A Chlamydial Major Outer Membrane Protein Extract as a Trachoma Vaccine Candidate

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Purpose. As shown in infected humans and in animal models of chlamydial infection, the major outer membrane protein (MOMP) of Chlamydia trachomatis is immunogenically potent. The purpose of this investigation was to test in the cynomolgus monkey model of trachoma a new extract of MOMP as a candidate vaccine against ocular chlamydial infection.

Method. The nonionic detergent octyl-β-D-glucopyranoside (OGP) was used to extract MOMP from purified C. trachomatis (serovar C) elementary bodies. Protective immunization with OGP–MOMP by mucosal and systemic routes was compared in the cynomolgus monkey model of trachoma. All control and immunized monkeys were challenged by topical application of infectious C. trachomatis to the conjunctivae 35 days after the initiation of immunization.

Results. Immunization with OGP-extracted MOMP successfully induced chlamydia-specific local and systemic immunity to MOMP and to whole organism before challenge and early clearance of infection by systemically immunized monkeys. Although ocular disease was not significantly reduced in either immunized group compared to control animals, the lowest clinical and microbiologic disease scores developed in two animals in the mucosal group with the highest immunoglobulin A tear antibody titers at days 0 to 14, whereas higher tear and serum immunoglobulin G correlated with reduced disease in the systemically immunized group.

Conclusions. These data demonstrate that despite evidence of vigorous MOMP-specific and other chlamydia-specific serologic and cell-mediated immunity, as well as anamnestic serologic responses to chlamydia, vaccination with OGP–MOMP was only partially protective against chlamydial ocular disease. The partial protection correlated best with tear immunoglobulin A responses after mucosal immunization and with local and systemic immunoglobulin G responses after peripheral immunization, suggesting that alternative chlamydial antigens may have to be considered in future vaccine development to induce more effective protective immunity and that evaluation of efficacy must be appropriate to route of immunization. Invest Ophthalmol Vis Sci. 1995;36:1477–1491.

Trachoma is one of the most common infectious diseases in humans, among the most prevalent of ocular infections, and the leading cause of infectious blindness. Chlamydial genital tract infection, which is often accompanied by conjunctivitis, is three times more common than gonorrhea, and Chlamydia trachomatis is the leading cause of tubal infertility and pelvic inflammatory disease. A successful antichlamydial vaccine not only would prevent these sequelae of infection, it also would substantially reduce health care costs accompanying these infections.
The chlamydial major outer membrane protein (MOMP) has been favored as an antichlamydial vaccine candidate because vigorous anti-MOMP responses are seen in naturally infected patients and animal models.\textsuperscript{10-12} The major outer membrane protein is a 394-amino acid residue protein with four variable domains that have been immunologically identified.\textsuperscript{13-16} Monoclonal antibodies that recognize surface-exposed epitopes of the MOMP also neutralize elementary body (EB) infectivity if EB are exposed to antibodies before challenge.\textsuperscript{17,18} Several methods have been used to obtain purified whole MOMP or individual MOMP variable domains for vaccine testing: high-pressure liquid chromatography purification of sodium dodecyl sulfate-extracted proteins\textsuperscript{19} and recombinant MOMP subunits obtained either from expression vectors\textsuperscript{20,21} or as fusion protein products\textsuperscript{19} (also Whittum-Hudson et al, manuscript in preparation). Sodium dodecyl sulfate-extracted MOMP was poorly immunogenic in monkeys, possibly because important conformational epitopes were destroyed during extraction.\textsuperscript{19} Recombinant MOMP subunits of one or more variable domains were only weakly immunogenic,\textsuperscript{20,21} even when these were conjugated to cholera toxin.\textsuperscript{20}

Recent studies by Batteiger et al showed that an MOMP preparation, obtained from \textit{C. psittaci} by sequential extraction with Sarkosyl\textsuperscript{22} and the nonionic detergent octyl-\(\beta\)-D-glucopyranoside (OGP), induced serologic and cell-mediated immune responses against whole organisms and was partially protective in the guinea pig model of genital infection.\textsuperscript{23,24} Similar results were seen with an OGP-extracted MOMP (OGP-MOMP) vaccine against the sheep abortion strain of \textit{C. psittaci},\textsuperscript{25} for which protection was measured by an increase in live births. These successful results have been attributed to preservation of more of the native three-dimensional structure of the MOMP molecule during extraction with the nonionic OGP detergent.\textsuperscript{26}

Our studies were undertaken to test the ability of OGP-MOMP prepared from a human biovar of \textit{C. trachomatis} (serovar C) to induce a protective immune response(s) in the cynomolgus monkey model of trachoma. Two routes of immunization were tested, mucosal (oral and topical ocular) and systemic because \textit{C. trachomatis} infects mucosal tissues and induction of mucosal immunity is thought to be important for protective immunity\textsuperscript{11,26} and because MOMP has been shown to be highly immunogenic when delivered systematically.\textsuperscript{18,24}

