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Original Article

Immunogenicity and protection against genital *Chlamydia* infection and its complications by a multisubunit candidate vaccine

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Background and Purpose: Genital infections due to *Chlamydia trachomatis* pose a considerable public health challenge worldwide and a vaccine is urgently needed to protect against these infections. We examined whether a vaccine composed of a combination of the major outer membrane protein (MOMP) and porin B protein (PorB) of *C. trachomatis* would have a protective advantage over a single subunit construct.

Methods: Single and multisubunit vaccines expressing MOMP and PorB were constructed and evaluated in the mouse model of genital infection. Thus, groups of female C57BL/6 mice were immunized intramuscularly with recombinant *Vibrio cholerae* ghosts (VCG) expressing the vaccine antigens or VCG alone and humoral and cell-mediated immune responses were evaluated.

Results: Significant levels of *Chlamydia*-specific secretory immunoglobulin A and immunoglobulin G2a were detected in vaginal washes and serum of immunized mice. The multisubunit construct induced a significantly higher level of T-helper Type 1 response than the single subunits as measured by the amount of interferon-gamma produced by immune T cells in response to re-stimulation with ultraviolet-irradiated elementary bodies in vitro. Three weeks after the last immunization, animals were challenged intravaginally with 10^7 inclusion-forming units of *C. trachomatis* serovar D. There was a significant difference in the intensity and duration of vaginal shedding between the vaccine-immunized mice and controls. All the animals immunized with the multisubunit vaccine had completely resolved the infection 2 weeks post-challenge. Higher numbers of embryos were observed in vaccinated animals than in controls, indicating protection against infertility.

Conclusion: These results underscore the potential, albeit moderate, vaccine advantage of the multisubunit formulation.

Key words: Bacterial outer membrane proteins; Bacterial vaccines; *Chlamydia* infections; Porins; Vaccination

Introduction

Chlamydia trachomatis genital infection is the most common bacterial sexually transmitted disease in several industrialized nations, including the United States. The infections constitute a major public health challenge due to their significant morbidity, that includes pelvic inflammatory disease, ectopic pregnancy and infertility [1,2]. Although *Chlamydia* genital infection can be treated with antibiotics, the frequent asymptomatic infection, especially in women, precludes early diagnosis and treatment, making clinical presentation of sequelae often the first indication of infection. Consequently, it has been suggested that the development and administration of a prophylactic or therapeutic vaccine capable of protecting against infection or even ameliorating

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severe disease would be the most promising and effective strategy to control *Chlamydia* [3-5].

The current immunologic paradigms for designing and evaluating chlamydial vaccines include the obligatory requirement for a T-helper Type 1 (Th1) immune response [5,6]. A number of studies have demonstrated that the presence of antibodies during natural or experimental infection does not correlate with protection. Such studies include the findings that in vitro neutralizing antibodies do not transfer protective passive immunity to mice in vivo [7], and limited microbial clearance in experimental and clinical infections even in the presence of high levels of secretory and systemic antibodies [4, 8-11]. However, recent findings indicate that antibodies of the immunoglobulin G2a (IgG2a) and immunoglobulin A (IgA) isotypes enhance Th1 activation against chlamydiae [12-14]. Furthermore, the selection of a suitable vaccine candidate capable of inducing the required immune effectors and the development of an effective delivery system to boost such immune responses are additional requirements for developing an efficacious chlamydial vaccine. The use of whole chlamydial agents as vaccines appears to be unattractive due to the potential existence of immunopathogenic components [15]. Thus, the development of vaccines based on chlamydial subunit components is the current focus of chlamydial vaccine design.

The major outer membrane protein (MOMP) is one of the leading subunit vaccine candidates and has been tested in various delivery systems [16-18]. However, experience with purified or recombinant MOMP as a protective antigen in several animal models [7,19,20] indicated that MOMP alone is inadequate, suggesting a need for a multisubunit approach or a more effective delivery system that will optimize the protective immune response. The focus on a multisubunit approach in chlamydial vaccine design imposes a major challenge; to determine the appropriate combination of immunogenic components that could be delivered to the immune system to elicit an optimal immune response. Other serologically defined and molecularly characterized chlamydial antigens include the cysteine-rich outer membrane proteins (OMPs), OMP2 (60 kDa) and OMP3 (15 kDa) [21], which are recognized during human infection [15,22,23]. In addition, recent advances in chlamydial genomics have predicted several other immunogenic proteins [24-26] that may serve as potential vaccine candidates. These include the polymorphic OMPs (POMPs or Pmps) [27-29], the adenosine diphosphate/adenosine triphosphate translocase of *Chlamydia pneumoniae* [24,25] and the conserved porin B protein (PorB) family of membrane proteins [30-32]. PorB has several immunologic and biochemical features that support its further evaluation as a potential vaccine candidate, including being: (1) highly conserved among *C. trachomatis* serovars as well as *C. pneumoniae*, suggesting structural conservation; (2) expressed on the outer membrane surface and thus easily accessible to the immune system; and (3) immunogenic [33]. Based on these qualities, PorB was considered for inclusion in the design of a multisubunit vaccine to induce sterilizing immunity or improve existing levels of protection.

We have recently shown that the recombinant *Vibrio cholerae* ghosts (rVCG) platform is an effective carrier and delivery system for cloned *C. trachomatis* proteins, eliciting chlamydial-specific immune responses following immunization [19]. We demonstrated that the rVCG platform is capable of delivering multiple antigens in a multisubunit chlamydial vaccine that induced a significantly higher protective immunity compared to a single vaccine construct [34]. The present study evaluated the protective advantage of a heterologous subunit candidate vaccine composed of the chlamydial OMPs, MOMP and PorB delivered as a multisubunit regimen using the rVCG platform technology in the mouse model of genital chlamydial infection.

Methods

Chlamydia stocks and antigens

Stock preparations of *C. trachomatis* serovar D strain were generated by propagating elementary bodies (EBs) in HeLa cells as previously described [35]. All stocks were titrated on HeLa cell monolayers followed by purification of EBs over renografin gradients [35] and stored at –70°C. Chlamydial antigens were prepared by ultraviolet-inactivation of EBs for 3 h and stored at –70°C.

