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Induction of protection against vaginal shedding and infertility by a recombinant *Chlamydia* vaccine

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Abstract

A vaccine formulated with the *Chlamydia muridarum* recombinant major outer membrane protein, plus the adjuvants CpG and Montanide, was tested for its ability to protect BALB/c mice against a vaginal challenge. Mice were immunized by mucosal [intravaginal (i.vag.) plus colonic (col.), or intranasal (i.n.) plus sublingual (s.l.)], or systemic [intramuscular (i.m.) plus subcutaneous (s.c.)] routes, and a combination of mucosal priming/systemic boosting routes. A negative control group was vaccinated with the *Neisseria gonorrhoeae* porin B (Ng-rPorB) and a positive control group was inoculated in the nares with live *Chlamydia*. The strongest *Chlamydia*-specific humoral and cell-mediated immune responses were observed in the groups immunized by a combination of mucosal and systemic routes. Following the vaginal challenge, groups immunized using mucosal priming followed by systemic immunization had a significant decrease in the number of mice with positive vaginal cultures. For example, of the mice immunized i.n./s.l.+i.m./s.c., 24% had positive cultures during the six weeks of the experiment versus 69% for the negative control group immunized with Ng-rPorB ($p<0.05$). Similarly, the groups of mice primed by the mucosal routes and boosted by the systemic routes had significantly less IFU in the vaginal cultures when compared to the Ng-rPorB animals ($P<0.05$). These combination groups were also protected against infertility. The two groups had fertility rates of 100% (i.n./s.l.+i.m./s.c.) and 81% (i.vag./col.+i.m./s.c.) equivalent to the positive-control group immunized with live *Chlamydia* (100% fertility; $P>0.05$). These results show the importance of the schedule and routes of vaccination and represent the first study to show protection against infertility by a *Chlamydia* recombinant subunit vaccine.

Keywords

Chlamydia; rMOMP; immunization; mucosal; mice; infertility

Introduction

Chlamydia trachomatis is the most prevalent sexually transmitted bacterial pathogen in the world, with an estimated 100 million cases each year [1, 2]. Acute symptoms in women

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Disclosure Statement

None of the authors have a conflict of interest.

include cervicitis and salpingitis, and in men, urethritis and epididymitis [3, 4]. Although *Chlamydia* is treatable with antibiotics, up to 70% of cases in women are asymptomatic; thus they go undiagnosed and untreated [4, 5]. Nonetheless, even in antibiotic-treated patients, many long-term or chronic sequelae can develop; in females, this includes pelvic inflammatory disease, ectopic pregnancy and infertility [6]. For these reasons, development of a vaccine serves as the best approach for effective control and eradication of *Chlamydia*.

In the 1960's live and inactivated whole organism *Chlamydia* vaccines were tested both in humans and in non-human primates to protect against trachoma [3, 7, 8]. Some of the vaccination protocols elicited a protective immune response. However, the protection was found to be relatively short lived, usually waning by 2–3 years post-vaccination. In addition, the protection appeared to be serovar or subgroup specific. An apparent detrimental effect was also observed in individuals immunized with a low dose vaccine. In these subjects, re-exposure to *C. trachomatis* resulted in a hypersensitivity reaction. Although still of unknown etiology this hypersensitivity reaction is thought to be due to a chlamydial component present in the whole organism and therefore, prompted the search for the formulation of a subunit vaccine.

In the 1970's the recognition of a major role for *C. trachomatis* in sexually transmitted infections (STI) reignited an interest in the pathogenesis of these infections and in the development of a vaccine [8, 9]. Recent studies in mouse models have focused on utilizing the *C. muridarum* major outer membrane protein (MOMP) as a subunit vaccine [10, 11]. This protein, which accounts for 60% of the mass of the outer membrane, is considered a strong candidate due to its antigenic properties with many T- and B-cell epitopes [12, 13]. Immunization with the native form of MOMP (nMOMP) has produced significant levels of protection in mice against genital and respiratory challenges and in monkeys against ocular infections [14–16]. However, nMOMP is very costly to produce in large quantities, and the use of a recombinant form (rMOMP) is preferred, although rMOMP was shown to not provide as strong of protection as nMOMP [17]. Regardless, the use of rMOMP is a desirable alternative and having a vaccine that is only 50% efficacious or protects for only a short time can still make a significant impact on reducing the prevalence of the disease [18].

Here, to enhance protection, we decided to use rMOMP utilizing mucosal, systemic, and a combination of mucosal priming/systemic boosting immunization routes. Our results show that with mucosal priming and systemic boosting, rMOMP provides significant protection against a vaginal challenge; in fact, the observed fertility rates were equivalent to those in the fertility control group and in the mice immunized with live *Chlamydia*. This represents the first study to show protection by a subunit vaccine against infertility induced by a chlamydial vaginal challenge, and the first to utilize combination mucosal and systemic immunization routes.