\textbf{MATERIALS AND METHODS}

\textbf{Animals}

Ten wild-caught young adult cynomolgus monkeys (\textit{Macaca fascicularis}) were used in these studies (Charles River Primates, Port Washington, NY). All procedures were performed on deeply anesthetized monkeys (ketamine hydrochloride), and those procedures adhered to National Institutes of Health guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

\textbf{Protein Purification}

Elementary bodies were purified from \textit{C. trachomatis} serovar C/TW-3 by density gradient centrifugation in Percoll (Pharmacia, Uppsala, Sweden), as previously described.\textsuperscript{27,28} The major outer membrane protein was purified from EB by extraction using the nonionic detergent, octyl-\(\beta\)-D-glucopyranoside (OGP; Sigma Chemical, St. Louis, MO) and dithiothreitol (DTT; Sigma) according to the method of Batteiger et al.\textsuperscript{24} For each extraction, 10 ml of EB (~10 mg total protein) in HEPES saline buffer (pH 7.4) were suspended in an equal volume of 4% Sarkosyl (Sigma).\textsuperscript{29} The solution was incubated for 30 minutes at 37°C, sonicated briefly, and centrifuged at 100,000g for 1 hour at 18°C. The pellet was dissolved in 20 ml of 2% Sarkosyl and 40 mM DTT in HEPES buffer, incubated 30 minutes at 37°C, and centrifuged again at 100,000g for 1 hour at 18°C. The pellet was resuspended in 2.5 ml of 2% OGP-40 mM DTT. After 1 hour at 37°C, the solution was centrifuged at 100,000g for 1 hour. The supernatant contained primarily MOMP and was designated OGP-MOMP (Fig. 1). Yields of outer membrane proteins comprised predominantly of the MOMP were approximately 2% of the total starting proteins. Because MOMP was lost during each extraction step, five separate OGP extraction procedures (80 mg of total starting protein) were required to immunize eight monkeys with a total of 360 \(\mu\)g of chlamydial protein over 5 weeks. Fractions of OGP-MOMP from the individual extractions were pooled before immunization, concentrated on a Speed Vac (Savant Instruments, Farmingdale, NY) without heat, and dialyzed to remove residual detergent.

\textbf{Immunization}

Monkeys were immunized either mucosally or systemically over a period of 5 weeks using the dosage protocols shown in Table 1. The mucosally immunized group of four monkeys (animal numbers 641, 767, 355, and 409) received oral and topical ocular applications of OGP-MOMP mixed with cholera toxin (CTox) (List Biological Laboratories, Campbell, CA) as adjuvant at days −35 and −21. A final ocular boost of 20 \(\mu\)g OGP-MOMP alone was administered 14 days before challenge. The systemically immunized group of four monkeys (animals 907, 635, 600, and 646) received OGP-MOMP with complete Freund’s adjuvant intramuscularly (100 \(\mu\)g of each) at −35 days and
Test of OGP–MOMP as a Chlamydial Vaccine

FIGURE 1. Analysis of the octyl-β-d glucopyranoside-extracted major outer membrane protein (OGP–MOMP) vaccine preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and by immunoblotting. (A) SDS–PAGE profile demonstrating enrichment for the MOMP during a typical sequential purification: (1) solubilized homologous C serovar elementary body (parent sample); (2) Sarkosyl total; (3) Sarkosyl-soluble fraction (supernatant); (4) Sarkosyl + dithiothreitol total; (5) Sarkosyl + dithiothreitol supernatant; and (6) OGP supernatant (OGP–MOMP). Molecular mass markers are at left. Gel was stained with Coomassie blue. (B) Enrichment for MOMP during the sequential extraction procedure as visualized by immunoblotting from a duplicate of the gel in A. See legend to A for lane designations. The positions of MOMP and low molecular weight markers are indicated. A high-titer polyclonal anti-chlamydial antibody was used to detect the presence of MOMP in each fraction.

additional boosts of 100 μg each of OGP–MOMP in incomplete Freund’s adjuvant at days −21 and −14. Two control monkeys (animals 78 and 657) received OGP-buffer mixed with CTox or Freund’s adjuvant at times and by routes in parallel with the respective test groups. The last immunizations were 14 days before infectious challenge. Additional historic control monkeys receiving only adjuvants and infectious ocular challenges with C serovar were included in analyses of clinical and microbiologic disease.29,30 These latter control animals were graded clinically by one of the same ophthalmologists involved in the current experiments.

Infectious Ocular Challenge
All monkeys were challenged topically in each eye with infectious C. trachomatis serovar C (TW-3) EB on day 0, as previously described.29,30 Approximately 5 x 10^3 inclusion-forming units (IFU) in 20 μl of sucrose-phosphate-glutamic acid buffer were applied topically to each conjunctival sac. Inoculum concentration was verified by retitrination on McCoy cells using standard methods.