DNA preparation and polymerase chain reaction amplification of gene sequences

Genomic DNA was purified from 1×10^8 chlamydial EBs using the QIAGEN DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The full length coding sequences for *omp1* and *PorB* genes were amplified from genomic DNA using the Expand™ High Fidelity polymerase chain reaction (PCR) system (a unique mix of Taq and Pwo DNA polymerases; Roche, Mannheim, Germany) and

oligonucleotide primers flanked with specific restriction sites. The primer design was based on published chlamydial sequences (accession number X62920) [25,36-38]. The forward primer (EF-9F) for *omp1* amplification incorporated a *Sal*1 restriction enzyme site (5'-ggcgc**gtcgac**gatgaaaaaactct-3'), while the reverse primer (EF-6R) incorporated a *Pst*I site, 5'-acaat**ctgcag** tattagaagcggaa-3'. *PorB* was amplified using the forward primer, EF-34F incorporating a *KpnI* restriction enzyme site (5'-cgtc**ggtacc**gtaatgagtagcaagc-3'), while the reverse primer (EF-35R) contained a *Dra*II site (5'-gcac**gggacct**cttagaattggaatc-3'). The amplification reaction was carried out in an Eppendorf Gradient Mastercycler (Eppendorf, Hamburg, Germany) and the amplified PCR products of the correct sizes, ~1200 bp (*omp1*) and 1047 bp (*PorB*) were isolated from a 1% agarose gel and purified with the QlAquick PCR purification kit (Qiagen, Valencia, CA, USA).

Construction of plasmid vectors harboring MOMP, PorB and a combination of MOMP and PorB coding sequences

Chlamydial MOMP and PorB were selected for co-delivery in this study because of their immunogenic and structural properties. Thus, plasmid expression vectors were designed harboring the coding sequences of single and multisubunit antigens. Construction of the single subunit plasmid, pCOM2 (plasmid vector harboring MOMP) containing the entire coding sequence for the mature Omp1 protein was achieved by inserting the *omp1* gene in frame with the *E'* and *L'* anchors of vector pKSEL5-2, as previously described [34]. The pPorB (plasmid vector harboring PorB) expression vector was constructed by inserting the amplified *PorB* PCR product (1047 bp) containing the full length *PorB* coding sequence, without the signal peptide, between the *LacZ'* and *E'* genes of vector pKSEL5-2, following digestion with *Kpn*1 and *Dra*II restriction endonucleases. T_4 DNA ligase-mediated ligation was carried out and the ligation mixture was transformed into *Escherichia coli* DH5alpha. The resultant plasmid was designated as pPorB and expresses the PorB protein from the N-terminal -E' anchor. To construct the multisubunit vaccine vector, pOMP-B (plasmid vector harboring a combination of MOMP and PorB coding sequences) carrying the *omp1* and *PorB* genes, the amplified *omp1* PCR product (1200 bp) containing the entire coding sequence, without the signal peptide, and the pPorB plasmid vector were digested with *SalI and PstI* restriction endonucleases (Roche, Indianapolis, IN, USA). The DNA fragments were separated by agarose gel electrophoresis, and purified by using the QlAquick PCR purification kit (Qiagen). The purified *omp1* DNA and pPorB plasmid were ligated with T_4 DNA ligase (Roche) and transformed into *E. coli* DH5alpha. Plasmid preparations from transformants were analyzed by restriction endonuclease digestion and sequencing of the junctions and coding regions of *omp1* and *PorB* genes. The resultant expression plasmid, designated as pOMP-B (Fig. 1), simultaneously expresses PorB and MOMP.

Expression of recombinant MOMP and PorB in *V. cholerae* 01

To generate recombinant *V. cholerae* expressing MOMP and PorB, the pOMP-B plasmid was introduced into *V. cholerae 01* strain HI (HI) by electroporation and positive clones were isolated and designated HMPB. The expression of MOMP or PorB by the transformed *V. cholerae* clones was evaluated by immunoblotting analysis of lysates of recombinant HMPB. Cultures of HMPB and control HI (harboring the parent plasmid pKSEL5-2 only) were grown to mid-log phase in brain heart infusion broth at 37°C and recombinant MOMP expression was induced by the addition of IPTG (isopropyl-beta-D-thiogalactopyranoside; Roche) to a final concentration of 2 mM; the cultures were then incubated for a further 2 h. Culture samples containing expressed proteins were solubilized in sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as previously described [39]. Purified MOMP subjected to the same conditions was included as a positive control. Following protein transfer, recombinant MOMP was detected using the mouse anti-MOMP antibodies kindly provided by Dr. Sukumar Pal, University of California, Irvine, CA, USA. Due to the unavailability of antibodies to PorB, the expression of PorB was evaluated by subcloning the *PorB* gene in pRSET-A to generate pRS-PorB. The expressed protein was then affinity purified using the Probond nickel column chromatography (Invitrogen, Carlsbad, CA, USA) and expression of the recombinant fusion protein was detected using the horseradish peroxidase (HRP)-conjugated Anti-Xpress™ antibody (Invitrogen).

