Published in final edited form as:

FEMS Immunol Med Microbiol. 2009 March ; 55(2): 280–291. doi:10.1111/j.1574-695X.2008.00493.x.

A *Vibrio cholerae* ghost-based subunit vaccine induces cross-protective chlamydial immunity that is enhanced by CTA2B, the nontoxic derivative of cholera toxin

Eno E. Ekong¹, Daniel N. Okenu¹, Jayanti Mania-Pramanik^{1,2}, Qing He¹, Joseph U. Igietseme^{1,3}, Godwin A. Ananaba⁴, Deborah Lyn¹, Carolyn Black³, and Francis O. Eko¹

¹ Morehouse School of Medicine, Atlanta, GA, USA

² National Institute for Research in Reproductive Health, Mumbai, India

³ Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA

⁴ Clark Atlanta University, Atlanta, GA, USA

Abstract

The *Vibrio cholerae* ghost (rVCG) platform is an effective carrier and delivery system for designing efficacious *Chlamydia* vaccines. We investigated whether CTA2B, the nontoxic derivative of cholera toxin, can augment protective immunity conferred by an rVCG-based chlamydial vaccine and enhance cross-protection against heterologous chlamydial strains. An rVCG vaccine coexpressing chlamydial major outer membrane protein and CTA2B was genetically constructed and antigens were targeted to the inner membrane of *V. cholerae* before ghost production by gene *E*-mediated lysis. Effective immunomodulation by CTA2B was demonstrated by the ability of the vaccine construct to enhance the activation and maturation of dendritic cells *in vitro*. Also, C57BL/6 mice immunized via mucosal and systemic routes showed increased specific mucosal and systemic antibody and T-helper type-1 (Th1) responses, irrespective of the route. The enhanced production of IFN- γ , but not IL-4 by genital mucosal and splenic T cells, indicated a predominantly Th1 response. Clearance of the *Chlamydia muridarum* vaginal infection was significantly enhanced by codelivery of the vaccine with CTA2B, with the intravaginal route showing a moderate advantage. These results indicate that the rVCG-based vaccine is capable of inducing cross-protection against heterologous chlamydial serovars and that incorporation of mucosal adjuvants, such as CTA2B in the rVCG delivery platform, may enhance protective immunity.

Keywords

vaccination; adjuvant; antibodies; cytokines; protection

Introduction

Chlamydia trachomatis genital infections constitute a major public health challenge due to the significant morbidity that includes pelvic inflammatory disease, ectopic pregnancy and infertility (Schachter & Grayston, 1998; Brunham & Zhang, 1999). The frequent asymptomatic infection, especially in women, precludes early diagnosis and treatment,

making clinical presentation of sequelae often the first indication of infection. In the United States alone, more than \$2 billion is spent annually in the management of chlamydial genital infections (Igietseme *et al.*, 2003). Consequently, a vaccine capable of protecting against infection or even ameliorating severe disease would be the most promising and effective strategy to control *Chlamydia* (Stagg, 1998; Igietseme *et al.*, 2002, 2003). The use of whole chlamydial agents as vaccines is unattractive due to the potential existence of immuno-pathogenic components (Brunham & Peeling, 1994) and the inability to genetically modify chlamydiae to produce safe, attenuated vaccine strains. Thus, the current focus is to develop vaccines based on chlamydial subunit components. The major outer membrane protein (MOMP) is one of the leading subunit vaccine candidates and has been tested in various delivery systems (Tuffrey *et al.*, 1992; Batteiger *et al.*, 1993; Knight *et al.*, 1995). The *Vibrio cholerae* ghost (rVCG) platform is an effective carrier and delivery system for cloned *C. trachomatis* proteins, eliciting chlamydial-specific immune responses and protection following immunization and challenge (Eko *et al.*, 2003, 2004). So far, results from our laboratory and others have shown that vaccines based on single subunits are only partially protective (Eko *et al.*, 2003, 2004; Pal *et al.*, 2003), suggesting that an efficacious chlamydial vaccine may require more efficient delivery vehicles and effective immunomodulation.

The failure of *Chlamydia* vaccines to induce sterilizing immunity may partly be due to their inability to induce an adequate immunostimulatory and favorable cytokine environment. Thus, vaccine strategies that incorporate an effective delivery system and an immunomodulator (mucosal adjuvant) may induce superior protective immunity. Cholera toxin (CT) is one of the most powerful mucosal adjuvants, consisting of one A and five B subunits (Spangler, 1992). High-affinity binding of the B subunit (CTB) to GM1 ganglioside receptors found on most mammalian cells is thought to be essential for adjuvant activity (Elson *et al.*, 1995; George-Chandy *et al.*, 2001). The A subunit is proteolytically cleaved into a toxic A1 and a linking nontoxic A2 polypeptide. The enteric toxicity of the CT holotoxin has precluded its use as a mucosal adjuvant in humans (Hajishengallis *et al.*, 1995), and considerable attention has been devoted to nontoxic mutants or derivatives, such as the nontoxic B (CTB) and A2/B (CTA2B) subunits, as potential adjuvants (Hajishengallis *et al.*, 1995; Pizza *et al.*, 2001). Notably, CTB is a specific protective antigen component of a widely registered oral cholera vaccine (Holmgren *et al.*, 1990). Chemical coupling (Wu & Russell, 1998) or genetic fusion (Martin *et al.*, 2001) of protein antigens to CTB induces mucosal and systemic immune responses to the coupled antigens. CTB induces a polarization of T-helper type-1 (Th1) responses following transcutaneous (Anjuere *et al.*, 2003) and intravaginal immunization (Luci *et al.*, 2006). The highly improved adjuvanticity of CTB when coupled to antigens is thought to be due to efficient presentation of the coupled antigen by antigen-presenting cells (APCs) (Luci *et al.*, 2006). Dendritic cells (DCs) are professional APCs with the unique ability to initiate and control antigen-specific immune responses. Upon activation, immature DCs migrate from peripheral tissues to draining lymph nodes and influence the type of immune response they stimulate through expression of a combination of cell surface and secreted molecules. Secretion of Th1-driving cytokines, such as IL-12, by DCs favors the induction of a Th1-biased response.

