INFECTION AND IMMUNITY, Feb. 2004, p. 1019–1028 Vol. 72, No. 2
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.2.1019–1028.2004

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

Linda J. Berry,[1] Danica K. Hickey,[1] Kathryn A. Skelding,[1] Shisan Bao,[2] Amanda M. Rendina,[3] Philip M. Hansbro,[1] Christine M. Gockel,[4] and Kenneth W. Beagley[1]*

Discipline of Immunology and Microbiology, School of Biomedical Science, The University of Newcastle and Hunter Medical Research Institute,[1] and School of Environmental and Life Sciences, Faculty of Science and Information Technology, The University of Newcastle,[3] Newcastle, and Department of Pathology, The University of Sydney, Sydney,[2] Australia, and Department of Microbiology, University of Buffalo, Buffalo, New York[4]

Received 14 July 2003/Returned for modification 28 August 2003/Accepted 20 October 2003

***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. Infection induces 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-

* Corresponding author. Mailing address: Discipline of Immunology and Microbiology, School of Biomedical Science, Faculty of Health, The University of Newcastle, Callaghan, New South Wales 2308, Australia. Phone: 61-2-49236157. Fax: 61-2-49236488. E-mail: Ken.Beagley@newcastle.edu.au.

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{pMMM3}) 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-containing medium (Luria-Bertani broth; Bacto, Liverpool, Australia). This 20 ml was then used to inoculate 1 liter of ampicillin-containing medium, which was then grown overnight at 37°C on a shaker incubator at 200 rpm. The next day, 3 ml of 0.1 M isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, Castle Hill, Australia) was added to the culture, which was incubated for a further 2 h. Bacteria were harvested by centrifugation and the pellet was washed twice with phosphate-buffered saline (PBS) (Trace Biosciences, Sydney, Australia). The pellet was resuspended in 40 ml of PBS and then sonicated on ice eight times, for 15 s on and 15 s off, at an amplitude of 7 (Soniprep 150-MSE; Townson and Mercer Pty. Ltd.). After centrifugation at 8,500 rpm with a Beckman J-12 centrifuge, the pellet was washed twice with PBS and resuspended in 10 ml of 8 M urea containing 100 μl of protease inhibitor cocktail (Sigma-Aldrich) for 2 h at 37°C. The solution was centrifuged to recover the soluble supernatant containing the MOMP. The MBP-MOMP protein was purified using a PD-10 Sephadex column (Amersham Biosciences, Baulkham Hills, Australia) followed by a Sephadex G-75 column (Sigma-Aldrich) and concentrated using an Amicon Centriprep concentrator (Millipore, North Ryde, Australia) with a 50,000-molecular-weight-cutoff filter. The protein concentration of each batch was determined using a Pierce protein estimation kit (Progen Industries, Darra, Australia). Characterization of the MOMP was performed for all batch samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 15% sodium dodecyl sulfate gels (Bio-Rad Laboratories Pty Ltd., Reagents Park, Australia) to ensure the presence of the predicted 80-kDa MBP-MOMP polypeptide consistent with the combined mass of MBP (42 kDa) and MOMP (39 kDa). MBP-MOMP samples were frozen at −20°C until required.

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 cm^2) of each mouse was shaved, with care taken not to break the skin. The shaved area was swabbed with acetone followed by sterile PBS and allowed to dry. A 50-μl volume of PBS containing 200 μg of MBP-MOMP admixed with 10 μg of CpG-ODN (5′-TCC ATG ACG TTC CTG ACG 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 immunization. After 30 min the application site was swabbed thoroughly with PBS. Immunizations were administered weekly for three consecutive weeks, followed by a 6-week booster. Nonimmunized 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 corpus. The ovary and oviducts were removed, and each uterine horn was flushed with 100 μl of PBS containing protease inhibitors. Uterine 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 flexiplates 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 0.05% fetal calf serum (FCS) (100 μl/well) in PBST 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 twofold from 1/100 to 1/800 in PBST. Background wells contained 50 μl of PBST alone. 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, Australia), 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. Horseradish 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 10^3 inclusion-forming units (IFU) of *C. muridarum* (*C. trachomatis* mouse pneumonitis biovar, 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% CO_2. 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 twice with PBS and then blocked for 30 min at 37°C using diluted goat serum (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 10 ml of PBS), 200 μl was added to each well, and then the plate was incubated 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 Immunopure metal-enhanced 3,3-diaminobenzidine substrate (200 μl per well; Progen), 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 ethanol 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% H_2O_2, 75 ml of PBS, 75 ml of methanol). MOMP at 16 mg/ml was diluted 1/300 in 10% FCS–PBS, and 150 μl was added to each slide. The sections were covered with a coverslip, and the slides were incubated for 30 min at 37°C prior to washing twice in PBS. The rabbit anti-MOMP antiserum (see 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 topped 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 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 antiserum. Rabbits (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 bled 1 week after the final immunization. Serum was isolated from clotted whole blood by centrifugation and incubated for 1 h at 4°C with 10^7 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 by T cells in CLN following in vitro 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 two times in Hanks buffered salt solution and suspended in complete DMEM (DMEM supplemented with 10% FCS, L-glutamine, 5×10^{-5} M 2-mercaptoethanol, HEPES buffer, penicillin-streptomycin, and amphotericin B [Fungizone], all from Trace Biosciences) at a concentration of 2×10^6 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% CO_2. After overnight incubation, cells were washed two times 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 which was added 360 μl of 2-mercaptoethanol (Sigma-Aldrich). To each sample was added 100 μl of 2 M sodium acetate made up in 0.1% diethyl pyrocarbonate water (Sigma-Aldrich), 1 ml of phenol, and 200 μl of chloroform-isoamyl alcohol (Sigma-Aldrich), mixing well after each addition. Tubes were vortexed vigorously for 10 s and then centrifuged at 8,000 rpm for 20 min at 4°C. The aqueous phase was then transferred to a fresh tube and 1 ml of isopropanol (BDH, Sydney, Australia) was added, the solution was mixed, and the tube was placed at −20°C for at least 1 h. The mixture was then centrifuged at 8,000 rpm 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

