Transcutaneous Immunization with Combined Cholera Toxin and CpG Adjuvant Protects against Chlamydia muridarum Genital Tract Infection

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Chlamydia trachomatis is a pathogen of the genital tract and ocular epithelium. Infection is established by the binding of the metabolically inert elementary body (EB) to epithelial cells. These are taken up by endocytosis into a membrane-bound vesicle termed an inclusion. The inclusion avoids fusion with host lysosomes, and the EBs differentiate into the metabolically active reticulate body (RB), which replicates by binary fission within the protected environment of the inclusion. During the extracellular EB stage of the C. trachomatis life cycle, antibody present in genital tract or ocular secretions can inhibit infection both in vivo and in tissue culture. The RB, residing within the intracellular inclusion, is not accessible to antibody, and resolution of infection at this stage requires a cell-mediated immune response mediated by gamma interferon-secreting TH1 cells. Thus, an ideal vaccine to protect against C. trachomatis genital tract infection should induce both antibody (immunoglobulin A [IgA] and IgG) responses in mucosal secretions to prevent infection by chlamydial EB and a strong TH1 response to limit ascending infection to the uterus and fallopian tubes. In the present study we show that transcutaneous immunization with major outer membrane protein (MOMP) in combination with both cholera toxin and CpG oligodeoxynucleotides elicits MOMP-specific IgG and IgA in vaginal and uterine lavage fluid, MOMP-specific IgG in serum, and gamma interferon-secreting T cells in reproductive tract-draining caudal and lumbar lymph nodes. This immunization protocol resulted in enhanced clearance of C. muridarum (C. trachomatis, mouse pneumonitis strain) following intravaginal challenge of BALB/c mice.

Chlamydia trachomatis is the leading cause of bacterial sexually transmitted disease worldwide. It is an obligate intracellular bacterial pathogen that infects the epithelium of the genital and ocular mucosa. In 1999, the World Health Organization estimated that 92 million cases of C. trachomatis genital infections occurred worldwide, with an estimated 4 to 5 million new cases occurring annually in the United States (69). The majority of infections occur in teenagers and people under 30 years of age. The majority of genital tract infections (>85%) in women are asymptomatic (reviewed in reference 6), masking the serious pathological consequences that can result from infection. In males, 45% of infections are asymptomatic. For women, the vaginal-cervical infection can spread to the upper reproductive tract, leading to serious complications which include chronic and recurrent pelvic inflammatory disease, fallopian tube scarring, ectopic pregnancy, chronic pelvic pain, and infertility (66). A recent report (13) by the Institute of Medicine estimates the annual direct and indirect costs of non-human immunodeficiency virus sexually transmitted diseases in the United States to be $10 billion, with approximately $4 billion of this due to C. trachomatis infections.

Although effective antimicrobial treatment is available, this has been largely unsuccessful in halting the spread of infection, most likely due to the high rate of asymptomatic infections, which may persist for months to years (57). Multiple-antibiotic-resistant strains of Chlamydia have also been reported recently (56). Furthermore, it has been suggested that antibiotic treatment can result in persistent infections with aberrant forms of C. trachomatis that may be reactivated at a later date (19). Recent studies also suggest that Chlamydia infection of the lower genital tract may be an important risk factor facilitating sexual transmission of human immunodeficiency virus infection (23). Thus, improved means of prevention and control of C. trachomatis genital tract infections are urgently required, particularly the development of a vaccine to prevent infection by inducing protection at mucosal surfaces. Vaccine-induced immunity will need to be more effective than that produced by natural infection. Induction of both antibody- and cell-mediated immunity that declines over time and is insufficient to prevent reinfection with other serovars.