In this study, we investigated the ability of CTA2B to augment protective immunity and enhance cross-protection against heterologous chlamydial strains. Thus, we initially characterized the immunomodulatory effect of C2A2B by measuring cytokine production and costimulatory molecule expression by DCs treated *in vitro* with rVCG-MOMP with and without CTA2B. We then evaluated the ability of CTA2B to enhance the protective immunity induced by the *C. trachomatis* serovar D-derived vaccine construct against the heterologous *Chlamydia muridarum* strain in a mouse model of genital chlamydial infection. Our results demonstrated that the codelivery of the vaccine construct with CTA2B induced

enhanced DC maturation and proliferation, as shown by increased expression of major histocompatibility complex II (MHC II) and costimulatory molecules, and production of proinflammatory cytokines. Codelivery of the vaccine construct with CTA2B further enhanced the Th1-inducing capacity and cross-protective ability of the rVCG vaccine construct, irrespective of the route of immunization. These results may have major implications in the design and development of genital and ocular chlamydial vaccines targeted for human use.

Materials and methods

Chlamydia stocks and antigens

Stock preparations of *C. trachomatis* serovar D strain and *C. muridarum* were generated by propagating elementary bodies (EBs) in HeLa cells as described previously (Ramsey *et al.*, 1988). All stocks were titrated on HeLa cell monolayers, followed by purification of EBs over renografin gradients (Ramsey *et al.*, 1988), and stored at -70°C . Chlamydial antigens were prepared by UV inactivation of EBs for 3 h and stored at -70°C .

Mice

Five- to eight-week-old female C57BL/6 mice (Jackson laboratory) 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 Institutional Animal Care and Use Committee (IACUC), American Association for Laboratory Animal Care (AALAC) and National Institutes of Health (NIH) guidelines. The studies were reviewed and approved by Morehouse School of Medicine's IACUC.

DNA preparation and PCR amplification of gene sequences

Genomic DNA was purified from 1×10^8 *C. trachomatis* serovar D EBs using the QIAGEN DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The full-length coding sequence for *omp1* was amplified from genomic DNA using the Expand High Fidelity PCR System (a unique mix of Taq and Pwo DNA polymerases) (Roche, Mannheim, Germany) as described previously (Eko *et al.*, 2004). Also, the *omp1-cta2B* gene fragment containing sequences coding for *C. trachomatis* MOMP genetically fused to CTA2B was similarly amplified from plasmid pUAB084 (a gift from Dr S.R. Singh, Alabama State University). The amplification reactions were carried out in an Eppendorf Gradient Mastercycler (Eppendorf, Hamburg, Germany), and the amplified PCR products of the correct sizes, *c.* 1200 bp (*omp1*) and 1857 bp (*omp1-cta2B*), were isolated from a 1% agarose gel and purified with the QIAquick PCR purification kit (Qiagen).

Construction of plasmid vectors pKS–MOMP and pKSM1–CTA2B harboring MOMP and a fusion of MOMP and CTA2B

The pKS–MOMP expression vector was constructed by inserting the full-length *omp1* coding sequence, without the signal peptide, between the *LacZ'* and the *L'* genes of the inner membrane targeting vector, pKSEL5-2, using KpnI and PstI restriction endonucleases. Following ligation, the ligation mixture was transformed into *Escherichia coli* NM522. The resultant plasmid was designated as pKS–MOMP. Utilizing KpnI and BamHI restriction sites incorporated into the oligonucleotide primers, the amplified *cta2B* fragment was cloned between the *LacZ'* and the *L'* anchors of vector pKSEL5-2 to yield plasmid pKSM1–CTA2B (Fig. 1). Expression of *omp1* and the *omp1-cta2B* fragment from these plasmids is under the transcriptional regulation of the *Lacpo* promoter and the recombinant proteins are expressed as *LacZ'*–*L'* fusion proteins. Thus, unlike conventional adjuvants, CTA2B is

encoded by the vector along with the encoded antigen (MOMP) in a bicistronic construct and delivered by rVCG.

Detection of recombinant MOMP and MOMP–CTA2B by Western blot analysis

Plasmids pKS–MOMP and pKSM1–CTA2B were isolated from *E. coli* NM522 and separately introduced into *V. cholerae* 01 strain H1 by electroporation, and clones containing the respective plasmids were isolated. Expression of the recombinant proteins (rMOMP or rMOMP–CTA2B) by the *Vibrio* clones was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and confirmed by Western blot analysis as described previously (Eko *et al.*, 2000) using the mouse anti-MOMP monoclonal antibodies (MoPn 40, a generous gift from Dr Sukumar Pal).

Production of rVCG

Production of rVCG was carried out essentially as described previously (Eko *et al.*, 2003). Briefly, competent H1 cells (harboring plasmid pKS–MOMP or pKSM1–CTA2B) were cotransformed with the lysis plasmid pDKLO1 (Kloos *et al.*, 1994) and bacterial cells were grown at 37 °C to an A_{600 nm} of 0.30. The expression of recombinant proteins was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 2 mM and cell lysis was achieved 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 vibrios was estimated by plating serial dilutions of samples on brain heart infusion agar as described previously (Eko *et al.*, 2000). Results indicated a 100% killing efficiency (i.e. no CFU were observed on plates at all dilutions). Lyophilized VCGs were weighed and the number of CFU per milligram of VCG was estimated based on the total number of CFU in the culture medium at the highest absorbance attained before lysis. Ghost preparations were stored at room temperature until use.

Phenotypic analysis of DCs pulsed with rVCG–MOMP vaccine constructs

DCs were generated from the bone marrow (BMDC) by a standard procedure as described previously (Su *et al.*, 1998). Briefly, bone marrow cells were depleted of RBC and lymphocytes and cultured for 5 days in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, 2-ME, HEPES and gentamicin and containing recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF, 10 ng mL⁻¹; PeproTech, Rocky Hill, NJ) and IL-4 (5 ng mL⁻¹; PeproTech) (DC medium). Following DC enrichment and washing, purified DCs were resuspended in DC medium at a concentration of 5 × 10⁵ cells mL⁻¹ and cultured for 24 h with or without 1 mg of rVCG–MOMP/CTA2B. Staining was carried out using PE-conjugated anti-CD11c, fluorescein isothiocyanate-conjugated anti-I-Ab, anti-CD14, anti-CD40, anti-CD71, anti-CD80, anti-CD83 or anti-CD86 antibodies (all from PharMingen, San Diego, CA), and cells were analyzed by flow cytometry. Marker expression was assessed on gated CD11c⁺ cells.