TABLE 1. Sequences of primers used in this study

Gene product	Primer	Sequence
Beta-actin	Forward	5′ AGA GGG AAA TCG TGC GTG AC 3′
	Reverse	5′ CAA TAG TGA TGA CCT GGC CGT 3′
IFN-γ	Forward	5′ TCA AGT GGC ATA GAT GTG GAA GAA 3′
	Reverse	5′ TGG CTC TGC AGG ATT TTC ATG 3′
IL-4	Forward	5′ GGC ATT TTG AAC GAG GTC ACA 3′
	Reverse	5′ AGG ACG TTT GGC ACA TCC AT 3′
C. muridarum MOMP (bp 206–281)	Forward	5′ GCC GTT TTG GGT TCT GCT T 3′
	Reverse	5′ CGT CAA TCA TAA GGC TTG GTT CA 3′

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 260/280 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 for 5 min at 95°C and then cooled to 4°C and frozen at −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 Biosciences). The sequences of all primers used in these studies are provided in Table 1. The relative increase in reporter fluorescent dye emission was monitored in real time during PCR amplification using the sequence detection system and software (ABI PRISM 7000 sequence detection system and software; PE Applied Biosystems Inc., Foster City, Calif.) and PCR was performed as described previously. Briefly, the 25-μl reaction volume contained 2 μl of cDNA from the reverse transcription reaction, 0.5 μl of primers and antisense primers, 0.25 μl of 100× Sybergreen dye, and 12.5 μl of Platinum qPCR supermix-UDG (Invitrogen). Beta-actin RNA was used as an internal standard to control for variability in amplification due to differences in initial RNA concentrations. The level of the cytokine RNA, relative to beta-actin RNA, was calculated using the following formula: relative RNA expression = $2^{-(\text{Ct of cytokine} - \text{Ct of beta actin})} \times 10^{10}$, where Ct is on the threshold cycle value (70). Since the level of cytokine RNA was considerably lower than the level of beta-actin RNA, all RNA values were arbitrarily multiplied by 10^{10}.

Intravaginal challenge with *C. muridarum*. Seven days prior to intravaginal challenge all mice received 2.5 mg of medroxyprogesterone acetate (Depo-Ralovera; Kenral, Rydalmere, New South Wales, Australia) subcutaneously. For intravaginal challenge mice were anesthetized intraperitoneally with Rompun and ketamine, and 5×10^4 IFU of *C. muridarum* in 10 μl of sucrose-phosphate-glutamate (SPG) solution was administered into the vaginal vault with the mouse in a head down position. When mice had recovered from the anesthesia, the genital region was swabbed to remove any *Chlamydia* cells from the external area to avoid oral inoculation as a result of grooming. The mice were then returned to their cages and housed under biosafety PC2 conditions.

Monitoring clearance of intravaginal inoculation by real-time PCR. Vaginal swabs were collected at 3-day intervals from day 0 to 21 of infection. The vagina was swabbed using a nasopharyngeal Calgiswab (Modular Medical Products, Alice Springs, Australia) moistened with ice-cold sterile SPG. The swab was then placed into an Eppendorf tube containing 500 μl of SPG and a single glass bead, the end of the swab was removed, and the tube was left on ice for immediate DNA extraction or frozen at −20°C. Total DNA was extracted using an Amplicore CT/NG specimen preparation kit (Roche, Castle Hill, Australia) according to the manufacturer's instructions. The swab sample was vortexed vigorously for 30 s, the swab was removed, and 100 μl of sample was removed to a new Eppendorf tube. This aliquot was combined with 50 μl of CT/NG lysis buffer, vortexed for 30 s, and incubated at room temperature for 10 min. One hundred microliters of CT/NG specimen diluent was then added, vortexed briefly, incubated at room temperature for 10 min, and then stored at 4°C overnight. Real-time PCR was used to quantify the amount of *Chlamydia* present in the vaginal swabs. The real-time PCR used flanking nucleotide primers specific for *C. muridarum MOMP*, as can be seen in Table 1, and Sybergreen dye with ROX reference dye. The relative increase in reporter fluorescent 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 mM 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 *C. muridarum* 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.

