 25 days from the start of second mating. At the time that the female mice were killed, the number of embryos in each uterine horn was counted.

Statistics. The statistical analyses were performed with the Statview software package on a Macintosh computer. The two-tailed unpaired Student *t* test, the Mann-Whitney U test, and the Fisher exact test were employed to assess the significance of differences between the groups.

RESULTS

Vaginal shedding. Following intrabursal challenge with 10⁵ *C. trachomatis* IFU, vaginal swabs were collected and cultured on a weekly basis for a period of 6 weeks. As shown in Fig. 1A, of the sham-immunized mice that were challenged 60 days later, 76.5% (13 of 17) shed *C. trachomatis* during the second week following the challenge (Fig. 1A). This is in contrast to only 5% (1 of 20) of the mice that had been intranasally immunized with 10⁴ *C. trachomatis* IFU (*P* < 0.05). Overall, 82.4% (14 of 17) of the sham-immunized animals shed during the 6 weeks of the observation, while only 10% (2 of 20) of the immunized animals shed (*P* < 0.05). Furthermore, the number of IFU per mouse was significantly higher in the sham-immunized mice. For example, on the second week the average number of IFU per mouse in the sham-immunized group was 14,734 (±39,504) while in the immunized group it was 50 (±224) (*P* < 0.05) (Fig. 1A). Similar results were observed with the challenged mice at 120 and 180 days (Fig. 1B and C). Of the sham-immunized mice that were challenged 120 days later, 65% (13 of 20) shed, while none of the 21 mice immunized with *C. trachomatis* shed (*P* < 0.05). In the groups challenged at 180 days postimmunization 73.7% (14 of 19) and 27.2% (6 of 22) of the sham and immunized mice shed, respectively (*P* < 0.05). All mice that were immunized and challenged at 180 days had negative cultures by the third week, while the sham-immunized mice were still positive by the fourth week postchallenge. The average number of IFU per mouse was also higher in the sham-immunized group; e.g., on the second week postchallenge the sham-immunized group shed on the average 23,080 (±51,639) IFU per mouse, while the immunized mice shed on the average 32 (±151) IFU per mouse (*P* < 0.05). All cultures from the control mice that were sham immunized and nonchallenged were negative.

Characterization of the immune response. To characterize the immune response from the mice inoculated with 10⁴ *C. trachomatis* IFU, serum and vaginal samples were collected prior to intrabursal challenge at 60, 120, and 180 days postimmunization. In addition, spleens were harvested and assayed for the presence of antibody-specific cells and for lymphoproliferative assay. As shown in Table 1, high-chlamydia-specific serum IgG antibodies remained fairly stable throughout the period of observation. In general, the IgG2a immune response was predominant over the IgG1 response. High levels of IgG3 were also detected in the course of the experiment. IgA serum levels increased fourfold over the 6 months of observation. High

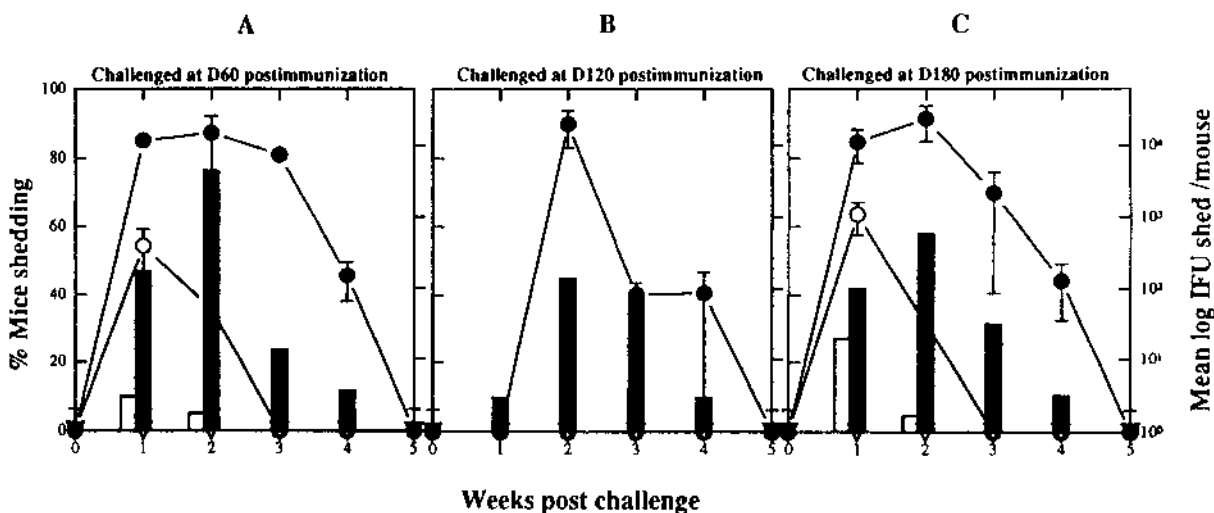


FIG. 1. Vaginal shedding observed in the *C. trachomatis* mice challenged at 60, 120, and 180 days postimmunization. The percentage of mice shedding for a particular group is shown as a bar graph, and the mean number \pm standard error of *C. trachomatis* MoPn IFU per mouse is shown as a line drawing. The black bars and the black circles correspond to the sham-immunized, challenged mice, and the open bars and open circles correspond to the immunized, challenged mice.

neutralizing antibody titers, ranging from 5,400 to 6,600, were detected in the three groups of mice tested. Stable levels of IgG and IgA chlamydial antibody were also measured in the vaginal secretions at the three time points tested.