Materials and Methods

C. muridarum stocks

The *C. muridarum* strain Nigg II (also called *C. trachomatis* mouse pneumonitis (MoPn)), was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and was grown as previously described [19, 20]. 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 (SPG) [21]. The stocks were titrated in HeLa-229 cells.

Preparation of rMOMP and *Neisseria gonorrhoeae* recombinant Porin B (Ng-rPorB)

Genomic DNA from *C. trachomatis* MoPn strain Nigg II was extracted using the Wizard genomic DNA Purification Kit (Promega; Madison, WI) [17, 22]. The MoPn MOMP gene

(GenBank, accession No. AE002272, X63409) was amplified without the leading sequence with *Pfu* Turbo DNA Polymerase (Stratagene) using the following primers. Forward primer: 5' ACGCCCATGGCACTGCCTGTGGGAATCCTGCT 3', and reverse primer: 5' AGCGGTCGACTTAGAAACGGAAGTGAAGCATT 3'. The MOMP DNA was cloned into the pET-45b vector (Novagen) at the *Nco* I and *Sal* I sites using T4 DNA ligase (New England Biolab), and transformed into TOP10 competent cells. After confirmation of positive clones by sequencing, the plasmid was transformed into BL21 (DE3) competent cells for expression in the presence of 0.4 mM IPTG. The efficiency of the protein induction was checked by SDS-PAGE.

N. gonorrhoeae strain FA1090, from the ATCC, was grown on GC agar plates and genomic DNA was extracted with the Wizard genomic DNA Purification Kit (Promega). The recombinant *PorB* gene (36 kDa; 330 AA) without the leading sequence (reference: GenBank ID: AAW90430) was amplified by the PCR with the following primers: Forward primer, Ngo-F2: 5'TATGCCATGGCCGATGTCACCTG 3' and reverse primer, Ngo-R1: 5'GCGGATCCCTTAGAATTTGTGGCGCAG 3'. The PCR product was cloned into pET 45b vector at the *Nco* I and *Bam*H I sites and transformed into TOP10 cells for sequencing. The plasmid carrying Ng-rPorB was transformed into BL21 (DE3) competent cells and the protein production was induced by 0.4 mM IPTG.

Purification of the *C. trachomatis* rMOMP and the *N. gonorrhoeae* rPorB from inclusion bodies

E. coli pellets with rMOMP and Ng-rPorB were treated with a combination of protease inhibitors, lysozyme, deoxycholic acid and DNase I as previously described [23]. This was followed by consecutive washes with a Tris-based buffer containing 2% Triton-X, 2% CHAPS with 2 M Guanidine HCl, and 2% Sarkosyl [24]. The rMOMP was solubilized in 8M urea, Tris buffer with 5% Anzergent 3–14 (*n*-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate) (Z3–14; Anatrace). The rMOMP protein was further purified by size exclusion gel filtration on Sephacryl-S-300 gel following the protocol by Qi et al. [25]. The collected fractions were concentrated using PEG 8000, and dialyzed extensively against 0.02M phosphate buffer (pH 7.4), 150mM NaCl, and 0.05% Z3–14, and stored at –80°C.

The Ng-PorB pellet was solubilized in 0.2M Tris, pH 8.2, 6M Gdn-HCl, 2 mM EDTA, 1 mM PMSF and 20 mM DTT and was centrifuged at 12,000-x g for 20 min. The supernatant was collected, and incubated at 50°C for 3 h with Z3–14 at a final concentration of 0.1% [15]. The protein was dialyzed against PBS buffer (pH 7.4) containing 0.05% Z3–14, 0.2 mM DTT, 1 mM PMSF, 1 mM EDTA and 4 M of Gdn-HCl. The buffer was changed every 5 hours with gradual decrease in the concentration of Gdn-HCl from 3 to 0 M. The Ng-rPorB was centrifuged at 12,000 × g for 30 min and the supernatant was dialyzed, against PBS (pH 7.4) with 0.05% Z3–14, stored at –80°C and used for immunization.

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. The animal protocols were approved by the UCI Animal Care and Use Committee. All experiments were repeated.

