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Immunization with the *Chlamydia trachomatis* major outer membrane protein, using adjuvants developed for human vaccines, can induce partial protection in a mouse model against a genital challenge

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Abstract

To test several vaccines for *Chlamydia trachomatis* we vaccinated BALB/c and C3H/HeN female mice with a purified preparation of the native mouse pneumonitis (MoPn) major outer membrane protein (MOMP). The MOMP was formulated with anyone of three different adjuvants MF59, LT-K63 or LT-R72. As a negative control the animals were immunized with ovalbumin. Positive controls were inoculated intranasally (i.n.) with 10^4 inclusion-forming units (IFU) of *C. trachomatis* MoPn. High levels of *Chlamydia*-specific antibodies were detected in the serum and vaginal washes of the mice immunized with MOMP. Using a lymphoproliferative assay (LPA) a significant response was obtained in splenocytes from most of the groups of mice vaccinated with MOMP. Two weeks after the last immunization the mice were challenged in the left ovarian bursa with 10^5 IFU of *C. trachomatis* MoPn and vaginal cultures were collected for a period of 6 weeks. Overall, BALB/c and C3H/HeN mice immunized with MOMP showed a decrease in the severity and length of the infection but the difference with the controls was not statistically significant. Following mating the percentage of mice with bilateral fertility was not significantly different between mice vaccinated with MOMP and their respective ovalbumin-immunized controls. However, the C3H/HeN mice immunized with MOMP using MF59 or LTR72 as adjuvants had significantly more embryos per mouse than the control groups. In conclusion, mice immunized with native MOMP and adjuvants developed for human vaccines showed significant *Chlamydia*-specific immune response and a limited protection against infection and long-term sequelae.

Keywords: *Chlamydia trachomatis*; Vaccines; Adjuvants

1. Introduction

Chlamydia trachomatis produces pelvic inflammatory disease (PID) that can lead to long-term sequelae including chronic abdominal pain, ectopic pregnancy and infertility [1–6]. Infertility is a major problem in the Western World and several studies have assessed the role of *C. trachomatis* in this disease. For example, Westrom et al. [5] followed a cohort of women with PID proven by laparoscopy. Tubal occlusion was found in 12.8% of women who had a single episode of PID and in 75% of individuals who had three or more attacks of PID. Women with confirmed PID developed infertility in almost 20% of the cases, chronic pelvic pain in 18%, and ectopic pregnancy in 9%.

Although chlamydial infections can be treated with antibiotics, over 50% of the genital infections in females are asymptomatic [2,3,6]. Furthermore, unless therapy is implemented in a timely fashion, adverse outcomes can result [6]. Therefore, vaccines are the only practical way to effectively protect against these infections and their long-term sequelae [7–11]. Among the potential immunogens the major outer membrane protein (MOMP) of *C. trachomatis* has been considered as the most likely candidate for a vaccine [12]. Clinical and experimental evidence indicates that the MOMP can induce

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strong humoral and cell mediated immune (CMI) responses in humans and in animal models.

Attempts to vaccinate with preparations of the MOMP have so far yielded for the most part negative results. Following vaccination the animals mounted a strong immune response but protection against a chlamydial genital challenge was limited [13,14]. Recently, an immunization protocol utilizing a preparation of the MOMP obtained by direct extraction of this protein from the organism was described [15]. This vaccine afforded significant protection in a mouse model against a genital challenge [15]. However, the vaccine was formulated with Freund's adjuvant. Unfortunately, due to it's side effects, Freund's adjuvant cannot be utilized in humans. For this reason we decided to test the natural preparation of the *C. trachomatis* MOMP in conjunction with adjuvants that are currently used in humans or have been developed for human use. Here, we show that this type of immunization can elicit significant humoral and cell-mediated immune responses and can result in partial protection against the long-term sequeale of a chlamydial genital infection.

2. Materials and methods

2.1. Organisms

The *C. trachomatis* MoPn strain Nigg II was obtained from the American Type Culture Collection (ATCC; Manassas, VA) [16]. The organism was grown in HeLa-229 cells with Eagle's minimal essential medium supplemented with 10% fetal bovine serum [17]. Elementary bodies (EB) were purified using Hypaque-76 (Nycomed Inc., Princeton, New Jersey) and stored in 0.2 M sucrose, 0.020 M sodium phosphate (pH 7.2) and 0.005 M glutamic acid (SPG) [17].

2.2. Purification of the C. trachomatis MoPn MOMP

To purify the MOMP the *C. trachomatis* MoPn was grown in HeLa-229 cells washed with PBS pH 7.4, centrifuged, and the pellet treated with DNAse as previously described [15]. After centrifugation the pellet was resuspended in 0.2 M phosphate buffer pH 5.5, containing 0.1 M DTT, and 0.001 M each of EDTA and phenylmethylsulfonyl fluoride (PMSF). MOMP was extracted using (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS; Anatrace Inc. Maumee OH), and subsequently Anzerget 3–14 (Z3–14; Anatrace Inc.) as previously described [15]. The MOMP was further purified using a hydroxyapatite column [17]. The purified 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 [15]. The preparation was concentrated, fixed with 2% glutaraldehyde for 2 min. at room temperature and subsequently, 2 M glycine was added to halt the reaction. The MOMP was concentrated using Centricon-10 filters and dialyzed against 0.02 M phosphate buffer pH 7.4, 0.15 M NaCl and 0.05% Z3–14 before inoculation [15].