Production of rVCG co-expressing MOMP and PorB

Production of rVCG was carried out as described previously [19]. Briefly, competent HPB and HMPB cells (harboring the cloned *PorB*, and *omp1 and PorB* genes, respectively) were cotransformed with the lysis

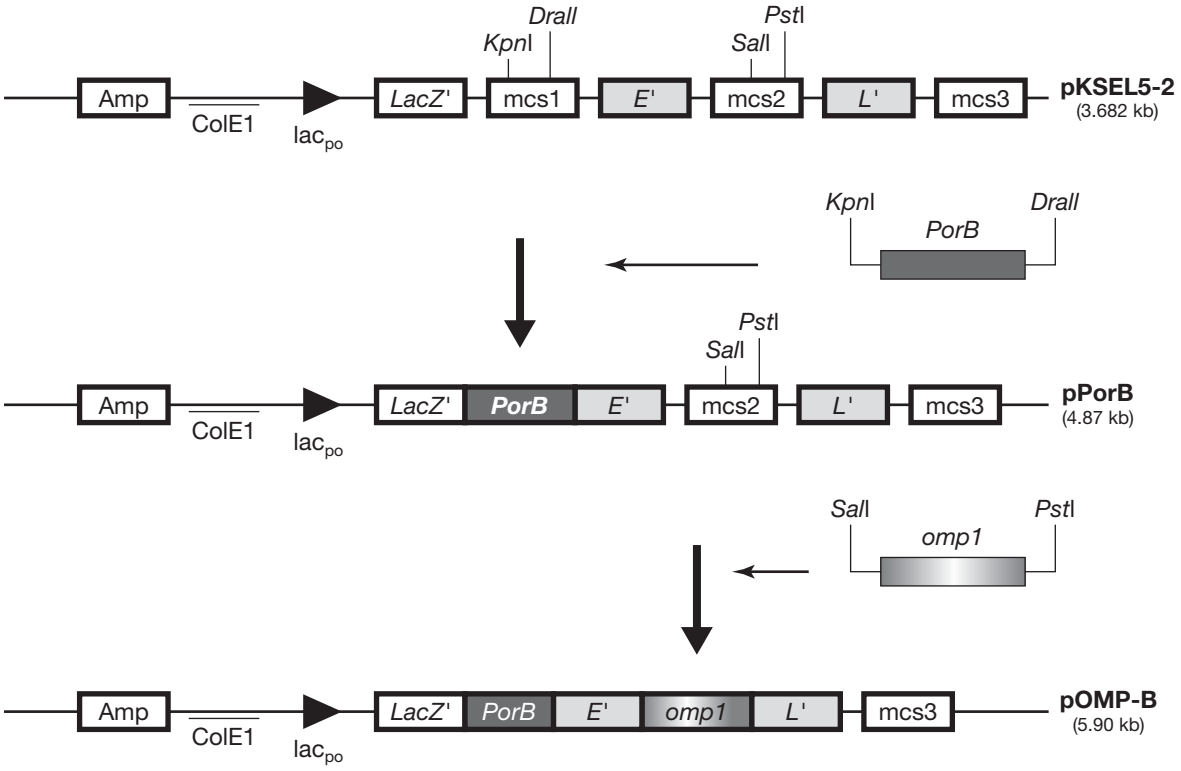

Fig. 1. Construction of the vaccine vectors pPorB (plasmid vector harboring porin B protein [PorB]) and pOMP-B (plasmid vector harboring a combination of major outer membrane protein and PorB coding sequences). The full length coding regions of *PorB* and *omp1,* without the leader sequences, were each amplified by polymerase chain reaction (PCR) from the chromosomal DNA of *Chlamydia trachomatis* serovar D. The *PorB* PCR product was cloned into the membrane targeting vector pKSEL5-2 at the *Kpn*I/*Dra*II sites to generate the plasmid pPorB. The *omp1* PCR product was subsequently cloned into the *Pst*I/*Sal*I sites of pPorB to generate the recombinant plasmid pOMP-B. Expression of *PorB* and *omp1* in pOMP-B are under the transcriptional regulation of the *lac* promoter (*lac$_{po}$*).

plasmid pDKLO1 [40] and the bacterial cells were grown at 37°C to an absorbance of 0.3 at 600 nm. The expression of recombinant proteins was achieved by adding IPTG to a final concentration of 2 mM and grown for 45 min at 37°C. Cell lysis was initiated by the addition of 3-methyl benzoate to induce gene *E* expression. After lysis, cultures were harvested, washed with phosphate-buffered saline (PBS) or a low ionic buffer and lyophilized. The efficiency of E-mediated killing of *V. cholerae* was estimated by plating serial dilutions of samples on BHI agar, as previously described [41]. Results indicated a 100% killing efficiency (i.e., no colony-forming units [CFU] were found on plates at any dilution). Lyophilized VCGs were weighed and the number of CFU/mg of VCG was estimated based on the total number of CFU in the culture medium at the highest absorbance attained prior to lysis. All VCG preparations are stored at room temperature and remain stable for several years without loss of activity.

Mice and immunization

Five- to eight-week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine, USA) were used for all experiments. The animals were housed in laminar flow racks under pathogen-free conditions at a constant temperature of 24°C with a cycle of 12 h of light and 12 h of darkness and were fed mouse chowder and water ad libitum. Mice were otherwise treated in accordance with IACUC (Institutional Animal Care and Use Committee; Memphis, TN, USA), AALAC (American Association for Laboratory Animal Care; Memphis, TN, USA) and NIH (National Institutes of Health; Bethesda, MD, USA) guidelines. Four groups of mice (10 mice per group) were vaccinated intramuscularly with lyophilized rVCG in PBS, as previously described [34]. Briefly, 3 mg of rVCG vaccine formulations or control (VCG alone) in 50 µL PBS was administered on days 0, 14 and 28. The dose of the vaccine was formulated such that 1 mg of lyophilized rVCG or VCG

corresponded to about 2×10^9 CFU. All immunizations were administered under phenobarbitol anesthesia [35].

