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A Novel Recombinant Multisubunit Vaccine against

**Chlamydia**

Francis O. Eko,* Qing He,* Teresa Brown,§ Lucinda McMillan,* Godwin O. Ifere,* Godwin A. Ananaba,‡ Deborah Lyn,* Werner Lubitz,† Kathryn L. Kellar,§ Carolyn M. Black,§ and Joseph U. Igietseme*§

The administration of an efficacious vaccine is the most effective long-term measure to control the oculogenital infections caused by *Chlamydia trachomatis* in humans. *Chlamydia* genome sequencing has identified a number of potential vaccine candidates, and the current challenge is to develop an effective delivery vehicle for induction of a high level of mucosal T and complementary B cell responses. *Vibrio cholerae* ghosts (VCG) are nontoxic, effective delivery vehicles with potent adjuvant properties, and are capable of inducing both T cell and Ab responses in mucosal tissues. We investigated the hypothesis that rVCG could serve as effective delivery vehicles for single or multiple subunit chlamydial vaccines to induce a high level of protective immunity. rVCG-expressing chlamydial outer membrane proteins were produced by a two-step genetic process, involving cloning of Omp genes in *V. cholerae*, followed by gene E-mediated lysis of the cells. The immunogenicity and vaccine efficacy of rVCG-expressing single and multiple subunits were compared. Immunologic analysis indicated that i.m. immunization of mice with either vaccine construct induced a strong mucosal and systemic specific Th1 response against the whole chlamydial organism. However, there was an immunogenic advantage associated with the multiple subunit vaccine that induced a higher frequency of Th1 cells and a relatively greater ability to confer protective immunity, compared with the single subunit construct. These results support the operational theory that the ability of a vaccine to confer protective immunity against *Chlamydia* is a function of the level of Th1 response elicited. *The Journal of Immunology*, 2004, 173: 3375–3382.

Genital infection with the obligate intracellular bacterium, *Chlamydia trachomatis*, poses a significant risk, especially in women, often leading to pelvic inflammatory disease, ectopic pregnancy, and infertility (1, 2). Although antibiotic therapy appears to effectively eliminate chlamydial infection, the frequent asymptomatic infection in women precludes early diagnosis and application of chemotherapy, making clinical presentation of sequelae often the first indication of a genital infection. Besides, antibiotic therapy does not always affect established pathology (3), and the rampant asymptomatic infections make treatment of symptomatic individuals alone unlikely to be a successful control strategy. The development and administration of a prophylactic or therapeutic vaccine capable of protecting against infection or even ameliorating severe disease remain the most promising and effective strategy for controlling chlamydial diseases that constitute a major public health challenge due to the significant morbidity worldwide (3–5).

Current challenges in chlamydial vaccine design and development include the identification of the elements of protective immunity, selection of a suitable vaccine candidate capable of inducing protective immunity, and the development of effective delivery systems to boost immune responses against potential subunit candidate Ags. Progress in the functional immunobiology of *Chlamydia* has established the essential immunologic paradigms for vaccine selection and evaluation, including the obligatory requirement for a vaccine to induce a Th1 immune response that controls chlamydiae (5). With respect to the choice of vaccine candidates, the use of whole chlamydial agents appears to be unattractive due to the potential existence of immunopathogenic components (6). Besides, progress made in molecular immunology and biotechnology in the last two decades has led to a gradual shift from the classical whole cell vaccines, consisting of inactivated or live-attenuated intact pathogens to peptide or subunit vaccines. 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. This 40-kDa immunodominant protein has been well characterized as a porin, an adhesin, a key determinant of chlamydial genus and species specificity, and a highly promising candidate vaccine. However, experience with purified or recombinant MOMP as a protective Ag in several animal models (7–11) suggests that MOMP alone is inadequate, calling either for a multisubunit approach or a more effective delivery system that will optimize the immune response.