2.3. Animal immunization and challenge

Seven- to eight-week-old female BALB/c (H-2^d) and C3H/HeN (H-2^k) mice were purchased from Charles River Laboratory (Wilmington, MA). Animals received a total of 20 μg of the *C. trachomatis* MoPn MOMP, or 20 μg ovalbumin (Sigma–Aldrich, St. Louis, MO) per mouse per immunization. The mice were vaccinated simultaneously by the intramuscular (10 μg of MOMP or ovalbumin), subcutaneous (5 μg of MOMP or ovalbumin) and intranasal (5 μg of MOMP or ovalbumin) routes (i.m. + s.c. + i.n.). Using a manual vortex, MOMP and ovalbumin were emulsified in 25 μl of MF59, 5 μg of LT-K63 or 5 μg of LT-R72 (the adjuvants were a gift from Chiron Vaccines, Siena and Marburg). The animals were immunized three times at 14 day intervals each time following the same protocol. Two weeks after the last boost the mice were challenged in the left ovarian bursa with 10^5 IFU of *C. trachomatis* MoPn in 20 μl of SPG while the right ovarian bursa was mock infected with HeLa cell extracts.

Control BALB/c mice were immunized i.n. with 10^4 inclusion forming units (IFU)/mouse of *C. trachomatis* MoPn, while C3H/HeN control mice received 10 IFU/mouse [15]. The number of IFU used for this inoculation was approximately 10-fold lower than the lethal dose (LD_{50}) for each strain of mice (unpublished data). Fertility control groups for both strains of mice were also included in the study. These animals were not immunized or challenged and were simply mated in parallel with the other groups. All protocols were approved by, the University of California, Irvine, Animal Care and Use Committee. All the experiments were repeated twice.

2.4. Detection of antibodies

Blood samples were collected from the orbital plexus on a weekly basis. Genital samples were collected by washing the vagina twice with 20 μl of PBS. Chlamydial specific antibodies were measured using an enzyme linked-immunosorbent assay (ELISA) [15]. Flat bottom 96-well plates were coated with *C. trachomatis* EB at a concentration of 10 μg per ml. A 1:2000 dilution of goat anti-mouse IgM, IgA, IgG (Cappel, Aurora, OH) and a 1:3000 dilution of goat anti-mouse IgGl, IgG2a, IgG2b and IgG3 (Southern Biotechnology Associates, Birmingham, AL) were used to determine subclass or isotype specific antibodies. ELISA titers were defined as the highest dilution of sera giving an absorbance (A_{405}) which was at least two-fold over that obtained with non-immune sera.

The ability of serum to neutralize in vitro the infectivity of *C. trachomatis* MoPn EB was determined as follows [18]. *C. trachomatis* MoPn (10^4 IFU) were added to two-fold serial dilutions of the serum made with 5% guinea pig

sera in Ca^{2+}, Mg^{2+} free PBS. After incubation at 37 °C for 45 min, the mixture was used to inoculate HeLa-229 cells by centrifugation. The cells were fixed at 30 h after infection with methanol, stained as described before, and the number of IFU were counted. Neutralization was defined as ≥50% inhibition of the number of IFU using as a control the sera from the animals inoculated with ovalbumin.

For immunoblotting *C. trachomatis* MoPn EB were resolved in 10% tricine-SDS-polyacrylamide gel electrophoresis [15]. Approximately, 250 μg of purified EB were loaded on a 7.5-cm wide slab gel. Following transfer to nitrocellulose membranes, the nonspecific sites were blocked with BLOTTO (Bovine Lacto Transfer Technique Optimizer: 5% (w/v) nonfat dried milk, 2 mM $CaCl_2$, and 50 mM Tris–HCl, pH 8.0), the serum samples diluted and incubated overnight at 4 °C. Antibody binding was detected using horseradish peroxidase-conjugated goat anti-mouse antibody developed with 0.01% hydrogen peroxide and 4-chloro-l-naphthol. Monoclonal antibody 40 (mAb 40) to the *C. trachomatis* MoPn MOMP was used as a control.

2.5. Lymphocyte proliferation assay

A T-cell lymphoproliferative assay (LPA) was performed using splenocytes as previously described [15]. Briefly, T-enriched cells were counted and 10^5 cells were aliquoted per well. UV inactivated *C. trachomatis* MoPn EB were added at a concentration of 10 EB to 1 antigen presenting cell (APC) which were prepared by irradiating splenocytes with 3300 rad. Negative control wells received medium alone, whereas the positive control wells received concanavalin A at a concentration of 5 μg/ml. Cell proliferation was measured by addition of 1 μCi of (methyl ^{3}H)thymidine per well. The mean count was obtained from triplicate cultures.

2.6. Genital challenge

Two weeks after the last immunization the mice were anaesthetized with xylazine and ketamine, a lateral abdominal incision was made, and 10^5 IFU of *C. trachomatis* MoPn were inoculated into the left ovarian bursa [15]. The right ovarian bursa was inoculated with mock-infected HeLa-229 cell extracts.

2.7. Genital cultures

Vaginal swabs for *C. trachomatis* MoPn were collected at weekly intervals following the genital challenge [15]. The swabs were vortex in 200 μl of SPG and two aliquots from each specimen (100 and 10 μl) were inoculated in McCoy cells grown in 48-well plates. The plates were centrifuged at 1000 × *g* for 1 h at room temperature. The cultures were incubated at 37 °C for 30 h when the inclusions were stained with a rabbit polyclonal anti-C. *trachomatis* MoPn serum prepared in our laboratory.