The number of *C. trachomatis*-specific IgG-secreting cells per 10^6 splenocytes was 150 at day 60 postimmunization and subsequently declined to 32 by 180 days postimmunization. On the other hand, the number of IgA specific B cells remained stable, between 150 and 188, for the 6 months following the intranasal immunization.

To further characterize the immune response, Western blots were probed with the murine sera and binding was detected with IgG-, IgG1-, and IgG2a-specific secondary antibodies (Fig. 2). Overall, the Western blot results correlated well with the ELISA data showing a predominant IgG2a over an IgG1 response. In addition to an overall quantitative increase of the IgG2a response, there were also qualitative differences between the antigens recognized by the two IgG subclasses. For example, relatively higher intensity bands at 42 (MOMP), 31, 27, 20, and 15 kDa were identified by the IgG2a subclass when compared with the IgG1 subclass. Furthermore, the reactivity to these bands was more prominent with the serum samples collected at 120 and 180 days postimmunization than with the specimens collected at 60 days postimmunization.

Serum samples from the immunized mice collected at 60, 120, and 180 days postimmunization were also reacted with synthetic octapeptides corresponding to the amino acid sequence of the *C. trachomatis* MoPn MOMP. Goat anti-mouse IgG1 or IgG2a was used as the secondary antibody. As shown in Fig. 3, discrete regions were bound in each of the four

variable domains (VD) of the *C. trachomatis* MoPn MOMP when the IgG2a was used as the secondary antibody. The consensus amino acid sequences reacting for each of the VD were as follows: VD1, TGDADLTAP; VD2, AVAADDI; VD3, QEFPLN; and VD4, SGSGIDVDTKIT. Overall, positive reactions to the four VD were obtained with the three sample sera. However, the two serum samples collected at 120 and 180 days postimmunization showed a strong decrease in reactivity with the VD3 and moderate decreases with the VD1 and VD4 relative to the reactivity of the sample from day 60. The reactivity to the VD2 was quantitatively similar for the three serum samples. No significant reactivity was observed when the peptides were probed for IgG1 antibodies, probably reflecting the overall lower titer of this Ig isotype.

The T-cell response was assayed at 60 days postimmunization. As shown in Table 2, in the animals that were immunized the proliferative response to MoPn EB was approximately 10-fold higher than in the mice that were sham immunized ($P < 0.05$). The production of IFN- γ and IL-4 was determined in the T-cell-enriched splenocyte supernatants after stimulation with MoPn EB. While the levels of IFN- γ were high, 298 ng/ml, the levels of IL-4 were below the level of detection in the immunized animals. In the sham-immunized control group, the levels of both IFN- γ and IL-4 were below the limits of detection for the assay.

Histopathological analyses. A significant acute inflammatory infiltrate consisting mainly of polymorphonuclear leukocytes was observed at 8 and 15 days postchallenge, in the oviducts of the mice that were sham immunized (data not shown). This acute inflammatory infiltrate was similar in the

TABLE 1. Systemic and local antibody response of the immunized mice before challenge

Days post-immunization	MoPn specific serum ELISA antibody titer						Vaginal wash IFA antibody titer		Serum neutralization titer	MoPn-specific ASC in spleen (10^6 /ml)	
	IgG	IgG1	IgG2a	IgG2b	IgG3	IgA	IgG	IgA		IgG	IgA
60	51,200	1,600	25,600	12,800	12,800	6,400	32	4	6,200	150 \pm 53	173 \pm 26
120	102,400	200	51,200	25,600	6,400	12,800	16	16	5,400	125 \pm 9	188 \pm 66
180	102,400	400	25,600	25,600	6,400	25,600	16	8	6,600	32 \pm 5	150 \pm 53