Other potential vaccine candidates in the *C. trachomatis* outer membrane complex include the cysteine-rich outer membrane proteins, OMP2 (60 kDa) and the 15-kDa OMP3 (12). The synthesis and incorporation of OMP2 and OMP3 in the *Chlamydia* outer...
membrane coincide with the transition of reticulate body to elementary body (EB) in the developmental cycle (13, 14), and these proteins are thought to contribute to cell wall rigidity and osmotic stability of the EB. Topological studies indicate that OMP2 and OMP3 are localized at the inner surface of the outer membrane (15, 16) and are encoded by a bicistronic operon (17). Although OMP2 shows V regions between different species, it is well conserved within a chlamydial species (18–21) and has been shown to be a highly immunoreactive Ag, inducing Ab responses in both humans and animals (21, 22), as well as a major immunogen in chlamydial infections (23). Besides the serologically defined and molecularly characterized chlamydial Ags recognized during human infection (6, 24, 25), recent advances in chlamydial genomics have predicted several immunogenic proteins (26–28) that may serve as potential vaccine candidates. The most prominent among these to date include the polymorphic OMPs (29) (30, 31), the conserved PorB family of membrane proteins (32–34), and the ADP/ATP translocase of Chlamydia pneumoniae (26, 27).

The development of effective delivery systems is a key challenge in chlamydial vaccine effort that has received less than adequate attention. We have recently shown that the recombinant Vibrio cholerae ghost (rVCG) platform is an effective carrier and delivery system for cloned Chlamydia trachomatis proteins, eliciting chlamydial-specific immune responses following immunization (11). Although the protection afforded by the single subunit design was partial, the results suggested that the rVCG system could be optimized into an effective method of delivering multiple chlamydial subunit Ags to elicit protective immune responses.

In this study, we have constructed a rVCG vector-based multi-subunit candidate vaccine expressing both MOMP and OMP2 (rVCG-M1-2) and directly compared the cellular and humoral immune responses with that of a single subunit construct, rVCG-MOMP. In addition, the ability of both vaccine constructs to confer protective immunity against a C. trachomatis genital infection was compared. The results show that the double rVCG subunit construct induced a greater specific Th1 response and conferred a greater level of protective immunity than the single subunit design. This result has an enormous implication in a future chlamydial vaccine targeted for human use.

Materials and Methods

Chlamydia stocks and animals

Stock preparations of C. trachomatis serovar D strain were generated by propagating 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. Five- to 8-wk-old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). 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 an approved institutional animal care and use protocol.

Genomic DNA preparation and PCR amplification of omp1 and omp2 sequences

Genomic DNA was purified from 1 × 10^8 chlamydial EBs using the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. The full-length omp1 and omp2 coding sequences were amplified from purified genomic DNA using the Expand High Fidelity 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 chlamydial sequences obtained from published data banks (36, 37). For omp1 amplification, the forward primer (EF-5F) incorporated a SalI restriction enzyme site with the sequence 5′-gagcggtagcgatgtataggaaagt-3′, and the reverse primer (EF-5R) incorporated a PstI site, 5′-acaattcaggtatgtaggaagag-3′. The forward primer (EF-19F) for omp2 amplification incorporated an Asp718 restriction enzyme site (5′-agaggggttagatcatgtagaaagt-3′), while the reverse primer (EF-20R) contained an ApaI site (5′-agaggggttagatcatgtagatggt-3′). The amplification reaction was conducted in an Eppendorf Gradient Mastercycler (Eppendorf, Hamburg, Germany), and the amplified PCR products of the correct sizes, −1200 bp (omp1) and 1689 bp (omp2), were isolated from a 1% agarose gel and purified with the QiAquick PCR purification kit (Qugen, Valencia, CA).

