Use of a primate model system to identify *Chlamydia trachomatis* protein antigens recognized uniquely in the context of infection

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A primate model system was used to identify *Chlamydia trachomatis* antigens uniquely recognized in the context of infection. Serum antibody titres were measured in cynomolgus monkeys challenged urethrally with *C. trachomatis* serovar L2 elementary bodies (EBs). High-titre sera from these primates were used, in parallel with antisera against killed *C. trachomatis* EBs, to differentially screen an expression library of *C. trachomatis* serovar U DNA. Four clones were recognized only by antisera from infected monkeys. Sequence analysis revealed that three of these immunoreactive clones overlap a common ORF, designated ORF D242 (encoding p242), in the *C. trachomatis* genome database. The fourth clone contains two complete ORFs, each encoding 32 kDa proteins that share identity with *Treponema pallidum* TroA and TroB (ORFs D067 and D068 in the *C. trachomatis* database, respectively). Immunoblot analysis of *Escherichia coli* lysates expressing *C. trachomatis* TroA, TroB and p242 fusion proteins showed that p242 and TroA, but not TroB, were detected by the sera collected from infected primates. Antibodies directed at TroA and p242 were also detected in sera from several *C. trachomatis*-infected patients, demonstrating that these proteins are also recognized by humans following infection. Immunoblot analysis with antibody against TroA and p242 also demonstrated that both antigens are present in higher abundance in infected ChoK1 cells relative to purified *C. trachomatis* EBs. Immunofluorescence microscopy shows that TroA and p242 are both localized to intracellular developmental forms at the margins of growing inclusions. Collectively, these studies identify two *C. trachomatis* proteins that are underrepresented in EBs and are recognized uniquely in the context of infection.

**Keywords**: bacterial pathogenesis, *Chlamydia*, primate, STDs

INTRODUCTION

*Chlamydiae* are obligate intracellular bacterial pathogens with a unique biphasic life cycle. They appear as two distinct cellular types, a small (0-3 μm) dense form with a rigid cell wall, termed the elementary body (EB), and a larger metabolically active but noninfectious reticulate body (RB). The life cycle begins with the attachment of EB developmental forms to the host cell, followed by endocytosis into a nascent vacuole (inclusion). After EB attachment and entry, the chlamydiae remain within this membrane-bound inclusion, which continues to expand as the developmental forms replicate. By 48–72 h post-infection (PI), this growth phase is terminated when the RBs reorganize back to EBs. The EBs are released upon host cell lysis and can then serve to initiate another infectious cycle. The difficulties in working with chlamydiae center on the obligate intracellular requirement for growth and the fact that no genetic system is in practice.

The genus *Chlamydia* includes two species that are primarily associated with human disease: *Chlamydia
trachomatis and Chlamydia pneumoniae. Sexually transmitted C. trachomatis leads to a spectrum of serious clinical conditions and conjunctival infection can cause trachoma, the leading cause of infectious blindness worldwide (Hillis et al., 1997; Thylefors et al., 1995). Additionally there is both laboratory and epidemiological evidence that chlamydial infection can facilitate the spread of HIV (Wasserheit, 1994). C. pneumoniae is a recently recognized species that causes acute respiratory diseases and is currently of interest because of its epidemiological association with asthma (Hahn et al., 1991) and cardiovascular disease (Danesh et al., 1997).

There is widespread interest in the identification of candidate antigens for protection against chlamydial disease, but the demonstration of any single protective antigen remains elusive. Recent reports have described protection mediated by a DNA vaccine using the omp1 gene, suggesting its gene product, the major outer-membrane protein (MOMP), may be a protective antigen. These studies are confounded by the inability of purified MOMP to protect in any subunit vaccine trial, and by the inability of omp1-mediated DNA vaccines to work at all mucosal sites (Zhang et al., 1997). There is also very recent evidence suggesting EB antigens presented to cytokine-stimulated dendritic cells can be used to confer protection in a murine model system (Su et al., 1998).

While a prior infection with C. trachomatis will provide specific, short-term protection against a subsequent challenge, many chlamydial diseases are exacerbated by an overaggressive immune response against EB structural components, possibly including either or both of two genus-common proteins (Morrison et al., 1992; Bachmaier et al., 1999). The distinction between the immune response in infected animals versus that seen in animals immunized with killed chlamydiae has been examined previously in a guinea pig model of chlamydial conjunctivitis, caused by Chlamydia psittaci strain Guinea Pig Inclusion Conjunctivitis (GPIC) (Rockey & Rosquist, 1994). This approach led to the identification of chlamydial proteins (IncA, B and C) localized to the inclusion membrane of both C. psittaci- and C. trachomatis-infected cells (Bannantine et al., 1998a, b; Rockey et al., 1995). In the present report, we have explored the use of a primate infection model of chlamydial genital infection for the purpose of uncovering novel C. trachomatis antigens recognized uniquely in the context of infection. Two previously unidentified antigenic proteins were identified, each under-represented in EBs and localized to developmental forms at the margins of the inclusion. Humans who have acquired a sexually transmitted chlamydial infection also recognize these proteins.