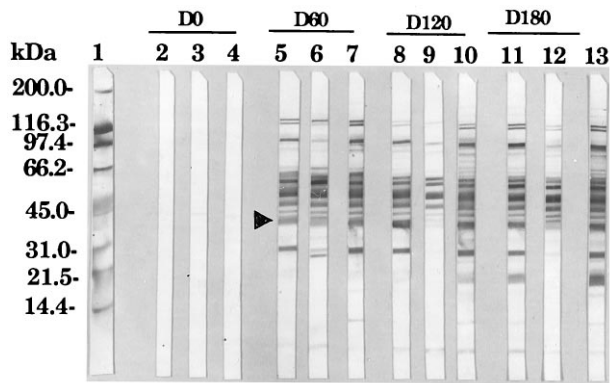


FIG. 2. Western blot of the sera collected at day 60, 120, and 180 postimmunization. Lane 1, molecular weight markers (biotinylated SDS-PAGE broad-range standard kit; Bio-Rad Laboratories). Lanes 2 to 13: *C. trachomatis* MoPn EB were probed with preimmune sera (lanes 2 to 4), or with sera from day 60 (lanes 5 to 7), day 120 (lanes 8 to 10), or day 180 (lanes 11 to 13) postimmunization, respectively. The second antibody was anti-IgG (lanes 2, 5, 8, and 11), anti-IgG1 (lanes 3, 6, 9, and 12), or anti-IgG2a (lanes 4, 7, 10 and 13). The arrowhead points to the MOMP band.

three groups of mice that were challenged at 60, 120, and 180 days post-sham immunization. By 21 days postchallenge the infiltrate was composed of a mixture of polymorphonuclear leukocytes and mononuclear cells including lymphocytes and

plasma cells. Specimens collected at 31 days postchallenge showed only few isolated foci of lymphocytes and plasma cells. In contrast, the mice that were intranasally immunized with *C. trachomatis*, and subsequently *C. trachomatis* challenged at 60, 120, and 180 days, had only a minimal reaction consisting mainly of mononuclear inflammatory cells, including lymphocytes and plasma cells, and a few polymorphonuclear leukocytes. The groups of mice that were sham immunized and nonchallenged showed no histopathological lesions.

Fertility studies. At 6 weeks following intrabursal challenge the mice were mated. The female mice were weighed, and those animals that gained weight were euthanized. Those that did not gain weight were mated a second time with a different male mouse and euthanized as the first group. The results of the two mating episodes are shown in Table 3. Overall, mice that were intranasally immunized with *C. trachomatis* and were subsequently challenged at 60, 120, or 180 days postimmunization had bilateral fertility rates similar to those of the control animals that were sham immunized and nonchallenged. For example, of the animals immunized with *C. trachomatis* that were challenged at 60, 120, and 180 days, 85.0% (17 of 20), 80.0% (16 of 20), and 54.5% (12 of 22) were bilaterally fertile, respectively, while in the sham-immunized nonchallenged group 92.3% (24 of 26), 75% (15 of 20), and 75% (12 of 16) of the mice, respectively, were bilaterally fertile ($P > 0.05$). This contrasts with 11.8% (2 of 17), 25% (5 of 20) and 27.8% (5 of