2.8. Fertility experiments

Six weeks after the genital challenge the female mice were caged with male breeder mice [15]. Starting at 10-day post-mating the female mice were weighed daily. Animals that gained weight were euthanized and the number of embryos was counted. Mice that did not gain weight were mated a second time with a different male mouse and the outcome of the mating was evaluated as indicated above.

2.9. Statistics

The two-tailed unpaired Student's *t*-test, the Fisher's exact test and the Mann–Whitney *U*-test were employed to determine the significance of differences between the groups using the Statview software program on a Macintosh computer (Apple Co, Cupertino, CA).

3. Results

3.1. Humoral immune response following vaccination

The BALB/c mice vaccinated with the *C. trachomatis* MoPn MOMP, plus anyone of the three adjuvants MF59, LT-K63 or LT-R72, had high chlamydial IgG titers in serum and a balanced Th1/Th2 response as determined by the levels of IgG2a and IgGl (Table 1A). In these three groups the ratio of IgG2a to IgGl was 0.5. On the other hand, BALB/c mice immunized i.n. with *C. trachomatis* EB had a predominant Th1 response as shown by the IgG2a/IgGl ratio of 16 (51,200/3200). *Chlamydia* specific IgA antibodies were detected in the serum of these groups of mice and were particularly high in the animals immunized i.n. with EB. Animals immunized with ovalbumin as a control had no *Chlamydia*-specific antibodies in serum.

Low titers of IgG and IgA *Chlamydia*-specific antibodies were detected in the vaginal washes of the BALB/c mice vaccinated with the MOMP and the three different adjuvants. In the mice immunized with EB, the titers of IgG and IgA in the vaginal washes where 320 and 1280. The neutralizing titer in serum of the mice vaccinated with MOMP and MF59, as well as in the control group immunized i.n. with EB was 1600 while lower or negative titers were determined in the other groups.

C3H/HeN mice vaccinated with MOMP using either one of the three adjuvants MF59, LT-K63 or LT-R72 had a high IgG antibody titer to *Chlamydia* in serum the day prior to the genital challenge (Table 1B). Similar titers of IgG2a and IgGl were observed in the three groups of mice indicating that the immunization resulted a balanced Th1/Th2 response. C3H/HeN mice immunized i.n. with *C. trachomatis* EB had a serum IgG titer of 51,200. These animals had a strong Th1 response as shown by an IgG2a/IgGl ratio of 8 (25,600/3200).

Table 1A
BALB/c mice: antibody response the day before the genital challenge

Mice immunized with	Adjuvant	*C. trachomatis* MoPn-specific ELISA antibody titer									Serum neutralizing titer
		Serum							Vaginal wash		
		IgM	IgG	IgGl	IgG2a	IgG2b	IgG3	IgA	IgA	IgG	
MOMP	MF59	400	12,800	12,800	6,400	6,400	12,800	800	80	20	1,600
Ovalbumin	MF59	<100	<100	<100	<100	<100	<100	<100	<10	<10	<25
MOMP	LT-K63	200	6,400	6,400	3,200	3,200	12,800	800	40	160	100
Ovalbumin	LT-K63	<100	<100	<100	<100	<100	<100	<100	<10	<10	<25
MOMP	LT-R72	400	12,800	12,800	6,400	6,400	6,400	400	40	40	<25
Ovalbumin	LT-R72	<100	<100	<100	<100	<100	<100	<100	<10	<10	<25
CT-MoPn	–	100	12,800	3,200	51,200	12,800	12,800	6,400	1,280	320	1,600

In the three groups immunized with MOMP and those inoculated i.n. with EB, the *Chlamydia* specific IgA antibody titers in serum were found to be high (1600 and 3200). The three controls groups of mice immunized with ovalbumin had negative *Chlamydia*-specific antibody titers.

Low titers of IgG and IgA to *C. trachomatis* MoPn were observed in the vaginal washes of the C3H/HeN mice vaccinated with MOMP (Table 1B). Control mice immunized i.n. with *C. trachomatis* MoPn EB had a 160 IgA antibody titer in the vaginal washes. No *Chlamydia*-specific antibodies were detected in the vaginal washes of the mice immunized with ovalbumin. The titers of neutralizing antibodies present in the C3H/HeN mice vaccinated with MOMP and either one of the three adjuvants were low or negative. Control C3H/HeN mice immunized i.n. with *C. trachomatis* MoPn EB had a neutralizing titer of 400.

Immunoblot analyses of the serum samples from the BALB/c and C3H/HeN mice vaccinated with *C. trachomatis* MoPn MOMP and either one of the three adjuvants demonstrated that the animals had developed antibodies to the MOMP (Fig. 1). Both strains of mice immunized i.n. with *C. trachomatis* EB developed antibodies against several chlamydial antigens, predominantly to proteins in the 75–100 kDa range, the 60 kDa cysteine rich protein, the 60 kDa heat shock protein, MOMP, and the 28 kDa protein. No antibodies against any chlamydial component were detected in the serum of the mice immunized with ovalbumin.