Clinical Examination and Specimen Collection
The clinical response of each eye was graded according to degree of conjunctival inflammation and follicular response, as described previously.19,30–32 All monkeys were examined weekly for 6 weeks and then biweekly in random order without examiner knowledge of group allocation. In addition, photodocumentation of each examination time for each monkey was read independently by two readers; discrepancies between grading scores were resolved by a third combined reading.32,33 The clinical response was graded on a scale of 0 to 3 for each of 10 signs of conjunctival inflammation to obtain a total inflammatory score for each monkey.29,30,33 A total clinical disease score
TABLE 1. Immunization Protocol for Groups of Cynomolgus Monkeys Immunized During a 5-Week Period With OGP-Extracted MOMP and Adjuvants

<table>
<thead>
<tr>
<th>Day of Experiment</th>
<th>Route (n = 4)</th>
<th>Mucosal (n = 2)</th>
<th>Systemic (n = 4)</th>
<th>Control (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-35</td>
<td>OC</td>
<td>OGP-MOMP + CTox</td>
<td>0</td>
<td>OGP buffer + CTox</td>
</tr>
<tr>
<td></td>
<td>PO</td>
<td>OGP-MOMP + CTox</td>
<td>0</td>
<td>OGP buffer + CTox</td>
</tr>
<tr>
<td></td>
<td>IM</td>
<td>0</td>
<td>OGP-MOMP + CFA</td>
<td>OGP buffer + CFA</td>
</tr>
<tr>
<td>-21</td>
<td>OC</td>
<td>OGP-MOMP + CTox</td>
<td>0</td>
<td>OGP buffer + CTox</td>
</tr>
<tr>
<td></td>
<td>PO</td>
<td>OGP-MOMP + CTox</td>
<td>0</td>
<td>OGP buffer + CTox</td>
</tr>
<tr>
<td></td>
<td>IM</td>
<td>0</td>
<td>OGP-MOMP + IFA</td>
<td>OGP buffer + IFA</td>
</tr>
<tr>
<td>-14</td>
<td>OC</td>
<td>OGP-MOMP</td>
<td>0</td>
<td>OGP buffer</td>
</tr>
<tr>
<td></td>
<td>PO</td>
<td>0</td>
<td>0</td>
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<td>IM</td>
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<tr>
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</tbody>
</table>

OC = ocular, 20 µg, 1:1 ratio; PO = oral, 100 µg, 1:1 ratio; IM = intramuscular, 100 µg, 1:1 ratio; OGP = octylglucopyranoside; MOMP = major outer membrane protein; CTox = cholera toxin; CFA = complete Freund’s adjuvant; IFA = incomplete Freund’s adjuvant. Controls received equal volumes of adjuvants or buffers.

(TCDS) was obtained by adding the scores of all eyes, and the mean of these scores was calculated for each treatment group for each time point.30 Conjunctival swabs were taken after clinical examination at each time point for chlamydial culture and for direct fluorescent antibody (DFA) cytology.34 Serum and tears were collected as previously reported.19

Proliferative Assay of Lymphocyte Function

Peripheral blood mononuclear cells, purified from heparinized blood as previously described,27 were tested for chlamydia-specific lymphoproliferative responses. Peripheral blood mononuclear cells (10⁶ cells/100 µl) were cultured in triplicate, round-bottom microwells with 100 µl of homologous C serovar EB (C-EB; 1 X 10⁴–1 X 10⁶ IFU/ml), concanavalin A (1 µg/well), or pokeweed mitogen (1 µg/well). Control wells received only cells and medium. After 72 hours of culture, cells were pulsed with 1 µCi of ¹³¹H-thymidine and then harvested 16 to 24 hours later onto glass fiber filters for scintillation counting. The stimulation index was determined by the standard formula (experimental cpm) ÷ (medium control cpm). A stimulation index of 4 or more was considered positive for monkey peripheral blood mononuclear cells (PBMC).27

Serology

Enzyme-linked immunosorbent assays (ELISA) were performed as previously described.27 Plates were coated overnight at 4°C with freshly thawed aliquots of EB (10⁶ IFU/well) diluted in phosphate-buffered saline (PBS; pH 7.4). Tears were eluted from cellulose sponges (Edward Weck, Research Triangle Park, NC) in 0.22 ml of PBS containing 1% bovine serum albumin (PBS–BSA) to yield an initial dilution of approximately 1:10. Serum or tears were diluted serially (1:20 to 1:2560) in the plates, and plates were incubated for 90 minutes at 37°C. After washes with PBS–0.5% Tween, alkaline phosphatase (AP)-conjugated anti-human immunoglobulin G (IgG; 1:1000) or anti-human immunoglobulin A (IgA; 1:500) (Kirkegaard and Perry, Gaithersburg, MD) diluted in PBS–BSA–0.05% Tween was added to each well, and plates were incubated for an additional 60 minutes at 37°C. The reaction was developed with paranitrophenylphosphate in diethanolamine buffer. Control wells contained no antigen but received all other reagents. When CTox was used as the ELISA test antigen, it was diluted to deliver 1 µg/well in 0.05 M carbonate–bicarbonate buffer (pH 9.6). Known positive and negative serum or tear samples for each antigen were included in each assay. The pretreatment samples from each monkey were used to determine the negative cutoff OD₄₀⁵ for each group of longitudinal specimens. All serum or tear samples from an individual monkey were assayed on the same plate. The entire plate was read on a Biotek (Winooski, VT) ELISA reader at OD₄₀⁵ and was analyzed using software provided by Dr. A. Donnenberg (University of Pittsburgh, Pittsburgh, PA).35 Results are expressed as endpoint titers.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting

Chlamydial proteins obtained from viable C-EB were separated under reducing conditions by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 12.5% preparative gels using standard meth-
Biotinylated low molecular weight standards (BioRad, Richmond, CA) were included in each gel. After electrophoresis, polypeptides were electrophoretically transferred to nitrocellulose paper (NCP) (Schleicher & Schuell, Keene, NH) using an LKB Multiphor II Transfer System (Pharmacia, Piscataway, NJ). Unoccupied binding sites on the NCP were blocked with BLOTTO (5% dry milk in 150 mM NaCl–10 mM Tris, 0.5% Tween) at room temperature for 1 hour. The NCP was then cut into 2.5-mm wide strips, and these were incubated overnight at 4°C with tears (1:50) or serum (1:100). All dilutions were made with BLOTTO. The next day, strips were washed and incubated with alkaline phosphatase-conjugated antihuman IgG (1:500) or IgA (1:250) for 60 minutes at room temperature; control strips containing biotinylated molecular weight protein standards were incubated with alkaline phosphatase-conjugated avidin (1:1000) (BioRad). After washing, strips were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) in 100 mM Tris–HCl with 100 mM NaCl and 5 mM MgCl₂ (pH 9.5). The reaction was stopped by adding 20 mM Tris–HCl with 5 mM ethylenediamine tetraacetic acid (pH 8.0). We also tested the effects of immunization and ocular challenge on serologic responses to two different extracts of chlamydial proteins obtained from solubilized elementary bodies—a Triton X-100 extract from C. trachomatis serovar C prepared as described previously40,41 to yield primarily the 57-kd heat shock protein (hsp60) and a Sarkosyl extract of C. trachomatis serovar C to yield the cysteine-rich 60 kd (omp2) protein in the Sarkosyl-insoluble fraction.42 Approximately 100 μg of chlamydial proteins obtained from these two extracts were resolved on separate 12.5% preparative gels and transferred to NCP membranes as described above for whole EB. Control strips were incubated with an anti-hsp60 monoclonal antibody (anti-GroEL, a gift of Dr. P. Bavoil, University of Rochester, Rochester, NY),42 or an anti-omp2 monoclonal antibody (a gift of W. J. Newhall V, Centers for Disease Control, Atlanta) to confirm the identity of the antigens. Duplicate serum samples were incubated with blots containing either hsp60 or omp2. Protein bands on all NCP strips were quantitated by densitometry (Hoeffer Scientific Instruments, San Francisco, CA) and analyzed using software purchased from Hoeffer. Densitometric results are expressed as mean peak heights ± SE in arbitrary units. Protein bands were identified by comparison with molecular weight standards run on the same gel. Twenty test strips were obtained per blotted minigel, and all samples from an individual monkey were tested on NCP strips prepared from the same gel.

Statistical Analyses

Clinical disease scores and microbiologic results for each group were compared by nonparametric Mann–Whitney rank sum tests. Unpaired Student's t-tests were used to compare the results of proliferative assays.

RESULTS

Biochemical Analyses of the Vaccine Candidate

The purity of OGP-MOMP was analyzed by comparing samples from each extraction step on a Coomassie blue-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (Fig. 1A) and by immunoblotting with an antichlamydial antibody (Fig. 1B). Results for a typical sequential purification of MOMP are shown in Figure 1A. Lane 6 shows the final purified MOMP (OGP-MOMP) used to vaccinate the monkeys. Although other proteins were seen in this final fraction, densitometric analyses of the immunoblot prepared from the same fractions shown in Figure 1B demonstrate that significant solubilization of EB was achieved with OGP. Approximately 30% to 50% of the MOMP in the starting whole EB solution was recovered while many of the chlamydial proteins were removed.

