A Recombinant *Chlamydia trachomatis* MOMP Vaccine Elicits Cross-serogroup Protection in Mice Against Vaginal Shedding and Infertility

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Background. *Chlamydia trachomatis* is the most common sexually transmitted bacterial pathogen worldwide. Here, we determined the ability of a *C. trachomatis* recombinant major outer membrane protein (rMOMP) vaccine to elicit cross-serogroup protection.

Methods. Female C3H/HeN mice were vaccinated by mucosal and systemic routes with *C. trachomatis* serovar D (UW-3/Cx) rMOMP and challenged in the ovarian bursa with serovars D (UW-3/Cx), D (UCI-96/Cx), E (IOL-43), or F (N.I.1). CpG-1826 and Montanide ISA 720 were used as adjuvants.

Results. Immune responses following vaccination were more robust against the most closely related serovars. Following a genital challenge (as determined by number of mice with positive vaginal cultures, number of positive cultures, number of inclusion forming units recovered, and number of days with positive cultures) mice challenged with *C. trachomatis* serovars of the same complex were protected but not those challenged with serovar F (N.I.1) from a different subcomplex. Females were caged with male mice. Based on fertility rates, number of embryos, and hydrosalpinx formation, vaccinated mice were protected against challenges with serovars D (UW-3/Cx), D (UCI-96/Cx), and E (IOL-43) but not F (N.I.1).

Conclusions. This is the first subunit vaccine shown to protect mice against infection, pathology, and infertility caused by different *C. trachomatis* serovars.

Keywords. cross-serogroup; protection; vaccine; *Chlamydia trachomatis*; MOMP; mice.

Chlamydia trachomatis is the leading bacterial sexual transmitted infection in the world [1, 2]. Acute and chronic chlamydial infections in women may lead to long-term sequelae including pelvic inflammatory disease, chronic abdominal pain, ectopic pregnancy, and infertility [3–6]. In countries with poor sanitary conditions it produces trachoma [7]. Screening and antibiotics treatment programs have not resulted in a decline in infections. Likely as a result of the antibiotic treatment, patients have halted natural immune responses that facilitate reinfections [8, 9]. Thus, there is a need for a vaccine to protect against this pathogen [10–13].

Based on immunological and protection studies, 15 major *C. trachomatis* serovars have been identified [14, 15]. These serovars were classified into 2 major immunocomplexes: B (B, Ba, E, D, L1, and L2) and C (C, J, H, I, and A). Serovars G and F are related to the B complex while K and L3 are related to the C

complex but bridge both the B and C complexes. In each complex there is a senior to junior relationship. The senior serovar, for example B in the B complex, protects against all the other serovars in the complex while the junior serovar, L2, elicits much limited cross-protection [15, 16]. When the sequence of *C. trachomatis* major outer membrane protein (MOMP) was determined, it was found to have variable domains (VD), regions of DNA unique to each serovar [17]. Phylogenetic analysis of the MOMP sequence supported the likelihood that the serovar/serocomplex protection elicited during the trachoma vaccine trials was due to MOMP [7, 18]. Therefore, we can hypothesize that a polyvalent vaccine formulated with the senior serovar of each complex will protect against all the individual serovars.

Here, we tested a vaccine formulated with the *C. trachomatis* serovar D (UW-3/Cx) rMOMP for its ability to induce protection against the homologous isolate, against a different isolate of serovar D (UCI-N96/Cx), and against 2 other serovars, E (IOL-43) and F (N.I.1). Our results showed robust protection against the homologous serovar, a different isolate of the same serovar, and against serovar E (IOL-43) from the same B-complex. No protection was induced against serovar F (N.I.1) from a different subcomplex. To our knowledge, this is the first time that cross-serogroup protection against *C. trachomatis*-induced infection,

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shedding, upper genital tract pathology, and infertility has been achieved with a subunit vaccine.

MATERIALS AND METHODS

Chlamydia trachomatis

C. trachomatis serovars D (UW-3/Cx), D (UCI-N96/Cx), E (IOL-43/GU), and F (strain N.I.1) were grown in HeLa-229 cells and elementary bodies (EBs) were purified as previously described [19]. Genomic DNA from serovar D (UW-3/Cx) was extracted and the *momp* gene was amplified, cloned, expressed, and purified as before [20–24] (Supplementary Figure 1). MOMP had less than 0.05 EU of lipopolysaccharide/mg of protein [25].