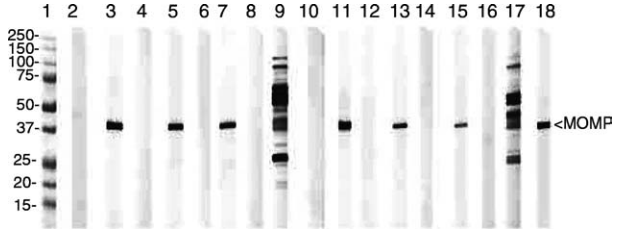

Fig. 1. Western blot of serum from vaccinated animals reacted with *C. trachomatis* MoPn EB. Lane 1: molecular weight markers. Lanes 2–8: sera from BALB/c mice. Lanes 10–16: sera from C3H/HeN mice. Lanes 2 and 10: pre-immunization sera. Lanes 3 and 11: sera from mice vaccinated with MOMP + MF59. Lanes 4 and 12: control sera from mice immunized with ovalbumin and MF59. Lanes 5 and 13: sera from mice vaccinated with MOMP and LT-K63. Lanes 6 and 14: control sera from immunized with ovalbumin and LT-K63. Lanes 7 and 15: sera from mice vaccinated with MOMP and LT-R72. Lanes 8 and 16: control sera from mice immunized with ovalbumin and LT-R72. Lanes 9 and 17: sera from mice immunized with EB. Lane 18: control monoclonal antibody to MOMP. Arrowhead: MOMP.

3.2. T-cell responses following vaccination

Spleens were harvested from immunized mice the day before the genital challenge and the preparations enriched for T cells were tested by a lymphoproliferative assay. As shown in Table 2 strong proliferative responses to EB were observed in the BALB/c vaccinated with MOMP using MF59, LT-K63 or LT-R72 as adjuvants. BALB/c mice immunized i.n. with *C. trachomatis* had also strong proliferative responses to EB.

Table 1B
C3H/HeN mice: antibody response the day before the genital challenge

Mice immunized with	Adjuvant	*C. trachomatis* MoPn-specific ELISA antibody titer									Serum neutralizing titer
		Serum							Vaginal wash		
		IgM	IgG	IgGl	IgG2a	IgG2b	IgG3	IgA	IgA	IgG	
MOMP	MF59	800	25,600	12,800	6,400	3,200	1,600	1,600	10	10	<25
Ovalbumin	MF59	<100	<100	<100	<100	<100	<100	<100	<10	<10	<25
MOMP	LT-K63	400	12,800	12,800	25,600	6,400	800	3,200	10	20	100
Ovalbumin	LT-K63	<100	<100	<100	<100	<100	<100	<100	<10	<10	<25
MOMP	LT-R72	200	51,200	12,800	12,800	25,600	1,600	1,600	<10	10	<25
Ovalbumin	LT-R72	<100	<100	<100	<100	<100	<100	<100	<10	<10	<25
CT-MoPn	–	400	51,200	3,200	25,600	12,800	3,200	3,200	160	10	400

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

Mice immunized with	Adjuvant	T-cell proliferation to (cpm × 10^3 ± 1S.D.)					
		BALB/c mice			C3H/HeN mice		
		EB[b]	Con A[c]	Medium	EB[b]	Con A[c]	Medium
MOMP	MF59	8.6 ± 3.1[d]	49.1 ± 10.7	0.3 ± 0.3	2.0 ± 0.8[d]	51.9 ± 7.5	0.3 ± 0.1
Ovalbumin	MF59	1.7 ± 0.3	33.1 ± 4.2	0.7 ± 0.4	0.3 ± 0.1	118.8 ± 14.8	0.1 ± 0.1
MOMP	LT-K63	8.3 ± 8.3[d]	34.1 ± 7.7	0.2 ± 0.1	1.0 ± 0.2[d]	74.8 ± 3.2	0.3 ± 0.1
Ovalbumin	LT-K63	1.7 ± 1.3	36.3 ± 3	0.2 ± 0.2	0.4 ± 0.1	118.3 ± 7.8	0.1 ± 0.1
MOMP	LT-R72	17.8 ± 1.8[d]	36.6 ± 4.2	0.2 ± .l	l.l ± 0.2	112.9 ± 11.3	0.1 ± 0.1
Ovalbumin	LT-R72	2.9 ± 2.7	38.6 ± 5.7	0.7 ± 0.7	l.l ± 0.4	76.7 ± 17.7	0.4 ± 0.1
CT-MoPn	–	17.2 ± 2.1[e]	57.3 ± 21.3	0.1 ± 0.1	29.2 ± 3.5[e]	69.2 ± 8.7	0.5 ± 0.2

[a] Results are means for triplicate cultures (±1 standard deviation). Data correspond to one of the experiments representatives of duplicate separate experiments.
[b] UV-inactivated *C. trachomatis* MoPn EB were added at a 10:1 ratio to the T cells.
[c] Concanavalin A (Con A) was added at concentration of 5 μg/ml.
[d] $P < 0.05$ by the Student's *t*-test compared to the corresponding group immunized with ovalbumin.
[e] $P < 0.05$ by the Student's *t*-test compared to the ovalbumin + MF59 immunized group.

C3H/HeN mice vaccinated with MOMP and MF59 or LT-K63 also showed significant *Chlamydia*-specific proliferative responses although weaker relative to the BALB/c mice. The C3H/HeN group immunized i.n. with EB had a strong proliferative response. Proliferative responses to concavalin A, used as a positive non-specific stimulant, and medium, used as the negative control, were similar among all groups of mice.