Mice were immunized either by mucosal or systemic routes or a combination of mucosal and systemic routes (Table 1). A total of four immunizations were performed at two-week intervals, using *C. muridarum* rMOMP (10 µg/immunization/mouse). To the rMOMP, we added 10 µg/immunization/mouse of CpG oligodeoxynucleotide 1826 (5' TCCATGACGTTTCCTGACGTT-3') (Coley Pharmaceutical Group, Kanata, Ontario,

Canada) for mucosal routes and CpG and Montanide ISA 720 VG (Seppic, Inc., Fairfield, NJ) (30:70 volume ratio of rMOMP/CpG to Montanide) for systemic routes. The positive-control group consisted of mice immunized once with 10^4 inclusion-forming units (IFU) of *C. muridarum* by the intranasal (i.n.) route. A negative-control group contained mice immunized by the intramuscular/subcutaneous (i.m./s.c.) routes with Ng-rPorB (10 µg/immunization/mouse) with CpG and Montanide. A fertility-control group included mice of the same age as and housed in the same location as the experimental mice.

For colonic (col.) immunization, mice were starved for food overnight and the immunization was performed using a 4.5 Fg dosing catheter (Harvard Apparatus, Holliston, MA) [26, 27]. After col. and intravaginal (i.vag.) immunization, mice were placed upside down at a slight angle for at least 10 min. in order to prevent leakage. Sublingual (s.l.) immunization was administered in two 5 µl dosages under the tongue [28]. Mice were treated s.c. with 1 mg of medroxy progesterone acetate (MPA) (Greenstone Ltd, Peapack, NJ) per mouse 4 days before the vaginal challenge [29]. The vaginal cytology was checked before the challenge. Mice were challenged vaginally, under xylazine/ketamine anesthesia, 4 weeks after the last immunization using 10^3 IFU of *C. muridarum*.

Immunoassays

Blood was collected from the periorbital region, and vaginal washes were collected in two 20 µl PBS samples (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 [30]. The following classes, or subclasses, of specific antibodies were used: immunoglobulin G (IgG), IgG1, IgG2a, IgG2b, IgG3, and IgA (Southern Biotechnology Associates, Inc., Birmingham, AL). In vitro neutralization assays were performed using HeLa-229 cell monolayers as described by Peterson et al. [31]. Western blots were performed using nitrocellulose membranes as previously described [30].

To measure the cell-mediated immune response we used a lymphoproliferative assay (LPA) as published [20]. LPA was performed on two mice per group from the following immunization schedules: i.m./s.c. (4X); i.vag./col. (2X) + i.m./s.c. (2X); i.n./s.l. (2X) + i.m./s.c. (2X); MoPn i.n. (1X); and Ng-rPorB: i.m./s.c. (4X).

Genital cultures for *C. muridarum*

Vaginal swabs were cultured at 7-day intervals for a period of 6 weeks following the genital challenge, and samples were stained and counted as described [20]. Briefly, HeLa cells grown in 48-well tissue culture plates were inoculated with 10-fold dilutions of the swabs and incubated for 30 h at 37°C. The monolayers were fixed with methanol and inclusions were measured by determining the incorporation stained using a pool of monoclonal antibodies (mAb) to the MOMP, the 60-kDa cysteine-rich protein (crp), the 150-kDa putative outer membrane protein and lipopolysaccharide (LPS) of MoPn [20]. The limit of detection was 2 IFU.

Fertility Studies

At 7 weeks following the challenge, female mice were housed with a proven breeder male mouse for a maximum of 18 days and then repeated if necessary [20]. All animals were euthanized and the number of embryos in each uterine horn counted. Fertility is defined as an embryo in at least one uterine horn.

Statistical analyses

The Mann-Whitney U test, Fisher's exact test, and the Student's *t*-test were used for statistical analysis with the program SigmaStat version 3.5.

Results

Immune response following vaccination with *C. muridarum* (MoPn) rMOMP

Table 2 shows the results of the humoral immune responses in sera from the day before the vaginal challenge. The highest *Chlamydia*-specific serum IgG antibody titer (409,600) was observed in the combination group i.vag./col. (2X) + i.m./s.c. (2X). Very high titers (204,800) were also observed in the groups immunized i.n./s.l. (2X) + i.m./s.c. (2X) and i.m./s.c. (4X). Four mucosal immunizations, i.vag./col. or i.n./s.l., did not provide as high of IgG levels as did two mucosal priming immunizations with the addition of two systemic boosts (<100 versus 409,600 for i.vag./col. and 12,800 versus 204,800 for i.n./s.l.). The positive-control group, mice immunized with live MoPn, had an IgG serum titer of 25,600; the negative-control group, mice immunized with Ng-rPorB, did not have detectable *Chlamydia*-specific antibodies.

For all immunization groups, the titer of IgG2a was greater than or equal to IgG1, indicative of a Th1 response. The group i.n./s.l. (2X) + i.m./s.c. (2X) had the highest IgG2a/IgG1 ratio (204,800: 12,800 = 16), equivalent to the MoPn positive-control group (51,200: 3,200 = 16).

