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Vaccination with the *Chlamydia trachomatis* Major Outer Membrane Protein Can Elicit an Immune Response as Protective as That Resulting from Inoculation with Live Bacteria

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BALB/c mice were vaccinated by the intramuscular (i.m.) and subcutaneous (s.c.) routes with a native preparation of the *Chlamydia trachomatis* mouse pneumonitis (MoPn) major outer membrane protein (MOMP), using Montanide ISA 720 and CpG-1826 as adjuvants. A negative control group was immunized with ovalbumin and the two adjuvants, and a positive control group was immunized intranasally (i.n.) with 10^4 inclusion-forming units (IFU) of *C. trachomatis*. Four weeks after the last i.m.-plus-s.c. immunization, mice were challenged in the ovarian bursa with 10^5 IFU of *C. trachomatis* MoPn. Six weeks after the genital challenge, animals were mated, and the pregnancies were monitored. After vaccination with MOMP, the mice developed strong *Chlamydia*-specific humoral and cellular immune responses. Following the genital challenge, of the mice vaccinated with the MOMP, only 15% (3/20) had positive vaginal cultures, while 85% (17/20) of the animals immunized with ovalbumin had positive cultures over the 6 weeks of observation ($P < 0.05$). Also, only 14% (3/21) of the animals inoculated i.n. with *Chlamydia* had positive vaginal cultures. After mating, 75% (15/20) of the mice vaccinated with MOMP carried embryos in both uterine horns. Of the animals vaccinated i.n. with the *Chlamydia*, 81% (17/21) had embryos in both uterine horns ($P > 0.05$). In contrast, only 10% (2/20) of the mice immunized with ovalbumin had embryos in both uterine horns ($P < 0.05$). In conclusion, immunization with a purified preparation of the MOMP is as effective as vaccination with viable *C. trachomatis* in eliciting a protective immune response against a genital challenge in mice.

Chlamydia trachomatis is the most prevalent sexually transmitted bacterial pathogen in the world, with an estimated 100 million clinically diagnosed cases occurring annually (22, 24, 41, 42, 44). In addition, a similar or greater number of asymptomatic cases go undetected (22, 41, 44). The most common clinical presentations are urethritis and cervicitis (41, 42, 44). In general, these acute manifestations resolve over a period of a few weeks. However, in certain patients, long-term sequelae may develop, including pelvic inflammatory disease, ectopic pregnancy, and infertility (55). Furthermore, in areas of the world with poor hygienic conditions, *C. trachomatis* causes trachoma and lymphogranuloma venereum (41, 42). Although effective antibiotic therapy is available, eradication of these organisms will most likely only be achieved through a vaccination program (10, 14, 15, 18, 47).

Decades ago, in an attempt to prevent trachoma, vaccine trials with viable or inactivated whole organisms were performed. Several conclusions resulted from those trials. The protection was of short duration and was serovar specific and, in some of the vaccinated individuals that were reexposed to *Chlamydia*, a severe disease developed (15, 42). This hypersensitivity reaction was thought to be due to a component present in the organism (26). Furthermore, the fact that the protection was serovar specific led to the conclusion that the antigenic component involved in eliciting the immune response was unique to each serovar. Based on these observations, recent efforts have focused on developing a subunit vaccine using the major outer membrane protein (MOMP) of *C. trachomatis* as the antigen (10, 18). The MOMP constitutes 60% of the mass of the outer membrane and is surface exposed, and numerous T- and B-cell epitopes have been mapped in this protein (2, 46). A proposed model of the secondary structure of the *C. trachomatis* mouse pneumonitis (MoPn) MOMP identified 16 transmembrane segments with large loops corresponding to the variable domains of this protein (40, 45).

The *C. trachomatis* MoPn serovar was originally recovered from mice inoculated with human respiratory tract specimens (30). This organism has extensively been used to characterize the pathogenesis of genital and respiratory infections in mouse models (27, 39). Inoculation of the genital tract of female mice with MoPn results in an infection that parallels that described in humans, including vaginal shedding for several weeks and development of infertility (9). The *C. trachomatis* MoPn model has also been utilized to test several vaccination strategies. For example, Pal et al. (33) showed that intranasal immunization of mice with viable *C. trachomatis* MoPn organisms elicited an immune response that protected against a subsequent genital challenge. The immunized and challenged mice had a significant decrease in the severity and length of vaginal shedding. Furthermore, the immunized animals were protected against infertility. Immunization of mice with *C. trachomatis* MoPn subunit vaccines has also provided some encouraging results. For instance, immunization with the outer membrane has been shown to induce significant protection (3, 34). A preparation of

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the MOMP, extracted directly from *Chlamydia* and formulated with Freund's adjuvant, was also shown to elicit partial protection (35). Unfortunately, Freund's adjuvant cannot be used in humans. In an attempt to test a subunit vaccine that could potentially be utilized in humans, we formulated the native MOMP using CpG and Montanide, two adjuvants that are now in clinical trials (8, 12, 17, 54). Here we show that, with this approach, we were able to achieve an immune response as protective as that induced by live organisms.