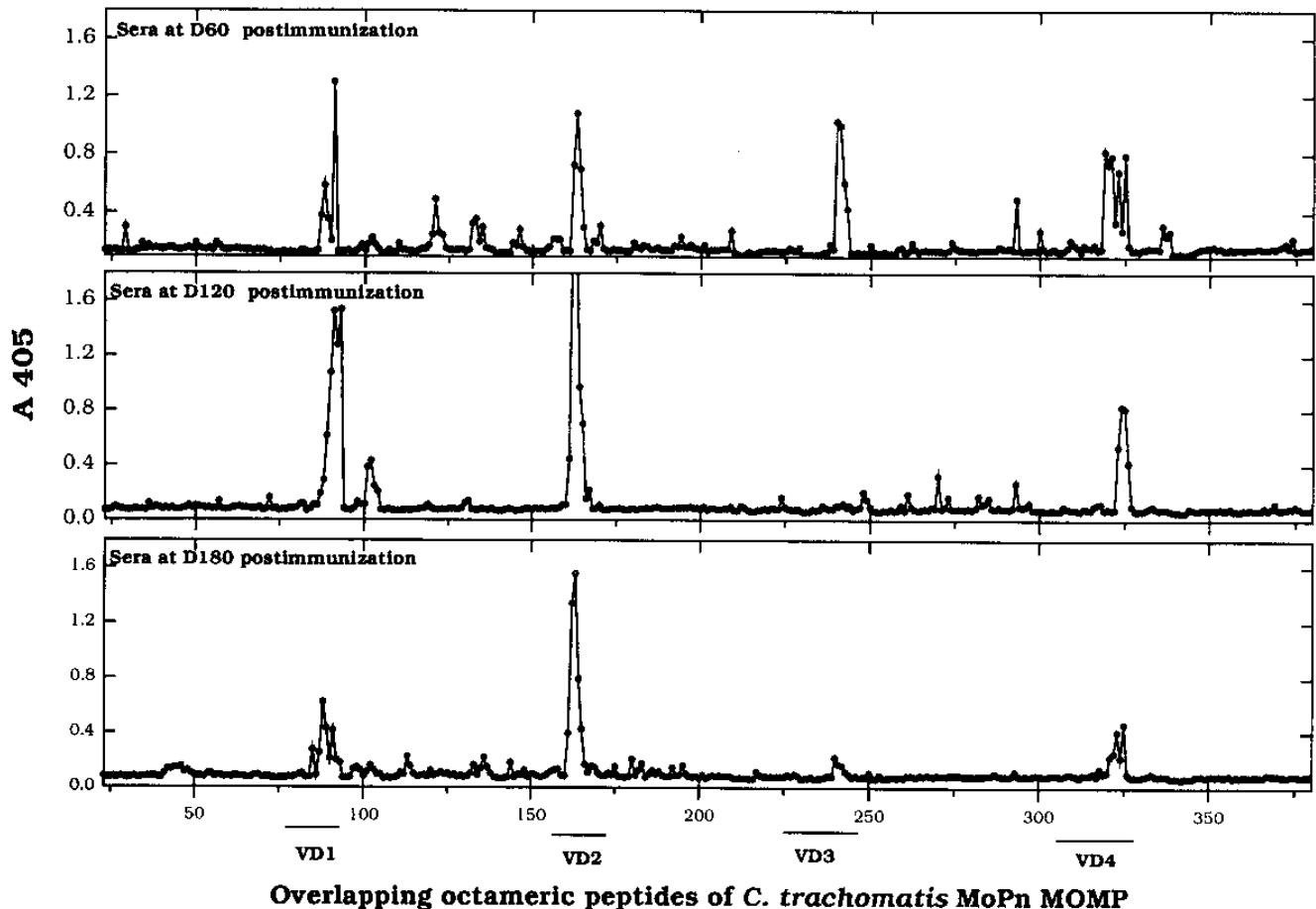


FIG. 3. Absorbance values obtained using octameric synthetic peptides corresponding to the *C. trachomatis* MoPn MOMP probed with serum collected at 60, 120, or 180 days following intranasal immunization with *C. trachomatis* MoPn. Goat anti-mouse IgG2a was used as the second antibody.

TABLE 2. T-cell response at day 60 postimmunization^a

Group	T-cell proliferative response (cpm ± 1 SD)			In vitro production of cytokines					
	EB ^b	Con A ^c	Medium	IFN-γ (ng/ml)			IL-4 (pg/ml)		
				EB ^b	Con A ^c	Medium	EB ^b	Con A ^c	Medium
Immunized	35,335 ^d ± 4,373	79,079 ± 11,769	117 ± 38	298 ^d	317	<0.1	<10	68	<10
Sham immunized	3,652 ± 101	35,124 ± 1,923	540 ± 159	<0.1	97	<0.1	<10	75	<10

^a Results are means for triplicate cultures. Data correspond to one of the experiments representative of duplicate separate experiments.

^b UV-inactivated *C. trachomatis* MoPn EB were added at a 1:1 ratio to the T cells.

^c Concanavalin A (Con A) was added at a concentration of 5 μg/ml.

^d $P < 0.05$ by Student's *t* test.

18) fertility rates for the mice that were sham immunized and challenged ($P < 0.05$) on the same dates. Similarly, the number of embryos in the left uterine horn, the site of the challenge, was similar in the immunized, challenged mice when compared with those of the sham-immunized, nonchallenged animals. For example, the groups that were immunized and challenged at 60, 120, and 180 days had an average of 2.3, 2.4, and 2.2 embryos, respectively, while the sham-immunized, nonchallenged group had on the average 2.8, 2.8, and 1.6 embryos in the left uterine horn ($P > 0.05$). This contrasts with 0.5, 0.7, and 0.8 embryos recovered from the sham-immunized, challenged group ($P < 0.05$).

DISCUSSION

Chlamydial genital infections affect individuals mainly in their early years of sexual activity (39, 47, 48). As a result, females acquire the disease during their teens to early twenties and some develop long-term sequelae following an asymptomatic or an acute episode of pelvic inflammatory disease (1, 3, 5, 16, 33, 42, 47, 48). As the individual grows older, either as a result of an increased natural or acquired immunity or due to a change in sexual behavior, the rate of sexually transmitted chlamydial infections decreases. Thus, the goal of a vaccine should be to protect young females during their initial sexual contacts. This "fertility window" period in the human female population spans only a period of 20 to 30 years, so that a chlamydial vaccine does not necessarily have to provide life time protection (10). Our ability to protect in this mouse model for a period of at least 180 days, equivalent to approximately a quarter of their life span, suggests that we should be able to produce vaccines for humans that protect for periods of two to three decades. Furthermore, although optimally a vaccine should protect against infection, this goal may be currently unrealistic and thus our efforts should concentrate on producing a vaccine that protects against disease, e.g., long-term sequelae, in particular infertility (10, 14, 15, 38, 46).