In the vaginal washes the highest IgG titer (640) was observed in the i.vag./col. (2X) + i.m./s.c. (2X) group, although the i.n./s.l. (2X) + i.m./s.c. (2X) and the i.m./s.c. (4X) groups also had high titers (320). The MoPn positive-control group had moderate IgA and IgG antibody levels of 80 and 40, respectively. The Ng-rPorB negative-control group had no detectable antibody titers.

A moderate titer (250) of neutralizing antibodies was observed only in the i.vag./col. (2X) + i.m./s.c. (2X) group (Table 2). The MoPn positive-control group showed a neutralizing titer of 1,250. The serum from the Ng-rPorB immunized group was used as negative control.

Immunoblot analyses of the serum samples, using EB as the antigen, are shown in Figure 1. All immunization groups, with the exception of i.vag./col. (4X), produced detectable antibodies against MOMP. MoPn-immunized mice developed antibodies against several high MW proteins (>100 kDa) and the 60 and 28 kDa proteins. No antibodies against *Chlamydia* were found in the serum of mice immunized with Ng-rPorB.

To determine the cellular immune response, T-cells were purified and stimulated with EB or medium, as a background control, and the amount of proliferation was measured by determining the incorporation of ³H-thymidine. As shown in Table 3, the combination groups and the MoPn-immunized mice had significant proliferative T-cell immune responses (*P*<0.05) compared to mice immunized with Ng-rPorB. The T-cell proliferation in the combination groups was greater than in the systemic-only immunization group. Due to the observed low, or lack, of antibody response, in the 4X mucosal immunization groups, no LPA analysis was performed on these animals.

Vaginal cultures for *C. trachomatis* MoPn

Four weeks after the last immunization the mice were challenged vaginally with 10³ IFU of *C. muridarum* and the infection was followed by weekly vaginal cultures. As a result of the MPA treatment all mice were in diestrus at the time of challenge. As shown in Table 4, over the six week period *Chlamydia* was recovered from 24% (5/21) of the mice immunized by i.n./s.l. (2X) + i.m./s.c. (2X) and 29% (6/21) of the i.vag./col. (2X) + i.m./s.c. (2X) group.

The number of mice that shed in the combination groups was significantly less ($P<0.05$) compared to the Ng-rPorB immunized mice, 69% (11/16). In contrast, 79% (11/14) of the mice immunized by the i.vag./col. (4X), 67% (10/15) vaccinated by i.n./s.l. (4X) and 43% (9/21) vaccinated by i.m./s.c. (4X) routes had positive vaginal cultures. None of these groups were significantly different from the Ng-rPorB immunized mice ($P>0.05$). The MoPn group was strongly protected with no mouse having a positive *Chlamydia* culture.

In the first two weeks, groups immunized by combination routes had significantly less mice shedding ($P<0.05$) than the Ng-rPorB group. The MoPn vaccinated group had less number of mice shedding than the Ng-rPorB group for the first three weeks ($P<0.05$). All immunization groups had negative vaginal cultures by week 5, with the i.n./s.l. (2X) + i.m./s.c. (2X) and the i.vag./col. (4X) groups being negative at week 4.

There were significantly less IFU ($P<0.05$) recovered from the vaginal cultures in the first two weeks for the i.n./s.l. (2X) + i.m./s.c. (2X) group and in weeks 2 and 3 for the i.vag./col. (2X) + i.m./s.c. (2X) group when compared with the Ng-rPorB group. The MoPn immunized animals had less number of IFU than the Ng-rPorB group for the first three weeks ($P<0.05$).

The schedule of immunization resulted in differences in the level of protection. There were significant differences in protection between mucosal only immunization and mucosal priming followed by systemic immunization. In addition, schedules that contained mucosal priming also showed better protection compared to schedules that contained systemic priming and mucosal boosting. For example, in week 1, both the number of mice that shed and the amount of IFU shed for the systemic-only and combination groups was significantly lower ($P<0.05$) than the i.vag./col. (4X) group; the combination groups were also significantly lower ($P<0.05$) for these values during week 2. Further, in week 1 for the i.n./s.l. (2X) + i.m./s.c. (2X) group and in week 2 for both combination groups, there was significantly less *Chlamydia* IFU shed ($P<0.05$) than the mucosal i.n./s.l. (4X) immunized mice. Over all 6 weeks, significantly less mice shed ($P<0.05$) in the combination groups compared to the i.vag./col. (4X) and i.n./s.l. (4X) groups. The systemic-only group only had significantly less mice shedding ($P<0.05$) than the i.vag./col. (4X) group.