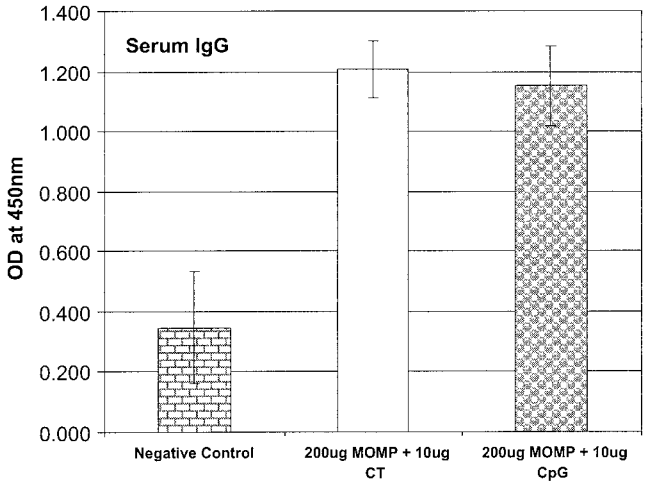

FIG. 1. CpG as adjuvants for TCI. Female BALB/c mice were immunized with MOMP mixed with either CT (10 μg) or CpG-ODN (10 μg) or control ODN by direct application to skin as described in Materials and Methods MOMP-specific IgG in serum (1:200 dilution) was determined by ELISA. Results are representative 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

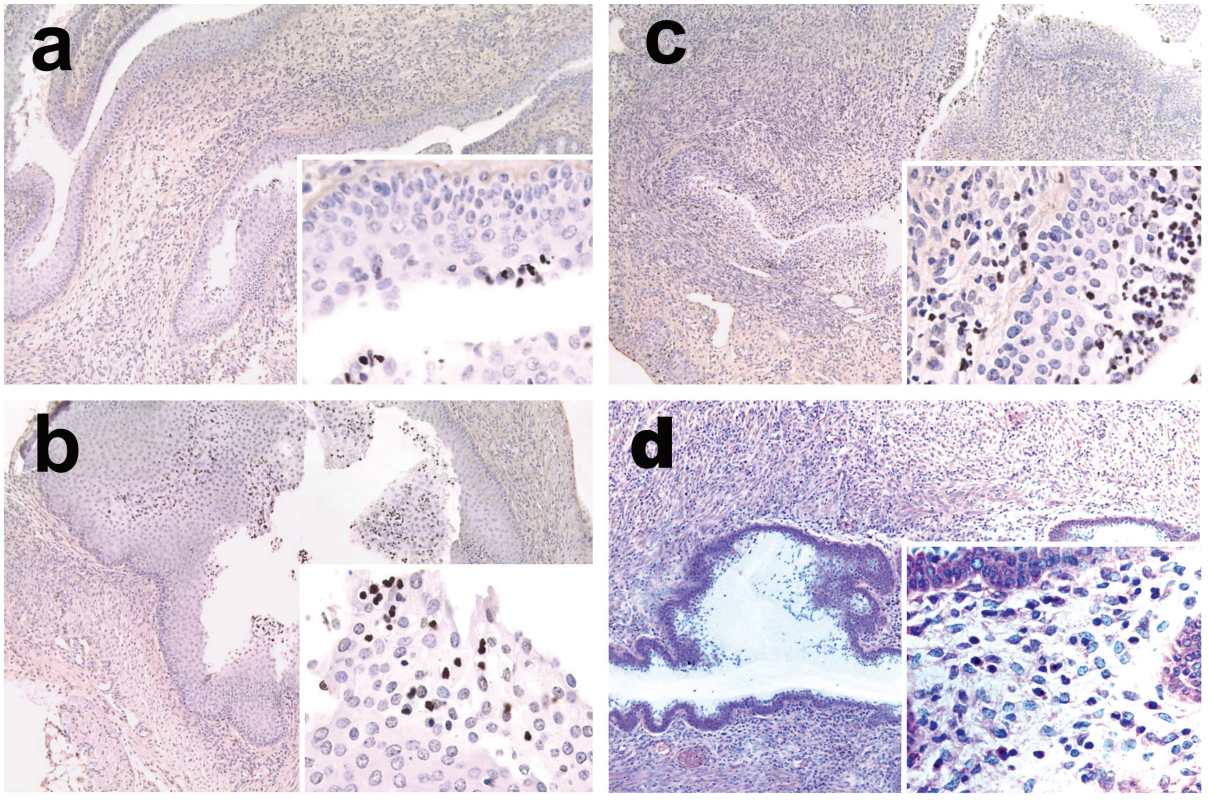

FIG. 2. MOMP-specific ASC in vaginal tissues. Vaginal tissues from mice immunized transcutaneously with MOMP alone (a), MOMP plus CT (b), MOMP plus CpG-ODN (c), and MOMP plus CT and CpG-ODN (d) were stained for MOMP-specific ASC as described in Materials and Methods. Magnification, ×10 (magnification for insets, ×40). The numbers of ASC per field in each of the large panels (mean ± standard error of the mean) were as follows: 2.2 ± 0.53, 4.4 ± 0.63, 7.5 ± 0.79, and 7.9 ± 1.4, respectively. Differences between numbers of ASC in panels a versus b and panels c versus d were not significant; differences between numbers of ASC in panels a versus c and a versus d were significant ($P < 0.01$]).