Several investigators have assessed the ability of vaccines to protect animal models against a chlamydial genital challenge. For example, Ramsey et al. (34) infected BALB/c mice intravaginally with *C. trachomatis* MoPn under the assumption that the natural route of infection could provide optimal protection. However, these authors found that mice were susceptible to reinfection, as shown by vaginal shedding, starting at 100 days following the initial intravaginal inoculation. Obviously, by using the intravaginal route for immunization it was not possible to evaluate the impact of the challenge results on the long-term sequelae. Cui et al. (7) orally immunized mice with viable *C. trachomatis* L2 and found that the animals had a significant degree of protection against an intravaginal challenge, as indicated by the absence of antigen shedding from the vagina. The intravaginal challenges, however, were performed

only at 12 and 24 days postimmunization, and thus the long-term protective effect of the oral immunization was not evaluated. Furthermore, no attempt was made to assess the impact of the immunization on fertility. Rank et al. (37) immunized guinea pigs with *Chlamydia psittaci* guinea pig inclusion conjunctivitis agent by intravenous, subcutaneous, oral, and ocular routes and challenged them intravaginally 2 weeks later. Overall, they saw a reduction in the intensity of the infection in all animals independent of the route of immunization, suggesting that both local and systemic immune responses may contribute to limit the infection. No attempt, however, was made to determine long-term protection.

We chose to use intranasal immunization with viable *C. trachomatis* in our animal model because chlamydial vaccines using viable organisms, like most other immunization protocols against microbial pathogens, have given overall better results than vaccines formulated with killed bacteria (37). Furthermore, *C. trachomatis* MoPn replicates well in the respiratory tract and thus we thought that more likely it could induce a strong mucosal immune response that may be critical for protection (2, 18). In general, local specific IgA and CD4⁺ responses appear to be critical for protection while CD8⁺ cells do not seem to play a substantive role in protection against chlamydial infections (2, 20). Here, we showed that an intranasal immunization induces a robust immune response that can protect mice against the long-term sequelae of an intrabursal challenge for at least 180 days. Thus, these results suggest that long-term effective vaccines are feasible against chlamydial genital infections and that we can use this animal model to identify the components of the host immune response that participate in the long-term protective response.

Work by Mosmann and Coffman (23) established the presence of Th1 and Th2 subsets of CD4⁺ T cells in the mouse. Th1 and Th2 cells subsets result from the stimulation of Th0 cells by IL-12 and IL-4, respectively. Th1 cells preferentially produce IFN-γ, IL-2, and tumor necrosis factor beta. Depending on several parameters, including the nature of the antigen and route of infection or inoculation, one particular type of the two Th subsets will predominate. Control of an infection with a particular pathogen may depend on the type of Th cell subset that predominates in the immune reaction (23). CD4⁺ T cells, and in particular the Th1 subset, appear to be critical for the clearance of a chlamydial genital infection (17, 20, 35, 36). Here, we have shown a significant increase in the levels of IFN-γ over IL-4, and of chlamydia-specific IgG2a over IgG1, following intranasal immunization, suggesting a predominance of Th1 over the Th2 cell type response. This specific IgG2a response was directed, among others, at particular regions of the variable domains of the MOMP that have been shown to correspond to neutralizable epitopes using monoclonal antibodies (27, 30, 32).

TABLE 3. Fertility results of mice challenged at 60, 120, or 180 days postimmunization

Group	Challenged at day 60			Challenged at day 120			Challenged at day 180			
	No. (%) bilateral fertile	Mean no. of embryos in the uterine horns ^a		No. (%) bilateral fertile	Mean no. of embryos in the uterine horns ^a		No. (%) bilateral fertile	Mean no. of embryos in the uterine horns ^a		
		Right	Left		Both	Right		Left	Both	Right
Immunized, challenged	17/20 (85.0)	3.3 ± 2.2	2.3 ± 1.8	5.6 ± 2.8	2.7 ± 1.9	2.4 ± 1.5	5.1 ± 2.6	2.3 ± 2.4	2.2 ± 2.4	4.5 ± 4.1
Sham immunized, challenged	2/17 ^b (11.8)	1.9 ± 2.3	0.5 ^c ± 0.8	2.4 ^c ± 2.2	2.0 ± 2.1	0.7 ^c ± 1.3	2.7 ^c ± 2.8	2.6 ± 1.5	0.8 ^c ± 1.7	3.4 ± 3.3
Sham immunized, nonchallenged	24/26 (92.3)	3.4 ± 1.6	2.8 ± 1.7	6.2 ± 2.6	2.4 ± 1.6	2.8 ± 1.9	5.2 ± 3.0	2.9 ± 2.0	1.6 ± 1.0	4.5 ± 2.9

^a The left ovarian bursa was inoculated with 10⁵ *C. trachomatis* MoPn IFU.

^b Significant by the Fisher exact test ($P < 0.05$) compared with the sham-immunized, nonchallenged group.

^c Significant by the unpaired Student's *t* test ($P < 0.05$) compared with the sham-immunized, nonchallenged group.