MATERIALS AND METHODS

***C. trachomatis* stocks.** The *C. trachomatis* MoPn strain Nigg II was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and was grown in HeLa-229 cells using Eagle's minimal essential medium supplemented with 10% fetal calf serum (33). Purified elementary bodies (EB) were stored at −70°C in 0.2 M sucrose, 20 mM sodium phosphate (pH 7.4), and 5 mM glutamic acid (6). The stocks were titrated in HeLa-229 cells.

Purification of the *C. trachomatis* MoPn MOMP. The extraction and purification of the *C. trachomatis* MoPn MOMP were performed as previously described (35). In brief, *C. trachomatis* MoPn grown in tissue culture was washed, resuspended in phosphate-buffered saline (PBS) (pH 7.4), and treated with 25 μg/ml of DNase for 2 h at 4°C. The preparation was centrifuged and the pellet was extracted twice with 2% of 3-[(3-cholamidopropyl)-dimethylammonio]-l-propanesulfonate (CHAPS; Anatrace, Maumee, OH) in 0.2 M phosphate buffer (pH 5.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF; Calbiochem, San Diego, CA), 1 mM EDTA, and 100 mM dithiothreitol (DTT; Roche Diagnostic Corporation, Indianapolis, IN). The pellet was again extracted with 2% Anzergent 3-14 (*n*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate) (Z3-14; Anatrace) in phosphate buffer (see above) at 37°C. To further purify the MOMP, the supernatant was applied to a 30- by 2-cm hydroxyapatite (Bio-Gel HTP gel; Bio-Rad Laboratories, Hercules, CA) column equilibrated with 0.1% Z3-14 in 0.02 M phosphate buffer (pH 5.5) and eluted with a linear gradient from 0.02 to 0.5 M in the same buffer (6). The fractions showing an increase in the absorbance at 280 nm were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and identified by Western blotting using the monoclonal antibody MoPn 40 to the *C. trachomatis* MoPn MOMP developed in our laboratory. Fractions containing a single band were pooled, and the MOMP was then electrophoresed and stained with silver nitrate according to the procedure described by the manufacturer (Bio-Rad Laboratories). To further assess the purity of the MOMP an N-terminal amino acid analysis was performed by the core facilities at the University of California, Irvine.

The purified *C. trachomatis* MoPn MOMP was refolded by dialysis in 0.1 M phosphate buffer (pH 7.8) containing 2 mM reduced glutathione, 1 mM oxidized glutathione (Sigma, St. Louis, MO), 1 mM EDTA, and 0.05% Z3-14. The protein was then concentrated and fixed with 2% glutaraldehyde (Sigma) at room temperature for 2 min. Glycine (Bio-Rad Laboratories) was then added to stop the reaction. The MOMP was concentrated using a Centricon-10 filter (Millipore Corp., Bedford, MA) and dialyzed before immunization against 20 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.05% Z3-14.

Immunization and challenge of mice. Three- to 4-week-old female BALB/c (H-2^d) mice were purchased from Charles River Laboratories (Wilmington, MA) and were housed at the University of California, Irvine, vivarium. All animal protocols were approved by the University of California, Irvine, Animal Care and Use Committee.

Animals were immunized by the intramuscular (i.m.) (10 μg/mouse) and subcutaneous (s.c.) (10 μg/mouse) routes with *C. trachomatis* MoPn MOMP in PBS (pH 7.4) containing 0.05% of Z3-14. To the MOMP, we added 10 μg of CpG oligodeoxynucleotide 1826 (5′-TCCATGACGTTCCTGACGTT-3′) (Coley Pharmaceutical Group, Kanata, Ontario, Canada) and Montanide ISA 720 (Seppic, Inc., Fairfield, NJ) at a 30:70 volume ratio of MOMP plus CpG to Montanide. In another group, 250 μg of alum (Alhydrogel 85; Superfos Biosectr A/S, Kvistgard, Denmark) was used as an adjuvant instead of Montanide ISA 720. The alum group was similarly inoculated with the antigens by both the i.m. and s.c. routes. The mice were boosted two times at 2-week intervals with the same vaccine preparation. As a negative control, the MOMP was replaced with ovalbumin (Sigma, St. Louis, MO) and another group was inoculated intranasally (i.n.) with Eagle's minimal essential medium (MEM-0). Positive control mice were immunized i.n. once with 10^4 inclusion-forming units (IFU) of the *C. trachomatis* MoPn strain. A fertility control group of mice of the same age was kept under the same conditions but was not immunized or challenged.

Mice were challenged in the ovarian bursa 4 weeks after the last i.m.-plus-s.c. boost (35). Briefly, animals were anesthetized, and a lateral abdominal incision was made. *C. trachomatis* MoPn (10^5 IFU) was inoculated into the left ovarian bursa, while the right ovarian bursa was inoculated with mock-infected HeLa-229 cell extracts. All experiments were performed twice.