Analysis of cytokine profile of antigen-pulsed DCs

Culture supernatants of DCs pulsed with rVCG vaccine constructs for 48 h were assayed for IL-2, IL-4, IL-5, IL-12 (p40), IFN-γ, TNF-α and GM-CSF by cytokine enzyme-linked immunosorbent assay (ELISA) as described below.

Immunization and sample collection

Groups of mice were vaccinated by the intramuscular, intravaginal and transcutaneous routes with 2 mg of lyophilized rVCG vaccine formulations in 50 μL PBS each on days 0, 14 and 28. The dose of the vaccine was formulated such that 1 mg of lyophilized rVCG

corresponded to about 2×10^9 CFU. Intramuscular and intravaginal immunizations were carried out as described previously (Eko *et al.*, 2004; #4135) (Igietsme *et al.*, 1998). For transcutaneous immunization, mice were shaved on the abdomen 24 h before skin inoculation and 50 μ L of the vaccine formulation was placed on the shaved abdominal skin over a 4-cm² surface area and left for 1 h. Each group/route included a control consisting of rVCG expressing glycoprotein D from HSV-2 (rVCG-gD2), a chlamydial irrelevant antigen. All immunizations were administered while under phenobarbitol (Sigma-Aldrich, Milwaukee, WI) anesthesia. Two weeks after the last immunization, serum and vaginal lavage samples were collected and stored as described previously (Macmillan *et al.*, 2007).

Purification of CD4⁺ T cells

Immune T cell-enriched cells were prepared from the lymphoid tissues [iliac lymph nodes (ILN) draining the genital tract and spleen for systemic draining] of immunized and control mice by the nylon wool enrichment procedure described previously (Igietsme & Rank, 1991). Positive selection of CD4⁺ T cells was achieved by a Midi magnetic bead-activated cell sorting (MidiMACS) purification system using CD4⁺-specific MACS microbeads (L3T4) (Miltenyi Biotech, Auburn, CA). The purity of the CD4⁺ T cell population was determined to be at least >95% by flow cytometry using an APC-conjugated anti-CD4 monoclonal antibody (PharMingen). A separate pool of splenocytes prepared from naive animals and treated with mitomycin C (25 μ g 10^{-7} cells) for 20 min was used as a source of APCs. The experiment was repeated to contain at least 12 mice per group.

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

The amounts of *Chlamydia*-specific antibodies (secretory IgA and IgG2a) in sera and vaginal washes were measured by a standard ELISA procedure described previously (Macmillan *et al.*, 2007). Results, generated simultaneously with the standard curve, display data sets corresponding to absorbance values as mean concentrations (ng mL⁻¹) $\pm$ SD and represent the mean values from triplicate experiments.

Detection of cytokine production by ELISA

The level of *Chlamydia*-specific Th1 or Th2 response was assayed by measuring the antigen-specific IFN- γ or IL-4 production by each cell population, respectively. Briefly, purified CD4⁺ T cells were plated in quadruplicate wells of 96-well tissue culture plates at 2×10^5 cells per well and cultured with APCs and UV-irradiated *C. trachomatis* EBs as antigen (10 μ g mL⁻¹) 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, in combination with the Bio-Plex Manager software (Bio-Rad, Hercules, CA). The mean and SD of all replicate cultures were calculated.

Intravaginal challenge by live *Chlamydia*

Two weeks after the last immunization, the animals were administered Depo Provera (2.5 mg per mouse; UpJohn Co., Kalamazoo, MI) to stabilize the estrous cycle and facilitate a productive infection and challenged intravaginally 1 week later with 10^7 inclusion forming units (IFUs) of live *C. muridarum* (MoPn). After the challenge, mice were observed twice daily for clinical signs of adverse reaction to infection. To assess the level of infection, cervicovaginal swabs were collected from each animal every 3 days following the challenge and *C. muridarum* was isolated from swabs in tissue culture by standard methods (Ramsey *et al.*, 1988). The experiment was repeated to contain 12–15 mice per group.

Statistical analysis

The results were expressed as mean $\pm$ SD. Data were analyzed by the Student's *t*-test and ANOVA using SIGMA STAT software (SPSS Inc.). A value of $P < 0.05$ was considered a significant difference.

Results

Construction of vaccine vectors coexpressing recombinant chlamydial MOMP and CTA2B

To test the effect of CTA2B as a compatible immunomodulator for rVCG-based chlamydial vaccines, we constructed the plasmid expression vectors pKS-MOMP and pKSM1-CTA2B by placing the *Omp1* coding region, with and without CTA2B sequences, respectively, under the transcriptional control of the *lac* promoter (*lac_{po}*) in pKSEL5-2 and in frame with the *LacZ'* and *L'* anchors (Fig. 1). The full-length *ompl* and *ompl-cta2b* fusion genes were thus expressed as *LacZ'*-*L'* fusion proteins, respectively (Fig. 1). 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. Expression of the recombinant fusion proteins, MOMP-*L'* (54 kDa) and MOMP/CTA2B-*L'* (74 kDa), was confirmed by Western blot analysis using mouse mAbs to MOMP (Fig. 2).

The rVCG-MOMP vaccine constructs promote DC maturation and proliferation *in vitro*

The levels of costimulatory molecules and MHC on the surface of DCs influence the induction of a T cell response as well as the magnitude and pattern of cytokine response (Lenschow *et al.*, 1996). To determine whether rVCG vaccine constructs influenced the phenotype of DCs, DCs were cultured for 24 h with or without vaccine constructs and quantified for expression of costimulatory molecules and MHC II by flow cytometry. Treatment of DCs with vaccine constructs resulted in the induction of phenotypic changes in the DCs. DCs pulsed with rVCG-MOMP resulted in the upregulated expression of MHC II, CD40, CD80 and CD86 that was further enhanced by codelivery with CTA2B (Fig. 3a). On the other hand, the expression of CD83 and CD14 was downregulated following treatment with rVCG-MOMP with and without CTA2B. Furthermore, expression of CD71 was upregulated when DCs were pulsed with rVCG-MOMP but was downregulated when pulsed with rVCG-MOMP coexpressing CTA2B (Fig. 3a). These results indicate that rVCG-MOMP induces the maturation and proliferation of DCs that is further enhanced by coadministration with CTA2B.