Immunization Protocols

Three-week-old female C3H/HeN (H-2^k) mice (Charles River Laboratories; Wilmington, MA) were vaccinated twice by the colonic (10 µg protein/mouse/immunization) route, followed by 2 intramuscular (6.6 µg protein/mouse/immunization) plus subcutaneous (3.3 µg protein/mouse/immunization) immunizations [26–28]. Two adjuvants were used: CpG-1826 (10 µg mouse/immunization; Tri-Link BioTechnologies LLC, San Diego, CA) and Montanide ISA 720 VG (Seppic Inc., Fairfield, NJ) [27, 28]. Montanide was delivered only systemically.

Two negative controls received phosphate-buffered saline (PBS) with adjuvants or minimum essential medium eagle (MEM) intranasally. Positive-control mice were immunized intranasally with 1×10^6 inclusion forming units (IFU) of serovar D (UW-3/Cx). A fertility control group was only mated. The experiment was replicated. The University of California Irvine Institutional Animal Care and Use Committee approved the protocols.

Immunological Assays

Blood and vaginal washes were collected before immunization and the day before the challenge. EB and rMOMP were used as antigens and levels of antibodies were determined using an enzyme-linked immunosorbent assay (ELISA) [29]. The in vitro neutralization assay was performed as previously described using HeLa-229 cells [30]. Neutralization was defined as greater than or equal to 50% decrease in the number of IFU as compared to the control incubated with preimmunization sera. Serological assays were performed in 6 independent samples.

A T-cell lymphoproliferative assay was performed the day before the challenge using nylon-purified splenic T cells (>95% purity) [31]. The mean counts per minute (cpm) were determined from triplicate culture wells. Levels of interferon-γ (IFN-γ) were assessed in supernatants from stimulated T cells as before (BD Biosciences, San Diego, CA) [31]. Samples from 4 different mice/group were assayed.

Intrabursal Challenge

Four weeks after the last immunization, mice were challenged in the left ovarian bursa [28]. The number of IFUs for

the challenge was chosen to induce at least 80% infertility in the sham-immunized animals [28]. To synchronize the estrus cycle in diestrus, 4 days before the challenge, mice were treated subcutaneously with 2 mg/mouse of medroxyprogesterone acetate (MPA; Greenstone Ltd, Peapack, NJ) [28, 32]. Following the challenge vaginal swabs were cultured twice a week for the weeks 2 and 3, and once a week for the rest of the experiment. The number of IFU was determined in HeLa-229 monolayers [28, 30, 33]. The limit of detection was 2 IFU/culture.

Fertility Studies

Female mice were housed with male mice at 6 weeks following the intrabursal challenge [29, 31]. Fertility was determined based on the number of embryos in the left and right uterine horn. Hydrosalpinx formation was assed visually.

Statistics

The 2-tailed unpaired Student *t* test, the Fisher exact test, and the Mann-Whitney *U* test, with Prism version 6 (GraphPad Software Inc.), were employed to determine the statistical significance between groups.

RESULTS

Vaccine-Induced Humoral Responses

Following vaccination, serum and vaginal washes were collected before the intrabursal challenge and probed against *C. trachomatis* EB from the 4 isolates and against *C. trachomatis* serovar D (UW-3/Cx) rMOMP (Table 1, Supplementary Table 1, and Supplementary Figure 2). The IgG geometric mean titers (GMTs) were higher against the homologous than against the heterologous serovars and the lowest titers were against serovar F (N.I.1), the most distantly related serovar. For example, in mice vaccinated with D (UW-3/Cx)-rMOMP, the IgG GMT to EBs from the 4 *C. trachomatis* isolates were: D (UW-3/Cx) GMT, 12 800; D (UCI-N96/Cx) GMT, 6400; E (IOL-43) GMT, 3805; and F (N.I.1) GMT, 1131. Mice vaccinated with serovar D (UW-3/Cx) EBs also had high IgG GMT to D (UW-3/Cx) EB (GMT, 6400).

Mice vaccinated with serovar D (UW-3/Cx) rMOMP had very high IgG GMT against D (UW-3/Cx)-rMOMP (GMT, 819 200) when compared to the control D (UW-3/Cx) EB vaccinated group (GMT, 1600; *P* < .05). The IgG2a/IgG1 ratio was 8 for both groups vaccinated with D (UW-3/Cx) rMOMP or EB.