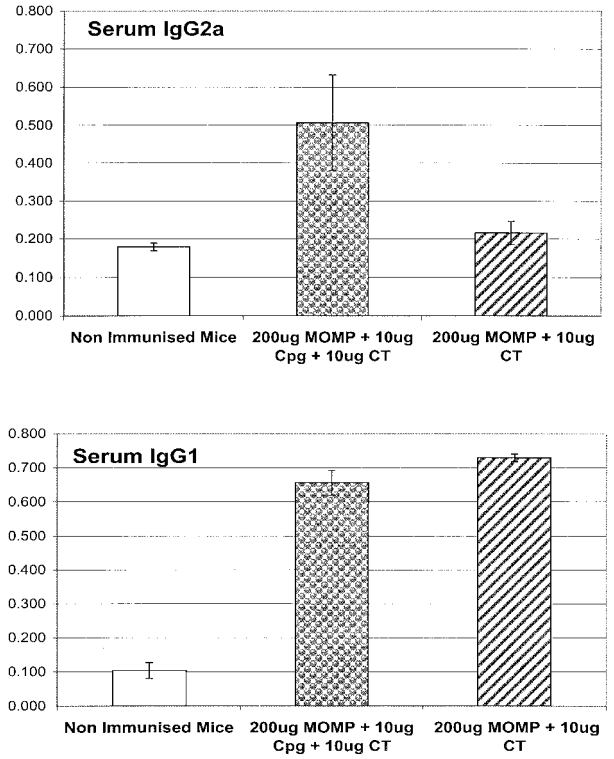

FIG. 3. Combination CT plus CpG-ODN adjuvant enhances IgG2a production. Female BALB/c mice were immunized with MOMP mixed with either CT alone or both CT and CpG-ODN by direct application to skin as described in Materials and Methods. MOMP-specific IgG2a (upper panel) and IgG1 (lower panel) in serum (1:200 dilution) were determined by ELISA. Results are representative of three separate experiments. The *y* axis shows optical density at 450 nm. Error bars, standard deviations.

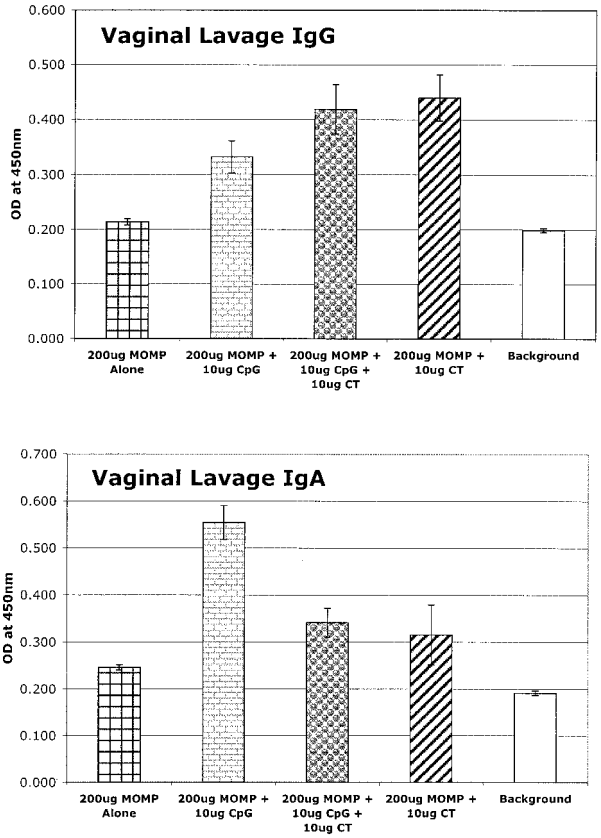

FIG. 4. MOMP-specific IgG and IgA in vaginal lavage fluid following TCI. Female BALB/c mice were immunized with MOMP mixed with either CT alone, CpG alone, or both CT and CpG-ODN by direct application to skin as described in Materials and Methods. MOMP-specific IgG (upper panel) and IgA (lower panel) in vaginal lavage fluid (1:40 dilution) were determined by ELISA. Results are representative of three separate experiments. Error bars, standard deviations.

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 2. McCoy cells containing inclusions[a]

Lavage fluid sample	Expt no.	% McCoy cells containing inclusions (mean ± SD)		*P*
		Nonimmune	Immune	
Vaginal	1	57.6 ± 3.7	31.7 ± 3.6	<0.0002
	2	67.6 ± 6.7	24.4 ± 11.1	<0.01
Uterine	1	78.9 ± 1.2	39.3 ± 1.3	<0.0001
	2	75.3 ± 0.5	33.9 ± 1.6	<0.0001

[a] *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.

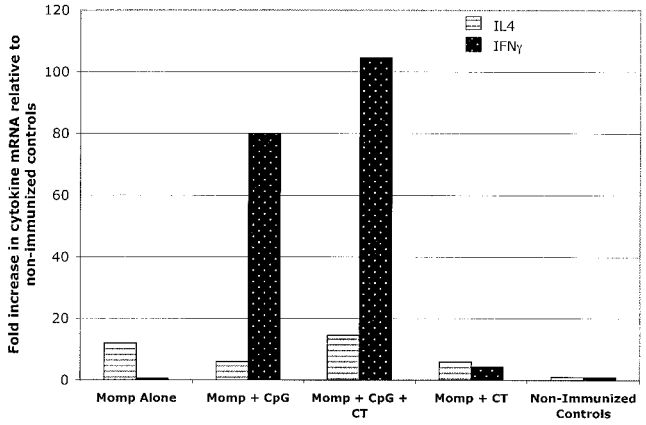

FIG. 5. IFN-γ and IL-4 mRNA expression by CLN cells. CLN cells from mice immunized transcutaneously with MOMP alone, MOMP plus CT, MOMP plus CpG, and MOMP plus CT and CPG-ODN were stimulated overnight with MOMP. mRNA was isolated from cultures, and levels of IFN-γ (dark bars) and IL-4 (light bars) mRNA, relative to nonimmunized control cultures, were determined by real-time PCR (70). Results are representative of three 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-