The rVCG vaccines induce enhanced Th1-promoting cytokine secretion *in vitro*

We evaluated the ability of DCs to secrete cytokines when pulsed with rVCG vaccines and assessed the effect of CTA2B on the magnitude of cytokines produced. Cytokine secretion was measured in 72-h culture supernatants from DC incubated with rVCG vaccines. The rVCG-MOMP vaccine induced modest levels of IL-2, IL-4, IL-5 and IFN- γ , and appreciable levels of IL-10, IL-12p40, GM-CSF and TNF- α that were increased in the presence of CTA2B (Fig. 3b).

Induction of *Chlamydia*-specific T cell-mediated immune responses

Cell-mediated immune responses were evaluated 2 weeks postimmunization. Purified CD4⁺ T cells from the ILN and spleens of immunized mice were cultured *in vitro* with or without chlamydial antigen and Th1 or Th2 response was measured in terms of the amount of antigen-specific IFN- γ or IL-4 produced by activated T cells, respectively. Compared with T cell cultures from mice immunized with the control vaccine construct, rVCG-gD2, cells from mice immunized with rVCG-MOMP/CTA2B or rVCG-MOMP responded significantly to restimulation with chlamydial antigen. The amount of *Chlamydia*-specific

IFN- γ produced by both mucosal and systemic immune T cells from rVCG-MOMP/CTA2B-immunized mice was significantly higher ($P > 0.05$) than that from mice immunized with rVCG-MOMP alone (Fig. 4a). To determine whether immunization with the human *C. trachomatis* MOMP-based vaccine constructs will induce cross-reactivity against the heterologous *C. muridarum* (MoPn) strain, immune T cells were restimulated in culture with UV-irradiated *C. muridarum* (MoPn) antigen. The results showed that the immune T cells responded significantly to restimulation with the heterologous *C. muridarum* antigen (Fig. 4b), suggesting a broad specificity of the CMI response. In contrast with IFN- γ , the amount of *Chlamydia*-specific IL-4 produced by ILN and splenic T cells from mice vaccinated via the different routes remained at baseline levels (data not shown).

Local mucosal and humoral antibody responses to rVCG vaccines

The humoral immune responses induced 2 weeks following immunization with the vaccine constructs and control (rVCG-gD2) were measured by titrating the serum and vaginal secretions of vaccinated mice against chlamydial antigen. Compared with controls, mice immunized with rVCG-MOMP or rVCG-MOMP/CTA2B developed significant antigen-specific secretory IgA and IgG2a antibodies in both vaginal secretions and serum, irrespective of the route of immunization ($P < 0.05$) (Fig. 5). In addition, antibody levels were higher when the vaccine was codelivered with CTA2B, irrespective of the route of immunization (Fig. 5). In general, the mucosal secretory IgA antibody levels were significantly higher ($P < 0.05$) than IgG2a levels (Fig. 5a). On the other hand, the amount of IgG2a antibody elicited in serum was significantly higher ($P < 0.05$) than IgA levels, irrespective of the route of immunization (Fig. 5b). The results indicated that the inclusion of CTA2B boosted antibody induction against chlamydial antigens.

Effect of CTA2B adjuvant on the efficacy of the rVCG vaccine in mice

Two weeks after the last immunization, the animals were challenged intravaginally with 10^7 IFU of live *C. muridarum*. Protection against the challenge was assessed by isolation of chlamydiae from cervicovaginal swabs and comparing the number of IFUs recovered from vaccinated and control mice at the indicated time points. The data are presented as Log_{10} IFU $\text{mL}^{-1} \pm \text{SD}$ for each experimental group (Fig. 6). Immunized mice were highly resistant to infection as indicated by the significant reduction in the numbers of chlamydial IFUs shed as early as 3 days postchallenge and the abbreviated time course of the infection compared with controls, irrespective of the route of immunization (Fig. 6a). The protective ability of the vaccine was significantly enhanced by codelivery with CTA2B, with the intravaginal route showing a protective advantage (Fig. 6b). All the animals in this group had completely resolved the infection 15 days postchallenge (Table 1), accompanied by a 4.5-log difference in the number of IFUs recovered compared with controls (Fig. 6b). In contrast, 50% of the rVCG-MOMP-immunized and 100% of the control mice were still infected at this time point (Table 1). Taken together, the results indicate that codelivery of the vaccine with CTA2B significantly enhanced protective immunity and that the serovar D-derived vaccine construct was capable of inducing cross-protection against the heterologous *C. muridarum* strain.

Discussion

The novel recombinant rVCG delivery platform is an effective carrier and delivery system for cloned *C. trachomatis* proteins, inducing significant immune responses and protection in the absence of added adjuvants (Eko *et al.*, 2003, 2004; Jechlinger *et al.*, 2005). In this study, we tested the hypothesis that the incorporation of a mucosal adjuvant into the rVCG platform will improve antigen delivery, enhance protective immunity and lead to cross-protection against heterologous chlamydial strains. Previous studies suggested that CTA2B

may constitute a suitable adjuvant for inducing local Th1 immune responses in the genital tract (Hajishengallis *et al.*, 1995; Pizza *et al.*, 2001). Our results demonstrate that the codelivery of the vaccine construct with CTA2B induced enhanced DC maturation and proliferation, as shown by increased expression of MHC II and costimulatory molecules, and production of proinflammatory cytokines. In addition, codelivery of the vaccine with CTA2B accelerated the maturation process as indicated by the upregulated expression of CD71. These results confirm the potent immunomodulatory ability of CTA2B in activating DC to express the molecules critically involved in T cell activation, expansion and differentiation.

Irrespective of the route of immunization, codelivery of the vaccine construct with CTA2B further enhanced the Th1-inducing capacity of the rVCG vaccine construct. The cross-reactivity of *C. trachomatis*-specific immunity against the heterologous *C. muridarum* (MoPn) suggested the potential of rVCG–MOMP/CTA2B as a broad-spectrum chlamydial vaccine. In addition, codelivery of the vaccine with CTA2B enhanced the clearance of *C. muridarum* from the genital tract of immunized mice, with the intravaginal route of immunization showing a protective advantage. A number of studies have shown that immunization via the intragastric, intranasal, intravaginal or transcutaneous route using the nontoxic B subunit of CT induces mucosal and systemic immune responses to antigen across many species (Johansson *et al.*, 1998; Gockel *et al.*, 2000; Luci *et al.*, 2006; Singh *et al.*, 2006). Also, intranasal and intravaginal delivery of chlamydial MOMP genetically linked with CTA2B resulted in enhanced clearance of *C. trachomatis* following an intravaginal challenge (Singh *et al.*, 2006).