Measurement of anti-chlamydial mucosal and systemic IgA and IgG antibodies

Blood was collected by periorbital puncture and the serum pooled for each group of animals. Mucosal secretions (vaginal samples) were collected by washing the vagina of each mouse with 100 μL of PBS (pH 7.2) [42]. Trypsin inhibitor (10 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) and ethylenediamine tetra-acetic acid (5×10^{-4} M, Sigma-Aldrich) were added to the samples, followed by centrifugation at 10,000 *g* for 10 min at 4°C to remove the debris. Supernatants were collected and 10^3 M phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 0.01% sodium azide (Sigma-Aldrich) were added. Samples were stored at –80°C until analyzed. The *Chlamydia*-specific antibody titer (secretory IgA and total IgG) in sera and vaginal washes was measured by a modification of the standard enzyme-linked immunosorbent assay procedure described previously [43]. Briefly, Maxisorb 96-well plates (Costar, Cambridge, MA, USA) were coated overnight with 10 μg/mL of *C. trachomatis* serovar D EB in 100 μL of PBS at 4°C. For generating a standard calibration curve, wells were similarly coated in triplicate with IgA or IgG2a standard (0.0, 12.5, 25, 50, 100, 250, 500 and 1000 ng/mL). After washing (PBS-0.05% Tween 20), plates were blocked with 1% bovine serum albumin containing 5% goat serum in PBS and then incubated with 100 μL of serum or 50 μL of vaginal wash in two-fold serial dilutions at 37°C for 2 h. Plates were again washed and incubated with 100 μL of horseradish peroxidase-conjugated goat anti-mouse IgA or IgG2a (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) for 1 h at room temperature. Peroxidase substrate, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) was added and the absorbance was measured at 490 nm on a Spectra Max 250 Microplate Autoreader (Molecular Devices Corp., Sunnyvale, CA, USA). Results correspond to absorbance values as mean concentrations (ng/mL) ± standard deviation (SD) and represent the mean of triplicate wells for each sample set.

Measurement of *Chlamydia*-specific T-cell responses

At two and eight weeks after the last immunization, animals designated for immunogenicity studies were sacrificed, and immune T cell-enriched cells were prepared from the lymphoid tissues (the iliac lymph nodes [ILN], draining the genital tract and the spleen for systemic draining) by nylon wool enrichment procedure, as previously described [44,45]. Purified lymphoid cells contained at least 95% CD3+ cells, as determined by fluorescence-activated cell sorting analysis. Purified T cells were re-stimulated in vitro with antigen-presenting cells (APCs) and chlamydial antigen and the level of *Chlamydia*-specific Th1 or Th2 response was assayed by measuring the antigen-specific interferon-gamma (IFN-γ) or interleukin (IL)-4 production by each cell population, respectively. Briefly, purified T cells were plated in quadruplicate wells of 96-well tissue culture plates at 2×10^5 cells/well and cultured with wild-type APCs and chlamydial antigen (10 μg/mL) for 5 days. Control cultures contained APCs and T cells without antigen. At the end of the incubation period, supernatants were harvested and assayed for cytokines using the Bio-Plex cytokine assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) in combination with the Bio-Plex Manager software (Bio-Rad Laboratories Inc.). The mean and SD of all replicate cultures were calculated.

Protection studies

Groups of mice (10 mice/group) were immunized with vaccine constructs as described above. Three weeks after the last immunization, the animals were challenged intravaginally with 10^7 inclusion-forming units (IFUs) of live *C. trachomatis* serovar D. After challenge, mice were observed twice daily for clinical signs of adverse reaction to infection. To assess the level of infection, cervico-vaginal swabs were collected from each animal every 3 days following the challenge and *C. trachomatis* was isolated from swabs in tissue culture by standard methods [35]. The experiment was repeated twice.

Fertility studies

Groups of mice were immunized with vaccine constructs and challenged intravaginally with live *C. trachomatis* serovar D, as described above. Approximately 6 weeks after the challenge, groups of five female mice were placed in a cage together with a proven breeder male mouse for 18 days [43]. Pregnancy was assessed by determining the increase in weight of the mice, as compared to non-mated mice. Pregnant mice were euthanized at days 14 to 17 of gestation. Non-pregnant mice were euthanized 2 weeks post-mating. The number of embryos in both uterine horns of pregnant mice was counted at the termination point.

Statistical analysis

The levels of IFN-γ in samples from different experiments as well as the level of protection conferred by the vaccine constructs were compared by student's *t* test. Probability values of <0.05 were taken as significant. The results were analyzed for statistical significance with SigmaStat® software (SPSS Inc., Chicago, IL, USA).

Results

Construction of plasmid vectors expressing recombinant chlamydial OMPs

Construction of the multisubunit plasmid expression vector, pOMP-B was accomplished by placing the entire Omp1 coding region, without the signal peptide, under the transcriptional control of the *lac* promoter (lac_{po}) in pPorB and in frame with the *E'* and *L'* anchors (Fig. 1). This resulted in the simultaneous expression of Ompl and PorB as full length E'-L' and lacZ'-E' fusion proteins, respectively. DNA sequencing confirmed that the cloned genes were in frame with the fusion anchors and that the integrity of the newly generated plasmid constructs was maintained. Plasmid pOMP-B (expressing both MOMP and PorB) was introduced into *V. cholerae* O1 strain H1 by electroporation and expression of the recombinant MOMP was confirmed by immunoblotting using mouse monoclonal antibodies to MOMP (Fig. 2). The expression of PorB was evaluated by subcloning the *PorB* gene in pRSET-A to generate pRS-PorB and the 38 kDa PorB protein was detected using the HRP-conjugated Anti-Xpress™ antibody (Invitrogen). Neither chlamydial MOMP nor PorB were detected in the control harboring the pKSEL5-2 targeting vector alone. Recombinant MOMP was detected as a 64 kDa E-MOMP-L fusion protein. These results confirmed that transformants co-expressed the different chlamydial proteins as efficiently as those harboring single subunit constructs.