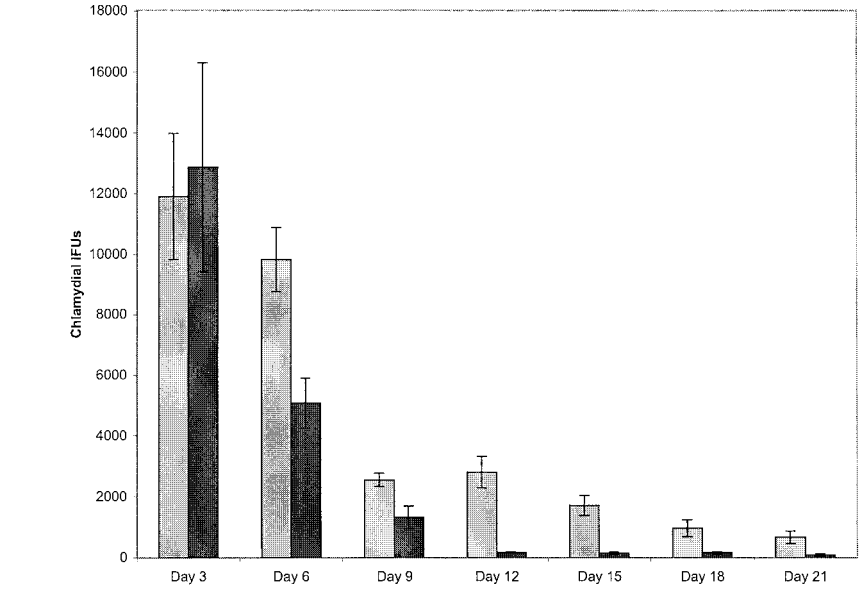

FIG. 6. TCI enhances clearance of vaginal *C. muridarum*. Vaginal swabs were collected at 3-day intervals from immunized and control mice following intravaginal challenge with *C. muridarum*. DNA was extracted from vaginal swabs, and relative levels of *Chlamydia* DNA were determined by real-time PCR. The significance of differences in the rates of clearance of *C. muridarum* between immunized (dark bars) and control (light bars) mice was determined using Kaplan-Meier survival curves and the log rank test. Results are representative of three separate experiments. Error bars, standard deviations.

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 such as 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 10^2 to 10^5 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 10^1 to 10^3, 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 (PIgR), has been shown to be present on epithelial cells in the rodent (67) and human (34) FRT. PIgR-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 PIgR. 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 chlamydiae are able to inhibit phagolysosome 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 $PIgR^+$ 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.

ACKNOWLEDGMENTS

This work was supported by grants from the Ronald Geoffrey Arnott Foundation, the Hunter Medical Research Institute, and the University of Newcastle Research Management Committee.

We thank Peter Timms for review of the manuscript.