Serum and vaginal antibody titers. Blood was collected by periorbital or heart puncture, and genital samples were collected by washing the vagina twice with 20 μl of PBS (pH 7.2). All immunoassays were performed with the pooled sera or vaginal washes from each group.

The *Chlamydia*-specific antibody titers in sera and vaginal washes were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described (33). Briefly, 96-well plates were coated with 100 μl/well of *C. trachomatis* MoPn EB containing 10 μg/ml of protein in PBS, and 100 μl of serum or 50 μl of vaginal wash was added per well in serial dilutions. Following incubation at 37°C for 1 h, the plates were washed, and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG), IgG1, IgG2a, IgM, or IgA (BD Pharmingen, San Diego, CA) was added. The plates were incubated and washed, and the binding was measured in an ELISA reader (Labsystem Multiscan; Helsinki, Finland) using 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) as the substrate.

In vitro neutralization assays. The in vitro neutralization assay was performed according to the protocol described by Peterson et al. (36). Briefly, twofold serial dilutions of the serum were made in PBS. Duplicate dilutions were incubated for 45 min at 37°C with 1×10^4 IFU of *C. trachomatis* MoPn. The mixtures were then inoculated by centrifugation onto HeLa-229 cell monolayers grown in 15- by 45-mm glass shell vials. The monolayers were incubated for 30 h and subsequently fixed with methanol, and the inclusions were stained using a cocktail of monoclonal antibodies to *Chlamydia* prepared in our laboratory. A horseradish peroxidase conjugated goat anti-mouse antibody was added and developed with 0.01% H_2O_2 and 4-chloro-naphthol (Sigma, St. Louis, MO). The results were each expressed as percent inhibition relative to the control sera.

Western blots. Immunoblotting was performed using 250 μg of protein from purified *C. trachomatis* MoPn EB, electrophoresed in Tricine-SDS polyacrylamide gels (10% acrylamide, 0.3% bisacrylamide [wt/vol]) (43). Following transfer to nitrocellulose membranes, the nonspecific sites were blocked with BLOTTO (Bovine Lacto Transfer Technique Optimizer: 5% [wt/vol] nonfat dried milk, 2 mM $CaCl_2$, and 50 mM Tris-HCl, pH 8.0). Serum samples were diluted with PBS and incubated overnight at 4°C. As a positive control we used the monoclonal antibody MoPn-40. Antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse antibody and the reaction detected with 0.01% hydrogen peroxide and 4-chloro-1-naphthol (35).

Lymphocyte proliferation assay. The lymphocyte proliferation assay was performed as previously described (33). In brief, the spleens of two to four mice from each group were collected and splenocytes enriched for T cells by passage over a nylon wool column. Accessory cells for antigen presentation were prepared by irradiating (3,000 rads; ^{137}Cs) syngeneic unseparated spleen cells and incubating them at various ratios with *C. trachomatis* MoPn EB. Concanavalin A (ConA; Sigma) was used as a positive stimulant at a concentration of 5 μg/ml, and HeLa-229 cell extracts and tissue culture media served as negative controls. At the end of 4 days of incubation, 1.0 μCi of [*methyl*-^{3}H]thymidine (47 Ci/mmol; Amersham, Arlington Heights, IL) in 25 μl of RPMI 1640 was added per well, and the incorporation of [^{3}H] was measured using a scintillation counter (Beckman Instruments, Fullerton, CA).

Measurement of cytokines. Levels of gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin-4 (IL-4) were determined using commercial kits (BD Pharmingen, San Diego, CA) with supernatants from splenic T cells stimulated as described above (35).

Genital cultures for *C. trachomatis* MoPn. Vaginal swabs were collected and cultured at 7-day intervals for a period of 6 weeks following the genital challenge (33). The swabs were vortexed in 200 μl of sterile sucrose-phosphate-glutamic acid medium, and two aliquots from each specimen (100 and 10 μl) were inoculated into McCoy cells grown in 48-well plates with centrifugation at $1{,}000 \times g$ for 1 h at room temperature. Following incubation at 37°C for 30 h, the chlamydial inclusions were stained as described above.

Fertility studies. At 6 weeks following the intrabursal challenge, groups of four female mice were housed in the same cage with a proven breeder male mouse for a maximum of 18 days (33). Pregnancy was assessed by determining the weight of each mouse. Mice that gained 7 to 10 g of weight by, or before, 18 days postmating were considered to be pregnant. These mice were euthanized, and the number of embryos in each uterine horn was counted. Following the first mating, mice that did not gain weight were mated a second time with a male mouse that had successfully mated with another group of female mice. Pregnancy