Despite many studies of animal models and infected humans, the type(s) of immunity needed to protect against genital tract chlamydial infection has not been well defined (3). CD4 TH1 responses, characterized by high levels of gamma interferon (IFN-γ) production (10, 48), are implicated as the major mechanism of clearance of Chlamydia in mouse and primate models, with antibody being of lesser importance. Only mice deficient in T-cell receptor αβ and major histocom-
patibility complex class II (MHC-II) genes are incapable of resolving genital infections (44, 48). Complete resolution of infection in the guinea pig model appears to require both antibody and cell-mediated immunity (CMI) (1, 51). Antibody, both serum immunoglobulin G (IgG) and local IgA, has been shown to play a role in clearance of *Chlamydia* from the genital tract in the mouse, guinea pig, primate, and marmoset models (50). Antibody is particularly important in recall immunity, as mice deficient in antibody are less resistant to reinfection than mice with local antichlamydial antibody (60). This is supported by studies of infected humans, which showed an inverse correlation between IgA levels in cervical secretions and the amount of *Chlamydia* recovered from the cervix (4). Antibody may (i) neutralize *Chlamydia* and reduce ascending infection by blocking chlamydial attachment to epithelial cells; (ii) enhance killing of opsonized *Chlamydia* by phagocytic cells, thereby limiting dissemination to distant sites; or (iii) contribute to the resolution of intracellular infection by antibody-dependent cellular cytotoxicity (42). The role of CD8 cells is unclear. Adoptive transfer studies of fractionated CD4 and CD8 cells and the use of CD8-cell-deficient mice all suggest that CD8 cells play a minor role, if any, in protection against chlamydial genital infection (44, 45), and that any effect is likely to be due to production of IFN-γ (36). Furthermore, infection of mice genetically deficient in perforin, Fas, or Fas ligand or in double-knockout mice deficient in both perforin and Fas ligand showed that these mice cleared primary chlamydial infections with kinetics nearly identical to those observed in wild-type control mice and were resistant to reinfection (49). *Chlamydia* can also inhibit both constitutive and IFN-γ-induced MHC-I expression by infected cells, due to degradation of the transcription factor RFX5 by a chlamydial protease (74), and inhibit apoptosis in target cells by preventing caspase activation (14). This would also be expected to limit the effectiveness of cytotoxic T lymphocyte (CTL) killing. There are, however, reports of the identification of human CD8 CTLs specific for chlamydial peptides from individuals with documented *C. trachomatis* infection. Using HLA class I tetramers Kim et al. (31) identified major outer membrane protein (MOMP)-specific CD8 CTLs in the peripheral blood of infected individuals. Peripheral blood mononuclear cells from individuals infected with *C. trachomatis* (30) and *C. pneumoniae* (68) include CD8 cells that are able to lyse histocompatible *Chlamydia* peptide-pulsed epithelial cells and *Chlamydia*-infected cell lines following multiple rounds of in vitro stimulation with HLA-binding peptides derived from the chlamydial MOMP. These data show that CD8 CTLs are induced by natural infection and that, after in vitro stimulation with defined chlamydial peptides, expanded cells can kill *Chlamydia*-infected targets. Furthermore, large numbers of cloned CD8 CTLs can partially protect naive mice against challenge, due to production of IFN-γ (24, 30, 58, 59). However, a direct protective role for CD8 CTLs in vivo has not yet been demonstrated, and it is widely accepted that CD8 T cells are neither sufficient nor necessary to confer protective immunity in the murine model of chlamydial genital infection (27, 43). These data from both animal models and human immunity to chlamydial infection suggest that an ideal vaccine would be one that induces both a strong mucosal antibody response and a strong Th1 CD4-T-cell response. Current knowledge relating to induction of Th1 and Th2 responses and the reciprocal down-regulation of each response by an established T-cell response of the opposite type (reviewed in reference 46) suggests that this type of immune response may be difficult to induce. Furthermore, both the humoral and cell-mediated components of a vaccine-induced response would need to be targeted to the female reproductive tract (FRT), something not achieved by conventional injectable vaccines.

In this study we have used a novel immunization method, transcutaneous immunization (TCI) (16), that we have previously shown to efficiently target immune responses to the murine FRT (18). We have also used a combination of cholera toxin (CT) and CpG oligodeoxynucleotide (CpG-ODN) DNA as adjuvants in these studies. CpG-ODN are potent adjuvants in many species, and when they are administered by parenteral, oral, or intranasal route, they induce predominantly Th1 immune responses (40, 41). CT is a potent adjuvant for induction of mucosal IgA responses when administered by either the oral or intranasal route (11, 12) but elicits mainly IgG responses when administered transcutaneously (16, 17). TCI of female mice with *C. muridarum* (formerly the mouse pneumonitis strain of *C. trachomatis*) MOMP admixed with both CT and CpG-ODN induced MOMP-specific IgG and IgA in vaginal and uterine lavage fluid together with IFN-γ-secreting T cells in reproductive tract-draining caudal and lumbar lymph nodes (CLN). This immunization protocol resulted in enhanced clearance of *C. muridarum* following intravaginal challenge of BALB/c mice.

**MATERIALS AND METHODS**

**Mice.** Specific-pathogen-free female BALB/c mice were obtained from the Central Animal House, The University of Newcastle. Animals were housed under a standard day-night cycle and provided with sterile food and water ad libitum. All procedures were approved by the University of Newcastle Animal Care and Ethics Committee.

