Immunization with the *Chlamydia trachomatis* Mouse Pneumonitis Major Outer Membrane Protein Can Elicit a Protective Immune Response against a Genital Challenge

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Infertility, ectopic pregnancy, and chronic abdominal pain are frequent complications of genital infections with *Chlamydia trachomatis*. In an attempt to produce a vaccine to protect against this pathogen we purified and refolded the *C. trachomatis* mouse pneumonitis (MoPn) major outer membrane protein (MOMP). This preparation, mixed with Freund's adjuvant using vortexing or sonication, was used to immunize BALB/c mice that were subsequently challenged in the upper genital tract. Vaginal cultures were taken on a weekly basis, and mice were mated 6 weeks after the challenge. Gels of the vortexed MOMP showed a predominant band with a molecular size of 62 kDa and weaker bands at 42 and 132 kDa, while the sonicated MOMP had a single band with a molecular size of 42 kDa. Following immunization with these two preparations, strong humoral and cell-mediated immune responses were detected in the mice inoculated with the vortexed MOMP. On the other hand, mice immunized with the sonicated MOMP had a strong humoral immune response but a relatively weak cell-mediated immune response, as determined by a T-cell lymphoproliferative assay and level of cytokine production by splenocytes. Vaginal cultures showed that the mice immunized with the vortexed MOMP were significantly protected, as determined by a decrease in the number of animals with positive cultures, the length of time the mice shed viable organisms, and the number of inclusion-forming units recovered per mouse. Animals immunized with the sonicated MOMP, on the other hand, showed a weaker level of protection based on the same three parameters. After mating, the number of fertile animals and number of embryos per mouse were significantly higher for the mice immunized with vortexed MOMP, but not for the mice immunized with sonicated MOMP, compared to those of the control groups. In conclusion, immunization with a purified and refolded preparation of the *C. trachomatis* MoPn MOMP confers a significant level of protection in mice against a genital challenge.

Infections due to *Chlamydia trachomatis* impose a significant medical and economical burden throughout the world (12, 38, 54). In areas with poor hygienic conditions, infections with this bacterium result in trachoma, the most common cause of preventable blindness in the world (12, 38). In addition, throughout the world this pathogen is one of the leading causes of sexually transmitted diseases (12, 38, 42, 54). The acute genital infection generally subsides, but in some patients it may result in long-term sequelae. In females, among the long-term sequelae infertility, ectopic pregnancy, and chronic abdominal pain are relatively frequent (55, 56). Effective antimicrobial therapy is available to treat infected patients. This approach, however, has not been effective at controlling infection by this organism. There are several reasons for this disappointing outcome (25, 42). On one hand, a majority of the genital infections in women are asymptomatic, and thus patients do not seek treatment. In addition, in patients that are symptomatic, by the time the antimicrobial therapy is implemented permanent damage may have already occurred. Routine screening, followed by adequate therapy, in certain populations at high risk can decrease the overall prevalence of the disease, but this approach is temporary and can be practically applied only to limited groups. Thus, if we want to control the diseases pro-

duced by this pathogen the most effective approach will be to engineer a vaccine.

Attempts to implement a vaccine using whole organisms for *C. trachomatis* infections have a long history (8, 13, 41, 53). Four or five decades ago most efforts focused on developing a vaccine against trachoma using viable or inactivated whole organisms (13, 41, 53). More recently, adoptive immunization with dendritic cells pulsed ex vivo with inactivated whole chlamydial organisms has also been used as an experimental model to define the parameters of protection (19, 45). Several conclusions resulted from the earlier studies (13). The vaccine trials showed that an effective, although short-lived, protection could be achieved if the patients were immunized with sufficient antigen of the serovar that they were going to be subsequently exposed to. On the other hand, patients inadequately immunized suffered a hypersensitivity reaction when reexposed to *C. trachomatis*. This last observation has been interpreted as possibly an immune reaction to a chlamydial component, maybe the 60-kDa heat shock protein, that cross-reacts with some human antigen(s) (20). This possibility gave impetus for the development of a subunit chlamydial vaccine.

A total of 15 human serovars of *C. trachomatis* have been described (52). The trachoma biovar includes 12 serovars, A through K plus Ba, and the lymphogranuloma venereum group consists of the L1, L2, and L3 serovars. The A through C serovars have been isolated mostly from trachoma cases, while the D through K serovars mainly cause genital infections. The mouse pneumonitis (MoPn) biovar is the only isolate of *C. tra-*

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chomatis so far recovered from mice (24). The grouping of *C. trachomatis* isolates into distinct serovars appears to be based mainly on the differences in the amino acid sequence of the variable domains (VD) of the major outer membrane protein (MOMP) of this organism (10, 43). The MOMP is surface exposed, accounts for over 60% of the mass of the outer membrane, and has been shown to induce neutralizing antibodies (5, 41, 53). These characteristics have made the MOMP the preferred candidate for a subunit vaccine (10, 41, 53). In our laboratory we have utilized the *C. trachomatis* MoPn model, since this serovar, as a natural murine pathogen, can infect the genital tract of mice and produce a pathology that closely mimics that found in humans (27, 46). Here we show that vaccination with a highly purified preparation of the MOMP that was refolded in an attempt to reestablish the conformation of the native epitopes was able to protect mice against a genital challenge.

MATERIALS AND METHODS

Organisms. The *C. trachomatis* MoPn biovar (strain Nigg II) was purchased from the American Type Culture Collection (Manassas, Va.) and grown in HeLa 229 cells (27). The elementary bodies (EB) were prepared as described by Caldwell et al. (5). The organisms were frozen at -70°C in SPG (0.2 M sucrose, 20 mM sodium phosphate [pH 7.4], and 5 mM glutamic acid).