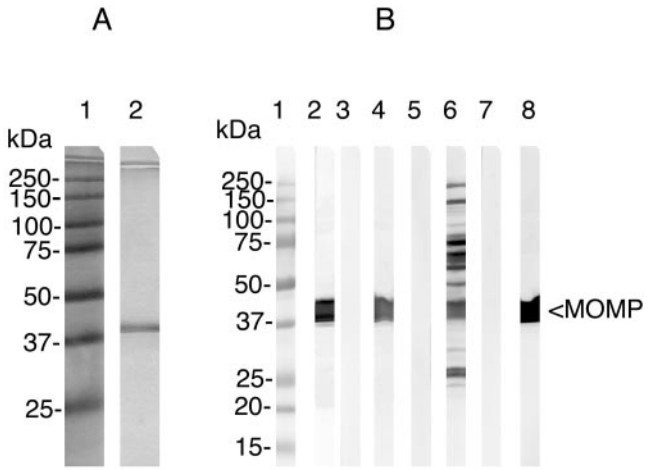

FIG. 1. (A) SDS-PAGE of *C. trachomatis* MoPn MOMP purified by hydroxyapatite chromatography and stained with silver nitrate. Lane 1, molecular mass standards; lane 2, *C. trachomatis* MoPn MOMP. (B) Immunoblot of *C. trachomatis* MoPn EB probed with serum samples collected the day before the genital challenge. Lane 1, molecular mass standards. Probe with serum from mice immunized with the following: MOMP plus CpG plus Montanide (lane 2), ovalbumin plus CpG plus Montanide (lane 3), MOMP plus CpG plus alum (lane 4), ovalbumin plus CpG plus alum (lane 5), *C. trachomatis* MoPn EB (lane 6), MEM-0 (lane 7), and control monoclonal antibody to the *C. trachomatis* MoPn MOMP (lane 8).

was followed as described above. All animals that had not gained weight were euthanized 25 days from the start of the second mating.

Statistical analyses. The Mann-Whitney U test, the Fisher exact test, and the Student *t* test were used for analysis with the Statview IV (Abacus, Berkeley, CA) software program using a Macintosh computer (Cupertino, CA).

RESULTS

Characterization of the *C. trachomatis* MOMP. The *C. trachomatis* MOMP was purified using a hydroxylapatite column and characterized by gel electrophoresis and amino acid sequencing. As shown in Fig. 1A, the MOMP migrated as a single band on an SDS-PAGE stained with silver. N-terminal amino acid analysis detected only the sequence L-P-V-G-N-P, corresponding to the mature *C. trachomatis* MoPn MOMP (data not shown). Based on these results, it was estimated that the *C. trachomatis* MOMP preparation was 99% pure.

Immune response following vaccination with the *C. trachomatis* MOMP. Table 1 shows the results of the characterization of the humoral immune response following vaccination. High *Chlamydia*-specific IgG antibody titers in serum were observed in the groups of mice immunized i.m. plus s.c. with MOMP plus CpG plus Montanide and MOMP plus CpG plus alum. The titer of total IgG was 409,600 in the mice vaccinated with MOMP plus CpG plus Montanide and 102,400 in the group immunized with MOMP plus CpG plus alum. In the group of mice vaccinated with MOMP plus CpG plus Montanide, the titer of IgG2a was 51,200 and the titer of IgG1 was 25,600, indicative of a Th1 response. The mice immunized with MOMP plus CpG plus alum had higher levels of IgG1 (25,600) than of IgG2a (6,400), suggestive of a Th2-biased response. Mice immunized i.n. with *C. trachomatis* MoPn EB had an IgG serum titer of 102,400. This control group had high levels of IgG2a (25,600) when compared to those of IgG1 (1,600) in serum, indicating a strong Th1 response. Control groups immunized with ovalbumin plus CpG plus Montanide, ovalbumin plus CpG plus alum, or MEM-0 had no detectable antibodies to *Chlamydia*.

The neutralizing antibody titer in the groups of mice vaccinated with MOMP plus CpG plus Montanide, MOMP plus CpG plus alum, and i.n. with *C. trachomatis* EB was 1,250 (Table 1). Sera from mice immunized using ovalbumin plus CpG plus Montanide, ovalbumin plus CpG plus alum, or MEM-0 served as controls.

The levels of *Chlamydia*-specific IgA antibodies in vaginal washes in the mice vaccinated with MOMP plus CpG plus Montanide or MOMP plus CpG plus alum were 160 and 40, respectively. Very high levels of IgA, a titer of 1,280, were detected in the vaginal washes of the mice inoculated i.n. with *C. trachomatis* EB. No *Chlamydia*-specific antibodies were detected in the control groups immunized with ovalbumin or MEM-0.

The Western blot using serum collected the day before the genital challenge is shown in Fig. 1B. Mice vaccinated with MOMP plus CpG plus Montanide or with MOMP plus CpG plus alum developed antibodies against MOMP. Animals immunized i.n. with EB had antibodies predominantly to bands of a high molecular weight (>100 kDa), the 60-kDa cysteine-rich protein (crp), the 60-kDa heat shock protein, MOMP, and the 28-kDa protein. Control mice immunized with ovalbumin plus CpG plus Montanide, ovalbumin plus CpG plus alum, or MEM-0 had no antibodies reactive with any of the chlamydial components.