3.3. Vaginal cultures following the genital challenge

The results of the vaginal cultures obtained in the BALB/c mice following the genital challenge with *C. trachomatis* MoPn are shown in Table 3A. Overall, animals vaccinated with MOMP and one of the three adjuvants, had less positive vaginal cultures than the respective controls immunized with ovalbumin. However, no statistically significant differences were observed between the mice immunized with MOMP and their respective controls. For example, 69% (11/16) of the mice immunized with MOMP and MF59 had positive vaginal cultures over the 6 weeks of observation while in the control group immunized with ovalbumin and MF59 78% (14/18; $P > 0.05$) of the animals shed. Also, the mice vaccinated with MOMP shed for shorter periods of time and had less IFU recovered per culture than the animals immunized with ovalbumin. Nevertheless, in most instances, these differences were not statistically significant. Only the group immunized with MOMP and LT-R72 had statistically signif-

Table 3A
BALB/c mice: results of vaginal cultures

Mice immunized with	Adjuvant	Number of mice that shed/total number of mice per group (% positive) and median (range) number of *C. trachomatis* IFU shed per group					Total number of mice that shed in 6 weeks (%)
		Week 1	Week 2	Week 3	Week 4	Week 5	
MOMP	MF59	8/16 (50) 10 (0–246,732)	9/16 (56) 3 (0–1730)	0/16 (0) 0	0/16 (0) 0	0/16 (0) 0	11/16 (69)
Ovalbumin	MF59	10/18 (56) 30 (0–4,460)	11/18 (61) 36 (0–29,240)	2/18 (11) 0 (0–420)	0/18 (0) 0	0/18 (0) 0	14/18 (78)
MOMP	LT-K63	5/18 (28) 0 (0–1,564)	6/18 (33) 0 (0–4,000,000)	3/18 (17) 0 (0–1,982)	1/18 (6) 0 (0–10)	1/18 (6) 0 (0–25,160)	8/18 (44)[c]
Ovalbumin	LT-K63	8/18 (44) 0 (0–582,800)	9/18 (50) 1 (0–75,480)	5/18 (28) 1 (0–4,460)	0/18 (0) 0 (0–0)	0/18 (0) 0 (0–0)	14/18 (78)
MOMP	LT-R72	9/18 (50) 9 (0–43,180)	2/18 (11)[a] 0 (0–40)[b]	2/18 (11) 0 (0–10)	0/18 (0) 0 (0)	0/18 (0) 0 (0)	10/18 (56)[c]
Ovalbumin	LT-R72	11/18 (61) 18 (0–41,380)	8/18 (44) 0 (0–20,740)	1/18 (6) 0 (0–85,080)	0/18 (0) 0	0/18 (0) 0	15/18 (83)
CT-MoPn	–	2/16 (13)[d] 0 (0–2)[e]	0/16 (0)[d] 0[e]	1/16 (6) 0 (0–16)	0/16 (0) 0	0/16 (0) 0	3/16 (19)[d]

[a] $P < 0.05$ by the Fisher's exact test compared to the corresponding ovalbumin immunized groups.
[b] $P < 0.05$ by the Mann–Whitney Utest compared to the corresponding ovalbumin immunized groups.
[c] $P < 0.1$ by the Fisher's exact test compared to the corresponding ovalbumin immunized groups.
[d] $P < 0.05$ by the Fisher's exact test compared to the ovalbumin + MF59 immunized group.
[e] $P < 0.05$ by the Mann–Whitney *U*-test compared to the ovalbumin + MF59 immunized group.

Table 3B
C3H/HeN mice: results of vaginal cultures

Mice immunized with	Adjuvant	Number of mice that shed/total number of mice per group (% positive) and median (range) number of *C. trachomatis* IFU shed per group						Total number of mice that shed in 6 weeks (%)
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	
MOMP	MF-59	4/11 (36) 0 (0–86,360)	4/11 (36) 0 (0–133,263)	7/11 (64) 744 (0–14,464)	2/11 (18) 0 (0–85,680)	0/11 (0) 0	0/11 (0) 0	8/11 (73)
Ovalbumin	MF-59	12/18 (67) 61 (0–22,780)	13/18 (72) 473 (0–378,606)	13/18 (72) 525 (0–32,920)	3/18 (17) 0 (0–413)	1/18 (6) 0 (0–165)	1/18 (6) 0 (0–165)	17/18 (94)
MOMP	LT-K63	4/18 (22) 0 (0–39,100)	11/18 (61) 264 (0–8,260)	8/18 (44) 0 (0–195,684)	7/18 (39) 0 (0–80,580)	1/18 (6) 0 (0–1,090)	0/18 (0) 0	16/18 (89)
Ovalbumin	LT-K63	7/14 (50) 91 (0–198,520)	9/14 (64) 516 (0–27,540)	12/14 (86) 1,276 (0–75,480)	2/14 (14) 0 (0–41,620)	0/14 (0) 0 (0)	0/14 (0) 0 (0)	13/14 (93)
MOMP	LT-R72	2/17 (12)[a] 0 (0–4,791)[b]	12/17 (71) 952 (0–306,288)	10/17 (59) 826 (0–54,230)	9/17 (53) 12 (0–48,280)	2/17 (12) 0 (0–273)	1/17 (6) 0 (0–8)	16/17 (94)
Ovalbumin	LT-R72	9/17 (53) 2 (0–53,380)	14/17 (82) 859 (0–1,600,000)	11/17 (65) 2,924 (0–14,790)	10/17 (59) 102 (0–202,436)	1/17 (6) 0 (0–165)	1/17 (6) 0 (0–2)	17/17 (100)
CT-MoPn	–	4/16 (25)[c] 0 (0–100)[d]	3/16 (19)[c] 0 (0–2,065)[d]	0/16 (0) 0	1/16 (6) 0 (0–165)	0/16 (0) 0	0/16 (0) 0	4/16 (25)[c]

[a] $P < 0.05$ by the Fisher's exact test compared to the corresponding ovalbumin immunized groups.
[b] $P < 0.05$ by the Mann–Whitney U-test compared to the corresponding ovalbumin immunized groups.
[c] $P < 0.05$ by the Fisher's exact test compared to the ovalbumin + MF59 immunized group.
[d] $P < 0.05$ by the Mann–Whitney U-test compared to the ovalbumin + MF59 immunized group.

icant differences with the control group during the second week of observation.