Furthermore, the role of IFNs, particularly IFN- γ , as an inhibitor of the growth of chlamydiae, both in vitro and in vivo, is well established (9, 49, 50). Hence, is not surprising that a predominant Th1 cell response would help to control a chlamydial infection. However, in addition to their role in protection it is possible that Th1 cells may be involved in the immunopathology of the disease (19, 21, 22). It has been proposed that in response to the chlamydial 60-kDa heat shock protein (hsp), Th1 cells preferentially increase IFN- γ production, macrophage activation, and delayed-type hypersensitivity. An increase in IFN- γ may result in a persistent infection with an augmented production of chlamydial 60-kDa hsp. The presence of epitopes in the human 60-kDa hsp that cross-react with epitopes in the chlamydial 60-kDa hsp could result in an immunopathological reaction with subsequent tissue damage, inflammation, and scarring (22, 26). In this respect, patients with infertility have been found to have antibodies to the 60-kDa hsp more frequently than control patients (6, 44, 45). Although this sequence of events may happen in certain cases, we think that infertility occurs in most individuals as a result of the damage produced by the acute infectious episode. Replacement of the connective tissue present in the normal fallopian tube by fibrotic scarring tissue as a result of the acute infectious episode could result in occlusion of the lumen of the tube. It is important to point out that in humans a majority of the patients with infertility had only a single pelvic inflammatory episode (47). Furthermore, recently Morrison et al. (20) showed that following an intravaginal challenge with *C. trachomatis* MoPn, mice with disrupted B2-microglobulin, I-A, or CD4 genes develop hydrosalpinx. Thus, the pathogenesis of tubal infertility does not appear to be mediated by a chronic immunological mechanism. The fact that in the *C. trachomatis*-immunized animals only a minimal inflammatory reaction was observed following the intragenital challenge and that the animals were protected against infertility suggest that a robust immunization protocol should be able to block the deleterious effects of a delayed-type hypersensitivity type reaction upon reexposure, as was previously observed in vaccinated individuals (14, 15).

In addition, to the role of the Th1 cells in protection, Th2 cells may also be important by helping B cells to produce specific neutralizing antibodies; e.g., IL-6 could induce mucosal B cells to secrete IgA-specific antichlamydial antibodies. The role of local vaginal antibody may be critical at the time of the initial infection, and subsequently antibodies may help to limit the cell-to-cell spread of the organism (2, 18, 24). Here, we showed that intranasal immunization results in prolonged stimulation of IgA chlamydia-specific cells in the vaginal mucosa. Furthermore, it is interesting to note that an increase in the antibody reactivity pattern by Western blot was observed in the serum samples collected at 120 and 180 days postimmunization when compared with the serum collected at 60 days postimmunization. A similar observation was described by Ramsey et al. (34) in mice inoculated intravaginally with *C. trachomatis* MoPn. These authors suggested that this late antibody response could be explained by a prolonged latent infection or by a decrease over time in the number of suppressor cells. Attempts to culture specimens from the genital tract after the initial infectious episode have been unsuccessful in these models (8, 29, 37, 43). Similarly, histopathological analysis of the genital tract indicates that the inflammatory reaction subsides after 6 to 8 weeks (8, 29, 43). Thus, the increase in the reactivity pattern by Western blot at 120 and 180 days postimmunization is most likely due to a decrease in the suppressive immune response (34). This immunosuppressive period may

make individuals with a chlamydial infection more susceptible to reinfection with *C. trachomatis* or with other pathogens.

In conclusion, the fact that we have been able to protect mice for at least a period of 180 days, corresponding to approximately a quarter of their expected life span, proves the feasibility of developing vaccines for humans that will protect against the long-term sequelae resulting from chlamydial genital infections. Thus, this model could provide the basis for characterizing the pathways necessary to induce a long-term protective immune response in the host and the antigenic components of *C. trachomatis* that are critical for eliciting that protection.