As shown in Figure 1, mice immunized with serovar D (UW-3/Cx) rMOMP had higher neutralizing GMT against the homologous serovar D (UW-3/Cx) (GMT, 2540) and D (UCI-N96/Cx) (GMT, 2263) than against a heterologous serovar E (IOL-43) (GMT, 800; *P* < .05). Serovar F (N.I.1) was not neutralized (GMT, <50). The positive control immunized with serovar D (UW-3/Cx) EB had a GMT of 317 against the homologous isolate.

Vaginal IgG GMT in mice vaccinated with serovar D (UW-3/Cx) rMOMP were positive only in vaginal washes against EB

Table 1. Serum Antibody Geometric Mean Titers the Day Before Chlamydia trachomatis Challenge Probed Against Serovar D (UW-3/Cx), E (IOL-43), F (N.I.1) EB, and D (UW-3/Cx) rMOMP

Vaccine	D (UW-3/Cx) EB		D (UCI-N96/Cx) EB		E (IOL-43) EB		F (N.I.1) EB		D (UW-3/Cx) rMOMP			
	IgG		IgG		IgG		IgG		IgG		IgG2a	IgG2a/IgG1
D (UW-3/Cx) rMOMP	12 800 ^a (12 800–12 800)	6400 (3200–12 800)	6400 (3200–12 800)	3805 ^b (3200–6400)	819 200 ^a (819 200–819 200)	22 400 ^a (12 800–25 600)	179 200 ^a (102 400–204 800)	8				
PBS	<100	<100	NT	NT	<100	NT	NT	NT	<100	<100	<100	...
D (UW-3/Cx) EB	6400 (6400–6400)	NT	NT	NT	NT	NT	NT	NT	200 (200–200)	1600 (1600–1600)	1600 (1600–1600)	8
MEM	<100	NT	NT	NT	NT	NT	NT	NT	<100	<100	<100	...

Data are GMT (range).

Abbreviations: EB, elementary body; GMT, geometric mean titer; MEM, minimum essential medium eagle; NT, not tested; PBS, phosphate-buffered saline; rMOMP, recombinant major outer membrane protein.

^aP < .05 by Student t test compared to D (UW-3/Cx) EB immunized.

^bP < .05 by Student t test compared to D (UW-3/Cx) rMOMP-immunized group's anti-D(UW-3/Cx) EB GMT.

^cP < .05 by Student t test compared to D (UW-3/Cx) rMOMP immunized group's anti-D(UCI-N96/Cx) EB GMT.

^dP < .05 by Student t test compared to D (UW-3/Cx) rMOMP immunized group's anti-E(IOL-43) EB GMT.

from D (UW-3/Cx) (GMT, 28) and D (UCI-N96/Cx) (GMT, 12) (Supplementary Table 1). Like in serum, a major difference in IgG GMT was found in mice vaccinated with D (UW-3/Cx) rMOMP when tested against D (UW-3/Cx) rMOMP (GMT, 2153) versus mice vaccinated with D (UW-3/Cx) EBs (GMT, 14; $P < .05$). The highest IgA GMT was detected in mice vaccinated with D (UW-3/Cx) rMOMP probed with E (IOL-43) EB (GMT, 13). When probed with D (UW-3/Cx) rMOMP, mice vaccinated with D (UW-3/Cx) rMOMP had an IgA GMT of 80 while those immunized with D (UW-3/Cx) EB had a GMT of 40.

Thus, the results of the ELISA and neutralizing antibody titers in serum correlated with the known immunological and protective relationships among *C. trachomatis* serovars. Antibodies reacted strongly with the homologous serovars and the heterologous serovars of the same complex but reacted weakly with a serovar of another subcomplex.

Cell-Mediated Immune Responses Elicited by Vaccination

To determine *C. trachomatis*-specific T-cell memory responses elicited by vaccination, mice immunized with serovar D (UW-3/Cx) rMOMP were euthanized and their spleens collected the day before the intrabursal challenge. T cells were stimulated with D (UW-3/Cx), D (UCI-N96/Cx), E (IOL-43), or F (N.I.1) EB. Mice immunized with D (UW-3/Cx) rMOMP had a Δ cpm of 4348 when stimulated with D (UW-3/Cx) EB and 4796 when stimulated with D (UCI-N96/Cx) EB ($P < .05$; Table 2). The weakest proliferative responses were against the most distantly related serovar F (N.I.1) (Δ cpm, 2134; $P < .05$). The Δ cpm in control animals immunized with D (UW-3/Cx) EB was 8319. All groups vaccinated with D (UW-3/Cx) rMOMP had significant proliferative T-cell responses when compared to their negative PBS control groups ($P < .05$).