Preparation of the *C. trachomatis* MoPn MOMP. *C. trachomatis* MoPn grown in HeLa 229 cells was washed with 10 mM phosphate-buffered saline (PBS), pH 7.4, and centrifuged and the pellet was treated with DNase (31). Following centrifugation the pellet was resuspended in 0.2 M phosphate buffer, pH 5.5, containing 0.001 M concentrations (each) of EDTA and phenylmethylsulfonyl fluoride (PMSF) and was extracted with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS; Calbiochem-Novabiochem Corp., San Diego, Calif.) and Zwittergent 3-14 (Z3-14; Calbiochem-Novabiochem Corp.) as previously described (31). The MOMP was recovered in the supernatant and purified using a 1-cm by 35-cm hydroxylapatite column (5). Fractions containing the MOMP were pooled, run on a 5 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and stained with silver and Coomassie blue (Bio-Rad, Hercules, Calif.). In addition, an N-terminal amino acid analysis was performed on the purified MOMP by the core facilities at the University of California, Irvine.

The MOMP was refolded by dialysis against 0.1 M phosphate buffer, pH 7.8, containing 0.001 M EDTA, 0.002 M reduced glutathione, 0.001 M oxidized glutathione, and 0.05% Z3-14 at room temperature at a protein concentration of 30 to 150 $\mu\text{g}/\text{ml}$ (16). The MOMP was concentrated and fixed with 20% glutaraldehyde for 2 min at room temperature, and subsequently 2 M glycine was added to stop the reaction (47). Before inoculation, the preparation was concentrated using Centricon-10 filters and dialyzed against a solution containing 0.02 M phosphate buffer, pH 7.4, 0.15 M NaCl, and 0.05% Z3-14.

Animal immunization and challenge protocols. Seven- to 8-week-old BALB/c female mice (H-2^d) were obtained from Simonsen Laboratories (Gilroy, Calif.). All animal protocols were approved by the University of California, Irvine, Animal Care and Use Committee.

Mice were immunized intramuscularly (5 $\mu\text{g}/\text{mouse}$) and subcutaneously (5 $\mu\text{g}/\text{mouse}$) with purified, refolded, and glutaraldehyde-fixed *C. trachomatis* MoPn MOMP that was mixed with complete Freund's adjuvant by vortex (vortexed MOMP), and they were boosted twice at 2-week intervals with the same dose of the MOMP preparation in incomplete Freund's adjuvant (31). Another group of mice was immunized with the same MOMP preparation that was mixed with Freund's adjuvant by sonication (sonicated MOMP) on ice with three bursts of 10 s each at high output using a Braun-Sonic-2000 apparatus (B. Braun Instruments, Burlingame, Calif.). Negative controls were inoculated in a similar manner, except ovalbumin was used. As a positive control, mice were immunized once intranasally with 10^4 inclusion-forming units (IFU) of *C. trachomatis* MoPn. An uninoculated, nonchallenged control group was mated and kept in the vivarium under the same conditions to determine the normal fertility of this strain of mouse (27).

Two weeks after the last boost the mice were challenged in the left ovarian bursa with 10^5 IFU of *C. trachomatis* MoPn, while the right ovarian bursa was inoculated with mock-infected HeLa 229 cell extracts processed as the EB (27,

46, 50). Vaginal swabs were collected and cultured at 7-day intervals for a period of 6 weeks after the genital challenge as described previously (27). All experiments were repeated.

Immunoassays. Vaginal secretions and blood were collected before each immunization, and antibody titers were determined using an enzyme-linked immunosorbent assay (ELISA) as follows (27). Multiwell plates (96 flat-bottom wells; Corning Glass Works, Corning, N.Y.) were coated overnight with 1 μg of purified *C. trachomatis* MoPn EB per well. Serial dilutions of the serum samples were added to each well, and the plates were incubated for 2 h at 37°C . The antigen-antibody reaction was detected by adding horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies. The following class or subclass-specific antibodies were used: immunoglobulin G (IgG), IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM (Southern Biotechnology Associates, Inc., Birmingham, Ala.). The reaction was measured at 405 nm using an ELISA reader (Bio-Rad Corp.). The substrate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) was used for color development.

In vitro neutralization assays were performed using HeLa 229 cell monolayers as previously described (31). Briefly, 10^4 IFU of *C. trachomatis* MoPn was added to serial dilutions of the serum made with 5% guinea pig serum in PBS. After incubation for 45 min at 37°C , the mixture was inoculated in HeLa 229 cells by centrifugation. Following 24 h of incubation at 37°C the monolayers were fixed and stained with a rabbit polyclonal anti-*C. trachomatis* MoPn serum and a goat anti-rabbit peroxidase stain. The neutralization titer of a serum sample was the dilution that yielded 50% neutralization over the control serum.

Western blots were performed using nitrocellulose membranes as previously described (31, 39). Briefly, *C. trachomatis* MOMP was loaded on a 7.5-cm-wide mini slab-gel and resolved by 10% tricine SDS-PAGE. Serum samples, diluted 1:100 with PBS-0.005% Tween 20, were incubated for 2 h at room temperature, and then the strips were treated with a 1:500 dilution of HRP-conjugated goat anti-mouse IgG and the bands were visualized with 4-chloro-1-naphthol.

To assess the specificity of serum antibodies with regard to epitopes within the MOMP, serum samples were used to probe octameric peptides representing this protein. The peptides were synthesized with a kit (Cambridge Research Biochemicals Inc., Wilmington, Del.) (11, 31). Goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Inc.) conjugated to HRP were used as second antibodies, and ABTS was the substrate utilized. The ELISA was repeated twice, using sonication in hot SDS and 2-mercaptoethanol to remove the antibodies between assays.

The lymphoproliferative assay was performed as previously described (27, 31). Spleens from two mice from each group were harvested, and single-cell suspensions were prepared to enrich for T cells using a nylon wool column. Using a fluorescence-labeled monoclonal antibody to CD3⁺ (GIBCO-BRL, Grand Island, N.Y.), close to 90% of the suspension was found to be T cells. Antigen-presenting cells were prepared by irradiating (3,300 rad of ^{137}Cs) unseparated spleen cells and incubating them with various concentrations of *C. trachomatis* MoPn EB. The enriched T-cell suspensions were cultured in 96-well plates at a concentration of 2×10^5 cells per well in 0.2 ml of RPMI 1640 supplemented with 10% fetal bovine serum. The lymphocytes were cocultured for 5 days with 1.2×10^5 antigen-presenting cells with or without antigen and stimulated with *C. trachomatis* MoPn EB. Concanavalin A was added to some wells as a positive control. At the end of the fourth day of incubation, 1.0 μCi of [^3H]thymidine (47 Ci/mmol; Amersham, Arlington Heights, Ill.) in 25 μl of RPMI 1640 was added to each well and the uptake of the [^3H]thymidine was measured.