Fertility Studies

As shown in Table 5, the i.n./s.l. (2X) + i.m./s.c. (2X) and the live MoPn immunized groups had 100% fertility rates, and were the only immunization groups with significantly higher fertility rates ($P<0.05$) than the Ng-rPorB group (50% fertile). All the mice in the fertility control group were also fertile. In addition, both combination immunizations groups had fertility rates that were not significantly different ($P>0.05$) than the fertility control group or the MoPn-immunized mice that were both 100% fertile.

The i.n./s.l. (2X) + i.m./s.c. (2X) combination immunization group had significantly more embryos than the Ng-rPorB group (6.0 ± 3.0 versus 3.0 ± 3.8 ; $P<0.05$) and was not significantly different from the fertility control group (7.2 ± 3.0 ; $P>0.05$). All other immunization groups had statistically less number of embryos compared to the fertility control animals. In comparison with the MoPn immunized group, only the mice immunized i.vag./col (4X) had less number of embryos ($P<0.05$).

Discussion

This study shows that vaccination of mice with rMOMP, using a combination of mucosal priming and systemic boosting immunization routes, provides significant protection against a vaginal challenge with *Chlamydia*. An enhanced protection was observed in immunization

combination groups for they exhibited significantly less number of mice shedding, less *Chlamydia* IFU, and higher fertility rates compared to mucosal or systemic only immunization routes as well as the Ng-rPorB negative-control group. Combination immunizations by the i.n./s.l. (2X) + i.m./s.c. (2X) schedule provided the best overall protection, although i.vag./col. (2X) + i.m./s.c. (2X) immunization also elicited significant protection. These results represent the first study to show protection against infertility using a recombinant antigen and are the first use of combination immunizations against *C. muridarum*.

Vaccination with recombinant antigens, in particular those using rMOMP, have shown promising results in animal models [17, 32, 33]. For example, Hansen et al. [32] immunized mice with the MoPn rMOMP adjuvanted with alum or the cationic liposome 1 (CAFO1). In the mice vaccinated with rMOMP and CAF01 they observed protection against a vaginal challenge as determined by a reduced vaginal chlamydial load. However, no protection was observed against infertility as determined by hydrosalpinx formation. Hickey et al. [33] administered rMOMP orally with a novel lipid-based adjuvant, resulting in significant protection against vaginal shedding although *Chlamydia* was still present on the last culture day (day 18 post-infection). No difference in the inflammatory response in the genital tract was noticed at 28 days post-infection. The authors proposed that the lack of protection in the rMOMP-immunized animals against tissue pathology was due to the influence of the treatment with MPA before the vaginal challenge [33].

The utilization of a mucosal immunization route appears to be relevant for protection against mucosal pathogens like *Chlamydia*. Mucosal tissue comprises the largest source of immunity in the body and is the first line of defense against many pathogens [34]. Mucosal IgA elicited by local vaccine administration may be critical for protection against pathogens that utilize mucosal surfaces as the site of entry [35–37]. For example, data from studies on HIV-subunit vaccines demonstrate that i.n. priming followed by i.m. boosting enhances both mucosal and systemic immune responses [38]. Also, mucosal priming resulted in enhanced immunity compared to systemic-only or systemic priming in *Helicobacter pylori* [39]. Similar results were also seen with influenza, and two simultaneous i.n. and i.m. immunizations did not confer as strong as protection as i.n. priming followed by systemic immunization [36]. All these results are mirrored by studies on polio vaccines, where immunization with an inactivated vaccine enhanced immunity only in individuals with prior contact with live virus [40].

The mucosal tissues are highly compartmentalized in which specific inductive sites are associated with specific effector sites [34]. This helps explain why the selection of vaccination route affects the consequent immune response. However, determining the best mucosal route for immunization is crucial not only for eliciting the strongest protection but for ease of use, cost, safety and societal acceptance. Immunization by the i.vag. route seems the most appropriate choice for eliciting protection against a genital pathogen. Intravaginal immunization through use of a gel has been successful against cholera, and topical application has been utilized against HIV [41, 42]. Limitations of this route include patient acceptance and the need for proper adjuvants. Also, our results show the IgA titers in vaginal washes were low in vaginally immunized animals regardless of the immunization schedule. Other studies report similar findings of low IgA and IgG in the vaginal fluid after vaginal immunization, even if the amount of antigen and number of immunizations were increased [43, 44].