Chlamydia-Specific Antibody Production After Immunization

Sera and tears collected longitudinally during the OGP-MOMP immunization period and after infectious challenge were tested by ELISA against intact EB for the presence of chlamydial-specific IgA (tears) and IgG (serum and tears) antibodies (Fig. 2). High titers of antichlamydial serum IgG developed in 7 of 8 OGP-MOMP immunized monkeys before infectious ocular challenge on day 0 (1:100 -> 1:5000) (Fig. 2A). Maximum serum IgG responses were reached 2 weeks after infectious challenge, which is consistent with a secondary response to infectious challenge. These high titers persisted throughout the course of the experiment. In the control group, positive responses were first seen at day 21, and they remained lower than those in the immunized groups through day 84. By day 98, the control monkey’s serum IgG titers were no longer significantly different from those in the mucosally immunized group (Fig. 2A). Antichlamydial antibody titers of systemically immunized monkeys at day 0 were equal to the maximum titer of the control group at day 84. The pattern of tear IgG responses was similar to that observed for sera, except that titers were lower at day 0 (1:36 to 1:855). Antichlamydial tear IgG increased rapidly in both immunized groups after ocular challenge, reached peak levels at
FIGURE 2. Anti-chlamydial antibody responses in serum and tears after octyl-β-D glucopyranoside-major outer membrane protein immunization. Values represent group mean titer ± SEM for each time point as determined in an enzyme-linked immunoadsorbent assay with whole homologous C serovar elementary bodies. One control monkey died 35 days after challenge. (A) Anti-chlamydial serum immunoglobulin G; (B) antichlamydial tear immunoglobulin G; and (C) antichlamydial tear immunoglobulin A.
days 14 to 28, and then gradually decreased. Control group tear IgG was not detected until 4 weeks after ocular challenge and increased through day 98, which is typical of a primary infection30 (Fig. 2B). Antichlamydial tear IgA responses were lower than IgG but were positive (> 1:100) at day 0 in 3 of 8 immunized monkeys (one mucosally immunized and two systemically immunized monkeys). All eight immunized monkeys had peak IgA titers by day 14 and remained positive through day 98 (Fig. 2C). Control monkeys were negative for chlamydia-specific tear IgA until after day 28.

To verify that the mucosal immunization protocol was adequate, antibody responses in serum and tears to the CTox used as mucosal adjuvant were also determined by ELISA for several time points. All recipients of CTox showed increasing titers of CTox-specific serum antibody after CTox-immunization. On day —7, after a primary and boosting immunization, anti-CTox serum IgG titers reached their maximum (≥ 1:5000) and remained high at least through day 28 (later times were not tested). Tear anti-CTox IgG responses also increased during the immunization period and were highly positive by day 0 (1:500 to 1:2750) in all CTox recipients (data not shown). Tear IgA responses to CTox were lower, and only 2 of 6 monkeys showed positive anti-CTox IgA titers at day 0 (data not shown). However, anti-CTox tear IgA was detected 2 weeks after infection in 5 of 6 CTox-immunized monkeys, indicating successful mucosal priming by this adjuvant and demonstrating that no monkeys were nonresponders to exogenous antigens. Similar local anti-CTox B cell responses after oral priming, ocular priming, or both have been reported30,43

**Immunoblot Analysis of the Fine Antigenic Specificities of Humoral Responses**

Before infectious ocular challenge, ELISA and lymphoproliferative responses to whole EB were positive in both immunized groups. These results differ from previous studies with purified MOMP or MOMP subunit vaccines in which such responses were not observed, and they suggest that at least some of the native conformation of MOMP (and other surface proteins) was preserved during OGP extraction. Immunoblotting was used to compare the extent to which monkeys were specifically immunized against MOMP before and after challenge because ELISA titers would reflect a cumulative response to all surface molecules. All eight OGP-MOMP immunized monkeys had detectable anti-MOMP IgG responses in tears before infectious challenge, and 7 of 8 monkeys had anti-MOMP serum IgG. Serum IgG responses increased after challenge, suggesting an anamnestic response to infectious challenge. Only 1 of 4 mucosally primed monkeys had weakly detectable anti-MOMP tear IgA at day 0, whereas 2 of 4 systemically immunized monkeys were immunoblot positive for IgA before challenge. The one mucosally immunized monkey that never exhibited anti-MOMP tear antibody by immunoblotting was positive by ELISA against whole organism on day 0 and thereafter. Control monkeys exhibited no detectable anti-MOMP responses before challenge, and none had anti-MOMP IgA in tears before day 28 after challenge as measured by either blotting or ELISA. Serum and tear responses to other chlamydial proteins also were detected in several animals at day 0, presumably as a result of additional membrane proteins contained in the OGP extract (Fig. 5).

Serum antibody responses to the two 60-kd chlamydial proteins appeared to develop differently in the two immunized groups: The mean serum IgG responses against the chlamydial hsp60 were highest in the mucosally immunized group at all days tested (days —35, 0, 28, and 84); weak anti-hsp60 responses developed in systemically immunized animals (Fig. 4A). In each group, only two monkeys exhibited positive anti-hsp60 responses at any of the times tested. Responses to the cysteine-rich 60-kd protein (omp2) were weakly positive in the systemically immunized group at days 0, 28, and 84 after ocular challenge, and the mean responses by this group did not change significantly over the period of the experiment. In contrast, the anti-omp2 responses in the mucosally immunized group were negative at day 0 but increased between days 28 and 84, by which time 3 of 4 mucosally immunized animals exhibited positive responses (Fig. 4B). Individual clinical disease scores correlated more with the antibodies to omp2 than those to hsp60 at day 84 (data not shown). Control animals had no detectable responses against either the hsp60 or omp2 proteins at any of the times tested and probably relates to receipt of only a single antigenic challenge by infection (Fig. 4).