Figure 1. In vitro *Chlamydia trachomatis* neutralization titers. Serum samples collected the day before the genital challenge from mice immunized with D (UW-3/Cx) MOMP or EB were tested for their ability to neutralize in vitro EB from the 4 *C. trachomatis* isolates used to challenge the mice. Abbreviations: EB, elementary body; GMT, geometric mean titer; MOMP, major outer membrane protein. * $P < .05$.

Table 2. T-Cell Proliferative Responses and IFN- γ Production From the Day Before Challenge When Stimulated With Serovar D (UW-3/Cx), D (UCI-N96/Cx), E (IOL-43), and F (N.I.1) EB

	D (UW-3/Cx) EB		D (UCI-N96/Cx) EB		E (IOL-43) EB		F (N.I.1) EB	
Vaccine	Δ cpm ^a	IFN-γ, pg/mL	Δ cpm ^a	IFN-γ, pg/mL	Δ cpm ^a	IFN-γ, pg/mL	Δ cpm ^a	IFN-γ, pg/mL
D (UW-3/Cx) rMOMP	4348 ± 18 ^{b,c,d}	3496 ± 489 ^{b,d}	4796 ± 396 ^{b,c}	3182 ± 634 ^b	3970 ± 560 ^{b,c}	2711 ± 717 ^b	2134 ± 235 ^b	2137 ± 746 ^b
PBS	1029 ± 126	<15	363 ± 133	<15	739 ± 100	<15	856 ± 134	<15
D (UW-3/Cx) EB	8319 ± 1267 ^e	4870 ± 192 ^e	NT	NT	NT	NT	NT	NT
MEM	964 ± 192	<15	NT	NT	NT	NT	NT	NT

Data are mean $\pm$ 1 SE.

Abbreviations: IFN- γ , interferon- γ ; Δ cpm, change in counts per minute; EB, elementary body; MEM, minimum essential medium eagle; NT, not tested; PBS, phosphate-buffered saline; rMOMP, recombinant major outer membrane protein.

^aΔ cpm, difference in counts per minute between EB-stimulated and medium-stimulated T cells. EB were added at a ratio of 0.5:1 to Antigen presenting cells + T cells.

^b*P* < .05 by Student *t* test compared to PBS immunized group.

^c*P* < .05 by Student *t* test compared to D (UW-3/Cx) rMOMP immunized group stimulated with F (N.I.1) EB.

^d $P < .05$ by Student *t* test compared to D (UW-3/Cx) EB immunized group stimulated with D (UW-3/Cx) EB.

^a*P* < .05 by Student *t* test compared to MEM immunized group.

T cells from the serovar D (UW-3/Cx) rMOMP immunized group produced progressively decreasing levels of IFN- γ when stimulated with EB from D (UW-3/Cx), 3496 pg/mL; D (UCI-N96/Cx), 3182 pg/mL; E (IOL-43), 2711 pg/mL, or F (N.I.1), 2137 pg/mL. The differences, however, were not statistically significant ($P > .05$; [Table 2](#)). T cells from the D (UW-3/Cx) EB-immunized group also produced similar levels of IFN- γ when stimulated with EB from D (UW-3/Cx) (4870 pg/mL).

These results demonstrate that cell-mediated immune responses were more robust against the homologous than against distantly related serovars.

C. trachomatis Vaginal Cultures

Four weeks after the last immunization, mice were challenged in the left ovarian bursa with various dosages (10^3 , 10^4 , or 10^5 IFUs) of 4 different *C. trachomatis* serovars. Protection against

Figure 2. *Chlamydia trachomatis* vaginal cultures over the 6 weeks of testing. *A*, Percentage of mice with positive vaginal cultures. *B*, Percentage of positive vaginal cultures in the 6 weeks of testing. ^a*P* < .05 by Fisher exact test compared to own PBS control. ^b*P* < .1 by Fisher exact test compared to own PBS control. Abbreviations: EB, elementary body; IFU, inclusion forming unit; MEM, minimum essential medium eagle; PBS, phosphate-buffered saline; rMOMP, recombinant major outer membrane protein.

the intrabursal challenge was evaluated based on the number of mice with positive vaginal cultures, number of positive cultures, number of IFU recovered, and mean number of days to clearance (Figure 2, Figure 3, Supplementary Figure 3, and Supplementary Table 2).