**Purification of recombinant MOMP.** The transformed *Escherichia coli* (DH5α/pMM3) expressing the pMAL-c2 vector encoding recombinant maltose binding protein (MBP)-MOMP fusion protein was a generous gift from Harlan Caldwell (Rocky Mountain Labs, Hamilton, Mont.) (61). Following overnight culture on ampicillin-supplemented nutrient agar plates, a single colony was selected from the plate and grown overnight in 20 ml of ampicillin-contain

**TCI of female mice with *C. muridarum* (formerly the mouse pneumonitis strain of *C. trachomatis*) MOMP admixed with both CT and CpG-ODN induced MOMP-specific IgG and IgA in vaginal and uterine lavage fluid together with IFN-γ-secreting T cells in reproductive tract-draining caudal and lumbar lymph nodes (CLN). This immunization protocol resulted in enhanced clearance of *C. muridarum* following intravaginal challenge of BALB/c mice.
TCI, Female BALB/c mice, 6 to 10 weeks old, were anesthetized intraperitoneally with xylazine hydrochloride (Rompun; Bayer, Sydney, Australia) and ketamine (Parnell Laboratory, Castle Hill, Australia). The lower back region (~1 cm²) of each mouse was shaved, with care taken not to break the skin. The shaved area was cleaned with alcohol. After 30 min the mice were injected intravenously with sterile PBS thoroughly dried. A 50-µl volume of PBS containing 200 µg of MBP-MOMP admixed with 10 µg of CpG-ODN (5'-TCC ATG ACG TTC ATG TT-3'), control ODN 5'-TCC AGG ACT TTC CTC AGG TT-3' (Geneworks, Rundle Mall, Australia) (28), and 10 µg of CT adjuvant (Sigma-Aldrich) was applied to the shaved area. The mice were maintained under anesthesia for 30 min to prevent grooming and oral infection from 1/10. After 30 min the injection site was washed thoroughly with PBS. Immunizations were administered weekly for three consecutive weeks, followed by a 6-week booster. Unanesthetized controls were included in all experiments.

Collection of serum, vaginal lavage fluid, and uterine lavage fluid samples. Serum and vaginal lavage fluid samples were collected preimmunization, post-third immunization, and post-6-week booster. Vaginal lavage fluid samples were collected by flushing the vagina with 40 µl of sterile PBS. Blood was collected from the saphenous vein into nonheparinized capillary tubes and then transferred to sterile 0.5-ml Eppendorf tubes and allowed to clot. Serum was separated by centrifugation and frozen at −20°C until required. At sacrifice the entire FRT was removed and the uterine horns were cut at the level of the uterine cervix. Uterine lavage fluids were collected by flushing the uterus with 50 µl of sterile PBS and then with 50 µl of 0.1% diethylhydrazine HCl (Sigma). Vaginal lavage fluids were frozen at −20°C until assayed.

MOMP-specific IgG (IgG1/IgG2a) and IgA ELISA. Levels of MOMP-specific IgA and IgG (IgG1/IgG2a) in serum and vaginal lavage fluid were determined by enzyme-linked immunosorbent assay (ELISA). Falcon 96-well flat-bottomed plates were coated with MBP-MOMP (2 µg/well) diluted in borate-buffered solution at pH 9.6 overnight at 4°C. The following day the plates were washed three times with 0.05% Tween 20 in PBS (PBST) and then blocked with 0.5% fetal calf serum (FCS) (100 µg/ml) well) for 1 h at 37°C. Plates were washed three times with PBST, and 50 µl of sample was added to appropriate wells at 4°C. Vaginal lavage fluid was serially diluted twofold from 1/20 to 1/160 in PBST. Serum was diluted 1/10 in PBST and then incubated for 1 h at 37°C. The plates were covered and incubated at 37°C for 1 h and then washed three times with PBST. Biotin-conjugated anti-IgA (Southern Biotechnology Associates, Birmingham, Ala.), anti-IgG (Amersham Life Science, Castle Hill, Austalia), anti-IgG1 (Southern Biotechnology Associates), and anti-IgG2a (Southern Biotechnology Associates) antibodies were diluted 1/1,000 in PBST, and 50 µl was added to each well of the respective plates. The plates were incubated for 1 h at 37°C and then washed five times with PBST. Hors eradish peroxidase (HRP)- conjugated streptavidin (Amersham Life Science) diluted 1/1,000 in PBST was added at 50 µl per well. The plates were incubated for 1 h at 37°C and then washed seven times with PBS. The plates were then developed using a 50-µl/well concentration of tetramethylbenzidine (0.01 mg/ml; Sigma-Aldrich) substrate dissolved in dimethyl sulfoxide (Sigma-Aldrich) and diluted 1/100 in phosphate citrate substrate buffer with sodium perborate (Sigma-Aldrich). The reaction was stopped by addition of 1 M sulfuric acid (50 µl/well) after 10 min, and the optical density of each plate was read at 450 nm.