Levels of gamma interferon (IFN- γ) and interleukin 4 (IL-4) were determined by using a commercial kit (Endogen, Cambridge, Mass.) with supernatants from splenic T cells stimulated as described above and collected at 48 h of incubation (28).

Fertility studies. Six weeks after the intrabursal challenge, groups of four female mice were housed with a proven breeder male mouse for a maximum of 18 days and the pregnancies were assessed by measuring the weight of each mouse (27). Animals that had gained 5 to 10 g of weight by or before 18 days postmating were considered pregnant and were euthanized, and the number of embryos in each uterine horn was counted. After the first mating the female mice that did not gain weight were mated a second time with male mice that had successfully mated with another group of female mice and were monitored as described previously (27). All the animals that had not gained weight were euthanized 25 days from the start of the second mating. The number of embryos in each uterine horn was counted when the mice were killed.

Statistics. Statistical analyses were performed with the Statview software package on a Macintosh computer. The two-tailed unpaired Student's *t* test, the Mann-Whitney's U test, and the Fisher's exact test were employed to determine the significance of the differences between groups.

FIG. 1. Silver-stained SDS-PAGE (5 to 20%) of the purified *C. trachomatis* MoPn MOMP. Lane, 1 molecular size standards (in kilodaltons). Lanes 2 through 6, preparation of reduced MOMP. Lane 2, 8 µg; lane 3, 4 µg; lane 4, 2 µg; lane 5, 1 µg; lane 6, 0.5 µg.

RESULTS

Characterization of the vaccine candidate preparations.

The purity of the MOMP preparation, following the hydroxylapatite column, was first assessed by gel electrophoresis and amino acid sequencing. As shown in Fig. 1, only one band, corresponding to the *C. trachomatis* MoPn MOMP, was detected on a gradient gel stained with silver. By N-terminal amino acid analysis only the expected sequence, L-P-V-G-N-P, of the mature MoPn MOMP was obtained, indicating a greater than 99% purity of the protein. Figure 2A shows a Coomassie blue-stained SDS-PAGE of the folded *C. trachomatis* MOMP that was vortexed (lane 2) or sonicated (lane 5) and run under nonreducing conditions. The folded MOMP was also boiled for 10 min (lane 6), boiled for 10 min in the presence of 30 mM dithiothreitol (DTT) (lane 3), or treated with 30 mM DTT but not boiled (lane 4). Sonication and boiling of the folded MOMP under reducing or nonreducing conditions resulted in a predominant single band with an apparent molecular size of approximately 42 kDa. Under nonreducing conditions or reducing conditions but not boiling there is a prominent band of 62 kDa and faint bands with approximate molecular sizes of 42 and 132 kDa. The 62- and 132-kDa bands most likely represent homopolymers of the MOMP not linked by disulfide bonds, since boiling, without a reducing agent, resulted in a single band of 42 kDa. Figure 2B shows a Western blot of the *C. trachomatis* MoPn MOMP under boiled-reduced and nonboiled-nonreduced conditions probed with serum from mice immunized with the vortexed and sonicated MOMP preparations. Under reduced conditions only one band is detected with a molecular size of 42 kDa, while in addition to the 42-kDa band there is a band at 62 kDa, with faint bands above and below it under nonreduced conditions. The lack of an identifiable band at 132 kDa may be due to the poor transfer of large-molecular-size components during blotting. Alternatively, a paucity of antibodies was elicited by this antigen or epitopes to which antibodies were formed were partially blocked by the conformation of the 132-kDa molecules. Western blots probed with monoclonal antibody MoPn-40 to a linear epitope on VD1 appeared similar to those reacted with serum from mice immunized with vortexed or sonicated MOMP. A faint band corresponding to the molecular size of the lipopolysaccharide

(LPS) could be observed when the Western blots of the MOMP preparation were performed using low dilutions of the immune serum (data not shown).

Immune response following vaccination with the MOMP.

An antibody response was detected in serum and vaginal samples 2 weeks following the initial immunization with MOMP. Antibody titers continued to rise until the day before the intrabursal challenge (Table 1). At that point the total IgG and IgA antibody titers in serum of the mice immunized with the vortexed MOMP were 12,800 and 1,600, respectively, while in the vaginal wash the IgG titer was 100 and the IgA titer was 50. Animals immunized with the sonicated MOMP had IgG and IgA titers in serum of 218,000 and 4,050, respectively, while in the vaginal wash they were 128 and 8, respectively. Control mice immunized intranasally (i.n.) with EB had IgG titers in serum and the vaginal wash of 48,600 and 80, respectively, while the IgA titers were 5,400 and 320, respectively.

Determination of the different chlamydial-specific IgG subclasses in serum showed that the mice immunized with vortexed MOMP had an IgG2a/IgG1 ratio of 2 (25,600/12,800) (Table 1), while the mice immunized with the sonicated MOMP had a ratio of 0.2 (26,700/145,000). Animals inoculated i.n. with EB had a predominant Th1 response with an IgG2a/

FIG. 2. (A) SDS-PAGE of the purified *C. trachomatis* MoPn MOMP. Lane 1, molecular size standards. Lane 2, preparation of folded MOMP electrophoresed under vortexed-nonreducing conditions; lane 5, preparation of folded MOMP electrophoresed under sonicated-nonreducing conditions. Lanes 3, 4, and 6, folded MOMP electrophoresed after it was boiled for 10 min in the presence of 30 mM DTT (lane 3), treated with 30 mM DTT but not boiled (lane 4), or boiled for 10 min (lane 6). (B) Lane 1, molecular size standards. Lanes 2 through 7, *C. trachomatis* MoPn folded MOMP boiled in the presence of 30 mM DTT (lanes 2, 4, and 6) or not boiled and not reduced (lanes 3, 5, and 7). The blots were probed with serum from mice immunized with the vortexed MOMP preparation (lanes 2 and 3), the sonicated MOMP (lanes 4 and 5), or with the monoclonal antibody MoPn-40 that recognizes the VD1 of the MOMP (lanes 6 and 7).