The control BALB/c animals immunized i.n. with *C. trachomatis* EB were protected against the genital challenge as shown by the low number, 19% (3/16), of mice that had positive vaginal cultures during the 6 weeks of follow up. Furthermore, the number of IFU recovered from these mice and the length of time the animals had positive cultures was also significantly decreased in comparison with the control group immunized with ovalbumin and MF59.

Similar results were obtained with the C3H/HeN animals. During the 6 weeks of observation 73% (8/11) of the C3H/HeN mice vaccinated with the MOMP and MF59 had positive vaginal cultures (Table 3B). In the control group immunized with ovalbumin and MF59, 94% (17/18) of the mice had positive vaginal cultures ($P > 0.05$). Likewise, 89% and 94% of the mice immunized with the MOMP or LT-K63 and LT-R72, respectively, had positive cultures. Similar percentages of animals with positive vaginal cultures were observed in the control groups immunized with ovalbumin and the LT-K63 (93%) or LT-R72 (100%) ($P > 0.05$). Overall, the number of IFU recovered from the vaginal cultures and the length of time the mice had positive cultures were not significantly different between the mice vaccinated with MOMP and the controls inoculated with ovalbumin. Like in the case of the BALB/c mice, only the C3H/HeN group immunized with MOMP and LT-R72 had significant differences with the control group during the early stages of the infection.

In the control group of C3H/HeN mice immunized i.n. with *C. trachomatis* MoPn EB only 25% (4/16) of the animals had positive vaginal cultures during the 6 weeks of observation in comparison to 94% (17/18) of the mice immunized with ovalbumin and MF59 ($P < 0.05$). In addition, the number of IFU recovered per mouse was significantly lower in comparison with the control group.

3.4. Fertility studies

Six weeks after the genital challenge the female mice were placed in the same cage with male animals. Females that gained weight were euthanized and the number of embryos counted in both uterine horns. Mice that did not get pregnant were mated for a second time with a different male and were followed up as described above. As expected, in all the groups, the number of embryos present in the left uterine horn, the side inoculated with *Chlamydia*, was lower than in the right uterine horn that was mock-infected (Table 4A). Only 6% (1/16) of the BALB/c mice vaccinated with MOMP and MF59 had embryos in both uterine horns with an average of 0.4 pups in the *Chlamydia*-challenged left uterine horn and 2.8 in the right uterine horn. In the control group immunized with ovalbumin and MF59 11% (2/18) of the mice had embryos in both uterine horns and had 0.6 and 1.8 pups in the left and right uterine horns, respectively. Similar pregnancy rates were observed in the mice vaccinated with MOMP and LT-K63 or LT-R72. Overall, the pregnancy rates in these three groups vaccinated with MOMP and one of the three adjuvants were not significantly different from the controls groups immunized with ovalbumin.

On the other hand, the control BALB/c mice immunized i.n. with EB were strongly protected. All animals had pups in both uterine horns. Furthermore, the number of pups in the left and right uterine horn in these animals was similar to the fertility control group.

The results of the fertility studies performed with the C3H/HeN mice are shown in Table 4B. In general, there was a trend towards higher fertility rates in the mice vaccinated

Table 4A
BALB/c mice: results of fertility studies

Mice immunized with	Adjuvant	Number of mice that had embryos in uterine horns/total number of mice per group (%)			Mean (±1S.D.) number of embryos per mouse per uterine horn		
		Right	Left	Both	Right	Left[a]	Both
MOMP	MF59	13/16 (81)	2/16 (13)	1/16 (6)	2.8 ± 1.9	0.4 ± 1.3	3.2 ± 1.9
Ovalbumin	MF59	10/18 (56)	3/18 (17)	2/18 (11)	1.8 ± 2.0	0.6 ± 1.5	2.4 ± 2.8
MOMP	LT-K63	13/18 (72)	7/18 (39)[b]	6/18 (33)	2.9 ± 2.2	1.3 ± 1.7[c]	4.2 ± 2.8
Ovalbumin	LT-K63	13/18 (72)	2/18 (11)	2/18 (11)	2.7 ± 2.2	0.4 ± l.l	3.1 ± 2.8
MOMP	LT-R72	14/18 (78)	4/18 (22)	4/18 (22)	3.3 ± 2.2	1.1 ± 2.2	4.3 ± 2.9
Ovalbumin	LT-R72	12/18 (67)	4/18 (22)	3/18 (17)	2.2 ± 2.0	0.7 ± 1.5	2.8 ± 2.5
CT-MoPn	–	16/16 (100)[d]	16/16 (100)[d]	16/16 (100)[d]	4.0 ± 1.5[e]	2.9 ± 1.0[e]	6.9 ± 1.7[e]
Fertility control	–	18/18 (100)	16/18 (89)	16/18 (89)	3.3 ± 1.3	2.7 ± 1.7	6.0 ± 2.0

[a] *C. trachomatis* MoPn inoculated side.
[b] $P < 0.1$ by the Fisher's exact test compared to the corresponding ovalbumin immunized groups.
[c] $P < 0.1$ by Student's *t*-test compared to the corresponding ovalbumin immunized group.
[d] $P < 0.05$ by the Fisher's exact test compared to the ovalbumin + MF59 immunized group.
[e] $P < 0.05$ by the Student's *t*-test compared to the ovalbumin + MF59 immunized group.