In vitro neutralization of C. muridarum infectivity. McCoy cells were grown to 80% confluence in 48-well flat-bottom plates. Aliquots (40 µl) of diluted vaginal lavage fluid were incubated with 10¹ inclusion-forming units (IFU) of C. muridarum (C. trachomatis mouse pneumonitis biotype, ATCC VR-123) elementary bodies (EBs) for 1 h and then serially diluted 1/10 to 1/320 in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, gentamicin (5 µg/ml), and streptomycin (100 µg/ml) before addition to McCoy cell monolayers. Infected plates were centrifuged at 2,000 rpm for 30 min at room temperature to facilitate EB infection of cells. The plate was incubated for 2 h at 37°C with 5% CO₂. The plates were then washed with PBS to remove the unbound EBs. Cycloheximide at 1 µg/ml (Sigma) in DMEM was added to each well, and the plate was incubated at 37°C for 24 h. The next day, plates were spun at 2,000 rpm for 30 min at room temperature and fed fresh medium containing cycloheximide. After 28 to 42 h the plates were stained to visualize active inclusion bodies (IBs) within the cells. Using this dose of C. muridarum caused infection of approximately 70% of cells in medium control wells.

Staining of C. muridarum inclusions in infected McCoy cells. McCoy cell monolayers were washed with PBS and fixed with 100% methanol for 5 min and then air dried. Endogenous peroxidase was quenched by incubating the wells with 6% hydrogen peroxide for 5 min at room temperature. The plates were washed three times and then blocked with sterile PBS and diluted TBS (3 drops in 10 ml of PBS; Jomar Diagnostics, Stepney, Australia). The blocking solution was removed and 200 µl of the primary rabbit anti-C. trachomatis antibody (Pierce/Progen, Richlands, Australia), diluted 1 in 200 in PBS, was added to each well and incubated at 37°C for 1 h before washing twice with PBS. The secondary anti-rabbit antibody conjugated to HRP (Progen) was made up fresh (1 drop of secondary antibody conjugate, 3 drops of blocking serum in 1 ml PBS) and then added to each well with sterile PBS and diluted 1/100 in a humidified chamber at 37°C for 1 h. The plate was rinsed twice with PBS, excess fluid was removed, and then fresh ABC reagent (Pierce ImmunoPure ABC peroxidase kit; Progen) (2 drops of solution A and 2 drops of solution B in 10 ml of PBS) was added (200 µl per well) and the plates were incubated at 37°C for 30 min. The plate was washed once with PBS for 10 min, and then Immunoperoxidase reaction (3-3'-diaminobenzidine (DAB) hydrochloride; Sigma- Aldrich), diluted 1:10 in PBS was added. The plate was left to incubate at room temperature until the color developed. The reaction was stopped with the addition of 100 µl of water to each well, and C. muridarum-infected cells were visualized by light microscopy.

Detection of MOMP-specific ASC in the FRT. MOMP-specific B-cell numbers were determined by previously described methods (18). Briefly, vaginal and uterine tissues were fixed in ethanol and embedded in wax, and 5-µm-thick sections were cut. Sections were dewaxed and rehydrated through graded ethan- ool solutions to PBS. Immunoperoxidase staining was used to detect B cells secreting MOMP-specific antibody. Endogenous peroxidase was quenched by incubation of sections in blocking solution (4 ml of 30% H₂O₂, 75 ml of PBS, 75 ml of methanol). MOMP at 16 mg/ml was diluted 1/300 in 10% FCS–PBS, and 1 µl was added to each slide. The sections were covered with coverslips and the slides were incubated for 30 min at 37°C prior to washing twice in PBS. The rabbit anti-MOMP antibody (serum below) was diluted 1/100 with 10% FCS in PBS, and 150 µl was added to each slide. Control slides were incubated in 10% FCS in PBS. Slides were covered with coverslips, incubated for 1 h at 37°C, and then washed twice with PBS. HRP-conjugated sheep anti-rabbit IgG (Silenus, Melbourne, Australia) was diluted 1/100 in 10% FCS–PBS, and 150 µl was added to all slides for 1 h at 37°C. Slides were then washed twice with PBS, and sections were developed in 3,3-diaminobenzidine (Vector DAB kit) for 5 min and then counterstained with hematoxylin. Irrelevant antibody of the same species and isotype was used as a control for nonspecific antibody binding at each step. Sections were photographed using a Spot Junior camera (Diagnostic Instruments, Sydney, Australia), and numbers of MOMP-specific antibody-secreting cells (ASC) per 40× field were determined using Image Pro Plus 4.0 software (Media Cybernetics). For each experimental group vaginal tissues from three animals were embedded in a single block. For calculation of the numbers of ASC per field, 10 random fields per experimental group were counted.

Production of rabbit anti-MOMP antisera. Rabbis (specific pathogen free, outbred, lop-eared) were immunized subcutaneously with 0.5 mg of MOMP suspended in complete Freund's adjuvant (Sigma-Aldrich). Three and five weeks later animals were immunized with 0.5 mg of MOMP suspended in incomplete Freund's adjuvant (Sigma-Aldrich). Each animal was bleed 1 week after the final immunization. Serum was isolated from clotted whole blood by centrifugation and incubated for 1 h at 4°C with 10¹ BALB/c spleen cells per ml of serum. Aliquots of the adsorbed serum were stored at −20°C for later use in histology.