Construction of the single and multiple subunit vaccine vectors, pCOM2 and pMAP12

The construction of the pCOM2 expression vector (Fig. 1) was essentially conducted, as previously described for pCOM12 (11), except that in pCOM2 the C. trachomatis omp1 gene is inserted between the E’ and L’ genes of vector pKSEL5-2 (i.e., E’-L’ targeting). Construction of the membrane targeting vaccine vector, pMAP1-2, which carries both the omp1 and omp2 genes, is based on the presence of multiple cloning sites in pCOM2. For construction, the amplified omp2 PCR product (1689 bp) containing the full-length omp2 coding sequence, minus the signal peptide, and the pCOM2 plasmid were digested with ApaI and Asp718 restriction endonucleases (Roche). The DNA fragments were separated by electrophoresis, and the DNA was recovered from agarose and purified by using the QIAquick PCR purification kit (Qugen). The omp2 DNA and pCOM2 plasmid were ligated with T4 DNA ligase (Roche) and transformed into Escherichia coli JM109. Transformants were analyzed by restriction endonuclease digestion of the resultant expression plasmid, pMAP12 (Fig. 1), and sequencing of the junctions and coding regions of omp1 and omp2 genes to ensure that the plasmid had not been corrupted.

Expression of rMOMP and rOMP2 in V. cholerae 01

For expression of the recombinant proteins, the pMAP12 plasmid was introduced into V. cholerae 01 strain H1 by electroporation, and clones containing the plasmid were isolated and designated HM1-2. The expression of MOMP or OMP2 by the Vibrio clones was evaluated by immunoblotting analysis. Cultures of HM1-2 and control H1 (pKSEL5-2) were grown to mid-log phase under appropriate conditions, and rMOMP or rOMP2 expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG; Roche Diagnostics, Indianapolis, IN) to a final concentration of 2 mM; the cultures were then incubated for 1 h. Samples were removed at the indicated times, solubilized in sample buffer containing 2-ME (Invitrogen Life Technologies, Carlsbad, CA), and separated by SDS-PAGE, as previously described (38). Purified MOMP subjected to the same conditions was included as a positive control; purified OMP2 was unavailable. Following protein transfer, rMOMP or rOMP2 was detected using the mouse anti-MOMP or anti-OMP2 Abs kindly provided by S. Pal (University of California, Irvine, CA).

Production of rVCG coexpressing MOMP and OMP2

Competent V. cholerae 01 strain HM1-2 cells (harboring the cloned omp1 and omp2 genes) were cotransformed with the lysis plasmid pDKLO1 (39), and the bacterial cells were grown in brain heart infusion broth containing appropriate antibiotics at 37°C to an A600 of 0.3. To induce rMOMP and rOMP2 expression, IPTG was added to a final concentration of 2 mM; the cultures were then incubated for 2 h and cell lysis was achieved by the addition of 3-methyl benzene (5 mM) to induce gene expression. At the end of lysis, cultures were harvested by centrifugation and washed twice with PBS or a low ionic buffer. Harvested ghosts were resuspended in PBS and then lyophilized. The efficiency of E-mediated killing of vibrios was estimated by plating samples on brain heart infusion agar, as previously described (40). Results indicated a 100% killing efficiency (i.e., no CFU were found on plates at any dilution). Lyophilized VCGs were weighed, and the number of CFU/mg VCG was calculated.