It should be noted that in this study, unlike conventional adjuvant systems, CTA2B is encoded by the vector along with the antigen (MOMP) in a bicistronic construct. It would appear that the type of immune response elicited by antigens coadministered with nontoxic CT derivatives depends on factors such as the type of antigen coupling (genetic or chemical), the nature of the antigen and the delivery system. In support of this concept, recent studies indicate that immune responses induced by native or chimeric enterotoxins could differ if these enterotoxins are used as adjuvants for more complex antigens or antigens with intrinsic biological activity (Bowman & Clements, 2001; Boyaka *et al.*, 2003). Thus, the efficacy of the MOMP/CTA2B fusion protein in antichlamydial immunity observed in this study may partly be due to the immunomodulatory effects of the VCG carrier/delivery system (Eko *et al.*, 2003, 2004; Macmillan *et al.*, 2007; Riedmann *et al.*, 2007) or the combined effects of the VCG carrier and CTA2B. These findings are in agreement with the suggestion that the development of an efficacious chlamydial vaccine may require the use of more effective delivery systems and effective immunomodulation and provides useful information for designing an effective chlamydial subunit vaccine.

Our results show that the rVCG vaccine constructs exploited the natural (intravaginal) as well as an alternative systemic pathway (intramuscular or transcutaneous) for induction of mucosal immunity, showing that effective systemic delivery of antigens with and without adjuvants also stimulates significant mucosal immunity (Anjuere *et al.*, 2003; Eko *et al.*, 2004; Skelding *et al.*, 2006). In contrast with our findings that intravaginal immunization with rVCG–MOMP/CTA2B elicited significant protective immunity against a chlamydial genital challenge, a similar recent study (Singh *et al.*, 2006) reported no reduction in chlamydial IFUs. These differences could be due to the effect of the immunostimulatory properties of the VCG delivery system. Recently, transcutaneous immunization (TCI) with CTB was reported to favor the development of vigorous systemic Th1 cellular responses, but weak systemic antibody responses to the coadministered antigen (Anjuere *et al.*, 2003). Also, Skelding *et al.* (2006) demonstrated that both intranasal and transcutaneous immunization with MOMP combined with adjuvants elicited strong cell-mediated and

humoral immunity that protected mice against respiratory *Chlamydia* infection. While intranasal immunization induced the greatest protection, TCI also greatly reduced the degree of weight loss and recoverable *Chlamydia* IFU at the peak of infection (Skelding *et al.*, 2006).

Protection against *Chlamydia* following natural or experimental infection is strongly dependent on the production of IFN γ by Th1 CD4⁺ T cells (Morrison & Caldwell, 2002; Igietseme *et al.*, 2003; Kelly, 2003). A number of studies have demonstrated that *in vitro* neutralizing antibodies do not transfer passive protection to mice *in vivo* (Su *et al.*, 1995) and there is limited microbial clearance in experimental and clinical infections even in the presence of high levels of antibodies (Cotter *et al.*, 1997; Johansson *et al.*, 1997a, b; Igietseme *et al.*, 2002; Loomis & Starnbach, 2002). However, other studies (Rank & Batteiger, 1989; Zhang *et al.*, 1989; Cotter *et al.*, 1995) support an *in vivo* neutralization role for antibodies. Also, although protection is mediated primarily by IFN- γ -producing Th1 cells, the absence of antibodies results in inefficient microbial clearance (Morrison & Caldwell, 2002; Igietseme *et al.*, 2003). Furthermore, antibodies of the IgG2a and IgA isotypes have been shown to enhance Th1 activation against chlamydiae (Morrison *et al.*, 2000; Moore *et al.*, 2002; Igietseme *et al.*, 2004). Thus, the predominant role of antibodies in chlamydial clearance has been suggested to be in resistance to reinfection, by enhancing the uptake, processing and presentation of chlamydial antigens by APCs for rapid Th1 activation and clearance of infection (Moore *et al.*, 2002). Also, antibody may mediate protection against the extracellular chlamydial EB by blocking the initial attachment of *Chlamydia* to epithelial cells, thereby enhancing the killing of opsonized *Chlamydia* by phagocytic cells and promoting antibody-dependent cellular cytotoxicity against infected cells (Moore *et al.*, 2002, 2003). Therefore, while the significant reduction in chlamydial shedding as early as 3 days postchallenge, observed in this study, may suggest an *in vivo* neutralizing role for antibodies, it should be noted that a high frequency of *Chlamydia*-specific Th1 cells also appears in the genital mucosa as early as 1–3 days after challenge (He *et al.*, 2007). Thus, while the role of humoral immunity in chlamydial control is still unfolding, B cells and CD4⁺ T cells may function synergistically in providing immunity in the mouse model of chlamydial infection (Morrison *et al.*, 2000).

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. One of the significant findings from this study is the cross-protection afforded by the human serovar D-derived MOMP-based vaccine against the heterologous *C. muridarum*, suggesting that the vaccine may induce broad-based protection against other chlamydial serovars. Given the level of reduction in chlamydial shedding at very early time points and that neutralizing antibodies may play a role even against heterologous challenge, the data support the desirable possibility that in addition to T cell epitopes, conserved neutralizing B cell epitopes exist across chlamydial serovars. Thus, the current opinion towards the design of a chlamydial vaccine favors the development of a regimen that would induce an optimal mucosal and systemic Th1 as well as mucosal antibody responses.

Acknowledgments

We thank Dr Shree R. Singh for his gift of the plasmid vector pUAB084 and Sukumar Pal for providing us with mAbs MoPn 40 used for Western blot analysis.

This work was supported by a Public Health Service grant AI41231 from the National Institutes of Health. The investigation was conducted in a facility constructed with support from Research Facilities Improvement Grant #1 C06 RR18386 from the National Center for Research Resources, National Institutes of Health.