TABLE 1. Systemic and local immune response of mice before challenge

Immunogen	<i>C. trachomatis</i> MoPn-specific ELISA antibody titer								Serum neutralizing titer
	Serum (10 ³)						Vaginal wash		
	IgG	IgG1	IgG2a	IgG2b	IgG3	IgA	IgA	IgG	
MOMP (vortexed)	12.8	12.8	25.6	12.8	6.4	1.6	50	100	810 ± 490
MOMP (sonicated)	218	145	26.7	97.2	10.8	4.05	8	128	1,350 ± 0
Ovalbumin	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<10	<10	0
MoPn EB	48.6	5.4	48.6	16.2	16.2	5.4	320	80	1,350 ± 0

IgG1 ratio of 9 (48,600/5,400). The control mice immunized with ovalbumin had no detectable levels of chlamydial-specific antibodies.

No significant differences in the neutralizing antibody titer were observed between the groups inoculated with MOMP or EB. The neutralizing antibody titer in the serum from the mice inoculated with the vortexed and sonicated MOMPs were 810 and 1,350, respectively, while animals immunized with EB had a neutralizing titer of 1,350 (Table 1).

Serum samples were also tested for the presence of specific antibodies to octameric peptides of the MOMP. As shown in Fig. 3, IgG2a and IgG1 antibodies to the four VD of the *C. trachomatis* MoPn MOMP were detected in the animals

inoculated with both MOMP preparations. However, mice immunized with the sonicated MOMP had, overall, a weaker IgG2a than IgG1 response to the VD than the animals immunized with the vortexed MOMP. As previously shown, mice inoculated with EB had IgG2a but no IgG1 antibodies to the VD (18, 21, and data not shown).

A significant EB-specific T-cell proliferative response, as measured by a lymphoproliferative assay, was detected in the mice immunized with the *C. trachomatis* MoPn vortexed MOMP and in those inoculated i.n. with EB, while animals immunized with the sonicated MOMP had no significant T-cell response (Table 2). In vitro cytokine production by splenocytes showed a predominance of IFN- γ over IL-4 levels in the mice immunized

FIG. 3. Reactivity to octameric synthetic peptides of sera collected from mice immunized with the *C. trachomatis* MoPn vortexed and sonicated MOMP on the day before the genital challenge. The VDs of the MOMP are marked at the bottom of the figure.

TABLE 2. T-cell response of immunized mice before challenge^a

Immunogen	T-cell proliferative response (cpm [10 ³] ± 1 SD) to:			In vitro cytokine production (ng/ml)					
				IFN-γ			IL-4		
	EB	Con A	Medium	EB	Con A	Medium	EB	Con A	Medium
MOMP (vortexed)	5.2 ± 1 ^b	109 ± 48	0.3 ± 0.2	17.8 ± 1.8 ^b	16.5 ± 1.2	<0.03	<0.08	1.6 ± 1.4	<0.08
MOMP (sonicated)	0.7 ± 0.3	50.3 ± 34	0.3 ± 0.04	0.03 ± 0.01	17.1 ± 1.2	<0.03	<0.08	0.2 ± 0.3	<0.08
Ovalbumin	1.5 ± 0.8	56 ± 7	0.2 ± 0.01	0.4 ± 0.4	24.9 ± 8.4	<0.03	<0.08	0.5 ± 0.2	<0.08
MoPn EB	6.5 ± 1.5 ^b	27 ± 0.7	0.1 ± 0.01	19.3 ± 1.6 ^b	29.6 ± 11	<0.03	<0.08	0.05 ± 0	<0.08

^a Results are means of triplicate cultures. Data correspond to one of the experiments representative of duplicate separate experiments. UV-inactivated *C. trachomatis* MoPn EB were added at a 10:1 ratio to the T cells. Concanavalin A (Con A) was added at a concentration of 5 µg/ml.

^b $P < 0.05$, determined by the Student's *t* test compared to the ovalbumin-immunized group.

with the vortexed MOMP and in those inoculated i.n. with EB, while animals immunized with sonicated MOMP had background levels of both IFN-γ and IL-4 (Table 2).

Vaginal shedding of *C. trachomatis* following the intrabursal challenge. As shown in Table 3, 25 of the 28 (89.9%) control animals inoculated with ovalbumin shed *C. trachomatis* over the 6 weeks of the experiment. In contrast, only 7 of the 29 (24.1%) mice immunized with the vortexed MOMP had positive cultures during that period of time ($P < 0.05$). The duration and intensity of the shedding were also significantly different between these two sets of mice (Table 3). On average, the number of IFU recovered was 1 log₁₀ lower in the animals immunized with vortexed MOMP than in those inoculated with ovalbumin. A more limited protection was observed in the group of mice immunized with the sonicated MOMP. Of the 25 mice, 16 (64%) had positive cultures, and only during the first week was there a statistically significant difference in the number of IFU recovered from the mice immunized with the sonicated MOMP versus that recovered from ovalbumin. As expected, almost complete protection was obtained in the group inoculated i.n. with EB. Of the 18 mice in this group, only 1 (5.6%) had a positive culture on the second week, with a very low level of *C. trachomatis* MoPn IFU detected.