In Table 2 the results of the measurements of the cell-mediated immune response are shown. T lymphocytes from animals vaccinated with MOMP plus CpG plus Montanide or

TABLE 1. Antibody levels the day before genital challenge

Antigen	Adjuvant	*C. trachomatis* MoPn-specific ELISA antibody titer									Serum neutralizing titer
		Serum (10^3)							Vaginal wash		
		IgM	IgG	IgG1	IgG2a	IgG2b	IgG3	IgA	IgA	IgG	
MOMP	CpG + Montanide	0.1	409.6	25.6	51.2	204.8	25.6	3.2	160	160	1,250
Ovalbumin	CpG + Montanide	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<20	<20	<10
MOMP	CpG + alum	0.1	102.4	25.6	6.4	51.2	12.8	0.8	40	20	1,250
Ovalbumin	CpG + alum	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<20	<20	<10
C. trachomatis		0.1	102.4	1.6	25.6	25.6	3.2	3.2	1,280	320	1,250
MEM-0		<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<20	<20	<10

TABLE 2. T-cell response the day before genital challenge[a]

Antigen	Adjuvant	T-cell proliferation response (10^3 cpm) to:			In vitro cytokine production					
					IFN-γ (pg/ml)		IL-4 (pg/ml)		TNF-α (pg/ml)	
		EB[b]	ConA[c]	Medium	EB[b]	ConA[c]	EB[b]	ConA[c]	EB[b]	ConA[c]
MOMP	CpG + Montanide	10.3 ± 2.3[d]	66.5 ± 1.6	0.2 ± 0.05	3,973 ± 1[d]	2,510 ± 128	<0.8	58 ± 3	459 ± 41[d]	318 ± 0
Ovalbumin	CpG + Montanide	0.9 ± 0.1	81.0 ± 9.1	0.09 ± 0.01	561 ± 14	3,341 ± 34	<0.8	43 ± 2	302 ± 5	288 ± 8
MOMP	CpG + alum	6.1 ± 2.2[d]	73.7 ± 9.4	0.3 ± 0.3	2,097 ± 26[d]	3,266 ± 0	2 ± 0.7	52 ± 1	372 ± 34[d]	310 ± 24
Ovalbumin	CpG + alum	1.7 ± 0.5	40.3 ± 6.2	1.1 ± 0.5	1,361 ± 15	2,575 ± 334	<0.8	66 ± 3	262 ± 9	254 ± 15
C. trachomatis		17.2 ± 2.1[d]	57.2 ± 21.3	0.08 ± 0.03	3,081 ± 137[d]	2,309 ± 209	5 ± 0.2	75 ± 3	455 ± 14[d]	398 ± 10
MEM-0		2.2 ± 0.7	48.4 ± 6.6	0.2 ± 0.08	683 ± 10	2,957 ± 81	<0.8	50 ± 0.1	164 ± 6	236 ± 55

[a] Results are the means for triplicate cultures (±1 standard deviation). Data correspond to one of the experiments representative of duplicate separate experiments.
[b] UV-inactivated *C. trachomatis* MoPn EB were added at a 10:1 ratio to the antigen-presenting cells.
[c] Concanavalin A (ConA) was added at a concentration of 5 μg/ml.
[d] $P < 0.05$ by the Student *t* test, compared to the ovalbumin- or MEM-0-immunized group.

MOMP plus CpG plus alum showed a significant lymphoproliferative response when stimulated with EB, compared with the corresponding control groups immunized with ovalbumin instead of MOMP ($P < 0.05$). Control BALB/c mice immunized i.n. with *C. trachomatis* also showed a significant lymphoproliferative response to MoPn EB.

Levels of IFN-γ and TNF-α from supernatants of splenocytes stimulated with EB were significantly higher in the mice vaccinated with MOMP plus CpG plus Montanide and MOMP plus CpG plus alum and in the animals immunized i.n. with *C. trachomatis*, compared with their respective control groups immunized with ovalbumin plus CpG plus Montanide, ovalbumin plus CpG plus alum, or MEM-0. The levels of IL-4 were very low, or below the level of detection, in all the groups entered in the study.

Vaginal cultures for *C. trachomatis* MoPn. Four weeks after the last i.m.-plus-s.c. immunization, the mice were challenged in the genital tract with the *C. trachomatis* MoPn and the course of the infection was followed with weekly vaginal cultures. As shown in Table 3, *C. trachomatis* was recovered from only 15% (3/20) of the mice vaccinated with MOMP plus CpG plus Montanide. Similarly, only 14% (3/21) of the mice immunized i.n. with *C. trachomatis* had positive vaginal cultures. In contrast, 85% (17/20) of the animals immunized with ovalbumin plus CpG plus Montanide and 75% (15/20) of the controls immunized with MEM-0 had positive vaginal cultures ($P < 0.05$). The protection observed in the MOMP-plus-CpG-plus-alum-vaccinated group was not very strong. A total of 75% (15/20) of the mice in this group had positive vaginal cultures during the 6 weeks of observation, in comparison with 95% (19/20) in the group immunized with ovalbumin plus CpG plus alum ($P > 0.05$).