References

- Anjuere F, George-Chandy A, Auduant F, Rousseau D, Holmgren J, Czerkinsky C. Transcutaneous immunization with cholera toxin B subunit adjuvant suppresses IgE antibody responses via selective induction of Th1 immune responses. *J Immunol.* 2003; 170:1586–1592. [PubMed: 12538724]
- Batteiger BE, Rank RG, Bavoi PM, Soderberg LSF. Partial protection against genital reinfection by immunization of guinea-pigs with isolated outer-membrane proteins of the chlamydial agent of guinea-pig inclusion conjunctivitis. *J Gen Microbiol.* 1993; 139:2965–2972. [PubMed: 7510322]
- Bowman CC, Clements JD. Differential biological and adjuvant activities of cholera toxin and *Escherichia coli* heat-labile enterotoxin hybrids. *Infect Immun.* 2001; 69:1528–1535. [PubMed: 11179323]
- Boyaka PN, Ohmura M, Fujihashi K, Koga T, Yamamoto M, Kweon M-N, Takeda Y, Jackson RJ, Kiyono H, Yuki Y, McGhee JR. Chimeras of labile toxin one and cholera toxin retain mucosal adjuvanticity and direct Th cell subsets via their B subunit. *J Immunol.* 2003; 170:454–462. [PubMed: 12496431]
- Brunham RC, Peeling RW. *Chlamydia trachomatis* antigens: role in immunity and pathogenesis. *Infect Agent Dis.* 1994; 3:218–233.
- Brunham RC, Zhang DJ. Transgene as vaccine for *Chlamydia*. *Am Heart J.* 1999; 138:S519–S522. [PubMed: 10539864]
- Cotter TW, Meng Q, Shen Z-L, Zhang Y-X, Su H, Caldwell HD. Protective efficacy of outer membrane protein-specific immunoglobulin A (IgA) and IgG monoclonal antibodies in a murine model of *Chlamydia trachomatis* genital tract infection. *Infect Immun.* 1995; 63:4704–4714. [PubMed: 7591126]
- Cotter TW, Ramsey KH, Miranpuri GS, Poulsen CE, Byrne GI. Dissemination of *Chlamydia trachomatis* chronic genital tract infection in gamma interferon gene knockout mice. *Infect Immun.* 1997; 65:2145–2152. [PubMed: 9169744]
- Eko F, Lubitz W, McMillian L, Ramey K, Moore T, Ananaba GA, Lyn D, Black CM, Igietseme JU. Recombinant *Vibrio cholerae* ghosts as a delivery vehicle for vaccinating against *Chlamydia trachomatis*. *Vaccine.* 2003; 21:1694–1703. [PubMed: 12639492]
- Eko FO, Mayr UB, Attridge SR, Lubitz W. Characterization and immunogenicity of *Vibrio cholerae* ghosts expressing toxin-coregulated pili. *J Biotechnol.* 2000; 83:115–123. [PubMed: 11000467]
- Eko FO, He Q, Brown T, et al. A novel recombinant multisubunit vaccine against chlamydia. *J Immunol.* 2004; 173:3375–3382. [PubMed: 15322201]
- Elson CO, Holland SP, Dertzbaugh MT, Cuff CF, Anderson AO. Morphologic and functional alterations of mucosal T cells by cholera toxin and its B subunit. *J Immunol.* 1995; 154:1032–1040. [PubMed: 7822780]
- George-Chandy A, Eriksson K, Lebens M, Nordstorm I, Schon E, Holmgren J. Cholera toxin B subunit as a carrier molecule promotes antigen presentation and increases CD40 and CD86 expression on antigen-presenting cells. *Infect Immun.* 2001; 69:5716–5725. [PubMed: 11500448]
- Gockel CM, Baob S, Beagley KW. Transcutaneous immunization induces mucosal and systemic immunity: a potent method for targeting immunity to the female reproductive tract. *Molecular Immunology.* 2000; 37:537–544. [PubMed: 11163403]
- Hajishengallis G, Hollingshead SK, Koga T, Russell MW. Mucosal immunization with a bacterial protein antigen genetically coupled to cholera toxin A2/B subunits. *J Immunol.* 1995; 154:4322–4332. [PubMed: 7722290]
- He Q, Martinez-Sobrido L, Eko FO, Palese P, et al. Live-attenuated influenza viruses as delivery vectors for *Chlamydia* vaccines. *Immunology.* 2007; 122:28–37. [PubMed: 17451464]
- Holmgren, J.; Jertborn, M.; Svennerholm, A-M.; Sack, D.; Clemens, J. The oral B subunit/inactivated whole cell vaccine. In: Woodrow, G.; Levine, M., editors. *New Generation Vaccines*. Marcel Dekker Inc; New York: 1990. p. 289-304.
- Igietseme JU, Rank RG. Susceptibility to reinfection after a primary chlamydial genital infection is associated with a decrease of antigen-specific T cells in the genital tract. *Infect Immun.* 1991; 59:1346–1351. [PubMed: 1900810]