The best protected groups were those challenged with the homologous or the heterologous serovars from the same complex. For example, over the 6-week period, of the mice challenged with 10^3 IFU of D (UW-3/Cx), only 19% (3/16) of the D (UW-3/Cx) rMOMP-vaccinated mice shed compared to 93% (14/15) of the PBS-immunized group ($P < .05$; Figure 2A and Supplementary Table 2). When challenged with 10^3 IFUs of D (UCI-N96/Cx), 13% (2/16) of the rMOMP-vaccinated mice were culture positive versus 50% (8/16) of the controls, approaching statistical significance ($P = .057$). In contrast, of the mice challenged with 10^3 IFUs of F (N.I.1.), 80% (12/15) of the rMOMP-vaccinated mice were culture positive versus 81% (13/16) of the controls ($P > .05$). Mice immunized intranasally with 10^4 IFU of D (UW-3/Cx) were solidly protected against a challenge with the homologous serovar (0% positive, 0/12). All MEM control mice had positive cultures (100%, 15/15).

From mice vaccinated with D (UW-3/Cx) rMOMP and challenged with 10^3 IFUs of D (UW-3/Cx), *C. trachomatis* was recovered from 3% (4/128) of all the vaginal cultures collected, compared to 40% (48/120) of the PBS-immunized group ($P < .05$) (Figure 2B and Supplementary Table 2). When challenged with 10^3 or 10^4 IFUs of D (UCI-N96/Cx), 2% (2/128) and 9% (12/128), respectively, of the cultures were positive in the D (UW-3/Cx) rMOMP-vaccinated groups versus 10% (13/128) and 20% (24/120) in the PBS-immunized mice, respectively ($P < .05$). Protection against a challenge with 10^5 IFUs of E (IOL-43) was also elicited by vaccination with D (UW-3/Cx) rMOMP when compared with the PBS controls (6% [8/128] positive cultures versus 15% [19/128], respectively; $P < .05$). No protection was achieved against a challenge with 10^3 or 10^4 IFUs of *C. trachomatis* serovar F (N.I.1.).

The total number of IFUs recovered per mouse during the 6 weeks was significantly lower for mice vaccinated with serovar D (UW-3/Cx) rMOMP and challenged with 10^3 IFU of D (UW-3/Cx), 10^3 IFUs of D (UCI-N96/Cx), or with 10^5 IFUs of E (IOL-43) when compared to the PBS controls: median 0 (range, 0–836) versus 3866 (range, 0–48 946); median 0 (range, 0–2) versus 3 (range, 0–3267); and median 0 (range, 0–39 847) versus 0 (range, 0–62 997), respectively ($P < .05$; Figure 3A and Supplementary Table 2). None of the other vaccinated groups had significant decreases in the total number of IFU shed. The controls vaccinated with EB had no positive vaginal cultures while the PBS control shed median 3273 (range, 688–26 510) IFU/mouse.

The length of time of shedding was calculated from the time of challenge until the first negative vaginal culture (first day of culture: day 7 postchallenge) (Figure 3B

and Supplementary Table 2). This parameter was significantly shorter in the D (UW-3/Cx) rMOMP-immunized mice versus PBS controls when challenged with 10^3 IFU D (UW-3/Cx) (10 days $\pm$ 1 vs 29 days $\pm$ 3), 10^3 IFU D (UCI-N96/Cx) (9 days $\pm$ 2 vs 23 days $\pm$ 4), or with 10^5 IFU E (IOL-43) (10 days $\pm$ 2 vs 22 days $\pm$ 4) ($P < .05$). In addition, the decrease in the length of shedding approached statistical significance between immunized and control mice in the 10^4 IFU E (IOL-43) challenged pair (9 days $\pm$ 1 versus 15 days $\pm$ 3; $P < .1$). No protection was observed in the 2 groups challenged with *C. trachomatis* serovar F (N.I.1.). The EB-immunized group shed for a shorter amount of time than the MEM group (7 days $\pm$ 0 versus 35 days $\pm$ 2; $P < .05$).