Fertility studies. Table 4 shows the results of the fertility studies. In the ovalbumin control group, only 3 of the 28 (10.7%) mice had embryos in both uterine horns. In contrast, of the mice immunized with vortexed MOMP, 16 of the 29 (55.2%) had embryos in both uterine horns ($P < 0.05$). However, animals immunized with the sonicated MOMP did not have a statistically significant increase in fertility. The group inoculated i.n. with EB and the fertility control group also had higher fertility rates than the ovalbumin control ($P < 0.05$). Furthermore, mice inoculated with ovalbumin or sonicated MOMP had significantly fewer embryos in the challenged left uterine horn than the fertility control animals ($P < 0.05$). In

contrast, no significant differences were observed between the number of embryos in the right and left uterine horn in the animals immunized with vortexed MOMP, or in those inoculated i.n. with EB, compared with the number of embryos in the fertility control group.

DISCUSSION

In this report we have shown that immunization with a preparation of the *C. trachomatis* MOMP, extracted directly from the organism, can induce significant protection against a genital challenge. This is, to our knowledge, the first time that a highly purified antigen of *C. trachomatis* has provided protection against a genital infection and infertility. This parallels the encouraging results recently reported using an anti-idiotypic antibody to the exoglycolipid antigen of *C. trachomatis* to protect mice against an ocular challenge (57) and the positive data obtained with DNA plasmids coding for the MOMP against an intranasal challenge (58).

Of the several antigens present in the chlamydial outer membrane, the MOMP appears to be the most obvious candidate for a vaccine against chlamydial genital infections (10, 41, 53). Based on that premise, various groups have used preparations of the MOMP, peptides corresponding to the VD, or DNA plasmids coding for the MOMP as an antigen in an attempt to induce protection (26, 31, 36, 44, 48, 49). Unfortunately, the results have been disappointing. Several explanations could account for these negative results, including the route of delivery, dose of antigen, and adjuvant preparations. On the other hand, a factor that has been considered by several investigators as potentially critical in inducing a protective immune response is the structural conformation of the epitopes of the MOMP (26, 36, 44, 49). In this respect, we have shown that a purified preparation of the chlamydial outer membrane complex, consisting mainly of the MOMP, the 60-kDa cys-

TABLE 3. Results of vaginal cultures

Immunogen	No. of mice that shed/total no. of mice per group (%), mean log ₁₀ <i>C. trachomatis</i> IFU shed ± 1 SE per group in week:					Total no. of mice that shed in 6 wks (%)
	1	2	3	4	5	
MOMP (vortexed)	2/29 (6.9), ^a 0.24 ± 0.19 ^b	4/29 (13.8), ^a 0.57 ± 0.29 ^b	4/29 (13.8), 0.29 ± 0.14	0/29 (0), 0	0/29 (0), 0	7/29 (24.1) ^a
MOMP (sonicated)	5/25 (20), 0.3 ± 0.16 ^b	12/25 (48), 1.4 ± 0.35	5/25 (20), 0.6 ± 0.26	2/25 (8), 0.2 ± 0.17	0/25 (0), 0	16/25 (64) ^a
Ovalbumin	12/28 (42.9), 1.57 ± 0.38	18/28 (64.3), 2.1 ± 0.36	8/28 (28.6), 0.50 ± 0.22	1/28 (3.6), 0.10 ± 0.10	0/28 (0), 0	25/28 (89.9)
MoPn EB	0/18 (0), ^a 0 ^b	1/18 (5.6) ^a 0.04 ± 0.03 ^b	0/18 (0), ^a 0 ^b	0/18 (0), 0	0/18 (0), 0	1/18 (5.6) ^a

^a Significant ($P < 0.05$) by the Fisher's exact test when compared with the ovalbumin-immunized mice.

^b Significant ($P < 0.05$) by the Mann-Whitney U test when compared with the ovalbumin-immunized mice.

TABLE 4. Results of fertility studies

Immunogen	No. of mice that had embryos in right, left, or both uterine horns/ total no. of mice per group (%)			Mean (± 1 SD) no. of embryos in right or left uterine horn	
	Both	Right	Left ^a	Right	Left ^a
MOMP (vortexed)	16/29 (55.2) ^{b,d}	26/29 (89.7)	19/29 (65.5) ^{b,d}	3.7 $\pm$ 2.3	2.0 $\pm$ 1.9 ^{c,e}
MOMP (sonicated)	8/25 (32.0)	21/25 (84)	9/25 (36)	2.6 $\pm$ 1.8	1.0 $\pm$ 1.5
Ovalbumin	3/28 (10.7)	21/28 (75.0)	6/28 (21.4)	3.1 $\pm$ 2.4	0.5 $\pm$ 1.0
MoPn EB	14/18 (77.8) ^{b,d}	16/18 (88.9)	15/18 (83.3) ^{b,d}	3.0 $\pm$ 2.0	2.8 $\pm$ 1.8 ^{c,e}
Fertility control	19/24 (79.2) ^b	21/24 (87.5)	20/24 (83.3) ^b	3.1 $\pm$ 1.7	2.7 $\pm$ 1.9 ^c

^a *C. trachomatis* MoPn-inoculated side.

^b $P < 0.05$ by the Fisher's exact test compared to the ovalbumin-immunized group.

^c $P < 0.05$ by the Student's *t* test compared to the ovalbumin-immunized group.

^d $P > 0.05$ by the Fisher's exact test compared to the fertility control group.

^e $P > 0.05$ by the Student's *t* test compared to the fertility control group.

teine-rich protein, and LPS, can induce protective immunity against a genital challenge (31). Similar results have also been reported using a preparation of the outer membrane to protect sheep and guinea pigs against *Chlamydia psittaci* (2, 47). It was, in part, on the basis of these results that we considered the need to refold the MOMP before vaccination in an attempt to reconstitute the protective epitopes present in the native outer membrane of *C. trachomatis*. Here, our data suggest that epitopes that are formed by refolding the MOMP may be critical for inducing a protective immune response. Refolding may reconstitute some of the nonlinear protective epitopes present in a single MOMP molecule in the native EB and also those formed by homopolymers of MOMP (23). The fact that sonication of the folded MOMP resulted in a decrease in its protective ability supports this interpretation, although an alteration of linear epitopes by sonication cannot completely be excluded. This conclusion is also upheld by the finding that a MOMP preparation extracted and purified in the same manner as that reported here but not refolded failed to induce protection (31). Obviously, until the crystal structure of the MOMP and its conformation in the EB are elucidated, no definitive conclusions can be reached.