Oral immunization is attractive for it is easy to administer and readily acceptable. Yet, the need for a successful delivery system and the large amounts of antigen required for an immune response limits this approach and increases its cost [34]. The sublingual route may

be a more desirable alternative and has been successful in mouse models, including this study [45, 46]. However, colonic immunization may be a more appropriate vaccination target since it has been shown to induce higher vaginal IgA levels than oral and even intramuscular immunization, as well as higher serum IgA levels than the oral route [27]. This is relevant for STI like *Chlamydia* since the colon is a source of IgA plasma effectors for the vaginal tract [27]. The ease of use and patient acceptance can be improved by utilizing oral delivery, which targets the colon and protects the antigen through the upper gastrointestinal tract.

The i.n. route combined with i.m. immunization has been shown to induce mucosal IgA and stimulate immunity not only in local tissues but also in the genital tract [34, 37]. In our study the i.n./s.l. (2X) + i.m./s.c. (2X) combination group showed the best overall protection with the lowest amount of IFU shed and a significantly higher fertility rate than the negative-control group. Finally, i.n. immunization is easy and inexpensive to administer. Therefore, i.n. may be the best mucosal route to confer protection against a genital infection, when used in conjunction with systemic boosting. Previous studies on mucosal pathogens, including the genital pathogens SHIV and herpes simplex virus (HSV), show that i.n. immunization provides a strong immune response even in genital tissues [36–40]. Unfortunately, i.n. immunization may negatively affect the central nervous system, and this safety issue is why some vaccines have been pulled from the market [47, 48]. As more research is performed, we may find that each mucosal route may be beneficial for a specific application or pathogen, allowing for a more efficient and safer vaccine.

In addition to the antigen, route and immunization schedule, another factor that may have contributed to the efficacy of our protocol, is the dose and schedule of MPA we used. Tuffrey and Taylor-Robinson [29] showed that treatment of CBA/H mice with MPA resulted on a long-lasting infection with a "fast" strain of *C. trachomatis* (SA₂f). In animals that were not treated with MPA, *Chlamydia* was not recovered from vaginal cultures. The authors stated that: "prolonged infection depends on progesterone treatment, which presumably prevents the loss of target epithelial cells during a 5-day oestron cycle". Since then, pretreating animals with MPA has been shown to increase susceptibility to various sexually transmitted pathogens including *N. gonorrhoeae*, HSV and simian immunodeficiency virus (SIV) [49–52].

A concern with the use of MPA is that sex hormones are potent modulators of the immune response. For example, Kaushic et al. [50] showed that treatment with MPA prevents induction of a protective response in mice following intravaginal immunization with HSV-2. Similarly, Abel et al. [49] demonstrated that MPA abrogated the protection induced in rhesus macaques by a lentivirus vaccine against a vaginal challenge with SIV mac239. However, there is also evidence that mice pretreated with MPA, and subsequently immunized intravaginally with live MoPn, were protected even when they were pretreated again with MPA before the challenge [53, 54]. Work by Gillgrass et al. [55] helped to clarify these apparent contradictory findings. These authors treated mice with MPA at 5 or 15 days before they were immunized intravaginally with HSV-2. Gillgrass et al. [55] showed that mice that had been treated with MPA for 15 days before the immunization were not protected against the HSV-2 challenge while animals that had been pre-treated for only 5 days were fully protected. Furthermore, they showed that prolonged exposure to MPA leads to poor innate and adaptive immune responses. Therefore, it appears that the amount of MPA and time the animals are under its influence is critical for masking the protective efficacy of a vaccine. Here, for that reason, we treated the mice with only 1 mg of MPA at 4 days before the vaginal challenge rather than using the standard treatment of 2.5 mg of MPA each at 14 and 7 days before the challenge [29].

In this study, we started to immunize the mice at three weeks of age with the purpose of completing all the vaccinations before the animals reached sexual maturity. A similar protocol should be implemented in humans since most of the chlamydial infections are acquired soon after the individual becomes sexually active. Tubal infertility in humans can result from a single or from multiple episodes of pelvic inflammatory disease. For example, Westrom et al. [56] followed, by clinical assessment and laparoscopy, 1,732 patients and 601 control individuals for a total of 13,400 and 3,958 woman-years, respectively. A total of 141 of women developed tubal infertility during the observation period. Of these, 109 became infertile after a single episode of PID, 36 after two events and 26 after three or more episodes of PID. Similarly, BALB/c, C3H/HeN and C57BL/6 mice can become infertile after a single vaginal inoculation with MoPn [57]. For example, only 40% of BALB/c mice infected once with MoPn were fertile while 100% of the control non-infected animals were fertile. Therefore, if we want to avoid long-term sequelae, a chlamydial vaccine should be implemented before the individual becomes sexually active.

In conclusion, the immunization schedule that uses mucosal priming in combination with systemic boosting appears important for developing an effective subunit vaccine against *C. muridarum*. We plan on exploring the mucosal immunization routes and schedules in more detail, in hopes of elucidating which individual mucosal route is more effective, as well as determining the minimal number of immunizations and doses required to produce optimal protection.