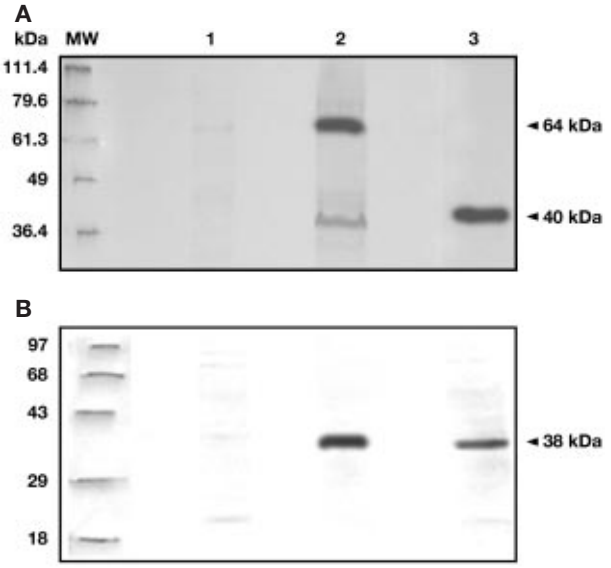

Fig. 2. Expression of major outer membrane protein (MOMP) and porin B protein (PorB). The expression of MOMP or PorB by the *Vibrio* clones was evaluated by immunoblotting analysis. Mid-log phase cultures were induced with isopropyl-beta-D-thiogalactopyranoside for 45 min and cell lysates assayed for recombinant MOMP or recombinant OMP2 expression by Western blot. Expression of MOMP from plasmid pOMP-B (plasmid vector harboring a combination of major outer membrane protein and PorB coding sequences) was detected using the mouse MOMP-specific monoclonal antibody MoPn 40. (A) Lane 1, *Vibrio cholerae* H1 cells containing the plasmid vector pKSEL5-2; lane 2, *V. cholerae* H1 cells harboring the recombinant plasmid, pOMP-B, expressing rMOMP; lane 3, purified MOMP from *Chlamydia trachomatis* elementary bodies. Expression of PorB was evaluated by subcloning the *PorB* gene in pRSET-A to generate pRS-PorB. The expressed protein was affinity purified using the Probond nickel column chromatography and the recombinant fusion protein was detected using the horseradish peroxidase-conjugated Anti-Xpress™ antibody. (B) Lane 1, lysate of *V. cholerae* O1 harboring pRSET-A; lane 2, Probond nickel column affinity-purified PorB; lane 3, lysate of *V. cholerae* O1 expressing PorB from pRS-PorB. Numbers to the left are approximate molecular masses, in kilodaltons (kDa).

Vaccination with rVCG expressing single or multiple chlamydial OMPs

Evaluation of immunogenicity at 2 and 8 weeks post-immunization revealed that all rVCG vaccine constructs harboring chlamydial antigens induced significant local mucosal Th1 responses, detectable in the ILN draining the genital tissues ($p<0.001$). The levels of *Chlamydia*-specific IFN-γ produced by mucosal immune T cells, as a measure of Th1 response, are shown in Fig. 3. The highest response was induced by the rVCG-MOMP/PorB multisubunit vaccine construct indicating, as hypothesized, that the multisubunit vaccine construct harboring MOMP and PorB would induce a superior Th1 immune response compared to either of the single subunit vaccines (rVCG-MOMP or rVCG-PorB). The similarly high concentrations of IFN-γ in spleens of mice immunized with the vaccine constructs sampled at 2 and 8 weeks post-immunization contrast with the significantly lower concentrations of this cytokine in spleens of mice immunized with VCG alone ($p<0.001$) [Fig. 4]. There was no statistical difference between the amounts of IFN-γ produced by mucosal immune

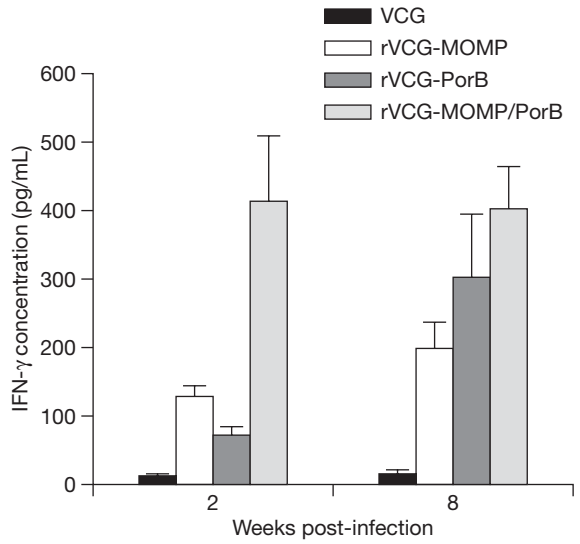

Fig. 3. Induction of local genital mucosal *Chlamydia*-specific T-cell response by recombinant *Vibrio cholerae* ghosts (rVCG) vaccines. Groups of mice were vaccinated intramuscularly, three times, 2 weeks apart with the rVCG vaccine constructs and local interferon-gamma (IFN-γ) response was assessed at 2 and 8 weeks after immunization. The amounts of IFN-γ contained in culture supernatants derived from culture-stimulated T cells from the iliac lymph nodes of immunized mice and controls were measured using Bio-Plex cytokine assay kit in combination with the Bio-Plex Manager software. The concentration of the cytokine in each sample was obtained by extrapolation from a standard calibration curve generated simultaneously. Data were calculated as the mean values (± standard deviation) for triplicate cultures for each experiment. The control cultures without antigen did not contain detectable levels of IFN-γ and so the data were excluded from the results. The results are from three independent experiments. MOMP = major outer membrane protein; PorB = porin B protein.