REFERENCES

1. **Batteiger, B. E., R. G. Rank, P. M. Bavoil, and L. S. Soderberg.** 1993. 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. **139:**2965–2972.
2. **Bauer, M., K. Heeg, H. Wagner, and G. B. Lipford.** 1999. DNA activates human immune cells through a CpG sequence-dependent manner. Immunology **97:**699–705.
3. **Beagley, K. W., and P. Timms.** 2000. Chlamydia trachomatis infection: incidence, health costs and prospects for vaccine development. J. Reprod. Immunol. **48:**47–68.
4. **Brunham, R. C., C. C. Kuo, L. Cles, and K. K. Holmes.** 1983. Correlation of host immune response with quantitative recovery of *Chlamydia trachomatis* from the human endocervix. Infect. Immun. **39:**1491–1494.
5. **Burns, J. W., M. Siadat-Pajouh, A. A. Krishnaney, and H. B. Greenberg.** 1996. Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity. Science **272:**104–107.
6. **Burst, H. V.** 1998. Sexually transmitted diseases and reproductive health in women. J. Nurse Midwif. **43:**431–444.
7. **Chen, B., R. Stout, and W. F. Campbell.** 1996. Nitric oxide production: a mechanism of Chlamydia trachomatis inhibition in interferon-gamma-treated RAW264.7 cells. FEMS Immun. Med. Microbiol. **14:**109–120.
8. **Chu, R. S., O. S. Targoni, A. M. Krieg, P. V. Lehmann, and C. V. Harding.** 1997. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J. Exp. Med. **186:**1623–1631.
9. **Cotter, T. W., Q. Meng, Z. L. Shen, Y. X. Zhang, H. Su, and H. D. Caldwell.** 1995. Protective efficacy of major outer membrane protein-specific immunoglobulin A (IgA) and IgG monoclonal antibodies in a murine model of *Chlamydia trachomatis* genital tract infection. Infect. Immun. **63:**4704–4714.
10. **Cotter, T. W., K. H. Ramsey, G. S. Miranpuri, C. E. Poulsen, and G. I. Byrne.** 1997. Dissemination of *Chlamydia trachomatis* chronic genital tract infection in gamma interferon gene knockout mice. Infect. Immun. **65:**2145–2152.
11. **Elson, C. O.** 1996. Mucosal vaccines, p. 59–72. Academic Press Inc., New York, N.Y.
12. **Elson, C. O., and M. T. Dertzbaugh.** 1999. Mucosal adjuvants, p. 817–838. *In* P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, and J. R. McGhee (ed.), Mucosal immunology, 2nd ed. Academic Press, San Diego, Calif.
13. **Eng, T. R., and W. T. Butler (ed.).** 1997. The hidden epidemic: confronting sexually transmitted disease. National Academies Press, Washington, D.C.
14. **Fan, T., H. Lu, H. Hu, L. Shi, G. A. McClarty, D. M. Nance, A. H. Greenberg, and G. Zhong.** 1998. Inhibition of apoptosis in chlamydia-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. J. Exp. Med. **187:**487–496.
15. **Fujihashi, K., T. Koga, F. W. van Ginkel, Y. Hagiwara, and J. R. McGhee.** 2002. A dilemma for mucosal vaccination: efficacy versus toxicity using enterotoxin-based adjuvants. Vaccine **20:**2431–2438.
16. **Glenn, G. M., M. Rao, G. R. Matyas, and C. R. Alving.** 1998. Skin immunization made possible by cholera toxin. Nature **391:**851.
17. **Glenn, G. M., T. Scharton-Kersten, R. Vassell, G. R. Matyas, and C. R. Alving.** 1999. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins as antigens and adjuvants. Infect. Immun. **67:**1100–1106.
18. **Gockel, C. M., S. Bao, and K. W. Beagley.** 2000. Transcutaneous immunization induces mucosal and systemic immunity: a potent method for targeting immunity to the female reproductive tract. Mol. Immunol. **37:**537–544.
19. **Hammerschlag, M. R.** 2002. The intracellular life of chlamydiae. Semin. Pediatr. Infect. Dis. **13:**239–248.
20. **Hartmann, G., R. D. Weeratna, Z. K. Ballas, P. Payette, S. Blackwell, I. Suparto, W. L. Rasmussen, M. Waldschmidt, D. Sajuthi, R. H. Purcell, H. L. Davis, and A. M. Krieg.** 2000. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. J. Immunol. **164:**1617–1624.
21. **Hawkins, R. A., R. G. Rank, and K. A. Kelly.** 2002. A *Chlamydia trachomatis*-specific Th2 clone does not provide protection against a genital infection and displays reduced trafficking to the infected genital mucosa. Infect. Immun. **70:**5132–5139.
22. **Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira.** 2000. A Toll-like receptor recognizes bacterial DNA. Nature **408:**740–745.
23. **Ho, J. L., S. He, A. Hu, J. Geng, F. G. Basile, M. G. Almeida, A. Y. Saito, J. Laurence, and W. D. Johnson, Jr.** 1995. Neutrophils from human immunodeficiency virus (HIV)-seronegative donors induce HIV replication from HIV-infected patients' mononuclear cells and cell lines: an in vitro model of HIV transmission facilitated by Chlamydia trachomatis. J. Exp. Med. **181:**1493–1505.