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REFERENCES

- Brunham, R. C., B. Binns, J. McDowell, and M. Paraskevas. 1986. *Chlamydia trachomatis* infection in women with ectopic pregnancy. *Obstet. Gynecol.* **67**:722-726.
- 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.
- Brunham, R. C., I. W. Maclean, B. Binns, and R. W. Peeling. 1985. *Chlamydia trachomatis*: its role in tubal infertility. *J. Infect. Dis.* **152**:1275-1282.
- Caldwell, H. D., J. Kromhout, and J. Schachter. 1981. Purification and characterization of the major outer membrane protein of *Chlamydia trachomatis*. *Infect. Immun.* **31**:1161-1176.
- Chow, J. M., M. L. Yonekura, G. A. Richwald, S. Greenland, R. L. Sweet, and J. Schachter. 1990. The association between *Chlamydia trachomatis* and ectopic pregnancy. *JAMA* **263**:3164-3167.
- Coles, A. M., H. A. Crosby, and J. H. Pearce. 1991. Analysis of the human serological response to *Chlamydia trachomatis* 60 kDa proteins by two dimensional electrophoresis and immunoblotting. *FEMS Microbiol. Lett.* **81**:299-304.
- Cui, Z.-D., D. Tristram, L. J. LaScolea, 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.
- de la Maza, L. M., S. Pal, A. Khamesipour, and E. M. Peterson. 1994. Intravaginal inoculation of mice with the *Chlamydia trachomatis* mouse pneumonitis biovar results in infertility. *Infect. Immun.* **62**:2094-2097.
- de la Maza, L. M., M. J. Plunkett, E. J. Carlson, E. M. Peterson, and C. W. Czarniecki. 1987. Ultrastructural analysis of the anti-chlamydial activity of recombinant murine interferon- γ . *Exp. Mol. Pathol.* **47**:13-25.
- de la Maza, M. A., and L. M. de la Maza. 1994. A new computer model for estimating the impact of vaccination protocols and its application to the study of *Chlamydia trachomatis* genital infections. *Vaccine* **13**:119-127.
- Fielder, T. J., S. Pal, E. M. Peterson, and L. M. de la Maza. 1991. Sequence of the gene encoding the major outer membrane protein of the mouse pneumonitis biovar of *Chlamydia trachomatis*. *Gene* **106**:137-138.
- Geysen, H. M., S. J. Rodda, T. J. Mason, G. Tribbick, and P. Schoofs. 1987. Strategies for epitope analysis using peptide synthesis. *J. Immunol. Methods* **102**:259-274.
- Grayston, J. T., and S. P. Wang. 1975. New knowledge of *Chlamydiae* and the diseases they cause. *J. Infect. Dis.* **132**:87-105.
- Grayston, J. T., and S. P. Wang. 1978. The potential for vaccine against infection of the genital tract with *Chlamydia trachomatis*. *Sex. Transm. Dis.* **5**:73-77.
- Grayston, J. T., S. P. Wang, Y. F. Yang, and R. L. Woolridge. 1962. The effects of trachoma vaccine on the course of experimental trachoma infection in blind volunteers. *J. Exp. Med.* **115**:1009-1022.
- Jones, R. B., B. R. Ardery, S. L. Hui, and R. E. Cleary. 1982. Correlation between serum antichlamydial antibodies and tubal factor as a cause of infertility. *Fertil. Steril.* **38**:553-558.
- Landers, D. V., K. Erlich, M. Sung, and J. Schachter. 1991. Role of L3T4-bearing T-cell populations in experimental murine chlamydial salpingitis. *Infect. Immun.* **59**:3774-3777.
- Mestecky, J. 1987. The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J. Clin. Immunol.* **7**:265-276.
- Morrison, R. P., R. J. Belland, K. Lyng, and H. D. Caldwell. 1989. Chlamydial disease pathogenesis. The 57 kD chlamydial hypersensitivity antigen is a stress response protein. *J. Exp. Med.* **170**:1271-1283.
- 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.
- Morrison, R. P., K. Lyng, and H. D. Caldwell. 1989. Chlamydial disease pathogenesis. Ocular hypersensitivity elicited by a genus specific 57 kD protein. *J. Exp. Med.* **169**:663-675.
- Morrison, R. P., D. S. Manning, and H. D. Caldwell. 1992. Immunology of *Chlamydia trachomatis* infections. In T. C. Quinn (ed.), *Sexually transmitted diseases*. Raven Press Ltd., New York.
- Mosmann, T. R., and R. L. Coffman. 1989. Th1 and Th2 cells; different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7**:145-163.
- Murray, E. S., L. T. Charbonnet, and A. B. MacDonald. 1973. Immunity to chlamydial infections of the eye. I. The role of circulatory and secretory antibodies in resistance to reinfection with guinea pig inclusion conjunctivitis. *J. Immunol.* **110**:1518-1525.
- Nichols, R. L., E. S. Murray, and P. E. Nisson. 1978. Use of enteric vaccines in protection against chlamydial infections of the genital tract and the eye of guinea pigs. *J. Infect. Dis.* **138**:742-745.
- Paavonen, J., M.-L. Lahdeaho, M. Puolakkainen, M. Maki, P. Parkkonen, and M. Lehtinen. 1994. Antibody response to B cell epitopes of *Chlamydia trachomatis* 60-kDa heat-shock protein and corresponding mycobacterial and human peptides in infants with chlamydial pneumonitis. *J. Infect. Dis.* **169**:908-911.
- Pal, S., X. Cheng, E. M. Peterson, and L. M. de la Maza. 1993. Mapping of a surface exposed B-cell epitope to the variable sequent 3 of the major outer membrane protein of *Chlamydia trachomatis*. *J. Gen. Microbiol.* **139**:1565-1570.
- Pal, S., T. J. Fielder, E. M. Peterson, and L. M. de la Maza. 1993. Analysis of the immune response in mice following intrauterine infection with the *Chlamydia trachomatis* mouse pneumonitis biovar. *Infect. Immun.* **61**:772-776.
- Pal, S., T. J. Fielder, E. M. Peterson, and L. M. de la Maza. 1994. Protection against infertility in a BALB/c mouse salpingitis model by intranasal immunization with the mouse pneumonitis biovar of *Chlamydia trachomatis*. *Infect. Immun.* **62**:3354-3362.
- Peterson, E. M., X. Cheng, B. Markoff, T. J. Fielder, and L. M. de la Maza. 1991. Functional and structural mapping of *Chlamydia trachomatis* species-specific major outer membrane epitopes by use of neutralizing monoclonal antibodies. *Infect. Immun.* **59**:4147-4153.
- Peterson, E. M., R. Oda, P. Tse, C. Gastaldi, S. C. Stone, and L. M. de la Maza. 1989. Comparison of a single-antigen microimmunofluorescence assay and inclusion fluorescent-antibody assay for detecting chlamydial antibodies and correlation of the results with neutralizing ability. *J. Clin. Microbiol.* **27**:350-352.
- Peterson, E. M., G. Zhong, E. Carlson, and L. M. de la Maza. 1988. Protective role of magnesium in the neutralization by antibodies of *Chlamydia trachomatis* infectivity. *Infect. Immun.* **56**:885-891.
- Punnonen, R., P. Terho, V. Nikkanen, and O. Meuraman. 1979. Chlamydial serology in infertile women by immunofluorescence. *Fertil. Steril.* **31**:656-659.
- Ramsey, K. H., W. J. Newhall, and R. Rank. 1989. Humoral immune response to chlamydial genital infection of mice with the agent of mouse pneumonitis. *Infect. Immun.* **57**:2441-2446.
- Ramsey, K. H., and R. Rank. 1991. Resolution of chlamydial genital infection with antigen specific T-lymphocyte lines. *Infect. Immun.* **59**:925-931.
- Ramsey, K. H., L. S. F. Soderberger, and R. G. Rank. 1988. Resolution of chlamydial genital infection in B-cell-deficient mice and immunity to reinfection. *Infect. Immun.* **56**:1320-1325.
- Rank, R. G., B. E. Batteiger, and L. S. F. Soderberg. 1990. Immunization against chlamydial genital infection in guinea pigs with UV-inactivated and viable *Chlamydiae* administered by different routes. *Infect. Immun.* **58**:2599-2605.
- Roizman, B. 1991. Introduction: objectives of herpes simplex virus vaccines seen from historical perspective. *Rev. Infect. Dis.* **13**:S892-S894.
- Schachter, J., and C. Dawson. 1978. *Human chlamydial infections*. PSG Publishing Company, Inc., Littleton, Mass.
- Schagger, H., and G. Von Jagow. 1987. Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis for the separation of protein range 1 to 100 kDa. *Anal. Biochem.* **166**:368-379.
- Sedgwick, J. D., and P. G. Holt. 1983. A solid phase immunoenzymatic technique for the enumeration of specific antibody secreting cells. *J. Immunol. Methods.* **57**:301-309.
- Svensson, L., P. A. Mardh, M. Ahlgren, and F. Nordenskjold. 1985. Ectopic pregnancy and antibodies to *Chlamydia trachomatis*. *Fertil. Steril.* **44**:313-317.
- Swenson, C. E., and J. Schachter. 1984. Infertility as a consequence of