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Figure 1. Western blots of sera from immunized mice using EB as the antigen

Lane 1. Molecular weight standards; lane 2, pooled pre-immunization serum; lanes 3–9 serum from mice immunized by: lane 3: i.vag./col. (4X) diluted 1:500; lane 4: i.n./s.l. (4X) diluted 1:1,000; lane 5: i.m./s.c. (4X) diluted 1:10,000; lane 6: i.vag./col. (2X) + i.m./s.c. (2X) diluted 1:10,000; lane 7: i.n./s.l. (2X) + i.m./s.c. (2X) diluted 1:10,000; lane 8: i.n. (1X) with EB diluted 1:250; lane 9: i.m./s.c. (4X) with Ng-rPorB diluted 1:500; lane 10: mAb MoPn-40 to the *C. muridarum* MOMP.

Table 1

Immunization routes and schedules

Antigen	Adjuvant	Immunization 1	Immunization 2	Immunization 3	Immunization 4
rMOMP	CpG	i.vag./col.	i.vag./col.	i.vag./col.	i.vag./col.
rMOMP	CpG	i.n./s.l.	i.n./s.l.	i.n./s.l.	i.n./s.l.
rMOMP	CpG + Montanide	i.m./s.c.	i.m./s.c.	i.m./s.c.	i.m./s.c.
rMOMP	CpG: i.vag./col. CpG + Montanide: i.m./s.c.	i.vag./col.	i.vag./col.	i.m./s.c.	i.m./s.c.
rMOMP	CpG: i.n./s.l. CpG + Montanide: i.m./s.c.	i.n./s.l.	i.n./s.l.	i.m./s.c.	i.m./s.c.
MoPh	none	i.n.	None	None	None
Ng-rPorB	CpG + Montanide	i.m./s.c.	i.m./s.c.	i.m./s.c.	i.m./s.c.

Table 2

Humoral immune response in pooled serum and vaginal wash from the day before the vaginal challenge

Antigen	Immunization route/(times)	<i>Chlamydia trachomatis</i> MoPn-specific ELISA antibody titer ^d									
		Seru m					Vaginal Wash		In vitro Neutralization titer		
		IgG	IgG1	IgG2a	IgG2b	IgG3	IgA	IgG			IgA
rMOMP	i.vag./col. (4X)	<100	<100	<100	<100	<100	<100	<100	<10	<10	50
rMOMP	i.n./s.l. (4X)	12,800	6,400	6,400	12,800	<100	200	20	80	<50	
rMOMP	i.m./s.c. (4X)	204,800	51,200	102,400	204,800	12,800	6,400	320	80	<50	
rMOMP	i.vag./col. (2X) + i.m./s.c. (2X)	409,600	51,200	204,800	204,800	25,600	12,800	640	80	250	
rMOMP	i.n./s.l. (2X) + i.m./s.c. (2X)	204,800	12,800	204,800	204,800	51,200	12,800	320	40	50	
MoPn	i.n. (1X)	25,600	3,200	51,200	25,600	12,800	1,600	40	80	1,250	
Ng-rPorB	i.m./s.c. (4X)	<100	<100	<100	<100	<100	<100	<10	<10	<50	

^a Limit of detection in serum: 100; vaginal wash: 10; in vitro neutralization 50.

Table 3T-cell responses of immunized mice from the day before intravaginal challenge with *C. trachomatis* MoPn^a

Antigen	Immunization route (times)	T-cell proliferative response ^a ($\times 10^3$ cpm) to:	
		EB ^{b,c}	Medium ^c
rMOMP	i.m./s.c. (4X)	1.9 $\pm$ 0.8	0.5 $\pm$ 0.70
rMOMP	i.vag./col. (2X) + i.m./s.c. (2X)	3.0 $\pm$ 0.8 ^d	0.2 $\pm$ 0.05
rMOMP	i.n./s.l. (2X) + i.m./s.c. (2X)	4.8 $\pm$ 2.3 ^d	0.2 $\pm$ 0.09
MoPn	i.n. (1X)	3.8 $\pm$ 0.8 ^d	0.1 $\pm$ 0.08
Ng-rPorB	i.m./s.c. (4X)	0.8 $\pm$ 0.4	0.1 $\pm$ 0.08

^aThe values are means $\pm$ 1 SD for the triplicate cultures.^bThe ratio of UV-inactivated *C. trachomatis* MoPn EB to antigen presenting cells was 10:1.^cValues are from a pooled sample of 2 mice run in triplicate.^dP<0.05 as determined by the Student's *t*-test compared to the Ng-rPorB immunized group.