**Cell-Mediated Immunity After Immunization**

Peripheral blood mononuclear cells were tested in proliferative assays to determine whether chlamydia-specific cell-mediated immunity to whole organism was induced by immunization. Proliferative responses of both OGP-MOMP immunized groups to several tenfold dilutions of EB were similar regardless of immunization protocol, and 5 of 8 immunized monkeys exhibited vigorous chlamydia-specific responses 14 days before infectious challenge. Mucosally primed monkeys exhibited a mean SI of 10, whereas PBMC cells from systemically primed monkeys responded to an optimal concentration of 10⁶ IFU/ml with a mean SI of 7 (Fig. 5). These values were not significantly different from each other, though both were signifi-
cantly higher than responses of control monkeys by Student's *t* tests (*P* < 0.05). Proliferative responses were apparently short lived because they returned to baseline levels by the day of challenge. Later, after the boosting effect of ocular infection, some animals in each vaccine group again exhibited positive cell-mediated immunity. Cell-mediated immunity for monkeys 907, 635, and 600 was positive more frequently after infectious challenge; in the mucosal group, positive cell-mediated responses by PBMC at day 0 and at 2 weeks after challenge correlated with reduced disease for one monkey (monkey 767). No animals in the mucosal group exhibited positive PBMC proliferation to whole organism after this time, although high frequencies of chlamydia-specific T cells were detected in conjunctivae and draining lymph nodes of one monkey–vaccine group each at 5 and 9 weeks after challenge. As expected, high frequencies of chlamydia-specific conjunctival lymphocytes were present at 5 weeks in more diseased conjunctivae (monkey
FIGURE 3. Immunoblot analysis of antichlamydial antibody in serum and tears from octyl-β-D glucopyranoside–major outer membrane protein (OGP–MOMP)-immunized and control monkeys. Elementary bodies were solubilized in Laemli buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis for transfer to nitrocellulose. Bound immunoglobulin G and immunoglobulin A were detected with alkaline phosphatase-conjugated antihuman reagents. (A,B) Antichlamydial serum immunoglobulin G; (C,D) antichlamydial tear immunoglobulin G; and (E,F) antichlamydial tear immunoglobulin A. Results for each group are shown for the three antibody assays. In each figure, lanes labeled A represent monkeys from the mucosal group; lanes labeled B represent monkeys from the systemic group; and lanes labeled C represent control monkeys. Blots for the same five monkeys are aligned in Figures 3A, 3C, and 3E (animal numbers: 641, 767, 967, 635, and 78); and the second five in Figures 3B, 3D, and 3F (animal numbers: 353, 409, 646, 600, and 657). Lanes 1 to 4 correspond, respectively, to preimmune (day –42), day 0, day +7, and day +28 of the experiment, where day 0 was day of infectious ocular challenge. The low molecular weight markers are indicated on the left margin. MOMP is indicated by an arrow.

641; 1.1 chlamydia-specific cells per 100 cells), compared to a systemically immunized and partially protected animal (monkey 635; 1.7 chlamydia-specific cells per 1000). The frequencies of chlamydia-specific cells in conjunctiva had fallen by 40- to 50-fold 4 weeks later when an additional monkey in each group was tested. Because of limited numbers of control monkeys, conjunctivae were not collected from unimmunized animals in this experiment, but results for lymph node cells were similar to both vaccine groups at 5 and 9 weeks (40/10⁶ cells to 50/10⁶ cells; not shown). These data are consistent with our previous report on chlamydia-specific T cells in blood and conjunctiva.44 Taken together, the results demonstrate that longer-lived cell-mediated immunity was induced by systemic immunization with OGP–MOMP. Cell-mediated immunity may correlate more with clearance of organism than with preventing initial infection and could explain why systemically immunized animals became culture negative earlier than other groups. This is consistent with cell-mediated immunity to other intracellular pathogens.

Clinical Responses to Immunization

Clinical disease scores and microbiologic effects of immunization were determined for the three groups of monkeys after infectious ocular challenge. Total clinical disease score was calculated for each animal, and a mean TCDS was determined for each group as shown in Figure 6. Conjunctival inflammation, including follicles, developed in mucosally immunized and mock mucosally primed control groups after the application of cholera toxin and OGP-containing samples at boosting immunization on day –14. This response resolved largely in the 14 days before infectious ocular challenge. However, residual conjunctival sensitivity might have influenced heightened clinical disease seen in the mucosally primed group after infectious challenge, similar to persistent sensitivity seen after the development of contact lens hypersensitivity.

After live chlamydial challenge, the mean TCDS of the two immunized groups did not differ significantly from that of the control group. The disease in the mucosally immunized group was marginally worse than that of systemically immunized animals during the first two weeks after challenge. One monkey from the control group died 35 days after challenge, and clinical disease worsened in the surviving control monkey during the last 3 weeks of the experiment. This increased inflammation was coincident with a culture-confirmed secondary bacterial infection with S. epidermidis.45 Control animal data shown in Figure 6 include 15 additional monkeys challenged with 5000 IFU of C. trachomatis (C-TW/3) from numerous experiments during the last 4 years.