In summary, based on the number of mice with positive vaginal cultures, number of positive vaginal cultures, number of IFU recovered, and days to clearance, *C. trachomatis* rMOMP elicited protective immune responses against genital challenges with various dosages of serovars of the same complex but not against a serovar from a different subcomplex.

Fertility Studies

To determine the ability of serovar D (UW-3/Cx) rMOMP to protect against upper genital tract pathology and infertility, vaccinated mice challenged in the left ovarian bursa were caged with males and the outcome of the mating evaluated (Table 3). Based on the number of fertile mice, embryos in the left uterine horn, and hydrosalpinges, mice challenged with 10^3 IFU of D (UW-3/Cx), 10^3 or 10^4 IFU of D (UCI-N96/Cx), or 10^4 IFU of E (IOL-43) were protected. For example, mice vaccinated with D (UW-3/Cx) rMOMP were protected against challenges with 10^3 IFU of D (UW-3/Cx) (38% [6/16] vs 7% [1/15]; $P = .08$), 10^3 IFU of D (UCI-N96/Cx) (56% [9/16] vs 6% [1/16]), and 10^4 IFU of E (IOL-43) (63% [10/16] vs 20% [3/15]) when compared to their respective PBS controls ($P < .05$). Serovar D (UW-3/Cx) rMOMP-immunized groups challenged with higher doses were protected but the differences were not significant: 10^4 IFU of D (UCI-N96/Cx) (31% [5/16] vs 7% [1/15]) or 10^5 IFU of E (IOL-43) (38% [6/16] vs 13% [2/16]). None of the groups challenged with serovar F (N.I.1.) were protected against infertility. Mice immunized with D (UW-3/Cx) EB had fertility rates similar to the fertility group ($P > .05$). No protection was observed in the MEM group.

The number of embryos in the left (challenged side) and the right (control) uterine horns were counted. As determined by the number of embryos in the left uterine horn, mice vaccinated with D (UW-3/Cx) rMOMP were protected against challenges with 10^3 IFU of D (UW-3/Cx) (1.2 ± 0.5 vs 0.2 ± 0.2), 10^3 IFU of D (UCI-N96/Cx) (2.3 ± 0.6 vs 0.2 ± 0.2), and 10^4 IFU of E (IOL-43) (2.2 ± 0.5 vs 0.4 ± 0.2) ($P < .05$) and approached significance when challenged with 10^4 IFU of D (UCI-N96/Cx) (1.1 ± 0.5 vs 0.1 ± 0.1) ($P = .06$). No protection was obtained against challenges with serovar F (N.I.1.).

Although mice were challenged in the left uterine horn, groups infected with 10^3 IFU of D (UW-3/Cx) and 10^4 IFU of D (UCI-N96/Cx) had significant decreases in number of embryos in the right uterine horn indicating that *C. trachomatis* migrated to that side. These 2 groups of mice were also protected when the total number of embryos in both uterine horns were analyzed following challenge with 10^3 IFU of D (UW-3/Cx) (4.2 ± 0.9 vs 0.7 ± 0.5) and 10^4 IFU of D (UCI-N96/Cx) (4.1 ± 0.9 vs. 0.7 ± 0.5) ($P < .05$). The number of embryos in these groups was not significantly different from mice vaccinated with D (UW-3/Cx) EB or the fertility control group ($P > .05$). Mice challenged with 10^4 IFU of F (N.I.1) also had decreases in the number of embryos in the right uterine horn but vaccination with D (UW-3/Cx) rMOMP was not protective.

[10/32]) ($P = .06$). Mice immunized with D (UW-3/Cx) EB and the fertility control group had no hydrosalpinx.

DISCUSSION

Table 3. Fertility of Mice Immunized With *Chlamydia trachomatis* Serovar D (UW-3/Cx) rMOMP or Controls and Challenged With *C. trachomatis* Serovars