24. **Holland, M. J., D. J. Conway, T. J. Blanchard, O. M. Mahdi, R. L. Bailey, H. C. Whittle, and D. C. Mabey.** 1997. Synthetic peptides based on Chlamydia trachomatis antigens identify cytotoxic T lymphocyte responses in subjects from a trachoma-endemic population. Clin. Exp. Immunol. **107:**44–49.
25. **Huang, D. S., S. N. Emancipator, M. E. Lamm, T. L. Karban, F. H. Blatnik, H. M. Tsao, and M. B. Mazanec.** 1997. Virus-specific IgA reduces hepatic viral titers in vivo on mouse hepatitis (MHV) infection. Immunol. Cell Biol. **75:**A12.
26. **Igietseme, J. U., G. A. Ananaba, D. H. Candal, D. Lyn, and C. M. Black.** 1998. Immune control of chlamydial growth in the human epithelial cell line RT4 involves multiple mechanisms that include nitric oxide induction, tryptophan catabolism and iron deprivation. Microbiol. Immunol. **42:**617–625.
27. **Igietseme, J. U., C. M. Black, and H. D. Caldwell.** 2002. Chlamydia vaccines: strategies and status. BioDrugs **16:**19–35.
28. **Jakob, T., P. S. Walker, A. M. Krieg, M. C. Udey, and J. C. Vogel.** 1998. Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. J. Immunol. **161:**3042–3049.
29. **Johansson, M., K. Schon, M. Ward, and N. Lycke.** 1997. 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. **65:**1032–1044.
30. **Kim, S. K., M. Angevine, K. Demick, L. Ortiz, R. Rudersdorf, D. Watkins, and R. DeMars.** 1999. Induction of HLA class I-restricted CD8+ CTLs specific for the major outer membrane protein of Chlamydia trachomatis in human genital tract infections. J. Immunol. **162:**6855–6866.
31. **Kim, S. K., L. Devine, M. Angevine, R. DeMars, and P. B. Kavathas.** 2000. Direct detection and magnetic isolation of Chlamydia trachomatis major outer membrane protein-specific CD8+ CTLs with HLA class I tetramers. J. Immunol. **165:**7285–7292.
32. **Krieg, A. M.** 2002. CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol. **20:**709–760.
33. **Krieg, A. M.** 2002. From A to Z on CpG. Trends Immunol. **23:**64–65.
34. **Kutteh, W. H.** 1999. Mucosal immunity in the human female reproductive tract, p. 1423–1434. *In* P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, and J. R. McGhee (ed.), Mucosal immunology, 2nd ed. Academic Press, San Diego, Calif.
35. **Kweon, M. N., M. Yamamoto, F. Watanabe, S. Tamura, F. W. Van Ginkel, A. Miyauchi, H. Takagi, Y. Takeda, T. Hamabata, K. Fujihashi, J. R. McGhee, and H. Kiyono.** 2002. A nontoxic chimeric enterotoxin adjuvant induces protective immunity in both mucosal and systemic compartments with reduced IgE antibodies. J. Infect. Dis. **186:**1261–1269.
36. **Loomis, W. P., and M. N. Starnbach.** 2002. T cell responses to Chlamydia trachomatis. Curr. Opin. Microbiol. **5:**87–91.
37. **Mazanec, M. B., C. S. Kaetzel, M. E. Lamm, D. Fletcher, and J. G. Nedrud.** 1992. Intracellular neutralization of virus by immunoglobulin A antibodies. Proc. Natl. Acad. Sci. USA **89:**6901–6905.
38. **Mazanec, M. B., J. G. Nedrud, C. S. Kaetzel, and M. E. Lamm.** 1993. A three-tiered view of the role of IgA in mucosal defense. Immunol. Today **14:**430–435.
39. **McCluskie, M. J., and H. L. Davis.** 2000. Oral, intrarectal and intranasal immunizations using CpG and non-CpG oligodeoxynucleotides as adjuvants. Vaccine **19:**413–422.
40. **McCluskie, M. J., R. D. Weeratna, P. J. Payette, and H. L. Davis.** 2001. The potential of CpG oligodeoxynucleotides as mucosal adjuvants. Crit. Rev. Immunol. **21:**103–120.
41. **McCluskie, M. J., R. D. Weeratna, P. J. Payette, and H. L. Davis.** 2001. The use of CpG DNA as a mucosal vaccine adjuvant. Curr. Opin. Investig. Drugs **2:**35–39.
42. **Moore, T., G. A. Ananaba, J. Bolier, S. Bowers, T. Belay, F. O. Eko, and J. U. Igietseme.** 2002. Fc receptor regulation of protective immunity against Chlamydia trachomatis. Immunology **105:**213–221.
43. **Morrison, R. P., and H. D. Caldwell.** 2002. Immunity to murine chlamydial genital infection. Infect. Immun. **70:**2741–2751.
44. **Morrison, R. P., K. Feilzer, and D. B. Tumas.** 1995. Gene knockout mice establish a primary protective role for major histocompatibility complex class II-restricted responses in *Chlamydia trachomatis* genital tract infection. Infect. Immun. **63:**4661–4668.
45. **Morrison, S. G., H. Su, H. D. Caldwell, and R. P. Morrison.** 2000. Immunity to murine *Chlamydia trachomatis* genital tract reinfection involves B cells and $CD4^+$ T cells but not $CD8^+$ T cells. Infect. Immun. **68:**6979–6987.
46. **Murphy, K. M., and S. L. Reiner.** 2002. The lineage decisions of helper T cells. Nat. Rev. Immunol. **2:**933–944.
47. **Murray, H. W., A. Szuro-Sudol, D. Wellner, M. J. Oca, A. M. Granger, D. M. Libby, C. D. Rothermel, and B. Y. Rubin.** 1989. Role of tryptophan degra-