That a specific conformation of the epitopes appears to be important for inducing a protective response suggests that the humoral immunity plays a substantial role in this animal model (6, 22). This supports the observation originally described with vaccines against trachoma in humans and monkeys, indicating that the protection was serovar specific (13). It is important, however, to consider that the development of protective CD4⁺ T-cell responses may also depend on the proper conformation of the inducing epitopes. For example, Sjolander et al. (40) have shown that the native surface antigen 2 from *Leishmania major* can induce a protective response, while the same antigen produced in *Escherichia coli* failed to elicit protection. Since protection in mice against *L. major* is dependent on T helper cells, the authors concluded that correct protein folding and/or posttranslation modification maybe needed for eliciting a protective CD4⁺ T-cell response.

Other than the innate immune factors and the natural anatomical barriers present in the genital tract, protection against infection, called sterilizing immunity, will have to be mediated by antibodies on the mucosal surface (41, 53). A protective role for local antibodies in humans was first suggested by Brunham et al. (3), who showed an inverse correlation between the titer of IgA in genital secretions and the quantity of infectious units

of *C. trachomatis* isolated from the cervix. Experimental proof that a monoclonal IgA antibody to a conformational epitope of MOMP could neutralize in vitro the infectivity of *C. trachomatis* MoPn has been reported (30). Subsequently it was demonstrated that passive immunization with this IgA antibody resulted in a significant decrease in the number of mice infected and in the intensity and duration of vaginal shedding following an intravaginal challenge (32).

Although IgA antibodies can block an infection at the site of entry, the challenge will be to develop a vaccination protocol that induces and maintains a high level of antibodies in the genital mucosa. Thus, in addition to inducing protective antibodies a vaccine most likely will also have to induce cell-mediated immunity. In this respect it appears that, at least based on experimental data with mice, a predominant Th1 response will be more efficient at eradicating a chlamydial infection than a dominant Th2 response (15, 21, 37). In general, immunization with viable organisms, including *C. trachomatis*, induces a predominant Th1 response, while nonviable antigens tend to elicit a Th2 dominant response (4, 18, 27, 31, 58). The vortexed MOMP preparation used here elicited a predominant Th1 response, while the sonicated MOMP induced mainly a Th2 response, as shown by the IgG2a/IgG1 ratio and the levels of IFN- γ and IL-4 produced by the splenocytes. These findings, in addition to the stronger T-cell proliferative response elicited by EB in mice immunized with the vortexed MOMP, suggest that the epitopes of this preparation more closely resemble those present in the native EB than those in the immunogenic sites of the sonicated MOMP.

There are presently several mouse models to test vaccine protocols and analyze the pathogenesis of a genital chlamydial infection (1, 9, 51). Each one of them has unique advantages, but not without shortcomings. The model of intrabursal inoculation used here was originally described by Barron et al. (1) and offers the advantage that all strains of mice so far tested, independent of age, develop infertility following a relatively low challenge with the *C. trachomatis* MoPn serovar (18). The obvious shortcoming of this model is the unnatural route of inoculation. The model in which the mice are challenged intravaginally requires a large dose of *C. trachomatis* MoPn, and certain strains of mice appear to be completely resistant to the development of infertility if they are inoculated past a certain age (9, 29). To overcome this limitation, an alternative model was developed in which the mice are treated with progesterone before they are challenged with *Chlamydia* (51). Treatment

with progesterone, however, not only makes the genital epithelium more susceptible to a chlamydial infection but also induces a significant alteration of the immune system (14, 17, 35). This, in our opinion, may be a significant shortcoming when testing a vaccine candidate, since treatment with progesterone may abrogate the protective immune response induced by the antigen. Thus, presently we do not have an ideal animal model to test vaccine candidates against chlamydial genital infections. We think, however, that if different groups of investigators test their vaccine candidates in several murine models, it is likely that, based on their accumulated experiences, conclusions could be reached that are applicable to a large segment of the human population by the time that a vaccine candidate is ready for clinical trials.

Several steps will have to be explored before we can implement a vaccination protocol in humans with a MOMP preparation. The most obvious is the need to obtain quantities of MOMP sufficient for vaccination. Although it is possible to increase the production of MOMP using tissue culture techniques, this issue can probably be better addressed by exploring some of the recombinant techniques now available. However, this will not be an easy task, since it will require not only the production of the MOMP but also the purification and refolding of the protein into the proper conformation. Structural characterization of the MOMP preparation that we have described, including its crystallization, could be very helpful to start addressing this problem. The use of MOMP will still pose another question, and that is the still-unresolved issue of whether or not this protein from one serovar can protect against all the other *C. trachomatis* serovars (34, 49). This will have to be addressed in the murine and monkey models before clinical trials are performed with humans (1, 9, 33, 51).

In addition, the issue as to whether or not the trace amounts of LPS present in this MOMP preparation may directly or indirectly play a role in protection will probably not be answered until a recombinant vaccine is tested. The fact that a nonrefolded preparation of MOMP that was extracted and purified using the same procedure utilized here did not induce protection suggests that if the LPS plays a role, it most likely will do so indirectly by modifying the MOMP (31). Alternatives to the use of Freund's adjuvant will also have to be explored. Various adjuvants are under intense investigation for other vaccines, so knowledge gained with other antigens may be applicable to the MOMP (7). In conclusion, we have shown that a purified and refolded preparation of the MOMP can induce protection against a genital challenge with *C. trachomatis* in a mouse model. A significant amount of work, however, is required before we can consider applying this approach to humans.