Determination of cytokine mRNA expression in T cells in CLIN following in vivo stimulation with MOMP. CLN were removed from immunized and control mice. Pooled CLN cells were isolated by passage of LN through a nylon cell strainer (Becton Dickinson, Sydney, Australia). The cells were washed twice in Hanks buffered salt solution and suspended in complete DMEM (DMEM supplemented with 10% FCS, 1-glutamine, 5 × 10⁻⁵ M 2-mercaptoethanol, HEPES buffer, penicillin-streptomycin, and amphotericin B [Fungizone], all from Trace Biosciences) at a concentration of 2 x 10⁶ cells per ml. Cells were divided into aliquots in 24-well plates (1 ml per well) and stimulated with MOMP (final concentration, 5 µg/ml) overnight at 37°C in 5% CO₂. After overnight incubation, cells were washed twice in Hanks buffered salt solution prior to isolation of RNA. Total RNA was isolated from stimulated and control LN cells by homogenization in 1 ml of solution A (50 ml of solution B [4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7, and 0.5% sarcosyl] [Sigma-Aldrich]) to the final concentration of 5 M and then removed by 2 M sodium acetate (pH 6.0) for 30 min at 4°C, the supernatant was discarded, and the pellet was resuspended in 300 µl of solution A. Three hundred microliters of isopropanol was added, and
the mixture was placed at −20°C overnight. The next day the RNA mixture was centrifuged at 8,000 rpm for 20 min at 4°C. The pellet was then washed twice, first with 100% ethanol to precipitate the RNA and then with 70% ethanol, and allowed to air dry to remove all traces of ethanol. The pellet was then dissolved in 100 μl of diethyl pyrocarbonate water. Samples were diluted 1:50, and absorbance at 260/290 nm was measured to determine the concentration. RNA (200 μg) was then reverse transcribed using 6 μg of random hexamers (Invitrogen, Mount Waverley, Australia), 1 μl of 10 mM PCR nucleotide mix (Invitrogen), 5 μl of 5× First-strand buffer (Invitrogen), and 5 μl of RNase OUT (Invitrogen). The reaction mixture was made up to 25 μl with RNase-free water, heated to 65°C for 5 min, and then transferred to 37°C for 5 min. Then, 1 μl of reverse transcriptase (Superscript II, Invitrogen) was added to each reaction tube, and the tubes were incubated for a further hour at 37°C. The tube was then heated to 70°C for 5 min and then cooled to 4°C and then cooled to 20°C or left on ice for immediate use in the PCR. Real-time PCRs were set up in PCR tubes with a final reaction volume of 25 μl. This technique used flanking nucleotide primers and Sybergreen with ROX reference dye. Primer sequences were designed using Primer Express 2.0 software (Applied Bioscienec). The sequences of all primers used in these studies are provided in Table 1. The relative increase in reporter fluorescence dye emission was monitored in real time during PCR amplification using the sequence detection system (ABI PRISM 7000 sequence detection system and software; PE Applied Biosystems, Inc.). PCR was performed in a 25-μl reaction volume that contained 2 μl of DNA extracted from vaginal swabs, 20 μM sense and antisense primers, 1× Sybergreen dye (0.25 μl of 100× stock), and Platinum qPCR Supermix-UDG with ROX added (12.5 μl). For each assay a standard curve using Chlamydia standards, determined by infection of McCoy cells as outlined above, was run simultaneously with the samples to allow for quantification using ABI PRISM 7000 software (PE Applied Biosystems).

Statistics. The significance of differences in the numbers of Chlamydia-positive McCoy cells between cultures preincubated with immune and nonimmune vaginal lavage and uterine lavage fluids was determined by unpaired two-tailed t test. The significance of differences in numbers of MOMP-specific ASC in vaginal tissues was determined by one-way analysis of variance with a Bonferroni post-test. The significance of differences in the rates of clearance of C. muridarum between immunized and control mice were determined using Kaplan-Meier survival curves and the log rank test.

![TABLE 1. Sequences of primers used in this study](image)

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>Beta-actin</td>
<td>Forward</td>
<td>5′ AGA GGG AAA TCG TGC GTG AC 3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′ CAA TAG TGA CCT GGC CGT 3′</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward</td>
<td>5′ TCA AGT GGC ATA GAT GTG GAA GAA 3′</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5′ TGG CTC TGC AGG ATT TTC ATG 3′</td>
</tr>
<tr>
<td>IL-4</td>
<td>Forward</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5′ AGG ACG TTG GCC ACA TCC AT 3′</td>
</tr>
<tr>
<td>C. muridarum MOMP (bp 206–281)</td>
<td>Forward</td>
<td>5′ GCC GTT TTG GGT TCT GCT T 3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′ CGT CAA TCA TAA GGC TTG GTT CA 3′</td>
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FIG. 1. CpG as adjuvants for TCI. Female BALB/c mice were immunized with MOMP mixed with either CT (10 μg) or CpG-ODN (10 μg) or control ODN by direct application to skin as described in Materials and Methods MOMP-specific IgG in serum (1:200 dilution) was determined by ELISA. Results are representative of four separate experiments. Error bars, standard deviations.
RESULTS