The lengths of time the vaginal cultures were positive and the numbers of *Chlamydia* IFU recovered were also significantly different between the groups. For example, the mice vaccinated with MOMP plus CpG plus Montanide and the controls immunized i.n. with *C. trachomatis* EB had positive cultures for only 2 weeks and 1 week, respectively, while the animals immunized with ovalbumin plus CpG plus Montanide and with MEM-0 had positive vaginal cultures for a period of 4 weeks. Furthermore, the number of *C. trachomatis* IFU recovered from the vaginal cultures during the first 2 weeks after the challenge was significantly lower in the MOMP-plus-CpG-plus-Montanide group and *C. trachomatis* EB i.n.-immunized groups when compared with the corresponding control animals ($P < 0.05$). In the group vaccinated with MOMP plus CpG plus alum, positive cultures were obtained for a period of 3 weeks after the vaginal challenge. The percentage of mice with positive cultures and the number of IFU recovered per animal were significantly lower for the first 2 weeks after the challenge, compared with the control mice immunized with ovalbumin plus CpG plus alum ($P < 0.05$).

Fertility studies. Six weeks after the animals were challenged in the genital tract, male mice were housed in the same cage with the females and the mice were followed over a course of two mating cycles. The results of the fertility studies are shown in Table 4. The mice vaccinated with MOMP plus CpG plus Montanide, the controls immunized i.n. with *C. trachomatis*, and the fertility control group all had equivalent fertility rates of 75% (15/20), 81% (17/21), and 83% (20/24), respectively ($P > 0.05$). The numbers of embryos in the challenged left uterine horn and the total numbers of embryos per mouse were also comparable for these three groups. For example, in the group vaccinated with MOMP plus CpG plus Montanide and the one immunized i.n. with EB, the mean number of embryos in the left uterine horn was 2.6, while in the fertility control the mean was 2.7 ($P > 0.05$). Fertility rates, however, were not maintained in the group vaccinated with MOMP plus CpG plus alum. Only 35% (7/20) of these mice had embryos in both uterine horns, in comparison to 83% (20/24) in the fertility control group ($P < 0.05$). Similarly, the numbers of embryos in the left uterine horn were significantly different between the two groups (0.9 versus 2.7; $P < 0.05$). The animals immunized with ovalbumin plus CpG plus Montanide and ovalbumin plus CpG plus alum had fertility rates similar to those of the group immunized with MEM-0, and all were significantly lower than the fertility rates of the mice vaccinated with MOMP plus CpG plus Montanide and with *C. trachomatis* EB.

DISCUSSION

Here we have shown that vaccination of mice with a purified preparation of the *C. trachomatis* MoPn MOMP, using two adjuvants that are currently in human clinical trials, can induce protection against a genital challenge. The level of protection against infection and disease obtained with the MOMP was equivalent to that resulting following i.n. immunization with viable *C. trachomatis* EB. In mice vaccinated with MOMP, the severity and duration of the infection were similar to those

observed in animals immunized with EB. In addition, the fertility rate in the animals vaccinated with MOMP paralleled that observed in mice immunized with *C. trachomatis* EB and in the fertility control group.

Work performed by several groups of investigators over the last 3 decades pointed to the MOMP as the antigen most likely involved in stimulating the protective immune response against a chlamydial infection (2, 10, 39, 46). However, attempts to induce protection in several animal models using recombinant MOMP, peptides corresponding to regions of this protein, or DNA plasmids containing the gene for the MOMP yielded, for the most part, disappointing results (4, 7, 31, 48, 50, 51, 57, 58). More encouraging results were obtained using outer membrane complexes of *Chlamydia* and with dendritic cells pulsed ex vivo with nonviable EB (3, 34, 49). These data suggest either that antigens other than the MOMP are necessary for inducing protection or that the conformation of the MOMP is critical for eliciting a protective immune response.

In an attempt to maintain the native conformation of the MOMP, we developed a protocol using zwitterionic detergents to extract this protein directly from *C. trachomatis* (32, 35). Blake and Gotschlich (5) described the ability of the zwitterionic detergent Z3-14 to maintain the native conformation of the porin A from *Neisseria gonorrhoeae*. Thus, we utilized Z3-14 to extract and to purify the MOMP directly from *C. trachomatis* EB. The outer membrane of *Chlamydia* has three cysteine-rich proteins, including the MOMP and the 60-kDa and 12-kDa proteins, that may be linked by disulfide bonds (16). We used DTT during the extraction procedure; thus, following purification, in an attempt to regain the native conformation of the MOMP, we incorporated a refolding step to oxidize the disulfide bonds. In this preparation we have now characterized two disulfide bonds with intramolecular pairs formed between Cys^{48} and Cys^{55} and between Cys^{201} and Cys^{203} (56). Furthermore, we have shown, by using monoclonal antibodies to conformational epitopes, that this natural MOMP maintains at least part of its native trimeric structure (56).