**METHODS**

**Animals, infections and antisera.** For this study, two male cynomolgus monkeys (Macaca fascicularis) weighing 5-4 and 5-2 kg were used. These animals, designated no. 102 and no. 894, were housed independently in the animal facilities at Rocky Mountain Laboratories. All experiments and immunizations were approved by an Institutional Animal Care and Use Committee as required by the Animal Use Welfare act. Monkeys anesthetized with ketamine-HCl were infected urethrally with a sterile calcium alginate swab saturated with a solution of 10^9 C. trachomatis LGV-434 serotype L2 EBs ml^-1. Animals were infected three times over the course of 18 months and allowed to recover between infections. Clinical signs were monitored weekly. Sera were collected every 2 weeks and analysed by immunoblotting (Rockey et al., 1995) and ELISA (Su et al., 1990) for reactivity with chlamydial antigens. High-titre serum from animal no. 894 collected 8 weeks after the secondary challenge (convalescent sera) was used to screen the genomic library.

Because of the expense associated with generating control antisera in primates, guinea pigs were used to produce antisera against C. trachomatis L2 EBs inactivated with 10% formalin-PBS. Fixed EBs (10^9 EBs ml^-1) were emulsified in Ribi trivalent adjuvant (Sigma) and injected twice over the course of 2 months using a protocol provided by the manufacturer. The resulting sera (z-EB antisera) were checked for reactivity to C. trachomatis EBs by immunoblotting and used to probe the C. trachomatis expression library. Antiseras against purified recombinant proteins were produced in New Zealand White rabbits and BALB/c mice using antisera emulsified in Ribi adjuvant. Anti-chlamydial Hsp60 mAb A57B9 was collected from hybridoma culture supernatants as described by Yuan et al. (1992).

Human sera that demonstrated high-titre antibody to C. trachomatis or C. pneumoniae by microimmunofluorescence assay were selected from stored serum specimens at the University of Washington. Both anti-C. trachomatis and control antisera were taken from patients in a clinical setting and analysed for reactivity by microimmunofluorescence against C. trachomatis and C. pneumoniae strain TWAR. The control antisera showed no detectable reactivity when using this assay.

**Bacteria, bacteriophage and library construction.** C. trachomatis LGV-434, serotype L2, was cultivated in HeLa 229 cells and purified using density centrifugation as previously described by Caldwell et al. (1981) and Hackstadt et al. (1992). *Escherichia coli* DH5x (Bethesda Research Laboratories) was used as the host strain for transformations with recombinant DNA. *E. coli* XL-1 Blue MR' (Stratagene) was used as the host strain for infection with lambda ZAPII bacteriophage vector. A C. trachomatis genomic library was constructed with the lambda ZAPII vector as previously described for C. psittaci (Bannantine et al., 1998a). Library plaques were plated, transferred to nitrocellulose filters (Schleicher & Schuell) in duplicate and probed with monkey convalescent antisera and guinea pig z-EB sera. Plaques that were recognized by the convalescent antisera, but not the z-EB sera, were selected for further study. Coinfection of *E. coli* SOLR (Stratagene) with purified candidate lambda clones and with ExAssist helper bacteriophage (Stratagene) resulted in SOLR clones harbouring pBluescript containing C. trachomatis inserts of interest.

**Nucleotide sequencing and sequence analysis.** Automated sequencing was performed with the Taq DyeDeoxy Terminator Cycle Sequencing kit (Perkin Elmer/Applied Biosystems Division) on an ABI377 sequencer (Oregon State University Central Services). Sequence assembly was performed using AssemblyLIGN software and sequence analysis
was performed with MacVector software (Oxford Molecular). Deduced amino acid sequences were compared with genomic databases using the BLAST program available from the National Center for Biotechnology Information on the World Wide Web.

Production of maltose-binding protein (MBP) fusion proteins. MBP fusions of TroA, TroB and p242 were constructed using the pMAL-c2 vector (New England Biolabs). The reading frame of each gene was amplified using Pfu DNA polymerase (Boehringer Mannheim) and serovar L2 genomic DNA as the template. The upstream and downstream oligonucleotides for these amplifications are as follows: troA, 5'-ATGAAATCGCATGATTGTGATGCCG-3' and 5'-GGCGGGATCCATTCAAGAACAGTCCCTCCACAAGTTCACTGTTTTTAAACCAGAC-3' and 5'-GGCGGGATCCACGATCCTAACGCTGAGCCGTATCC-3'; troB, 5'-ATGTCTTTGTAATACTTATTAGCATGTTAAT-3' and 5'-GGCGGGATCCCTCGATCATCTAGCAGATCC-3'. The reading frame of each gene was amplified using Pwo DNA polymerase (Biolabs). The resulting fusion proteins were overproduced in E. coli DH5α and subsequently purified by affinity chromatography using an amylase resin (New England Biolabs). The purified MBP fusion proteins were used as antigen for the production of monoclonal antibodies in mice and/or rabbits by the method of Rockey et al. (1995).