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REFERENCES

- Barron, A. L., H. J. White, R. G. Rank, B. L. Soloff, and E. B. Moses. 1981. A new animal model for the study of *Chlamydia trachomatis* genital infection of mice with the agent of mouse pneumonitis. *J. Infect. Dis.* **143**:63–66.
- Batteiger, B. E., R. G. Rank, P. M. Bavoi, and L. S. F. Soderberg. 1993. Partial protection against genital reinfection by immunization of guinea-pigs with isolated outer-membrane proteins of the chlamydial agent of guinea-pig inclusion conjunctivitis. *J. Gen. Microbiol.* **139**:2965–2972.
- 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.
- Cain, T. K., and R. G. Rank. 1995. Local Th1-like responses are induced by intravaginal infection of mice with the mouse pneumonitis biovar of *Chlamydia trachomatis*. *Infect. Immun.* **63**:1784–1789.
- 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.
- Cotter, T. W., Q. Meng, Z.-L. Shen, Y.-X. Zhang, H. Su, and H. D. Caldwell. 1995. Protective efficacy of major outer membrane protein-specific immunoglobulin A (IgA) and IgG monoclonal antibodies in a murine model of *Chlamydia trachomatis* genital tract infection. *Infect. Immun.* **63**:4704–4717.
- Cox, J. C., and A. R. Colter. 1997. Adjuvants—a classification and review of their modes of action. *Vaccine* **15**:248–256.
- de la Maza, M. A., and L. M. de la Maza. 1995. 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.
- 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.
- Fitch, W. M., E. M. Peterson, and L. M. de la Maza. 1993. Phylogenetic analysis of the outer membrane protein genes of *Chlamydiae* and its implications for vaccine development. *Mol. Biol. Evol.* **10**:892–913.
- 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.
- Grossman, J. C. 1985. Interactions between the gonadal steroids and the immune system. *Science* **227**:257–261.
- Igietsme, J. U., K. H. Ramsey, D. M. Magee, D. M. Williams, T. J. Kincy, and R. G. Rank. 1993. Resolution of murine chlamydial genital infection by the adoptive transfer of a biovar-specific Th1 lymphocyte clone. *Reg. Immunol.* **5**:317–324.
- Jaenicke, R., and R. Rudolph. 1989. Folding proteins, p. 191–223. In T. E. Creighton (ed.), *Protein structure—a practical approach*. IRL Press, Oxford, United Kingdom.
- Kausch, C., F. Zhou, A. D. Murdin, and C. R. Wira. Effects of estradiol and progesterone on susceptibility and early immune responses to *Chlamydia trachomatis* infection in the female reproductive tract. *Infect. Immun.* **68**:4207–4216.
- Kelly, K. A., E. A. Robinson, and R. G. Rank. 1996. Initial route of antigen administration alters the T-cell cytokine profile produced in response to the mouse pneumonitis biovar of *Chlamydia trachomatis* following genital infection. *Infect. Immun.* **64**:4976–4983.
- Lu, H., and G. Zhong. 1999. Interleukin-12 production is required for chlamydial antigen-pulsed dendritic cells to induce protection against live *Chlamydia trachomatis* infection. *Infect. Immun.* **67**:1763–1769.
- Morrison, R. P., R. J. Belland, K. Lyng, and H. D. Caldwell. 1989. Chlamydial disease pathogenesis. The 57-kDa chlamydial hypersensitivity antigen is a stress response protein. *J. Exp. Med.* **170**:113–122.
- Morrison, R. D., 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, S. G., H. Su, H. D. Caldwell, and R. P. Morrison. 2000. Immunity to murine *Chlamydia trachomatis* genital tract reinfection involves B cells and CD4⁺ T cells but not CD8⁺ T cells. *Infect. Immun.* **68**:6979–6987.
- Newhall, W. J., and R. B. Jones. 1983. Disulfide-linked oligomers of the major outer membrane protein of *Chlamydiae*. *J. Bacteriol.* **154**:998–1001.
- Nigg, C. 1942. An unidentified virus which produces pneumonia and systemic infection in mice. *Science* **99**:49–50.
- Paavonen, J., and P. Wolner-Hanssen. 1989. *Chlamydia trachomatis*: a major threat to reproduction. *Hum. Reprod.* **4**:111–124.
- Pal, S., K. M. Barnhart, Q. Wei, A. M. Abai, E. M. Peterson, and L. M. de la Maza. 1999. Vaccination of mice with DNA plasmids coding for the *Chlamydia trachomatis* major outer membrane protein elicits an immune response but fails to protect against a genital challenge. *Vaccine* **17**:459–465.
- 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.
- Pal, S., E. M. Peterson, and L. M. de la Maza. 1996. Intranasal immunization induces long-term protection in mice against a *Chlamydia trachomatis* genital challenge. *Infect. Immun.* **64**:5341–5348.
- Pal, S., E. M. Peterson, and L. M. de la Maza. 2001. Susceptibility of mice