Immunization of mice with this preparation of MOMP using Freund's adjuvant elicited significant protection against a genital challenge (35). Although Freund's is an adjuvant that has been extensively tested in animal models, due to its negative effects it cannot be utilized in humans. CD4 Th1 cells have been shown to play a critical role in protection against a primary and secondary challenge with *C. trachomatis* MoPn, while B cells and/or antibodies appear to be important only against a secondary challenge (27–29). Based on this information, we decided to test adjuvants that induce predominantly a Th1 immune response and are safe for humans. We initially showed that natural MOMP, administered with CpG plus alum, can induce significant protection against a respiratory challenge with *C. trachomatis* MoPn (32). Here, in addition to testing CpG plus alum as adjuvants, we also utilized a combination of CpG plus Montanide ISA 720. Alum is an adjuvant that favors a Th2 response (23). On the other hand, the combination of CpG plus Montanide ISA 720 has been found to enhance the Th1 response against malarial antigens (17). Montanide ISA 720 is an oil adjuvant that renders water in oil emulsions when mixed with an aqueous phase. The slow release of the antigen and its protection from proteolytic enzymes by this type of

TABLE 3. Results of vaginal cultures

Mice immunized with:	Adjuvant	No. of mice that shed[a] and no. of *C. trachomatis* IFU shed[b]										Total no. of mice that shed in 6 wk (% positive)
		wk 1		wk 2		wk 3		wk 4		wk 5		
		Mice shedding	*C. trachomatis* IFU shed	Mice shedding	*C. trachomatis* IFU shed	Mice shedding	*C. trachomatis* IFU shed	Mice shedding	*C. trachomatis* IFU shed	Mice shedding	*C. trachomatis* IFU shed	
MOMP	CpG + Montanide	1/20 (5)[c]	0 (0–960)[d]	2/20 (10)[c]	0 (0–12)[d]	0/20 (0)	0	0/20 (0)	0	0/20 (0)	0	3/20 (15)[c]
Ovalbumin	CpG + Montanide	12/20 (60)	2 (0–2,368,060)	13/20 (65)	14 (0–534,586)	5/20 (25)	0 (0–134,720)	1/20 (5)	0 (0–6)	0/20 (0)	0	17/20 (85)
MOMP	CpG + alum	7/20 (35)[c]	0 (0–36,868)[d]	10/20 (50)[c]	13 (0–2,272)[d]	4/20 (20)	0 (0–1,160)	0/20 (0)	0	0/20 (0)	0	15/20 (75)
Ovalbumin	CpG + alum	13/20 (65)	18 (0–891,922)	17/20 (85)	391 (0–302,034)	5/20 (25)	0 (0–309,124)	1/20 (5)	0 (0–7,820)	0/20 (0)	0	19/20 (95)
C. trachomatis		3/21 (14)[c]	0 (0–18)[d]	0/21 (0)[c]	0[d]	0/21 (0)	0	0/21 (0)	0	0/21 (0)	0	3/21 (14)[c]
MEM-0		11/20 (55)	2 (0–8,500)	10/20 (50)	327 (0–372,934)	1/20 (5)	0 (0–2,380)	1/20 (5)	0 (0–867)	0/20 (0)	0	15/20 (75)

[a] No. of mice that shed/total no. of mice per group (% positive).
[b] Median no. of *C. trachomatis* IFU shed per group (range).
[c] $P < 0.05$ by the Fisher exact test, compared with the corresponding ovalbumin- or MEM-0-immunized control group.
[d] $P < 0.05$ by the Mann-Whitney U test, compared with the corresponding ovalbumin- or MEM-0-immunized control group.

TABLE 4. Results of fertility studies

Antigen	Adjuvant	No. of mice that had embryos in uterine horns/total no. of mice/group (%)			Mean no. of embryos in uterine horns ± 1 SD		
		Both	Right	Left[a]	Both	Right	Left[a]
MOMP	CpG + Montanide	15/20 (75)[c,e]	20/20 (100)	15/20 (75)[c,e]	5.7 ± 3.0[d,f]	3.1 ± 1.4	2.6 ± 2.4[d,f]
Ovalbumin	CpG + Montanide	2/20 (10)	14/20 (70)	2/20 (10)	3.0 ± 2.5	2.9 ± 2.4	0.2 ± 0.5
MOMP	CpG + alum	7/20 (35)	17/20 (85)	8/20 (40)	4.2 ± 2.2[d,f]	3.3 ± 1.9	0.9 ± 1.3
Ovalbumin	CpG + alum	2/20 (10)	12/20 (60)	2/20 (10)	2.3 ± 2.4	2.0 ± 2.1	0.3 ± 0.8
C. trachomatis		17/21 (81)[c,e]	20/21 (95)	18/21 (86)[c,e]	5.7 ± 2.6[d,f]	3.1 ± 1.7	2.6 ± 1.5[d,f]
MEM-0		6/20 (30)	16/20 (80)	7/20 (35)	3.7 ± 2.1	3.0 ± 2.0	0.8 ± 1.2
Fertility control[b]		20/24 (83)	23/24 (96)	21/24 (88)	5.8 ± 2.5	3.2 ± 1.6	2.7 ± 1.8