CpG-ODN nucleotides as adjuvants for TCI. TCI requires that bacterial ADP-ribosylating exotoxins be mixed with antigen in order to elicit immune responses (17). The data in Fig. 1 show that CpG-ODN can also enhance responses to antigens applied directly to the skin. CpG-ODN, at a dose of 10 μg, mixed with 200 μg of MOMP, elicited levels of MOMP-specific serum IgG comparable to those elicited by MOMP administered with CT as an adjuvant. In preliminary studies (data not shown) increasing the CpG-ODN dose to 50 or 100 μg did not result in increased MOMP-specific serum IgG responses. In some experiments, control oligonucleotides (Fig. 1, negative control) that did not contain CG dinucleotides also had some adjuvant effect. Thus, skin-based immunization is also possible using adjuvants other than ADP-ribosylating exotoxins. Of greater importance for protection against Chlamydia infection, TCI using CpG-ODN adjuvant resulted in significant numbers of MOMP-specific ASC in vaginal tissues. Numbers of vaginal MOMP-specific ASC in animals immunized with CpG-ODN adjuvant (Fig. 2c) were twofold greater than ASC numbers in mice immunized with CT as adjuvant (Fig. 2b). MOMP-specific ASC numbers in tissues from animals immunized with MOMP combined with both CT and CpG (Fig. 2d) were not increased compared to those in tissues from animals immunized with MOMP plus CpG alone. Low numbers of MOMP-specific ASC were detected in vaginal tissues of animals immunized with MOMP alone (Fig. 2a), most likely due to the LPS present in the MOMP preparation.

TCI using combined CT plus CpG-ODN adjuvant induces both Th1 and Th2 responses. TCI using CT results in a serum IgG response that is predominantly IgG1, indicative of a Th2 response (Fig. 3), whereas CpG-ODN induce predominantly Th1 responses (40). However, when mice were immunized transcutaneously with both CT and CpG-ODN, the serum IgG response contained both IgG1 and IgG2a (Fig. 3), indicative of a mixed Th1-Th2 response.

CpG-ODN increase vaginal lavage fluid IgA and IgG levels following TCI. TCI, using CT as an adjuvant, induces antigen-specific IgG and low levels of IgA in vaginal lavage fluid (18) (Fig. 4). When mice were immunized with MOMP combined with either CT or CpG or with both CT and CpG-ODN, MOMP-specific IgG was detected in vaginal lavage fluid (Fig. 4a). Levels of IgG did not differ significantly depending on the adjuvant used. Interestingly, TCI with CpG plus MOMP resulted in higher levels of IgA in vaginal lavage fluid than that found in animals immunized with CT or CT plus CpG adjuvant (Fig. 4b). Furthermore, preincubation of C. muridarum with vaginal lavage fluid or uterine lavage fluid from mice immunized with MOMP mixed with both CT and CpG-ODN inhibited in vitro infection of McCoy cells by 45 to 60% at a dilution
of 1:160 (Table 2), demonstrating that antibody in both upper and lower reproductive tract secretions could potentially prevent attachment of *Chlamydia* and reduce sexual transmission.

Combination CT plus CpG-ODN adjuvant induces IFN-γ mRNA expression by CLN cells. Local Th1 responses have been implicated in clearance of *Chlamydia* from the genital tract. We therefore isolated mononuclear cells from the reproductive-tract-draining CLN of immunized mice, stimulated these in vitro with MOMP, and used real-time PCR to determine the relative levels of IFN-γ and interleukin 4 (IL-4) mRNA (Fig. 5). CLN cells from mice immunized with MOMP alone showed an 11-fold increase in IL-4 mRNA compared to cells from nonimmunized mice following in vitro stimulation. Immunization of mice with MOMP plus CT resulted in a six- to sevenfold increase in IL-4 mRNA levels in CLN cells, with a three- to fourfold increase in IFN-γ mRNA. In vitro stimulation of CLN cells from mice immunized with MOMP plus CpG resulted in a 75- to 80-fold increase in IFN-γ mRNA and a 5- to 6-fold increase in IL-4 mRNA. In vitro stimulation of CLN cells from mice immunized with MOMP plus combined CT and CpG-ODN adjuvant resulted in an even greater, 90- to 105-fold increase in IFN-γ mRNA expression, while IL-4 mRNA levels were 15-fold higher than levels seen in cells from nonimmunized control mice, levels still higher than those found in animals immunized with MOMP plus CT.