Vaccine	Challenge		No. Fertile Mice ^a / Total No. Mice (%)	No. Embryos in Uterine Horn, Mean ± SD			Total No. Hydrosalpinges/ No. Uterine Horns (%)
	Serovar	IFU/Mouse		Left ^b	Right	Both	
D (UW-3/Cx) rMOMP	D (UW-3/Cx)	10 ³	6/16 ^{c,d} (38)	1.2 ± 0.5 ^g	3.0 ± 0 ^g	4.2 ± 0.9 ^g	2/32 ^e (6)
PBS	D (UW-3/Cx)	10 ³	1/15 (7)	0.2 ± 0.2	0.5 ± 0.3	0.7 ± 0.5	22/30 (73)
D (UW-3/Cx) rMOMP	D (UCI-N96/Cx)	10 ³	9/16 ^e (56)	2.3 ± 0.6 ^g	2.4 ± 0.5	4.7 ± 0.9	2/32 (6)
PBS	D (UCI-N96/Cx)	10 ³	1/16 (6)	0.2 ± 0.2	2.9 ± 0.6	3.1 ± 0.7	6/32 (19)
D (UW-3/Cx) rMOMP	D (UCI-N96/Cx)	10 ⁴	5/16 ^f (31)	1.1 ± 0.5 ^h	3.0 ± 0.6 ^g	4.1 ± 0.9 ^g	4/32 ^e (13)
PBS	D (UCI-N96/Cx)	10 ⁴	1/15 (7)	0.1 ± 0.1	0.5 ± 0.5	0.7 ± 0.5	13/30 (43)
D (UW-3/Cx) rMOMP	E (IOL-43)	10 ⁴	10/16 ^e (63)	2.2 ± 0.5 ^g	3.4 ± 0.7	5.7 ± 1.1	2/32 ^e (6)
PBS	E (IOL-43)	10 ⁴	3/15 (20)	0.4 ± 0.2	3.5 ± 0.5	3.9 ± 0.6	9/30 (30)
D (UW-3/Cx) rMOMP	E (IOL-43)	10 ⁵	6/16 ^d (38)	1.2 ± 0.4	1.1 ± 0.4	2.5 ± 0.8	3/32 ^e (9)
PBS	E (IOL-43)	10 ⁵	2/16 (13)	0.3 ± 0.2	1.9 ± 0.6	2.2 ± 0.7	10/32 (31)
D (UW-3/Cx) rMOMP	F (N.I.1)	10 ³	3/15 ^{f,i} (20)	0.6 ± 0.4 ⁱ	2.3 ± 0.7	2.9 ± 0.9 ⁱ	5/30 ^d (17)
PBS	F (N.I.1)	10 ³	0/16 (0)	0.0 ± 0.0	2.8 ± 0.6	2.8 ± 0.6	11/32 (34)
D (UW-3/Cx) rMOMP	F (N.I.1)	10 ⁴	0/16 ^f (0)	0.0 ± 0.0 ⁱ	0.9 ± 0.5 ⁱ	0.9 ± 0.5 ⁱ	4/32 (13)
PBS	F (N.I.1)	10 ⁴	0/16 (0)	0.0 ± 0.0	1.5 ± 0.6	1.5 ± 0.6	8/32 (25)
D (UW-3/Cx) EB	D (UW-3/Cx)	10 ³	9/12 (75)	2.0 ± 0.6	3.1 ± 0.5	5.1 ± 1.0	0/24 (0)
MEM	D (UW-3/Cx)	10 ³	0/15 (0)	0.0 ± 0.0	0.9 ± 0.4	0.9 ± 0.4	18/30 (60)
Fertility control	...	...	11/15 (73)	2.6 ± 0.7	2.3 ± 0.5	4.9 ± 1.0	0/30 (0)

Abbreviations: IFU, inclusion forming units; MEM, minimum essential medium eagle; PBS, phosphate-buffered saline; rMOMP, recombinant major outer membrane protein.

^aFertility was defined as at least 1 embryo present in the any uterine horn.

^bMice were challenged in the left ovarian bursa.

^c $P < .1$ by Fisher exact test compared to PBS immunized group challenged with the same serovar.

^d $P < .1$ by Fisher exact test compared to fertility control.

^e $P < .05$ by Fisher exact test compared to PBS immunized group challenged with the same serovar.

^f $P < .05$ by Fisher exact test compared to fertility control.

^g $P < .05$ by Student t test compared to PBS immunized group challenged with the same serovar.

^h $P < .1$ by Student t test compared to PBS immunized group challenged with the same serovar.

ⁱ $P < .05$ by Student t test compared to fertility control.

^jD(UW-3/Cx) rMOMP immunized and challenged with F(N.I.1) 10³ group lost 1 mouse during first mating.

mice were significantly protected against serovars of the same immunocomplex but not from a different subcomplex. This is the first time that cross-serogroup protection against infection, vaginal shedding, upper genital tract pathology, and infertility has been achieved with a subunit *C. trachomatis* vaccine.