- chlamydial infection of the upper genital tract in female mice. *Sex. Transm. Dis.* **11**:64–67.
44. **Toye, B., C. Laferriere, P. Claman, P. Jessamine, and R. Peeling.** 1993. Association between antibody to the chlamydial heat-shock protein and tubal infertility. *J. Infect. Dis.* **168**:1236–1240.
45. **Wagar, E. A., J. Schachter, P. Bavoil, and R. S. Stephens.** 1990. Differential human serologic response to two 60,000 molecular weight *Chlamydia trachomatis* antigens. *J. Infect. Dis.* **162**:922–927.
46. **Ward, M. E.** 1992. Chlamydial vaccines—future trends. *J. Infect.* **25**:11–26.
47. **Westrom, L., R. Joesoef, G. Reynolds, A. Hagdu, and S. E. Thompson.** 1992. Pelvic inflammatory disease and fertility: a cohort study of 1,844 women with laparoscopically verified disease and 657 control women with normal laparoscopy. *Sex. Transm. Dis.* **19**:185–192.
48. **Westrom, L., and P. A. Mardh.** 1993. Chlamydial salpingitis. *Br. J. Bull.* **39**:145–150.
49. **Zhong, G., E. M. Peterson, C. W. Czarniecki, and L. M. de la Maza.** 1988. Recombinant murine gamma interferon inhibits *Chlamydia trachomatis* serovar L1 in vivo. *Infect. Immun.* **60**:3143–3149.
50. **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.

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