Table 4B
C3H/HeN mice: results of fertility studies

Mice immunized with	Adjuvant	Number of mice that had embryos in uterine horns/total number of mice per group (%)			Mean (±1S.D.) number of embryos per mouse per uterine horn		
		Right	Left	Both	Right	Left[a]	Both
MOMP	MF59	5/11 (46)	5/11 (46)	2/11 (18)	2.9 ± 3.7[b]	1.5 ± 2.7	4.5 ± 4.0[c]
Ovalbumin	MF59	3/18 (17)	4/18 (22)	2/18 (11)	0.8 ± 2.1	0.7 ± 1.5	1.6 ± 3.2
MOMP	LT-K63	6/18 (33)	1/18 (6)	1/18 (6)	1.6 ± 2.4	0.1 ± 0.2	1.7 ± 2.5
Ovalbumin	LT-K63	2/14 (14)	1/14 (7)	0/14 (0)	0.9 ± 2.4	0.2 ± 0.8	1.1 ± 2.4
MOMP	LT-R72	9/17 (53)[d]	3/17 (18)	2/17 (12)	3.0 ± 3.2[c]	0.9 ± 2.5	3.9 ± 3.9[c]
Ovalbumin	LT-R72	2/17 (12)	0/17 (0)	0/17 (0)	0.5 ± 1.5	0 ± 0	0.5 ± 1.5
CT-MoPn	–	25/26 (96)[e]	25/26 (96)[e]	24/26 (92)[e]	3.8 ± 1.5[f]	3.8 ± 1.6[f]	7.6 ± 2.1[f]
Fertility control	–	11/12 (92)	11/12 (92)	10/12 (83)	3.8 ± 1.9	3.7 ± 2.1	7.5 ± 2.4

[a] *C. trachomatis* MoPn inoculated side.
[b] $P < 0.1$ by the Student's *t*-test compared to the corresponding ovalbumin immunized groups.
[c] $P < 0.05$ by the Student's *t*-test compared to the corresponding ovalbumin immunized groups.
[d] $P < 0.05$ by the Fisher exact test compared to the corresponding ovalbumin immunized groups.
[e] $P < 0.05$ by the Fisher's exact test compared to the ovalbumin + MF59 immunized group.
[f] $P < 0.05$ by the Student's *t*-test compared to the ovalbumin + MF59 immunized group.

with MOMP when compared with the animals immunized with ovalbumin. However, this difference was not statistically significant in any of the groups. For example, in the group immunized with MOMP and MF59 18% (2/11) of the mice had embryos in both uterine horns while in the control group 11% (2/18) had pups. The percentage of fertile animals was 92% in the C3H/HeN mice immunized i.n. with EB and 83% in the fertility control group ($P > 0.05$).

Statistically significant differences were observed when the number of embryos was compared among the groups vaccinated with MOMP and those immunized with ovalbumin. For example, the mean number of embryos in both uterine horns was 4.5 in the group vaccinated with MOMP and MF59 while in the control group immunized with ovalbumin and MF59 was 1.6 ($P < 0.05$). Protection was also obtained in the mice vaccinated with MOMP and LT-R72. The mean number of embryos in both uterine horns was 3.9 in the mice immunized with MOMP and LT-R72 while in the control group was 0.5 ($P < 0.05$). The number of embryos in the right uterine horn was also different in some groups. For example, in the group immunized with MOMP and LT-R72 the mean number of embryos in the right uterine horn was 3 while in the control group immunized with ovalbumin and LT-R72 was 0.5 ($P < 0.05$).

In the control group immunized with *C. trachomatis* EB the fertility rate 92% (24/26) was equivalent to that observed in the fertility control animals (83%; 10/12). Also, the number of pups in the right and left uterine horns was equivalent to that observed in the fertility control group.

4. Discussion

Attempts to produce a vaccine against *C. trachomatis* were initiated several decades ago [7]. Based on the results obtained with the trachoma clinical trials and the animal models it was concluded that in poorly protected individuals, using the whole organism as a vaccine, there was a risk of eliciting a hypersensitivity reaction upon re-exposure to the organism [7,19]. Furthermore, protection was serovar spe-

cific [7]. As a result, most of the recent research has focused in producing a subunit vaccine [8,12–15]. Among the various antigens considered, the MOMP is one of the prime candidates. Based on the comparison of the DNA sequences of various serovars it was shown that this protein has four variable domains (VD) spaced between five constant domains (CD) [20,21]. The amino acid variability observed in the VD of the MOMP defines the uniqueness of each serovar and is most likely the result of the escape mechanisms developed by *Chlamydia* under the immunological pressure of the host [12]. This points to this protein as the most likely immunogen responsible for protection in the trachoma trials [7].