dation in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophages. Infect. Immun. **57:**845–849.
48. **Perry, L. L., K. Feilzer, and H. D. Caldwell.** 1997. Immunity to Chlamydia trachomatis is mediated by T helper 1 cells through IFN-gamma-dependent and -independent pathways. J. Immunol. **158:**3344–3352.
49. **Perry, L. L., K. Feilzer, S. Hughes, and H. D. Caldwell.** 1999. Clearance of *Chlamydia trachomatis* from the murine genital mucosa does not require perforin-mediated cytolysis or Fas-mediated apoptosis. Infect. Immun. **67:** 1379–1385.
50. **Rank, R. G.** 1994. Animal models for urogenital infections. Methods Enzymol. **235:**83–93.
51. **Rank, R. G., and A. L. Barron.** 1983. Effect of antithymocyte serum on the course of chlamydial genital infection in female guinea pigs. Infect. Immun. **41:**876–879.
52. **Rank, R. G., A. Bowlin, and T. Darville.** 2002. Infection dose as a critical factor in determining treatment effect in an animal model of chlamydial genital infection, p. 253–256. *In* J. Schachter et al. (ed.), Chlamydial infections. Proceedings of the 10th International Symposium on Human Chlamydial Infections. International Chlamydia Symposium, San Francisco, Calif.
53. **Rankin, R., R. Pontarollo, X. Ioannou, A. M. Krieg, R. Hecker, L. A. Babiuk, and S. van Drunen Littel-van den Hurk.** 2001. CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. Antisense Nucleic Acid Drug Dev. **11:**333–340.
54. **Rockey, D. D., M. A. Scidmore, J. P. Bannantine, and W. J. Brown.** 2002. Proteins in the chlamydial inclusion membrane. Microbes Infect. **4:**333–340.
55. **Scharton-Kersten, T., G. M. Glenn, R. Vassell, J. Yu, D. Walwender, and C. R. Alving.** 1999. Principles of transcutaneous immunization using cholera toxin as an adjuvant. Vaccine **17:**537–543.
56. **Somani, J., V. B. Bhullar, K. A. Workowski, C. E. Farshy, and C. M. Black.** 2000. Multiple drug-resistant Chlamydia trachomatis associated with clinical treatment failure. J. Infect. Dis. **181:**1421–1427.
57. **Stamm, W. E.** 1999. Chlamydia trachomatis infections: progress and problems. J. Infect. Dis. **179:**S380–S383.
58. **Starnbach, M. N., M. J. Bevan, and M. F. Lampe.** 1995. Murine cytotoxic T lymphocytes induced following *Chlamydia trachomatis* intraperitoneal or genital tract infection respond to cells infected with multiple serovars. Infect. Immun. **63:**3527–3530.
59. **Starnbach, M. N., M. J. Bevan, and M. F. Lampe.** 1994. Protective cytotoxic T lymphocytes are induced during murine infection with Chlamydia trachomatis. J. Immunol. **153:**5183–5189.
60. **Su, H., K. Feilzer, H. D. Caldwell, and R. P. Morrison.** 1997. *Chlamydia trachomatis* genital tract infection of antibody-deficient gene knockout mice. Infect. Immun. **65:**1993–1999.
61. **Su, H., L. Raymond, D. D. Rockey, E. Fischer, T. Hackstadt, and H. D. Caldwell.** 1996. A recombinant Chlamydia trachomatis major outer membrane protein binds to heparan sulfate receptors on epithelial cells. Proc. Natl. Acad. Sci. USA **93:**11143–11148.
62. **Van der Stede, Y., F. Verdonck, S. Vancaeneghem, E. Cox, and B. M. Goddeeris.** 2002. CpG-oligodinucleotides as an effective adjuvant in pigs for intramuscular immunizations. Vet. Immunol. Immunopathol. **86:**31–41.
63. **van Ginkel, F. W., R. J. Jackson, Y. Yuki, and J. R. McGhee.** 2000. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. J. Immunol. **165:**4778–4782.
64. **Vollmer, J., A. Janosch, M. Laucht, Z. K. Ballas, C. Schetter, and A. M. Krieg.** 2002. Highly immunostimulatory CpG-free oligodeoxynucleotides for activation of human leukocytes. Antisense Nucleic Acid Drug Dev. **12:**165–175.
65. **Weeratna, R. D., C. L. Brazolot Millan, M. J. McCluskie, and H. L. Davis.** 2001. CpG ODN can re-direct the Th bias of established Th2 immune responses in adult and young mice. FEMS Immunol. Med. Microbiol. **32:** 65–71.
66. **Westrom, L., and P. A. Mardh.** 1983. Definitions of infectious and infectious-like conditions in the lower genital tract of the female. Scand. J. Infect. Dis. Suppl. **40:**65–70.
67. **Wira, C. R., J. E. Stern, and E. Colby.** 1984. Estradiol regulation of secretory component in the uterus of the rat: evidence for involvement of RNA synthesis. J. Immunol. **133:**2624–2628.
68. **Wizel, B., B. C. Starcher, B. Samten, Z. Chroneos, P. F. Barnes, J. Dzuris, Y. Higashimoto, E. Appella, and A. Sette.** 2002. Multiple Chlamydia pneumoniae antigens prime CD8+ Tc1 responses that inhibit intracellular growth of this vacuolar pathogen. J. Immunol. **169:**2524–2535.
69. **World Health Organization.** 1996. Global prevalence and incidence of selected curable sexually transmitted diseases: overview and estimates. World Health Organization, Geneva, Switzerland.
70. **Xia, D., A. Sanders, M. Shah, A. Bickerstaff, and C. Orosz.** 2001. Real-time polymerase chain reaction analysis reveals an evolution of cytokine mRNA production in allograft acceptor mice. Transplantation **72:**907–914.
71. **Yamamoto, M., D. E. Briles, S. Yamamoto, M. Ohmura, H. Kiyono, and J. R. McGhee.** 1998. A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. J. Immunol. **161:**4115–4121.
72. **Yamamoto, S., H. Kiyono, M. Yamamoto, K. Imaoka, K. Fujihashi, F. W. Van Ginkel, M. Noda, Y. Takeda, and J. R. McGhee.** 1997. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc. Natl. Acad. Sci. USA **94:**5267–5272.
73. **Yamamoto, S., Y. Takeda, M. Yamamoto, H. Kurazono, K. Imaoka, K. Fujihashi, M. Noda, H. Kiyono, and J. R. McGhee.** 1997. Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrheagenicity but retain adjuvanticity. J. Exp. Med. **185:**1203–1210.
74. **Zhong, G., L. Liu, T. Fan, P. Fan, and H. Ji.** 2000. Degradation of transcription factor RFX5 during the inhibition of both constitutive and interferon gamma-inducible major histocompatibility complex class I expression in chlamydia-infected cells. J. Exp. Med. **191:**1525–1534.

Editor: J. T. Barbieri