[a] Ovarian bursae inoculated with *C. trachomatis* MoPn.
[b] Fertility control: mice were neither immunized nor challenged.
[c] $P < 0.05$ by the Fisher exact test, compared to the corresponding ovalbumin- or MEM-0-immunized control group.
[d] $P < 0.05$ by the Mann-Whitney U test, compared to the corresponding ovalbumin- or MEM-0-immunized control group.
[e] $P > 0.05$ by the Fisher exact test, compared to the fertility control group.
[f] $P > 0.05$ by the Mann-Whitney U test, compared to the fertility control group.

adjuvant may also have helped to induce a protective immune response (12). Using this approach, we were able to elicit an immune response against an intrabursal challenge that is as protective as that obtained with viable *Chlamydia*. These results now open the possibility of testing a vaccine formulated with MOMP to protect humans against trachoma and genital infections.

There are several limitations in this model that will have to be addressed before human trials can be implemented. The need to purify the MOMP directly from *Chlamydia* significantly curtails the feasibility of producing the antigen in large quantities. In addition to the cost of growing large amounts of *Chlamydia*, the extraction, purification, and refolding of the MOMP will add significant expense to the preparation of the vaccine. Furthermore, concerns about adventitious agents that may be present in the tissue culture system will have to be addressed. Some of these limitations could be solved if recombinant MOMP preparations became available. However, production of an efficacious recombinant protein may pose specific challenges. For example, formulating a recombinant MOMP may be difficult if conformational epitopes are required for protection. Similarly, the assembly of a biochemically engineered immunogen will require the identification and characterization of the protective epitopes.

Another potential shortcoming of using the MOMP as a vaccine is the questionable ability of this immunogen to offer broad protection against multiple *C. trachomatis* serovars. In our opinion, this limitation may only be relative and can be overcome. Work by Wang and Grayston (52, 53) using a murine model indicated that most of the human *C. trachomatis* serovars can be divided into two main groups: the C group, comprising serovars C, J, H, I, and A, and the B group that includes serovars B, Ba, E, D, L1, and L2. Serovars K and L3 are related to the C complex, while G and F are related to the B complex. Within each group there is a senior-to-junior relationship. For example, in the C group, the C serovar is the senior isolate. The C serovar cross-reacts and protects against the other four serovars in the group, including A, the most junior serovar (52, 53). Thus, the MOMP from serovar C, for the C group, and the MOMP from serovar B, for the B group, could potentially induce protection against the rest of the serovars in each group. It is then possible that a vaccine that included the MOMPs from the most senior serovars of the C and B groups and the MOMPs from the G and K serovars could protect against all the human *C. trachomatis* serovars (38, 52, 53). The apparent serovar specificity of the protection induced during the vaccination trials for trachoma was probably due to the fact that the two most common serovars causing this disease are C and B (15, 42). These two serovars are in different groups, and thus there is minimal or no cross-protection.

Furthermore, once the protective regions of the MOMP from a serovar are identified, it may be possible to use that information to construct a polyvalent vaccine with broad coverage. It is also important to point out that most of the urogenital infections are produced by serovars D, E, and F (25, 53). Even more important, a vaccine with limited antigenic determinants may significantly affect the epidemiology of these diseases (11). However, in this case, the possibility that the *Chlamydia* isolates not included in the vaccine will spread throughout the population will have to be considered. Including a protective antigen that is common to all the serovars will address this problem. Unfortunately, at this point, a shared protective immunogen has not been identified.

The intrabursal model that we have tested has shortcomings, since it does not parallel the natural route of infection. A particular concern is the fact that the mechanisms of protection in the lower genital tract may differ from those in the upper genital tract (21). On the other hand, directly challenging the site we are interested in protecting may provide more stringent proof of the efficacy of the vaccine. The feasibility of determining the ability of *Chlamydia* to disseminate from one uterine horn to the other is also one of the advantages of using the intrabursal versus the vaginal challenge. With the intravaginal model, in order to increase the susceptibility to infection and facilitate the development of infertility, the mice are treated with progesterone before they are challenged (39). Progesterone has a strong immunomodulatory effect in mice and humans. In particular, progesterone induces a shift from Th1 to a Th2 response (19, 20, 37). Unfortunately, a Th1 response is considered to be necessary for protection against a *C. trachomatis* infection (27, 29). As a result, treating mice with progesterone before the intravaginal challenge may mask the protective effects of a vaccine. For example, in the case of herpes simplex virus, it has been reported that prolonged exposure of mice to progesterone prevented the induction of

protective responses following immunization (13). Moreover, treatment of rhesus macaques with Depo-Provera before intravaginal challenge with simian immunodeficiency virus abrogated the protection induced by a vaccine with an attenuated lentivirus (1).

In conclusion, we have shown that vaccination with the *C. trachomatis* MOMP can induce an immune response in mice that is as protective as that resulting from immunization with live organisms. Furthermore, this protection was achieved using adjuvants that are effective and tolerated by humans. We realize that translating this finding from a murine model to a vaccine for humans is going to require a significant amount of work. Recent advances in genomics, proteomics, and molecular engineering should help address this challenge.

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