Immunization protocol

Animal immunizations were conducted using a 1-ml syringe fitted with a 27-guage needle. Groups of mice (10/group) were vaccinated i.m. with lyophilized rVCGs or PBS, as follows: groups 1 and 2 received 3 mg of rVCG-M1-2 and rVCG-MOMP in 50 µl of PBS per animal, respectively. Group 3 received an equivalent dose of VCG alone, while group 4 served as the negative control and received 50 µl of PBS per animal. The choice of this dosage was based on the results of a previous report (11) as well as unpublished data (F. O. Eko, L. McMillan, and J. U. Igietseme). The vaccine dose was formulated such that 1 mg of lyophilized rVCG or VCG corresponded to −2 × 10^8 CFU. All immunizations were administered while under phenobarbitol anesthesia (35), and animals were boosted twice at two weekly intervals.
Blood was collected by periorbital puncture, and the serum was pooled for each group of animals. Mucosal secretions (vaginal samples) were collected at 2, 4, 6, 8, and 10 wk after the first administration of vaccines (rVCG-M1-2 or rVCG-MOMP) by washing the vagina of each mouse with 200 μl of PBS (pH 7.2) (41). Trypsin inhibitor (10 μg/ml; Sigma-Aldrich, St. Louis, MO) and EDTA (5 × 10⁻⁴ M; Sigma-Aldrich) were added to the samples and centrifuged at 10,000 × g for 10 min at 4°C to remove the debris. Supernatants were collected, and 10⁻⁷ M PMSF (Sigma-Aldrich) and 0.01% sodium azide (Sigma-Aldrich) were added. Samples were stored at −80°C until analyzed. The Chlamydia-specific Ab titer (secretory IgA and IgG2a) in sera and vaginal washes was measured by a modified ELISA procedure, as previously described (42). Briefly, Maxisorb 96-well plates (Costar, Cambridge, MA) were coated overnight with 10 μg/ml C. trachomatis serovar D EB in 100 μl of PBS. A standard curve was obtained using purified mouse IgA or IgG2a serially diluted from 1.25 to 10 μg/ml PBS to coat the plates under the same conditions. After three washes in PBS containing 0.05% Tween 20, plates were blocked with 1% BSA containing 5% goat serum in PBS. After washing, 100 μl of serum or 50 μl of vaginal wash in 2-fold serial dilutions was added per well, while control wells contained PBS. After incubation at 37°C for 2 h and washing, plates were incubated with 100 μl of HRP-conjugated goat anti-mouse IgA or IgG2a (Southern Biotechnology Associates, Birmingham, AL) for 1 h at room temperature. Peroxidase substrate, ABTS, was added, and the reaction was stopped with 1% SDS. Colorimetric measurement was performed by measuring the OD at 490 nm on a Spectra Max 250 Microplate Autoreader (Molecular Devices, Sunnyvale, CA). Results represent the mean of triplicate wells for each sample set.

Measurement of the frequency of Chlamydia-specific Th1 cells (Th1 frequency) in mucosal and systemic draining tissues after immunization with rVCGs

The iliac lymph nodes (ILN) draining the genital tract and the spleens (systemic draining tissues) were harvested from immunized mice at 2 and 8 wk after the last immunization. Immune T cell-enriched cells were prepared from the lymphoid tissues by nylon wool enrichment procedure, as previously described (43, 44). Purified lymphoid cells contained at least 95% CD3⁺ cells, as determined by FACS. A modified procedure for the limiting dilution technique reported previously (45, 46) was used to measure the frequency of Chlamydia-specific Th1 cells (assayed as Ag-specific IFN-γ production) by each cell population. In brief, purified T cells were seeded in a serial doubling dilution into 96-well round-bottom tissue culture plates at 24 wells/dilution. The T cells were stimulated with APCs seeded in a serial doubling dilution into 96-well round-bottom tissue culture plates at 24 wells/dilution. The T cells were stimulated with APCs and Ag. After 5 days of incubation, the supernatants were assayed for IFN-γ 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 24 replicates of background cultures were calculated. Three times the value of the SD was added to the mean value, and the sum was the baseline for positive experimental wells. After determination of the number of positive and negative wells per dilution of each T cell preparation, the data were analyzed by a limiting dilution computer program (LIDIA) (45), which provided both the Th1 frequency and the conformity of the input data with a single-hit Poisson model. The data are expressed as the Th1 frequency per 10⁶ responding T cells in each cell preparation. T cells from naive wild-type mice have a Th1 frequency of 15 (range, 9–21) per 10⁶ cells

Protection studies

Groups of mice (10 mice/group) were immunized i.m. three times, 2 wk apart, as described above. Three weeks after the last immunization, mice from each group (five mice/subgroup) were either challenged directly with 10⁶ inclusion-forming units of live C. trachomatis serovar D, or T cells were isolated from draining lymph nodes and spleens of immunized mice (five mice/subgroup) and adoptively transferred into naive mice at 2.5 × 10⁶ cells/mouse (2.5 spleen equivalents), then challenged with serovar D 24 h after cell transfer, as previously described (11). Infections were monitored by cervicovaginal swabbing of individual animals every 3 days, and Chlamydia was isolated from swabs in tissue culture by standard methods (35) and confirmed by an ompA-based PCR method (47). The experiment was repeated twice.