Table 4

Results of vaginal cultures for six weeks after the vaginal challenge

Antigen	Immunization route/(times)	Percentage of mice with positive vaginal cultures and median number of <i>C. trachomatis</i> IFU ^a (range)										Total # mice that shed over 6 wks (% positive)
		Week 1		Week 2		Week 3		Week 4		Weeks 5/6		
		Mice	IFU	Mice	IFU	Mice	IFU	Mice	IFU	Mice	IFU	
rMOMP	i.vag./col. (4X)	79	440,586 (<2-2,529,340)	57	9,717 (<2-550,434)	29	<2 (<2-33,522)	0	<2	0	<2	11/14 (79)
rMOMP	i.n./s.l. (4X)	60	74,292 (<2-1,372,624)	53	6 (<2-420,288)	20	<2 (<2-60,894)	7	<2 (<2-2,478)	0	<2	10/15 (67)
rMOMP	i.m./s.c. (4X)	38 ^b	<2 (<2-790,550) ^c	38	<2 (<2-126,564)	19	<2 (<2-1,809)	5	<2 (<2-16)	0	<2	9/21 ^b (43)
rMOMP	i.vag./col. (2X) + i.m./s.c. (2X)	29 <i>b,d,e</i>	<2 (<2-1,610,848) ^c	19 <i>b,d</i>	<2 (<2-114,624) ^{c,f,g}	10	<2 (<2-595) ^f	5	<2 (<2-451)	0	<2	6/21 ^{b,d,e} (29)
rMOMP	i.n./s.l. (2X) + i.m./s.c. (2X)	19 <i>b,d,e</i>	<2 (<2-2,963,620) ^{c,f,g}	19 <i>b,d</i>	<2 (<2-365,844) ^{c,f,g}	19	<2 (<2-287,854)	0	<2	0	<2	5/21 ^{b,d,e} (24)
MoPn	i.n. (1X)	0 <i>b,d,e</i>	<2 <i>c,f,g</i>	0 <i>b,d,e</i>	<2 <i>c,f,g</i>	0 <i>b,d</i>	<2 <i>c,f,g</i>	0	<2	0	<2	0/15 ^{b,d,e} (0)
Ng-rPorB	i.m./s.c. (4X)	69	10,219 (<2-771,324)	63	5,186 (<2-767,742)	38	70 (<2-66,864)	13	<2 (<2-392)	0	<2	11/16 (69)

^aLimit of detection: 2 IFU.

^bP<0.05 by the Fisher's Exact test compared to the i.vag./col. (4X) immunization group

^cP<0.05 by the Mann Whitney U test compared to the i.vag./col. (4X) immunization group.

^dP<0.05 by the Fisher's Exact test compared to the Ng-rPorB immunization group.

^eP<0.05 by the Fisher's Exact test compared to the i.n./s.l. (4X) immunization group.

^fP<0.05 by the Mann Whitney U test compared to the Ng-rPorB immunization group.

^gP<0.05 by the Mann Whitney U test compared to the i.n./s.l. (4X) immunization group.

Table 5

Results of fertility studies

Antigen	Immunization route/(times)	Number of fertile mice (%)	Mean # embryos/mouse
rMOMP	i.vag./col. (4X)	6/14 (43) ^a	2.0±2.9 ^b
rMOMP	i.n./s.l. (4X)	11/15 (73) ^a	4.9±4.0 ^{b,c}
rMOMP	i.m./s.c. (4X)	14/21 (67) ^a	3.7±3.9 ^{b,c}
rMOMP	i.vag./col. (2X) + i.m./s.c. (2X)	17/21 (81) ^{a,d}	5.0±3.3 ^{b,c}
rMOMP	i.n./s.l. (2X) + i.m./s.c. (2X)	21/21 (100) ^{d,e}	6.0±3.0 ^{c,f,g}
MoPn	i.n. (1X)	21/21 (100) ^e	4.8±2.9 ^b
Ng-rPorB	i.m./s.c. (4X)	8/16 (50) ^a	3.0±3.8 ^{b,c}
---	Fertility control	23/23 (100) ^{d,e}	7.2±3.0 ^f

^aP<0.05 by the Fisher's Exact test compared to the fertility control group.

^bP<0.05 by the Student's *t* test compared to the fertility control group.

^cP>0.05 by the Student's *t* test compared to the MoPn immunization group.

^dP>0.05 by the Fisher's Exact test compared to the MoPn immunization group.

^eP<0.05 by the Fisher's Exact test compared to the Ng-rPorB immunization group.

^fP<0.05 by the Student's *t* test compared to the Ng-rPorB immunization group.

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