- Igietseme JU, Uriri IM, Kumar SN, Ananaba GA, Ojior OO, Momodu IA, Canadal DH, Black CM. Route of infection that induces a high intensity of gamma interferon-secreting T cells in the genital tract produces optimal protection against *Chlamydia trachomatis* infection in mice. *Infect Immun*. 1998; 66:4030–4035. [PubMed: 9712743]
- Igietseme JU, Black CM, Caldwell HD. Chlamydia vaccines: strategies and status. *BioDrugs*. 2002; 16:19–35. [PubMed: 11908999]
- Igietseme JU, Eko FO, Black CM. Contemporary approaches to designing and evaluating vaccines against Chlamydia. *Expert Rev Vaccines*. 2003; 2:129–146. [PubMed: 12901604]
- Igietseme JU, Eko FO, He Q, Black CM. Antibody regulation of T-cell immunity: implications for vaccine strategies against intracellular pathogens. *Expert Rev Vaccines*. 2004; 3:23–34. [PubMed: 14761241]
- Jechlinger W, Haller C, Resch S, Hofmann A, Szostak MP, Lubitz W. Comparative immunogenicity of the Hepatitis B virus core 149 antigen displayed on the inner and outer membrane of bacterial ghosts. *Vaccine*. 2005; 23:3609. [PubMed: 15855021]
- Johansson E-L, Rask C, Fredriksson M, Eriksson K, Czerkinsky C, Holmgren J. Antibodies and antibody-secreting cells in the female genital tract after vaginal or intranasal immunization with cholera toxin B subunit or conjugates. *Infect Immun*. 1998; 66:514–520. [PubMed: 9453604]
- Johansson M, Schon K, Ward M, Lycke N. Genital tract infection with *Chlamydia trachomatis* fails to induce protective immunity in gamma interferon receptor-deficient mice despite a strong local immunoglobulin A response. *Infect Immun*. 1997a; 65:1032–1044. [PubMed: 9038313]
- Johansson M, Schon K, Ward M, Lycke N. Studies in knockout mice reveal that anti-chlamydial protection requires TH1 cells producing IFN-gamma: is this true for human? *Scand J Immunol*. 1997b; 46:546–552. [PubMed: 9420616]
- Kelly KA. Cellular immunity and Chlamydia genital infection: induction, recruitment, and effector mechanisms. *Int Rev Immunol*. 2003; 22:3. [PubMed: 12710502]
- Kloos DU, Stratz M, Guttler A, Steffan RJ, Timmis KN. Inducible cell lysis system for the study of natural transformation and environmental fate of DNA release and cell death. *J Bacteriol*. 1994; 176:7352–7361. [PubMed: 7961508]
- Knight SC, Iqbal S, Woods C, Stagg A, Ward ME, Tuffrey M. A peptide of *Chlamydia trachomatis* shown to be a primary T-cell epitope *in vitro* induces cell-mediated immunity *in vivo*. *Immunology*. 1995; 85:8–15. [PubMed: 7543450]
- Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. *Annu Rev Immunol*. 1996; 14:233–258. [PubMed: 8717514]
- Loomis WP, Starnbach MN. T cell responses to *Chlamydia trachomatis*. *Curr Opin Microbiol*. 2002; 5:87. [PubMed: 11834375]
- Luci C, Hervouet C, Rousseau D, Holmgren J, Czerkinsky C, Anjuere F. Dendritic cell-mediated induction of mucosal cytotoxic responses following intravaginal immunization with the nontoxic B subunit of cholera toxin. *J Immunol*. 2006; 176:2749–2757. [PubMed: 16493030]
- Macmillan L, Ifere G, He Q, Igietseme JU, Keller K, Okenu D, Eko F. A recombinant multivalent combination vaccine protects against *Chlamydia* and genital herpes. *FEMS Immunol Med Microbiol*. 2007; 49:46–55. [PubMed: 17094789]
- Martin M, Hajishengallis G, Metzger DJ, Michalek SM, Connell TD, Russell MW. Recombinant antigen-enterotoxin A2/B chimeric mucosal immunogens differentially enhance antibody responses and B7-dependent costimulation of CD4⁺ T cells. *Infect Immun*. 2001; 69:252–261. [PubMed: 11119513]
- Moore T, Ananaba GA, Bolier J, Bowers S, Belay T, Eko FO, Igietseme JU. Fc receptor regulation of protective immunity against *Chlamydia trachomatis*. *Immunology*. 2002; 105:213–221. [PubMed: 11872097]
- Moore T, Ekworomadu C, Eko F, et al. Fc receptor-mediated antibody regulation of T cell immunity against intracellular pathogens. *J Infect Dis*. 2003; 188:617–624. [PubMed: 12898452]
- Morrison R, Caldwell H. Immunity to murine chlamydial genital infection. *Infect Immun*. 2002; 70:2741–2751. [PubMed: 12010958]

- Morrison SG, Su H, Caldwell HD, Morrison RP. Immunity to murine *Chlamydia trachomatis* genital tract reinfection involves B cells and CD4+ T cells but not CD8+ T cells. *Infect Immun*. 2000; 68:6979–6987. [PubMed: 11083822]
- Pal S, Luke CJ, Barbour AG, Peterson EM, de la Maza LM. Immunization with the *Chlamydia trachomatis* major outer membrane protein, using the outer surface protein A of *Borrelia burgdorferi* as an adjuvant, can induce protection against a chlamydial genital challenge. *Vaccine*. 2003; 21:1455–1465. [PubMed: 12615442]
- Pizza M, Giuliani MM, Fontana MR, Monaci E, Douce G, Dougan G, Mills KHG, Rappuoli R, Del Giudice G. Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants. *Vaccine*. 2001; 19:2534. [PubMed: 11257389]
- Ramsey KH, Soderberg LSF, Rank RG. Resolution of chlamydial genital infection in B-cell-deficient mice and immunity to reinfection. *Infect Immun*. 1988; 56:1320–1325. [PubMed: 3258586]
- Rank RG, Batteiger BE. Protective role of serum antibody in immunity to chlamydial genital infection. *Infect Immun*. 1989; 57:299–301. [PubMed: 2909492]
- Riedmann EM, Kyd JM, Cripps AW, Lubitz W. Bacterial ghosts as adjuvant particles. *Expert Rev Vaccines*. 2007; 6:241–253. [PubMed: 17408373]
- Schachter, J.; Grayston, JT. Epidemiology of human Chlamydial infections. In: Stephens, RS.; Byrne, GI.; Christiansen, G., et al., editors. *Chlamydial Infections*. Berkeley; San Francisco, CA: 1998. p. 3-10.
- Singh SR, Hulett K, Pillai SR, Dennis VA, Oh MK, Scissum-Gunn K. Mucosal immunization with recombinant MOMP genetically linked with modified cholera toxin confers protection against *Chlamydia trachomatis* infection. *Vaccine*. 2006; 24:1213. [PubMed: 16194585]
- Skelding KA, Hickey DK, Horvat JC, Bao S, Roberts KG, Finnie JM, Hansbro PM, Beagley KW. Comparison of intranasal and transcutaneous immunization for induction of protective immunity against *Chlamydia muridarum* respiratory tract infection. *Vaccine*. 2006; 24:355. [PubMed: 16153755]
- Spangler B. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol Rev*. 1992; 56:622–647. [PubMed: 1480112]
- Stagg AJ. Vaccines against *Chlamydia*: approaches and progress. *Mol Med Today*. 1998; 4:166–173. [PubMed: 9572058]
- Su H, Parnell M, Caldwell HD. Protective efficacy of a parenterally administered MOMP-derived synthetic oligopeptide vaccine in a murine model of *Chlamydia trachomatis* genital tract infection: serum neutralizing IgG antibodies do not protect against genital tract infection. *Vaccine*. 1995; 13:1023–1032. [PubMed: 8525685]
- Su H, Messer R, Whitmire W, Fischer E, Portis JC, Caldwell HD. Vaccination against Chlamydial genital tract infection after immunization with dendritic cells pulsed *ex vivo* with nonviable chlamydiae. *J Exp Med*. 1998; 188:809–818. [PubMed: 9730883]
- Tuffrey M, Alexander I, Conlan W, Woods C, Ward M. Heterotypic protection of mice against chlamydial salpingitis and colonization of the lower genital tract with a human serovar F isolate of *C. trachomatis* by prior immunization with recombinant serovar L1 major outer membrane protein. *Eur J Immunol*. 1992; 23:1169–1172.
- Wu H-Y, Russell MW. Induction of mucosal and systemic immune responses by intranasal immunization using recombinant cholera toxin B subunit as an adjuvant. *Vaccine*. 1998; 16:286. [PubMed: 9607044]
- Zhang Y-X, Stewart SJ, Caldwell HD. Protective monoclonal antibodies to *Chlamydia trachomatis* serovar- and serogroup-specific major outer membrane protein determinants. *Infect Immun*. 1989; 57:636–638. [PubMed: 2463971]

Fig. 1.