TCI enhances clearance of *C. muridarum* following vaginal challenge. Vaginal swabs were collected at 3-day intervals from mice immunized with MOMP admixed with both CT plus

<table>
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<tr>
<th>Lavage fluid sample</th>
<th>Expt no.</th>
<th>% McCoy cells containing inclusions (mean ± SD)</th>
<th>Nonimmune</th>
<th>Immune</th>
<th>p</th>
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<td>Vaginal</td>
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<td>31.7 ± 3.6</td>
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<td></td>
<td>2</td>
<td>67.6 ± 6.7</td>
<td>24.4 ± 11.1</td>
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<td>Uterine</td>
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<td>78.9 ± 1.2</td>
<td>39.3 ± 1.3</td>
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<td></td>
<td>2</td>
<td>75.3 ± 0.5</td>
<td>33.9 ± 1.6</td>
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<td>&lt;0.0001</td>
</tr>
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*C. muridarum* was incubated for 1 h with vaginal and uterine lavage fluids from immunized or nonimmunized mice prior to plating onto McCoy cell monolayers. After 24 to 40 h of incubation, McCoy cell monolayers were fixed and stained to reveal chlamydial IBs. Results represent McCoy cells containing chlamydial inclusions. The dose of *Chlamydia* used resulted in infection of approximately 70% of the McCoy cells in the medium-only control cultures. All samples were tested in triplicate, and the results are representative of four separate experiments.
CpG-ODN and nonimmunized control mice. Total DNA was extracted from swabs, and chlamydial DNA was determined by real-time PCR (Fig. 6). Standard curves were generated in each assay using *C. muridarum* standards that had been quantitated for IFU by in vitro infection of McCoy cells. At 6 days postinfection, levels of *Chlamydia* recovered from immunized animals were less than half that recovered from nonimmunized controls. By day 12 postinfection chlamydial DNA was below the limits of detection in swabs from four of five immunized mice but readily detected in five of five nonimmunized controls. All immunized mice had cleared the infection by day 15, and vaginal swabs from immunized mice remained negative out to day 21 when the experiment was terminated. At day 21 two of five control animals still had detectable chlamydial DNA in their vaginal lavage fluid. The rate of clearance of *Chlamydia* in immunized mice was significantly higher (*P* < 0.02) than that seen in nonimmunized controls.

**DISCUSSION**

The present studies show that CpG-ODN can function either alone or in combination with CT as effective adjuvants for TCI, inducing serum IgG responses and ASC in vaginal tissues. TCI with a combination of CT plus CpG-ODN induces both IgG1 and IgG2a in serum, indicative of a mixed Th1-Th2 response. The combination adjuvant also elicits antigen-specific IgG and IgA in vaginal lavage fluid. Vaginal and uterine lavage fluid from animals immunized transcutaneously with MOMP mixed with both CT and CpG-ODN inhibited in vitro chlamydial infection of McCoy cells. Stimulation of CLN cells from immunized mice resulted in high levels of IFN-γ mRNA production, indicative of a strong Th1 response, together with moderate levels of IL-4 mRNA, characteristic of Th2 responses. Immunized mice showed significantly enhanced clearance of *Chlamydia* following vaginal challenge. Bacterial DNA containing nonmethylated CG sequences is a strong activator of innate immunity, activating phagocytic cells by binding to Toll-like receptor 9 (22). This property has re-
sulted in the use of synthetic ODN containing CG motifs as adjuvants (32, 41). CpG-ODN has been shown to be an effective adjuvant when administered by subcutaneous, intramuscular, oral, rectal, and nasal routes (39) and induces immune responses that are predominantly Th1 (8, 65). For our studies, we chose CpG-ODN that had been shown to enhance the expression of the costimulatory molecules MHC-II and CD86 on cutaneous Langerhans cells and to increase IL-12 production and accessory cell function by Langerhans cells (28), because these cells are involved in initiation of immune responses following application of antigen to the skin (55). As shown in Fig. 1, CpG-ODN can also function as effective adjuvants for antigens applied directly to the skin, for induction of serum IgG responses. TCI using CpG-ODN also induced high numbers of ASC in vaginal tissues (Fig. 2c), higher numbers than those seen in animals immunized with MOMP plus CT (Fig. 2b). Interestingly, in some experiments we found that control non-CG-containing ODN also enhanced serum and mucosal responses to the coadministered MOMP. This effect has also been observed in other studies (64), in which methylated ODN longer than 24 nucleotides and with a phosphorothioate backbone retained immunostimulatory properties. ODN rich in thymidine residues were also shown to be immunostimulatory in the absence of CG dinucleotide sequences. While immunostimulatory CpG-ODN have been identified for humans and many animal species (2, 20, 30, 33, 44, 53, 62) and can therefore be used as adjuvants, the design of optimum CpG-ODN adjuvants for any particular application must consider features of the type of backbone, length, and thymidine content.