Measurement of anti-chlamydial mucosal and systemic IgA and IgG2a Ab

The levels of IFN-γ, secretory IgA, and IgG2a in the ILN and serum samples from different experiments as well as the level of protection conferred by the two vaccine constructs were compared by Student’s t test. The level of significance was judged at p < 0.05.

Results

Construction of the single and multiple subunit vaccine vectors expressing chlamydial rOMPs

The plasmid pCOM2 was constructed to contain the entire coding sequence for the mature Omp1 protein, omitting the 22-aa leader sequence and in frame with the E′ and L′ anchors. The multisubunit plasmid expression vector, pMAP12, was constructed from pCOM2 by placing the entire Omp2 coding region, minus the signal peptide, under the transcriptional control of the lac promoter and in frame with the LacZ′ and E′ anchors (Fig. 1). The full-length omp1 and omp2 genes were thus expressed as E′-L′ and lacZ′-E′ fusion proteins, respectively. Sequencing results confirmed that the constructed plasmids were not corrupted in any manner. Transformation of V. cholerae O1 strain H1 with pMAP12 by electroporation and expression of the recombinant proteins (rMOMP and rOMP2) was confirmed by Western immunoblotting analysis using mouse mAbs to MOMP or OMP2 (Fig. 2). Neither chlamydial MOMP nor OMP2 was detectable in the test strain harboring the pKSEL5-2 targeting vector alone. Two forms of rOMP were detected, with the lower band corresponding in size to the authentic mature MOMP purified from EBs, while the higher band represents the MOMP-EL fusion protein. These results confirmed that transformants coexpressed the different chlamydial proteins as efficiently as those harboring single subunit constructs.

Immunogenicity of rVCG-expressing single and multiple chlamydial OMPs

We tested the hypothesis that a multisubunit vaccine will induce a higher level of Th1 frequency in the genital tract compared with a single subunit vaccine. The results presented in Fig. 3 indicate that the frequency of Chlamydia-specific Th1 cells induced by the rVCG-M1-2 multisubunit vaccine construct harboring MOMP and the 60-kDa proteins in the ILN draining the genital mucosa was ∼3-fold higher than that of the single subunit vaccine (rVCG-MOMP) at 2 wk postimmunization. Overall, there were significantly higher levels of specific T cells induced by both vaccine constructs compared with the VCG control (p < 0.05). In addition, the frequency of specific Th1 cells elicited by the multisubunit vaccine 8 wk postimmunization was ∼2-fold higher than that of the single subunit construct. As previously reported (11), the frequency of Chlamydia-specific Th1 cells elicited by immunization with VCG alone was comparable to that of naive mice at all time points after immunization. Figs. 4 and 5 show the levels of IFN-γ secreted when 10⁵ purified T cells from the ILN and spleens of mice vaccinated with either the single or multiple subunit vaccine constructs were stimulated in culture with intact chlamydial elementary bodies, which also demonstrates the superiority of the specific Th1 response induced by the multiple subunit vaccine construct. The data presented in Fig. 4 indicate that the pattern of bulk Th1 response measured by Ag-specific IFN-γ secretion by Chlamydia-immune T cells correlates with the pattern of frequency of IFN-γ-producing T cells in the ILN (Fig. 3). In addition, there was no statistical difference between the amounts of IFN-γ produced by systemic immune T cells from the spleens of mice vaccinated with either vaccine construct, as measured at 2 and 8 wk postimmunization (p > 0.05) (Fig. 5). This is in contrast with the result obtained in the genital mucosa, which showed that the multisubunit vaccine had a significant immunologic advantage over the
single subunit construct at the two time points evaluated \( p < 0.05 \). Chlamydia-specific IL-4 levels produced by ILN and splenic T cells from vaccinated mice were also measured to assess whether Th2 responses were induced following immunization. IL-4 production was detected at very low levels, and there were no significant differences in IL-4 levels produced in the ILN \( (12.67 \pm 2.25 \text{ pg/ml}) \) and splenic T cells \( (10.97 \pm 0.67 \text{ pg/ml}) \) among the different groups of mice \( p > 0.05 \). Taken together, the data indicate that these vaccines have a predilection for inducing predominantly Th1 immune responses.