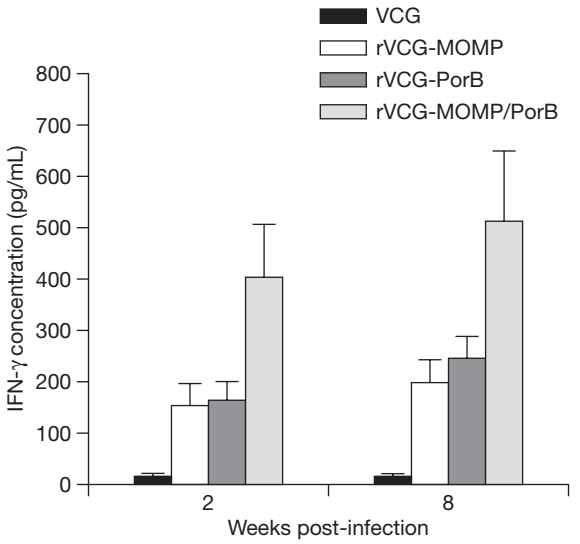

Fig. 4. Induction of *Chlamydia*-specific systemic T-cell response by recombinant *Vibrio cholerae* ghosts (rVCG) vaccines. Groups of mice were immunized as described in Fig. 3 and systemic interferon-gamma (IFN-γ) response was assessed at 2 and 8 weeks post-immunization. The level of T-helper type 1 response induced was determined by measuring the response of *Chlamydia*-specific, IFN-γ-secreting T cells from spleen cells of immunized mice. The amounts of IFN-γ contained in culture supernatants derived from culture-stimulated cells and controls were measured using a Bio-Plex cytokine assay kit in combination with the Bio-Plex Manager software. The concentration of the cytokine in each sample was obtained by extrapolation from a standard calibration curve generated simultaneously. Data were calculated as the mean values (± standard deviation) for triplicate cultures for each experiment. The control cultures without antigen did not contain detectable levels of IFN-γ and so the data were excluded from the results. The results are from three independent experiments. MOMP = major outer membrane protein; PorB = porin B protein.

T cells from the ILN of mice vaccinated with either rVCG-PorB or rVCG-MOMP/PorB, measured at 8 weeks post-immunization ($p>0.05$) [Fig. 3]. This contrasts with the result obtained in splenic tissue, in which the rVCG-MOMP/PorB multisubunit vaccine had a significant immunologic advantage over both single subunit constructs 8 weeks after immunization ($p<0.05$). To assess the magnitude of Th2 response induced following immunization, the amount of *Chlamydia*-specific IL-4 levels produced by ILN and splenic T cells from vaccinated mice was also measured. Very low levels of IL-4 were detected in the ILN and spleens of the different vaccinated groups of mice at 2 and 8 weeks post-immunization. There was no significant difference in IL-4 levels produced by both ILN and splenic T cells among the different vaccinated groups up to 8 weeks post-immunization ($p>0.05$) [Fig. 5]. These results support our previous findings that intramuscular delivery of rVCG-based subunit vaccines expressing chlamydial OMPs polarize towards a Th1 response.

Vaccination with rVCG expressing multiple chlamydial OMPs

Previous studies have indicated that secretory IgA and IgG may have protective roles during genital chlamydial infection [6,44,46]. Thus, IgA and IgG2a responses induced after immunization with rVCG vaccine constructs were measured in serum and vaginal wash samples from vaccinated and unvaccinated animals. There was no detectable antibody in the serum and vaginal washes of mice immunized with VCG alone (control). Although significant antibody levels were

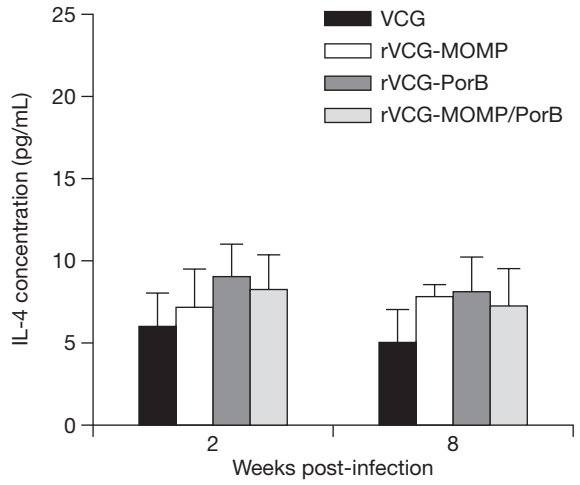

Fig. 5. Induction of local genital mucosal *Chlamydia*-specific T-helper Type 2 response by recombinant *Vibrio cholerae* ghosts (rVCG) vaccines. Groups of mice were immunized as described in Fig. 3 and mucosal interleukin (IL)-4 response was assessed at 2 and 8 weeks post-immunization. The level of T-helper type 2 response induced was determined by measuring the response of *Chlamydia*-specific, IL-4-secreting T cells from iliac lymph nodes of immunized mice. The amounts of IL-4 contained in culture supernatants derived from culture-stimulated cells and controls were measured using a Bio-Plex cytokine assay kit in combination with the Bio-Plex Manager software. The concentration of the cytokine in each sample was obtained by extrapolation from a standard calibration curve generated simultaneously. Data were calculated as the mean values (± standard deviation) for triplicate cultures for each experiment. The control cultures without antigen did not contain detectable levels of IL-4 and so the data were excluded from the results. The results are from three independent experiments. MOMP = major outer membrane protein; PorB = porin B protein.

detected in the vaginal washes of mice immunized with the rVCG vaccine constructs during the first 2 weeks following immunization, the levels of IgG2a in the genital washes were significantly lower ($p<0.01$) than those of IgA (Table 1). In general, antibody levels were significantly higher ($p<0.01$) in serum than in vaginal washes and there was no significant difference between the levels of IgA or IgG2a elicited in the genital tract and serum by the various vaccine constructs ($p>0.01$).

Induction of protective immunity against challenge by rVCG vaccines

We have previously shown that an rVCG vaccine construct expressing two chlamydial OMPs, MOMP and OMP2 conferred a greater level of protective immunity than a single subunit construct, although the protection was suboptimal [34]. Therefore, we examined whether other OMP combinations would induce enhanced protection against intravaginal challenge with the homologous *C. trachomatis* D strain. Protection against challenge was assessed by isolation of chlamydiae from cervicovaginal swabs and comparing the number of IFUs recovered from vaccinated and unvaccinated mice at the indicated time points. The data are presented as Log_{10} IFU/mL ± SD for each experimental group (Fig. 6). The results showed that the mice immunized with the rVCG vaccines were highly resistant to infection as indicated by the low number of IFUs shed and the time taken to resolve the infection, compared to the control mice that received VCG alone. The most significant level of protection was observed in the animals immunized with the rVCG-MOMP/PorB multisubunit construct. All the animals in this group had completely resolved the infection within two weeks after challenge. These results indicate that the multisubunit vaccine has a protective advantage over the single subunit constructs by shortening the time taken to clear the infection.