Protection against genital tract Chlamydia infection requires that immune responses be targeted to the FRT. We have previously shown that TCI is particularly effective at inducing immunity in the lower (vagina) (18) and upper (uterine horns [Skelding et al., unpublished data]) FRT. This has also been demonstrated in the present study. Furthermore, the type of immune response induced must be appropriate for the pathogen, in this case Chlamydia. Both Th1-cell-mediated immunity and antibody responses have been shown to protect against genital tract Chlamydia infection in mouse, guinea pig, and primate models. The relative role of cell-mediated versus humoral immunity will likely depend mainly on the numbers of infectious Chlamydia used in a particular study. For example, one study that claims to show that mucosal IgA is not protective (29) used a challenge dose of 10^7 C. trachomatis EBs. However there are numerous studies using challenge doses in the range of 10^5 to 10^7 IFU of Chlamydia that show a protective effect for both IgG and IgA antibodies (9, 43, 50, 60). With regard to the efficacy of any potential human vaccine for Chlamydia, it is not known how many IFU of Chlamydia are present in the ejaculate of an infected male. This will obviously have a major bearing on the effectiveness of vaccine-induced immunity. Using the guinea pig model of Chlamydia genital tract infection, Rank et al. (52) showed that the number of chlamydial agents of guinea pig inclusion conjunctivitis transmitted by infected males is in the range of 10^5 to 10^7, a dose at which vaginal antibodies are certainly protective. Thus, an ideal vaccine may be one that induces both Th1 CMI plus a strong mucosal antibody response. Th1-T-cell production of IFN-γ can protect against the intracellular reticulate body (RB) form of Chlamydia by one or more of three mechanisms. These include up-regulation of inducible nitric oxide synthase expression resulting in nitric oxide production by macrophages (7, 26); induction of indoleamine 2,3-dioxygenase, which degrades intracellular stores of tryptophan, an amino acid that Chlamydia has only a limited ability to produce (26, 47); and down-regulation of transferrin receptor expression on the surfaces of infected cells, which would decrease intracellular iron stores (26). Th1 cells secreting IFN-γ have been shown to be protective in animal models (10, 48), while Chlamydia-specific Th2 clones do not provide protection against genital infection (21). Antibody may also mediate protection by a number of mechanisms, including blocking the initial attachment of Chlamydia to epithelial cells and prevention of subsequent ascending infection, enhancing killing of opsonized Chlamydia by phagocytic cells, thereby limiting dissemination to distant sites and enhancing the resolution of intracellular infection by antibody-dependent cellular cytotoxicity (42). IgA in vaginal secretions may also be able to target the intracellular chlamydial RBs due to the fact that the IgA transporter molecule, the polymeric immunoglobulin receptor (PlgR), has been shown to be present on epithelial cells in the rodent (67) and human (34) FRT. PlgR-mediated transport of IgA across epithelial cells provides an opportunity to target intracellular pathogens (37, 38). Intraepithelial cell neutralization of viruses by IgA has been demonstrated both in vitro and in vivo (37). IgA antibodies against viral envelope proteins can neutralize Sendai and influenza virus infections in polarized epithelial cell monolayers that express the PlgR. IgA antibodies against murine rotavirus and murine hepatitis virus neutralize viruses in vivo inside intestinal epithelial cells and hepatocytes, respectively (5, 25). Thus, an immunization protocol that elicits IgA antibodies against both surface antigens on the EB and antigens associated with the inclusion membrane of Chlamydia, such as the Inc proteins (54), may provide increased protection against infection. Although chlamydialae are able to inhibit phagosome formation as a means of enhancing intracellular growth, it is not currently known if replication of Chlamydia within the IB is susceptible to neutralization by IgA during transepithelial transport. We are currently using in vitro models of chlamydial infection of PlgR^+ cell lines to determine if IgA can target the intracellular RB stage.

The present studies have demonstrated that TCI with combination CT plus CpG-ODN adjuvant fulfills all of the criteria for an effective vaccine against genital tract Chlamydia infection; specifically, the response is targeted to the site of infection, the FRT; neutralizing antibody (both IgG and IgA) is induced in vaginal and uterine secretions; and a strong Th1 IFN-γ response is elicited in the reproductive-tract-draining LN. This immunization strategy could easily be tested in humans due to the availability of CpG-ODN that are immunostimulatory for human lymphocytes and nontoxic mutant CT that retains adjuvant properties (35, 71–73). Furthermore, the application of CT, either wild-type or mutant forms, directly to skin further reduces the chance of toxic side effects that occur following oral ingestion of CT and also avoids potential toxicity of CT for the central nervous system (15, 63) that may occur following intranasal administration. Both CT and its β subunit have been shown to accumulate in the olfactory nerves or epithelium and olfactory bulbs of mice when given by the nasal
route, something that could limit the utility of intranasal immunization in humans.

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