**Immunization with rVCGs elicited Th1-associated humoral immune response**

The specificity of the IgG isotype generated during an immune response is indicative of the type of Th cell response, with Th1 cells inducing IgG2a, while Th2 cells facilitate IgG1 and IgG2b (48). Also, previous studies have indicated that secretory IgA and IgG have protective roles during genital chlamydial infection (43, 49), although protection against genital chlamydial infection by a dendritic cell-based cellular vaccine correlated better with the cell-mediated immunity-associated IgG2a than secretory IgA (46). When the IgA and IgG2a responses induced following immunization with rVCG-MOMP or rVCG-M1-2 were measured in sera and vaginal samples from vaccinated animals, comparable levels of secretory IgA and IgG2a were detected in the vaginal washes of mice immunized with both vaccine constructs during the first 2 wk following immunization (Fig. 6). There were no detectable Ab levels in the serum and vaginal washes of unimmunized control mice (data not shown). At 8 wk postimmunization, mice immunized with the double subunit vaccine construct \((rVCG-M1-2)\) elicited higher IgA and IgG2a Ab levels in the genital mucosa compared with the single subunit construct (Fig. 6). However, the levels of IgG2a in the genital washes remained low and were significantly lower \( p < 0.01 \) than those of IgA during the same time period. In general, Ab levels were significantly higher \( p < 0.01 \) in serum than in vaginal washes at 8 wk postimmunization (Figs. 6 and 7). Although there was no significant difference between the levels of IgG2a induced in serum at 2 and 8 wk by the rVCG-MOMP vaccine construct, the levels of IgA increased from barely detectable amounts during the first 2 wk of immunization to very high levels after 8 wk (Fig. 7).

**Induction of protective immunity by vaccine constructs**

The ability of an experimental chlamydial vaccine to confer protective immunity is a function of the level of Th1 response elicited. Therefore, we expected that Chlamydia-specific T cells from immunized mice showing a relatively higher frequency of Th1 cells would confer superior protection against a \( C. trachomatis \) challenge infection following adoptive transfer to naive mice. Isolation of chlamydiae from vaginal swab cultures obtained at 3-day intervals following intravaginal challenge and confirmation with a sensitive PCR method were used to monitor the course of infection and evaluate the number of mice that were positively infected. The results are presented as the percentage of mice that remained infected relative to the total number challenged at the indicated time points (Fig. 8). All of the mice in the control group \((10 of 10)\) that

![FIGURE 1. Construction of the expression plasmid vectors, pCOM2 and pMAP1-2. The coding regions of omp1 and omp2, minus the leader sequences, were each amplified by PCR from the chromosomal DNA of C. trachomatis serovar D. The omp1 PCR product was cloned into the membrane targeting vector, pKSEL5-2, at the PstI/SalI sites to generate plasmid, pCOM2. Subsequent cloning of the omp2 PCR product into the Apal/Asp718 sites of pCOM2 generated the recombinant plasmid, pMAP1-2. Expression of omp1 and omp2 in pMAP1-2 is under the transcriptional regulation of the lac promoter (lacO).](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org)
received T cells from mice immunized with VCG alone had positive cultures throughout the period of observation. The most significant level of protection was observed in the group that received T cells from mice immunized with the multisubunit construct, rVCG-M1-2. In this group, only 20% (2 of 10) of the animals shed chlamydiae vaginally \((p/< 0.05)\) by 2 wk after the challenge. However, 60% of the mice that were immunized with the single subunit vaccine remained infected. These results indicate that a multisubunit vaccine based on the rVCG platform technology has a significant advantage in protecting vaccinated recipients from chlamydial infection compared with the single subunit vaccine \((p/< 0.05)\).