Microbiologic Effects of Immunization

The frequency of positive chlamydial isolation cultures from the conjunctiva did not differ significantly for the two immunized groups, but the frequency of infectious chlamydia was lower after systemic immunization than in the other two groups. Systemic immunization with OGP–MOMP clearly reduced infection before day 21 (Fig. 7A). The surviving control monkey had peak positive culture results at 56 days, several weeks after both immunized groups had declining positive culture isolations. Because the infection in 15 historic control monkeys peaked by culture assay between 28 and 35 days after challenge, we think this late positive result is accounted for by complications of secondary conjunctival infection.45 Direct fluorescent antibody
FIGURE 4. Limited development of anti-hsp60 and anti-omp2 immunoglobulin G antibodies in serum. Values represent the mean peak height (±SEM) units obtained by densitometric analyses of immunoblots that contained either (A) chlamydial hsp60 or (B) omp2. Serum collected before (day —35 and 0) or after (days +28 and +84) ocular challenge were tested for reactivity to the two 60-kd chlamydial proteins.

results were similar to culture results, except at day 7 after challenge when systemically immunized monkeys had lower 15-field EB counts than the other groups (Fig. 7B). However, in comparison to historic controls, no statistically significant reduction in DFA scores was seen, and mucosal immunization appeared to result in higher DFA scores (Fig. 7C). Thus, systemic immunization induced an earlier culture-negative, but not a DFA-negative, state and may reflect nonviable organisms detectable by DFA.

When individual animals in each immunization group were examined for correlations between lower clinical and microbiologic disease scores and higher antibody responses, two animals in each group showed partial protective immunity: animals 767 and 409 in the mucosally immunized group and systemically immunized animals 907 and 600. Monkeys 767 and 907 consistently exhibited less disease by any criterion and had the highest antibody responses for their respective groups. Associations of other animals in each group varied, except that all animals in the systemic group had negative culture results 4 weeks earlier than those in the mucosal group. Of possible significance for future vaccine strategy, individual tear IgA titers and day 0 blots (rather than serum or tear IgG) correlated with partial protection for monkey 767 in the mucosal group; this animal was the one of two animals in this group that exhibited positive proliferative responses (stimulation indices > 4) at day 14 after challenge. In contrast, tear and serum IgG titers and blots (rather than tear IgA) correlated with partial protection for monkey 907 in the systemic group; proliferative responses for monkey 907 returned to baseline by day 0.

DISCUSSION

Failure of previous attempts to vaccinate against chlamydial infections has usually been attributed to the inability to induce an adequate antichlamydial immune response to whole organism before challenge. Our data demonstrate that vaccination with an OGP extract of MOMP induced strong humoral and cell-mediated immune responses to intact or solubilized organism before infectious challenge. These immune responses did not significantly protect either group...
against infection or clinical disease after ocular challenge with infectious chlamydia, although they were associated with a modest reduction in the recovery of viable *C. trachomatis* in cultures after challenge and, on an individual basis, with reduced clinical disease. Vaccine candidates tested in the cynomolgus monkey model of trachoma before the present studies included oral immunization with viable or killed *C. trachomatis* whole EB of various serovars, recombinant chlamydial lipopolysaccharide (LPS), a sodium dodecyl sulfate extract of MOMP, MOMP subunits, and chlamydial hsp70. Other tests of whole chlamydia as protective immunogens also generally failed to protect against disease. Although subunit vaccines prepared from one or more *C. trachomatis* antigens have been suggested as the most promising vaccine candidates, in the cynomolgus monkey model partial resistance to ocular challenge was induced previously only by oral administration of viable serovar L2 EB. Serologic responses before challenge were induced poorly by these latter vaccine candidates, and after challenge immune responses did not differ substantially, regardless of immunization status. No candidate vaccine to date has reduced clinical or microbiologic disease better than a previous ocular infection.

In contrast to these previous attempts, vaccination with OGP extract containing primarily MOMP (omp1) induced strong chlamydia-specific humoral and cell-mediated immune responses to whole organism in naive monkeys before infectious ocular challenge. However, only serologic responses were sustained in both immunization groups. In the current experiments, the immune responses induced by mucosal and systemic administrations of OGP-MOMP were impressive. It is generally thought that an effective vaccine against trachoma would require stimulation of local immunity at the mucosal surface of the conjunctiva. In humans, a significant inverse correlation between the titer of secretory IgA antibodies in genital secretions and the presence of chlamydia isolated from the cervix has been shown. Also, a secretory IgA antibody response in the genital tract of BALB/c mice is associated with protection against subsequent *C. trachomatis* infection. In the current study, low titers of IgA were detected in tears from the systemically primed animals before ocular infection. These eyes were uninflamed before challenge, suggesting that the IgA in tears before challenge had not leaked from serum. Of interest after infectious challenge, 2 of 4 mucosally immunized animals had higher levels of tear IgA that, although still of low titer, correlated with greater reduction in microbiologic and clinical disease. The neutralizing capability of these tears was not tested, and an absence of neutralization or inadequate IgA titers could explain the failure to protect eyes better from infectious challenge. Other studies have demonstrated the presence of protective chlamydial-neutralizing antibodies in tears.