- to a vaginal infection with *Chlamydia trachomatis* mouse pneumonitis is dependent on the age of the animal. *Infect. Immun.* **69**:5203–5206.
30. Pal, S., I. Theodor, E. M. Peterson, and L. M. de la Maza. 1994. Characterization of a neutralizing IgA monoclonal antibody directed against the *Chlamydia trachomatis* mouse pneumonitis biovar, p. 141–144. In J. Orfila, G. I. Byrne, M. A. Chernesky, J. T. Grayston, R. B. Jones, G. L. Ridgway, P. Saikku, J. Schachter, A. E. Stamm, and R. S. Stephens (ed.), *Chlamydial infections*. Societa Editrice Esculapio, Bologna, Italy.
 31. Pal, S., I. Theodor, E. M. Peterson, and L. M. de la Maza. 1997. Immunization with an acellular vaccine consisting of the outer membrane complex of *Chlamydia trachomatis* induces protection against a genital challenge. *Infect. Immun.* **65**:3361–3369.
 32. Pal, S., I. Theodor, E. M. Peterson, and L. M. de la Maza. 1997. Monoclonal immunoglobulin A antibody to the major outer membrane protein of the *Chlamydia trachomatis* mouse pneumonitis biovar protects mice against a chlamydial genital challenge. *Vaccine* **15**:575–582.
 33. Patton, D. L., C.-C. Kuo, S.-P. Wang, and S. A. Halbert. 1987. Distal tubal obstruction induced by repeated *Chlamydia trachomatis* salpingeal infections in pig-tailed macaques. *J. Infect. Dis.* **155**:1291–1299.
 34. Peterson, E. M., J. Z. You, V. Motin, and L. M. de la Maza. 1999. Intranasal immunization with *Chlamydia trachomatis* serovar E, protects from a subsequent vaginal challenge with the homologous serovar. *Vaccine* **17**:2901–2907.
 35. Piccinni, M.-P., M.-G. Giudizi, R. Biagiotti, L. Beloni, L. Giannarini, S. Sampognaro, P. Parronchi, R. Manetti, F. Annunziato, C. Livi, S. Romagnani, and E. Maggi. 1995. Progesterone favor the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. *J. Immunol.* **155**:128–133.
 36. Qu, Z., X. L. Cheng, M. de la Maza, and E. M. Peterson. 1994. Analysis of the humoral response elicited in mice by a chimeric peptide representing variable segments I and IV of the major outer membrane protein of *Chlamydia trachomatis*. *Vaccine* **12**:557–564.
 37. Ramsey, K. H., and R. G. Rank. 1991. Resolution of chlamydial genital infection with antigen specific T-lymphocyte lines. *Infect. Immun.* **59**:925–931.
 38. Schachter, J., and C. Dawson. 1978. Human chlamydial infections. PSG Publishing Company, Inc., Littleton, Mass.
 39. 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.
 40. Sjolander, A., T. M. Baldwin, J. M. Curtis, K. L. Bengtsson, and E. Handman. 1998. Vaccination with recombinant parasite surface antigen 2 from *Leishmania major* induces a Th1 type of immune response but does not protect against infection. *Vaccine* **16**:2077–2084.
 41. Stagg, A. J. 1998. Vaccines against *Chlamydia*: approaches and progress. *Mol. Med. Today* **4**:166–173.
 42. Stamm, W. E. 1999. *Chlamydia trachomatis* infections of the adult, p. 407–422. In K. K. Holmes et al. (ed.), *Sexually transmitted diseases*. McGraw-Hill, New York, N.Y.
 43. Stephens, R. S., R. Sanchez-Pescador, E. A. Wagar, C. Inouye, and M. S. Urdea. 1987. Diversity of *Chlamydia trachomatis* major outer membrane protein genes. *J. Bacteriol.* **169**:3879–3885.
 44. Su, H., M. Parnell, and H. D. Caldwell. 1995. Protective efficacy of a parenterally administered MOMP-derived synthetic oligopeptide vaccine in a murine model of *Chlamydia trachomatis* genital tract infection: serum neutralizing IgG antibodies do no protect against chlamydial genital infection. *Vaccine* **13**:1023–1031.
 45. Su, H., R. Messer, W. Whitmire, E. Fischeer, J. C. Portis, and H. D. Caldwell. 1998. Vaccination against chlamydial genital tract infection after immunization with dendritic cells pulsed ex vivo with nonviable chlamydiae. *J. Exp. Med.* **188**:809–818.
 46. 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.
 47. Tan, T.-W., A. J. Herring, L. E. Anderson, and G. E. Jones. 1990. Protection of sheep against *Chlamydia psittaci* infection with a subcellular vaccine containing the major outer membrane protein. *Infect. Immun.* **58**:3101–3108.
 48. Taylor, H. R., J. Whittum-Hudson, J. Schachter, H. D. Caldwell, and R. A. Prendergast. 1988. Oral immunization with chlamydial major outer membrane protein (MOMP). *Investig. Ophthalmol. Vis. Sci.* **29**:1847–1853.
 49. Tuffrey, M., F. Alexander, W. Conlan, C. Woods, and M. Ward. 1992. Heterotypic protection of mice against chlamydial salpingitis and colonization of the lower genital tract with a human serovar F isolate of *Chlamydia trachomatis* by prior immunization with recombinant serovar L1 major outer-membrane protein. *J. Gen. Microbiol.* **148**:1707–1715.
 50. Tuffrey, M., P. Falder, J. Gale, and D. Taylor-Robinson. 1986. Salpingitis in mice induced by human strains of *Chlamydia trachomatis*. *Br. J. Exp. Pathol.* **67**:605–616.
 51. Tuffrey, M., and D. Taylor-Robinson. 1981. Progesterone as a key factor in the development of a mouse genital-tract infection with *Chlamydia trachomatis*. *FEMS Microbiol. Lett.* **12**:111–115.
 52. Wang, S.-P., and J. T. Grayston. 1984. Micro-immunofluorescence serology of *Chlamydia trachomatis*, p. 87–118. In L. M. de la Maza and E. M. Peterson (ed.), *Medical virology III*. Elsevier Science Pub. Co., New York, N.Y.
 53. Ward, M. E. 1992. Chlamydial vaccines-future trends. *J. Infect.* **25**:11–26.
 54. Washington, A. F., and P. Katz. 1991. Cost and payment source of pelvic inflammatory disease. *JAMA* **266**:2565–2569.
 55. Westrom, L., R. Joesoef, G. Reynolds, A. Hagdu, and S. E. Thompson. 1992. Pelvic inflammatory disease and fertility: a cohort study of 1844 women with laparoscopically verified disease and 657 control women with normal laparoscopy. *Sex. Transm. Dis.* **19**:185–192.
 56. Westrom, L., and P. A. Mardh. 1990. Chlamydial salpingitis. *Br. J. Bull.* **39**:145–150.
 57. Whittum-Hudson, J. A., L.-L. An, W. M. Saltzman, R. A. Prendergast, and B. MacDonald. 1996. Oral immunization with an anti-idiotypic antibody to the exoglycolipid antigen protects against experimental *Chlamydia trachomatis* infection. *Nat. Med.* **2**:1116–1121.
 58. Zhang, D. J., J. Berry, C. Shen, G. McClarty, and R. C. Brunham. 1997. DNA vaccination with the major outer-membrane protein gene induces immunity to *Chlamydia trachomatis* (mouse pneumonitis infection). *J. Infect. Dis.* **176**:1035–1040.