**Discussion**

The development of effective, safe, and stable delivery systems and adjuvants remains an important objective in chlamydial vaccine research. On the basis of the current paradigms guiding vaccine design, the ideal delivery system for an efficacious *Chlamydia* vaccine should: 1) have the capacity to harbor a multisubunit vaccine; 2) induce a qualitatively and quantitatively ambient cytokine and chemokine environment, as well as boost the costimulatory function of the APCs that favors the induction of a robust Th1 response; and 3) be administered via a convenient route that fosters the activation of a high frequency of Th1 effectors as well as the complementary Ab response at the mucosal sites of chlamydial infection. The experimental chlamydial vaccine delivery strategies used to date include live or nonliving viral or bacterial vectors, DNA with or without cytokine genes, CpG-rich oligonucleotides, CFA, immunostimulating complexes, and dendritic cell-based

**FIGURE 3.** Relative frequency of *Chlamydia*-specific T cells in the ILN draining the genital mucosa. Groups of mice were vaccinated i.m., three times, 2 wk apart with either rVCG-MOMP (●) or rVCG-M1-2 (■) or VCG alone (□). Local Th1 response was assessed at 2 and 8 wk after immunization, by measuring the response of chlamydial-specific, IFN-γ-secreting T cells from the ILN of immunized mice. The amounts of IFN-γ contained in culture supernatants derived from culture-stimulated cells and controls were measured by Bio-Plex cytokine assay kit. Data were analyzed by a limiting dilution computer program (LIDIA) and expressed as the Th1 frequency per 10⁶ responding T cells in each cell preparation. The results are from three independent experiments. The difference between the frequencies of Th1 cells induced by the two vaccines in the ILN was statistically significant \((*, p/< 0.001)\).

**FIGURE 4.** Induction of local genital *Chlamydia*-specific T cell response by rVCG. Groups of mice were immunized, as described in Fig. 3, and local IFN-γ response was assessed at 2 and 8 wk after immunization. The amounts of IFN-γ contained in culture supernatants derived from culture-stimulated T cells from the ILN 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 \((±SD)\) for triplicate cultures for each experiment. The control cultures without Ag did not contain detectable levels of IFN-γ, and so the data were excluded from the results. The results are from three independent experiments. * Indicates statistically significant differences between the two vaccine constructs \((p/< 0.001)\).
cellular delivery (5). rVCG are effective bacterial delivery vehicles that exhibit potent adjuvant properties and are capable of inducing both T cell and Ab responses in mucosal tissues (11). The results from this study have verified the hypothesis that rVCG could serve as effective delivery vehicles for a single or multiple subunit chlamydial vaccine that induces a high level of protective immune responses. There was a comparative vaccine advantage associated with the multisubunit approach because rVCG harboring both chlamydial MOMP and the 60-kDa cysteine-rich OMP induced at least a 3-fold higher frequency of Th1 cells and conferred a greater

FIGURE 5. Induction of Chlamydia-specific systemic T cell response by rVCG. Groups of mice were immunized, as described in Fig. 3, and systemic IFN-γ response was assessed at 2 and 8 wk postimunization. The level of Th1 response induced was determined by measuring the response of chlamydial-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 (±SD) for triplicate cultures for each experiment. The control cultures without Ag did not contain detectable levels of IFN-γ, and so the data were excluded from the results. The results are from three independent experiments. The difference between the levels of systemic IFN-γ induced by the two vaccine constructs at weeks 2 and 8 postimmunization was not statistically significant (p < 0.11 and 0.36, respectively).