Table 1. Local and systemic anti-*Chlamydia* antibody response of mice 2 weeks after immunization with recombinant *Vibrio cholerae* ghosts (rVCG) vaccine candidates

Vaccine	*Chlamydia trachomatis*-specific antibody (μg/mL)			
	Vaginal wash		Serum	
	IgA	IgG2a	IgA	IgG2a
VCG	0.05	0.05	0.05	0.05
rVCG-MOMP	3.01	0.75	71.55	560.00
rVCG-PorB	1.60	0.24	178.00	456.00
rVCG-MOMP/PorB	1.77	0.24	170.00	570.00

Abbreviations: IgA = immunoglobulin A; IgG2a = immunoglobulin G2a; MOMP = major outer protein membrane; PorB = porin B protein

Fertility studies

The ultimate requirement of a chlamydial vaccine is to confer protection against infection. However, because clinical presentation of severe sequelae is often the first indication of infection, it has been suggested that even a therapeutic vaccine capable of ameliorating debilitating sequelae would be extremely useful [3-5]. Thus, having demonstrated that immunization with rVCG-based vaccines induced protection against genital chlamydial challenge infection in mice, we also evaluated whether this vaccine approach would protect against *Chlamydia*-induced complications, specifically infertility. Accordingly, six weeks after the intravaginal challenge, mice were allowed to mate with proven

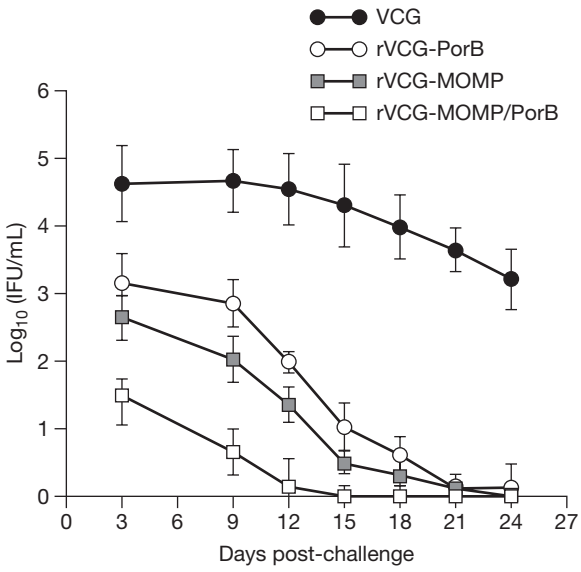

Fig. 6. Induction of protective immunity against challenge by recombinant *Vibrio cholerae* ghosts (rVCG) vaccines. Three weeks after the last immunization, mice immunized with rVCG vaccines, or VCG alone were challenged intravaginally with 10^7 inclusion-forming units (IFUs) of live *Chlamydia trachomatis* serovar D. Infections were monitored by cervicovaginal swabbing of individual animals every 3 days and *Chlamydia* was isolated from swabs in tissue culture. The data show the mean recoverable IFUs, expressed as $\log_{10}$ IFU/mL, for each experimental group ± standard deviation. Student's *t* test was used to compare differences between control and experimental groups at $p<0.05$. MOMP = major outer membrane protein; PorB = porin B protein.

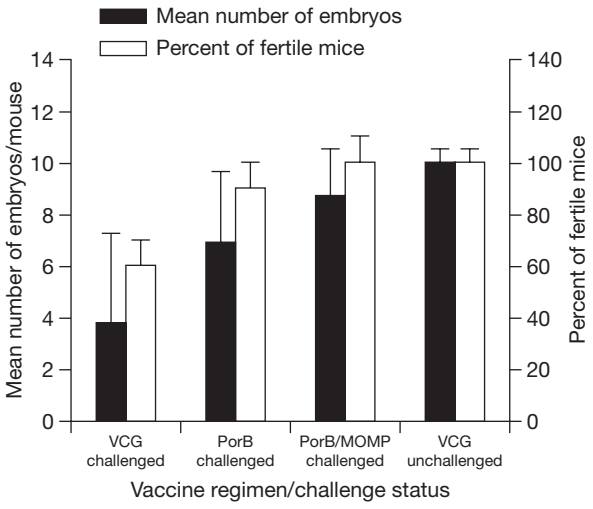

Fig. 7. Protection against *Chlamydia*-induced infertility. Twelve weeks after the intravaginal challenge, mice were mated with proven breeder male mice and the outcome of pregnancy was assessed 18 days later. Fertility rates were evaluated and used as indicators of protective endpoint. The results are presented as the percent of mice that became pregnant and the mean number of embryos in both uterine horns of pregnant mice. VCG = *Vibrio cholerae* ghosts; PorB = porin B protein; MOMP = major outer membrane protein.

breeder males and the outcome of pregnancy was assessed. Two fertility parameters were evaluated and used as indicators of protective immunity endpoint: first, the relative number of pregnant mice; and second, the number of embryos in both uterine horns of immunized and challenged pregnant mice. The results revealed that animals that were protected by vaccination had higher mean number of embryos per mouse and thus had a higher relative protection score (70-90%) compared to VCG-immunized and challenged (negative control) mice (38%) [Fig. 7]. There was a highly significant difference between the fertility parameters recorded for the mice immunized with VCG but left unchallenged (fertility control, 100%) and the negative control group that were similarly immunized and challenged intravaginally with 10^5 IFU of live *Chlamydia* (negative control). Mice immunized with the PorB and PorB/MOMP vaccines showed high fertility rates (90% and 100%, respectively) that were comparable with the positive fertility control group (100%). There was, however, a decrease in the mean number of embryos/mouse (6.9 and 8.7, respectively) compared to the positive control group (Fig. 7).

Discussion

Our results show that the rVCG multisubunit vaccine regimen induced a greater specific Th1 response and conferred a greater level of protective immunity, including protection from infertility, than the single subunit construct. These results confirm and extend previous findings that there is an immunogenic advantage associated with the multisubunit vaccine that induced a higher Th1 immune response and conferred greater protection compared with the single subunit construct [19,34] and has implications in the design and development of chlamydial vaccines targeted for human use.