Construction of the vaccine vectors, pKS-MOMP and pKSM1-CTA2B. The *omp1* cDNA was inserted between and in frame with the *LacZ'* and *L'* genes of vector pKSEL5-2 at the KpnI-PstI sites to generate plasmid pKS-MOMP. Also, the coding region of *omp1:cta2B* was amplified by PCR from plasmid pUAB084 and inserted between and in frame with the *LacZ'* and *L'* anchor sequences at the KpnI and BamHI restriction sites of vector, pKSEL5-2, to generate plasmid, pKSM1-CTA2B. Expression of *omp1* and *omp1-cta2B* are under the transcriptional regulation of the *lac* promoter (*lacpo*).

Fig. 2.

Expression of MOMP and MOMP-CTA2B. The expression of MOMP and MOMP/CTA2B by the *Vibrio* clones was evaluated by immunoblotting analysis. Mid-log phase cultures of H1 harboring the expression plasmids were induced with IPTG and cell lysates assayed for rMOMP or rMOMP/CTA2B expression by Western blot. MOMP and MOMP/CTA2B were detected using the mouse MOMP-specific monoclonal antibody MoPn 40. Lane 1, *Vibrio cholerae* HI cells harboring the recombinant plasmid, pKS-MOMP expressing rMOMP (54 kDa); lanes 2 and 3, H1 cells harboring the recombinant plasmid, pKSM1/CTA2B expressing rMOMP/CTA2B (74 kDa) at 1 and 2 h, respectively. Numbers to the left are approximate molecular masses, in kilodaltons (kDa).

Fig. 3.

Expression of costimulatory molecules and cytokine production by DCs after treatment with rVCG-MOMP with and without CTA2B. BMDCs were isolated from mice by established procedures using IL-4 and GM-CSF and characterized as loosely adherent mononuclear cells expressing high levels of CD11c but lacking B220 surface antigens. Harvested cells were pulsed with rVCG vaccines for 24 h, stained with conjugated monoclonal antibodies against CD11c, CD40, CD80, CD83, CD86, 1Ab, CD14 and CD71 or isotype-matched controls and quantified in triplicate by flow cytometry. Also, supernatants from control and experimental cultures were assayed for the synthesis of cytokines by the Multiplex Luminex technique. The data show (a) the mean fluorescence intensity of staining of the indicated molecules and (b) the amount of Th1-promoting cytokines secreted after treatment. The experiments were repeated twice.

Fig. 4.

Chlamydia-specific IFN- γ production by ILN and splenic T cells. Groups of mice were vaccinated by the IM, IVag or TC routes and boosted twice, 2 weeks apart, with the rVCG vaccine constructs. Two weeks after the last boost, Tcells were purified from the ILN and spleens of immunized mice and controls and restimulated *in vitro* with UV-irradiated *Chlamydia trachomatis* (a) or *Chlamydia muridarum* (b). Local mucosal and systemic IFN- γ response was assessed as a measure of Th1 response. The amounts of IFN- γ contained in the supernatants of culture-stimulated cells were measured using the 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 ($\pm$ SD) 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 two independent experiments. IM, intramuscular; IVag, intravaginal; TC, transcutaneous.

Fig. 5.

Genital mucosal and systemic *Chlamydia*-specific humoral immune response after immunization with rVCG vaccines. Groups of mice were immunized and boosted as described above and vaginal lavage and serum samples were obtained 2 weeks postimmunization. The amount of antibody elicited in genital lavage (a) and serum samples (b) was assessed by a modified antibody ELISA and expressed in ng mL⁻¹. Data were calculated and presented as the mean values $\pm$ SD of triplicate cultures for each experiment. The results are from two independent experiments. IM, intramuscular; IVag, intravaginal; TC, transcutaneous.

Fig. 6.

Protection against genital chlamydial challenge after immunization with rVCG vaccines. Two weeks after the last immunization, mice immunized with rVCG chlamydial and control (rVCG-gD2) vaccines were challenged intravaginally with 10^7 IFU of live *Chlamydia muridarum* (MoPn). One week before challenge, mice were administered Depo Provera to stabilize the estrous cycle and facilitate a productive infection. Infections were monitored by cervicovaginal swabbing of individual animals every 3 days and *Chlamydia* was isolated from swabs in tissue culture and enumerated. The data show the mean recoverable IFUs expressed as $\log_{10}$ IFU $\text{mL}^{-1} \pm \text{SD}$, for mice immunized with (a) rVCG-MOMP and (b) rVCG-MOMP/CTA2B. Differences between the control and the experimental groups were compared with Student's *t*-test at $P < 0.05$. The experiment was repeated to contain 10–12 mice per group. IM, intramuscular; IVag, intravaginal; TC, transcutaneous.

Table 1

Percentage of intravaginal-immunized mice shedding *Chlamydia* after genital challenge

Vaccine	% of mice shedding <i>Chlamydia</i> (n = 10) on the following days after IVag challenge*									
	3	6	9	12	15	18	21	24	27	
rVCG-gD2	100	100	100	100	100	100	100	100	100	100
RVCG-MOMP	100	100	100	90	50	40	0	0	0	0
RVCG-MOMP/CTA2B	100	100	90	40	0	0	0	0	0	0

* Mice (10 per group) were vaccinated and boosted twice with rVCG-MOMP, rVCG-MOMP/CTA2B or rVCG-gD2 (control). Two weeks after the final boost, mice were challenged intravaginally with 1×10^7 IFU of *Chlamydia muridarum*. On the indicated days after challenge, chlamydial shedding was measured. There were significant differences in the time taken to resolve the infection between vaccinated and control mice ($P < 0.05$, as determined by the Student's *t*-test). The results are representative of two independent experiments.

IVag, intravaginal.