Vaccination of mice with the native *C. trachomatis* MoPn MOMP using Freund's adjuvant resulted in a strong protective immune response against a genital challenge [15]. Due to the negative effects induced by Freund's, this adjuvant cannot be used in humans. For this reason we searched for adjuvants that were already approved for use in humans or that were developed for human vaccines. Using the outer surface protein A from *Borrelia burgdorferi* (OspA) as an adjuvant, we were able to show that the native MOMP was able to induce a protective immune response against a chlamydial genital challenge [22]. OspA has been used to immunize humans against Lyme's disease and was found to be safe [23–25]. However, utilizing OspA as an adjuvant for a vaccine may have potential shortcomings. For example, the non-specific immunostimulatory effects of the lipidated OspA may limit the use of this adjuvant, particularly if several booster doses are required [22]. For these reasons we decided to explore the feasibility of using MF59 and the heat-labile mutant toxins LT-R63 and LT-R72 of *Escherichia coli*.

MF59 is a detergent-stabilized oil-in-water emulsion containing squalene [26]. MF59 is currently a component of a licensed influenza vaccine and encouraging results have also been obtained in vaccines against several human pathogens [26–28]. The mode of action of this adjuvant is now under investigation but it appears to locally activate dendritic cells helping the internalization of the antigen. The other two adjuvants were derived from the heat-labile enterotoxin produced by the enterotoxigenic *E. coli* (LT) that causes diarrhea in humans [29,30]. This toxin has two functional domains. Subunit A has ADP-ribosylating activity that by enhancing cAMP levels alters ion transport resulting in water and chloride losses from the intestine. The B subunit contains the monosialoganglioside receptor-binding site. The wild-type LT can act as a potent mucosal adjuvant, however, its toxicity precludes its use in humans. To overcome this problem some investigators have used only the nontoxic B subunit while others have genetically produced detoxified mutants of LT. The LT-K36 has a serine-to-lysine substitution at position 63 in the A subunit while the LT-R72 has an alanine-to-arginine substitution at position 72 of the same subunit [29–31]. While the LT-R72 still has some toxic activity the LT-K63 is a completely nontoxic mutant.

The results obtained here are encouraging since with the three adjuvants tested the native MOMP induced significant *Chlamydia*-specific humoral and cellular immune responses in particular in the BALB/c mice. Based on the IgG2a/IgGl ratios in serum, the three adjuvants elicited a balanced Th1/Th2 response in the BALB/c and C3H/HeN mice a result consistent with reports from other studies [29–31]. In the case of chlamydial infections data obtained using mouse models indicates that, following a genital infection, a strong Th1 response is induced that is necessary for protection against reinfection of the genital tract [32]. However, a low level of IgA was detected in the vaginal washes of the mice immunized with MOMP in comparison to the animals immunized with EB. Furthermore, serum neutralizing titers in the mice immunized with MOMP were, for the most part, lower than the titers in the mice immunized with EB. This may affect the ability of the vaccine formulated with MOMP and these adjuvants to block the infectivity of *Chlamydia*.

Based on the results of the vaginal cultures, we noted a protective trend in both BALB/c and the C3H/HeN mice immunized with MOMP and either one of the three adjuvants when compared with the controls immunized with ovalbumin. However, overall the differences between the groups immunized with MOMP and the controls did not reach statistical significance. Only in isolated weeks the number of BALB/c and C3H/HeN mice with positive vaginal cultures, and the number of IFU recovered per mouse, was statistically significant different in the group immunized with MOMP and LT-R72 when compared with the group immunized with ovalbumin and LT-R72. The same trend was noted when the long-term sequelae were evaluated. In this case, the fertility rates in the mice immunized with MOMP and either one of the three adjuvants were higher than those observed in the ovalbumin immunized groups but, overall, the differences were not statistically significant. However, as in the case of the vaginal cultures, the groups of C3H/HeN mice immunized with MOMP and LT-R72, or MF59, were significantly protected, as shown by number of embryos in both uterine horns, when compared to the groups immunized with ovalbumin. Furthermore, in the animals vaccinated with MOMP and LT-R72, or MF59, the number of embryos in the right uterine horn was significantly higher than the number of embryos in the control groups immunized with ovalbumin. This indicates that vaccination with MOMP and LT-R72 limited the dissemination of the organisms from the left to the right uterine horn. The different amount of protection obtained with the two LT may reflect the fact that LT-R72 is a more potent adjuvant as compared to LT-K63.

The feasibility of determining the ability of *Chlamydia* to disseminate from one uterine horn to the other is one of the advantages of using the intrabursal versus the vaginal challenge. In addition, 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 [33]. Progesterone has a strong immunomodulatory effect in mice and humans. In particular progesterone induces a shift from Th1 to a Th2 response [34–36]. However, a Th1 response is considered to be nec-

essary for protection against a chlamydial infection [32]. As a result treating mice with progesterone before the intravaginal challenge may mask the protective effects of a vaccine. The intrabursal model has shortcomings particularly since it does not parallel the natural route of infection. However, by directly challenging the site we are interested in protecting, it may provide a more stringent proof of the efficacy of the vaccine.

In conclusion, formulating the natural MOMP with adjuvants that are currently used in humans, or were developed for use in humans, we were able to show that significant *Chlamydia*-specific immune responses can be elicited in mice. However, protection as measured by the number of positive vaginal cultures and rates of fertility was limited. More work is needed to formulate novel adjuvants that are safe for use in humans and favor the induction of Th1 immune responses.

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