FIGURE 6. Induction of secretory IgA and Th1-associated IgG2a Abs in the genital mucosa after immunization with rVCG. Female C57BL/6 mice were vaccinated i.m., three times, 2 wk apart with the rVCG vaccines or controls. Vaginal washes were performed at the indicated time points after immunization with 200 μl of PBS and stored at −80°C until assayed. The levels of Chlamydia-specific secretory IgA and IgG2a Abs in vaginal washes were measured by a modified ELISA procedure. Bars represent the mean (+SD) of triplicate wells for each set of samples obtained from 10 animals per group (*, p < 0.001).

FIGURE 7. Induction of systemic IgA and Th1-associated IgG2a Abs after immunization with rVCGs. Female C57BL/6 mice were vaccinated, as described above. Sera were collected from the immunized mice at the indicated time points by periocular puncture and stored at −80°C until assayed. The levels of Chlamydia-specific secretory IgA and IgG2a Abs in serum samples were measured by a modified ELISA procedure. Bars represent the mean (+SD) of triplicate wells for each set of samples obtained from 10 animals per group (*, p < 0.001).

FIGURE 8. Efficacy of protective immunity conferred by immune T cells from mice immunized with rVCG or VCG. T cells from mice immunized with rVCG vaccines, VCG alone, or naive mice were adoptively transferred into naive recipients at 2.5 spleen equivalents/mouse. The mice were challenged intravaginally with 10^5 inclusion-forming units of live C. trachomatis serovar D after 24 h of cell transfer. Infections were monitored by cervicovaginal swabbing of individual animals every 3 days, and Chlamydia was isolated from swabs in tissue culture and confirmed by PCR. The experiment was repeated twice with similar results. Bars represent the percentage of mice shedding chlamydiae at the indicated times following challenge. The differences between the levels of protection afforded by the two vaccinated groups at days 3, 6, 9, and 15 were statistically significant (*, p < 0.01).
level of protective immunity than the single rVCG subunit containing MOMP alone. These results support the operational theory that the ability of a vaccine to confer protective immunity against *Chlamydia* is a function of the level of Th1 response elicited. This finding is in consonance with predictions from previous studies that vaccine designs based on MOMP alone were only marginally protective against chlamydial infection in the genital, ocular, and respiratory infection models due to inadequate Th1 response (5). In addition, this and other results (50) would suggest that, in the absence of a more potent adjuvant, it is unlikely that a vaccine directed against a single subunit Ag will be protective (or provide sterilizing immunity).

It will be observed that no sterilizing protective immunity was achieved even with the current double subunit design. However, the level of protection achieved was at least comparable with or superior to the temporary protective immunity conferred by the intravaginal infection with the live organism, as recently reported (51). The significance of this level of protection can be appreciated considering that immunizations were conducted with recombinant lysophosphatidylcholine ghosts resuspended in saline without additional adjuvants. In addition, the theoretical amount of targeted chlamydial Ag in the rVCG-M1-2 is $\sim 0.1-0.3\%$ of the total envelope proteins (52). Thus, these results indicate that VCGs constitute an effective vaccine delivery vehicle and represent a novel approach to modern vaccine development.

The key element of protection against *Chlamydia* is the induction of a relatively high frequency of specific Th1 response and the accessory Abs such as the cell-mediated immunity-associated IgG2a and secretory IgA (53–56). The role of humoral immunity in chlamydial control is yet unfolding. Recent studies suggest that the predominant role of Abs in chlamydial clearance is in resistance to reinfection, by enhancing the uptake, processing, and presentation of a relatively high frequency of specific Abs (51). The significance of these results will be focused on selecting additional chlamydial proteins that can be coexpressed in rVCG because these delivery vehicles possess the capacatory power of being able to accommodate the expression of multiple heterologous Ags. Furthermore, besides immunogenicity and the ability to induce protective immunity, which is the focus of this study, the selection of subunits should be cognizant of the potential safety and toxicology concerns that may be associated with certain Ags with vaccine prospects (58).

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**References**

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