Attempts to elicit cross-serovar *C. trachomatis* protection in mice are limited. This is in part due to the lack of long-term sequelae, specifically tubal pathology and infertility, induced by *C. trachomatis* in mice following a vaginal challenge [32, 34]. Yang et al [35] showed that mice pretreated with MPA and infected vaginally with 10⁵ IFU of serovar D (UW-3/Cx; D-LC) have a short low-burden of infection, with minimal inflammatory responses and no seroconversion. Tuffrey et al [36] were the first to induce infertility by pretreating mice with MPA and challenging them in the ovarian bursae with *C. trachomatis* serovar F (N.I.1). Tuffrey et al [37] were also the first to attempt to elicit cross-serovar protection in mice with a subunit vaccine. In C3H mice immunized with a truncated rMOMP from *C. trachomatis* serovar L1 and challenged in the uterine horn with serovar F (N.I.1), there was a reduction in salpingitis and vaginal shedding but no protection against infertility. Olsen et al [38, 39] constructed a chimeric antigen (Hirep1) using the variable domain 4 (VD4) of MOMP and its surrounding constant

domains (CD) from *C. trachomatis* serovars D, E, and F. C3H/HeN mice immunized with Hirep1, using the adjuvant CAF01, were protected against vaginal challenges with *C. trachomatis* serovars D, E, or F, as determined by the number of IFUs in vaginal swabs and inflammatory responses in the upper genital tract. However, no protection against hydrosalpinx or infertility was reported.

A difficulty when characterizing *C. trachomatis* cross-serovar protection is that the in vitro infectivity and the in vivo virulence of a serovar could be different [28]. Even more, within the same serovar different isolates may have various levels of virulence [28]. For example, 10³ IFU of *C. trachomatis* serovar D isolate X versus isolate Y may or may not result in the same rate of infection, shedding, upper genital tract pathology, and/or infertility. This requires careful planning of the experiments and interpretation of results. To address this issue, and the lack of pathogenicity of the *C. trachomatis* serovars following a vaginal infection, we tested the ability of several *C. trachomatis* isolates to induce upper genital tract pathology and infertility following an intrabursal challenge in C3H/HeN mice [28]. We were particularly interested in identifying isolates from serovars D, E, and F because they are the most frequent serovars causing human genital infections [40]. Based on those results, here, we used

C. trachomatis isolates and challenge doses that induced at least 80% infertility in naive C3H/HeN mice [28]. High, rather than low, infectious doses in humans, like in mice, result in upper genital tract pathology [41, 42]. In most human infections, the number of *C. trachomatis* IFU transmitted is lower than some of the challenge dosages tested here and therefore, likely, a rMOMP vaccine will be protective for most exposed patients [42–44].

One limitation of this study is the treatment of mice with MPA before the intrabursal challenge. Tuffrey and Taylor-Robinson [45] demonstrated the need for pretreating mice with MPA for a *C. trachomatis* infection to elicit upper genital tract pathology. They postulated that by blocking the estrus cycle in diestrus MPA enhances the local infection. The major effect of MPA is likely a switch from Th1 to Th2 immune responses [46–49]. Protection against a chlamydial infection depends on CD4⁺ Th1 cells. Therefore, switching the immune response to Th2 before the challenge counteracts vaccine-induced protection.

The need to challenge mice in the ovarian bursa, or in the uterus, to induce upper genital tract pathology with the *C. trachomatis* serovars is also a limitation. However, it could be argued that direct inoculation in the ovarian bursa may be a more strictive approach for testing the protective effect of a vaccine than a vaginal challenge because *C. trachomatis* is directly inoculated at the site where upper genital tract pathology occurs [29]. Nevertheless, we realize that immune responses in the lower and upper genital tract of mice are different and this may affect protection depending on site of challenge [50].

In conclusion, immunization with *C. trachomatis* serovar D rMOMP protects mice against an intrabursal challenge with the homologous serovar, a different strain of same serovar, and a heterologous serovar from the same complex. No protection was observed against a serovar from a different subcomplex. Thus, a polyvalent vaccine formulated with rMOMP from the senior *C. trachomatis* serovar of each immunocomplex should elicit broad cross-serovar protection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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