Current opinion towards the design of a chlamydial vaccine favors the development of a multisubunit regimen that would induce an optimal mucosal and systemic Th1 as well as the accessory antibody responses. These immune effectors are required for a sterilizing immunity and for a rapid microbial clearance during reinfection [5,6]. In addition to a sterilizing protective immunity against a homologous chlamydial

strain, a vaccine capable of inducing an expanded protection against heterologous strains would be preferable. A major challenge, however, is to determine the appropriate combination of immunogenic components that would be co-delivered to the immune system to elicit an optimal immune response. Considering the profile of anti-chlamydial vaccine candidates, studies with chlamydial OMPs have demonstrated greater potential at inducing significant protective immune responses in experimental animals [5]. Current evidence suggests that the chlamydial MOMP is a key determinant of protective immunity with significant contributions from other OMPs, including OMP2 and OMP3 [21], POMPs [27-29] and PorB [30-33]. The identity of PorB was initially predicted by genomic analysis [25]. Expression of the protein has since been demonstrated in chlamydiae and shown to be highly conserved among *C. trachomatis* serovars, as well as *C. pneumoniae*, suggesting its structural conservation across the species. The PorB protein has been shown to be localized to the chlamydial outer membrane surface and functions as a porin [30]. This immunoreactive protein, like MOMP, elicits antibodies that neutralize chlamydial infectivity [33]. These important vaccine properties of PorB make it a potentially viable component of a multisubunit vaccine that may induce sterilizing immunity or improve existing levels of protection.

A multisubunit vaccine formulation would have numerous inherent advantages. Firstly, since results from previous studies revealed inadequacies in the ability of single subunit antigens to provide adequate protection or induce sterilizing immunity [5,34,47], multiple subunits should furnish more T-cell and antibody epitopes and thus increase the magnitude of T- and B-cell responses. Secondly, multivalent vaccines that combine antigens expressed at different stages of the pathogen's life cycle may be desirable to ensure that the organism is adequately confronted by the immune effectors after an infection [5]. Thirdly, although a single antigen could by itself induce protection when delivered with an effective delivery system and an appropriate adjuvant, a vaccine comprising several antigens would conceivably be a better regimen for human applications, especially if some of the subunits contain antigens that are highly conserved across species to confer both homologous and heterologous protective immunity. Thus, the identification of a complementary set of chlamydial subunit antigens that can be effectively administered with an efficient delivery system to induce an optimum protective immune response could meet the current objectives of an efficacious chlamydial vaccine. The rationale for selecting MOMP and PorB in this study was based on the complementary immunologic and structural characteristics of the proteins, including high immunogenicity, outer membrane localization and expression during different stages of the chlamydial developmental cycle.

The rVCG platform as a delivery system has a proven capacity to deliver multiple protein subunits to the immune system with high efficiency and adjuvant advantage. As reported previously [19,34,48], rVCG constitute a highly effective delivery and adjuvant system since they have been shown: 1) to be an efficient carrier of heterologous antigens; 2) to be immunogenic when applied mucosally and systemically; 3) to have an adjuvant effect in mice and rabbits, and possibly humans; and 4) to effectively stimulate both T- and B-cell responses. These properties make the rVCG system a superior alternative to the use of purified recombinant proteins and heat- or chemically-killed vaccines. VCG are non-living and non-pathogenic cell envelopes produced by expression of the cloned *E. coli*-specific lysis gene *E* [41]. Following oligomerization, gene *E* subunits fuse with the inner and outer membranes of the vibrios, forming an *E*-lysis tunnel through which the cytoplasmic contents are expelled [48]. VCG are highly flexible since proteins from any pathogenic organism can be expressed in the bacterial membrane or other cellular compartments prior to ghost production. Since ghosts are non-living entities, they pose no pathogenic threat as they are devoid of the cholera toxin machinery. Although VCG contain intact lipopolysaccharide, previous studies have shown that the dosage of ghosts needed to induce efficient immune responses can be administered without leading to endotoxin-related side effects [49]. The ability of ghosts to function as natural adjuvants is likely related to their surface properties; ghosts retain many of the immune-stimulating proteins, lipids, sugars, and membrane-associated structures of their living counterparts. These may facilitate the recognition of primary antigen-presenting cells by pattern-recognition and toll-like receptors following uptake [50].

Several studies have reported that natural human infection results in strong serological responses to MOMP, while antibodies to PorB are usually low or absent [32,33]. In addition, although mice experimentally challenged with live EBs produce weak anti-PorB antibody responses, immunization with purified PorB results in a potent antibody response [33]. Intramuscular

immunization of mice with single (rVCG-PorB, rVCG-MOMP) and multisubunit (rVCG-MOMP/PorB) vaccine constructs produced vigorous local and systemic Th1 responses, which are required for chlamydial immunity, in addition to significant local mucosal IgA and IgG2a antibody responses, detectable in vaginal secretions. Vaccinated animals were highly protected against intravaginal challenge, including protection from tubal factor infertility, a common *Chlamydia*-induced complication. In addition, there was a moderate protective advantage in vaccinating with the multisubunit design compared to the single subunit constructs. These studies support and extend our earlier observations [19, 34] that intramuscularly immunization of mice with rVCG-based subunit vaccines stimulated a protective Th1 immune response against genital chlamydial infection, with the multisubunit formulation showing a moderate protective advantage over the single subunit constructs. A recent report suggests that infection of mice with human strains of chlamydiae may not be a suitable model for studying immunity [51]. However, human strains have previously been used in the murine genital tract model, and have been shown to cause post-infection sequelae following inoculation with large doses of chlamydiae [18,52,53].

While this study has confirmed the relative advantage of multisubunit vaccine constructs over single subunit vaccines, future studies will compare the degree of long-term protective immunity conferred by several multisubunit vaccine combinations, using *Chlamydia muridarum* as the challenge strain. Such studies will assist in the identification of the ideal combination of subunit antigens that may lead to the development of a reliable chlamydial vaccine.

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