Either mucosal or systemic immunization with OGP-MOMP induced chlamydia-specific cell-mediated immunity as measured by lymphoproliferative responses by peripheral blood mononuclear cells to whole EB. Cell-mediated immunity to purified MOMP or other purified chlamydial antigens was not tested. The cell-mediated immune responses by PBMC were short lived before infectious challenge, although purified protein derivative of tuberculin skin test results for recipients of complete Freund’s adjuvant were positive for several months after challenge (data not shown). Ocular infection appeared to boost PBMC responses in the systemic group, but responses varied among the individual animals. Given the high antibody titers, particularly in systemically immunized animals, it is possible that peripheral blood T-cell responses were downregulated by immunization as responding lymphocytes localized to mucosal tissues or draining regional lymphoid tissues. The latter responses were not tested in all animals.

There is no firm evidence that anti-MOMP, cell-mediated immunity itself plays a major protective role in this disease, although CD4+ T cells are probably required for antibody and memory responses. There is evidence that local anti-hsp60 T-cell responses are
FIGURE 7. Microbiologic evidence of partial protective immunity. (A) Culture detection of chlamydial inclusion bodies in swabs of the conjunctiva for the three groups of monkeys. Mean numbers of inclusions detected in 15 high power fields (original magnification, ×400). (B) Direct fluorescence antibody assay results of 15 high-power field counts on conjunctival smears. (C) The direct fluorescent antibody scores based on a score of 1 to 4 for these groups. The control group includes 15 historical control monkeys.
deleterious because they are manifested as local delayed hypersensitivity reactions.\textsuperscript{40,41,57} Chlamydial hsp70 does not elicit such reactions in immune animals.\textsuperscript{57} The role for CD8+ T cells in either protection or pathogenesis is unknown, although many CD8+ cells are present in the inflamed conjunctiva during infection.\textsuperscript{53,58} The immunoblotting results against the two chlamydial 60-kd proteins suggested that both routes of immunization influenced the immunogenicity of additional non-MOMP chlamydial antigens. Because Sarkosyl detergent eliminates the hsp60 antigen from preparations, it is not unexpected that antibody to this antigen did not develop until after infectious challenge with whole chlamydia. Similarly, the amount of 60-kd omp2 protein in the OGP-extract may have been suboptimal to induce an antibody response after mucosal immunization. However, ocular infection after immunization was sufficient to boost a previously undetected response because detectable responses to either antigen did not develop in control animals through day 84.

Despite vigorous serologic responses before and after challenge, transiently positive cellular immune responses, and some microbiologic evidence for reduction in shedding of organism, there were no significant differences in clinical disease scores for immunized and nonimmunized groups of monkeys. This can be attributed in part to the individual variability of immune responses to chlamydia by outbred monkeys. However, when serologic responses by individual animals were compared to disease parameters, higher tear IgA titers corresponded to best reduction in microbiologic and clinical scores after mucosal immunization. Tear and serum IgG corresponded best to partial protection in the systemically immunized group. Cellular immunity persisted through day 14 after ocular challenge in two of the mucosally immunized animals, whereas responses in the systemic group remained low for three of four animals at this time. At 4, 9, and 14 weeks, PBMC from one to three systemically immunized animals had positive stimulation indices, with monkey 907 showing positive results at all three times. Mucosally immunized monkeys had negative stimulation indices of < 4 after 2 weeks. Although extrapolations to requirements for a human vaccine cannot be made from such small numbers of animals, these results suggest that more than one route of immunization and mechanism may be protective against chlamydial ocular infection and that a cocktail vaccine containing MOMP and additional antigen(s) may be required even to protect partially 100% of test subjects.

The conjunctival inflammation induced in the mucosally immunized group by concomitant topical inoculation of CTox with OGP or buffer may have influenced the clinical results. Instillation of OGP or CTox alone did not induce inflammation. It is possible that residual detergent altered the permeability of the conjunctivae so that the cholera toxin could penetrate and cause an inflammatory response. Similar observations have been made with Triton X-100 buffer preparations.\textsuperscript{58}

It is generally accepted that the ideal vaccine should completely prevent infection or, failing that, at least decrease the intensity and duration of infection. Recently, Allen and Stephens\textsuperscript{59} demonstrated differences in the ability of the MOMP variable segments to elicit T-cell help for antibody responses in mice. More information regarding such T-helper cell requirements may aid efforts to optimize an anti-trachoma vaccine candidate. The current study shows that the OGP–MOMP vaccine induced specific antibody and cell-mediated immunity responses before challenge, but this vaccine only partially protected against infectious challenge. Partial protection appeared to correlate with serologic and cellular immune responses, but antibody class and timing of cell-mediated immunity responses reflected the route of immunization. Additional study of vaccine candidates retaining sufficient native conformation to induce immune responses to whole organism will help clarify the requirements for protective immunity to chlamydia.

Key Words

\textit{Chlamydia trachomatis}, conjunctiva, major outer membrane protein, trachoma, vaccine

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References

38. Pal S, Taylor HR, Whittum-Hudson JA. Identification