Preparation of ChoK1 lysates for electrophoresis. Monolayers of ChoK1 cells in six-well trays were infected with 1 x 10⁶ C. trachomatis per well in Hanks' balanced salts solution (HBSS; Gibco-BRL) for 30 min on a rocker platform. The HBSS was replaced with RPMI1640 + 10% foetal calf serum and incubated at 37°C in 5% CO₂. Infected and mock-infected control cells were harvested at 20 h Pl. Monolayers were washed twice with PBS and then lysed in 600 μl x 1 x SDS sample loading dye (1% SDS; 50 mM Tris, pH 6.8; 1% 2-mercaptoethanol; 10% glycerol). Lysates were pooled, boiled for 5 min and stored at -20°C.

Electrophoresis and immunoblotting. PAGE and immunoblotting were conducted as described previously (Rockey & Rosquist, 1994; Rockey et al., 1995). Rabbit primary antibody was detected with [³⁵S]Protein A (4600 Bq ml⁻¹; Amersham) diluted 1:500 in PBS/0.1% Tween 20/2% BSA. Mouse antibodies were detected with peroxidase-conjugated secondary antibodies diluted 1:5000 and chemiluminescence (ECL reagent; Amersham). Slot-blots analyses of monkey test bleeds and human sera were performed using a Mini-Protein II Multiscreen apparatus (Bio-Rad) as described by Rockey et al. (1996). Autoradiographs were subsequently scanned into digital images and processed using Photoshop (Adobe Systems) and Canvas (Deneba Software) graphics programs.

Immunofluorescence studies. Chlamydiae grown in HeLa cells on sterile glass coverslips were methanol-fixed 20-35 h Pl. These fixed coverslips were immunostained using mouse anti-TroA, mouse anti-p242 and/or rabbit anti-IncA diluted in PBS containing 3% (w/v) BSA (PBS-BSA), followed by FITC-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG diluted in PBS-BSA. Immunostaining of the fixed coverslips was performed according to the method of Rockey & Rosquist (1994) and visualized with the ×63 objective of a Zeiss microscope equipped with an epifluorescence condenser and MC 63 C photomicrographic camera.

RESULTS

Characterization of primate response to infection

The C. trachomatis-infected cynomolgus monkeys displayed markedly enlarged inguinal lymph nodes beginning 2 weeks post-primary challenge and these remained enlarged until 10 weeks post-challenge. Serum antibody titres against C. trachomatis increased in both animals during the 12 weeks following the primary challenge (Fig. 1). A secondary challenge was administered 14 weeks post-primary. Inguinal lymph nodes were again enlarged in both animals following secondary infection. Serum antibody titres from animal no. 894 remained high following secondary challenge while anti-chlamydial titres dropped in animal no. 102. A tertiary challenge was administered to each monkey but this had no effect on antibody levels in either animal – titres of no. 102 remained low while no. 894 remained high throughout the course of the challenges. There was no urethral discharge, fever, or weight loss in either animal following any of the three chlamydial challenges.

Results obtained by immunoblotting with the primate antisera correlated well with that observed by ELISA (not shown). These blots were also used to identify unique antigens that might be recognized only in the context of infection. Two proteins appeared to be more abundant in the blots of infected cells – one of 28 kDa and one above 90 kDa.

Identification of antigens recognized by convalescent antisera

Because of the higher and more consistent antibody titre, serum from animal no. 894 collected 8 weeks after the secondary challenge was used to screen a bacteriophage expression library of C. trachomatis DNA. Four plaques from the C. trachomatis library were recognized by serum from animal no. 894, but not by z-EB sera. SDS-PAGE and immunoblot analysis of E. coli harbouring the chlamydial inserts showed that each produced one or more proteins that were recognized by serum from animal no. 894 but not by four independent guinea pig z-EB sera (Fig. 2). E. coli/pCt1 produced a 28 kDa antigenic protein, E. coli/pCt2 and E. coli/pCt3 produced a 20 kDa antigenic protein and E. coli/pCt4 produced multiple proteins at 32-34 kDa. Blotting was essentially identical when high-titre serum from infected animal no. 102 (collected 12 weeks post-primary infection) was used, indicating that these proteins were immunogenic following infection of two different monkeys.

Sequence analysis of immunoreactive genomic clones

Sequence analysis of recombinants revealed that three of four clones (pCt1, pCt2 and pCt3) were overlapping and contained only one common ORF. This common ORF is virtually identical to ORF D242 (contig 3.7) in the C. trachomatis serovar D genome sequence (Stephens et al., 1998) and it encodes a protein product lacking significant
Fig. 1. Serological response to *C. trachomatis* in urethrally infected cynomolgus monkeys as measured by ELISA. Two primates, numbered RML 102 (●) and RML 894 (○), were bled on biweekly intervals and their sera were analysed for anti-*C. trachomatis* antibodies. The week post-primary infection is indicated (x-axis). A secondary infection was given 14 weeks post-primary (arrow). The relative anti-*C. trachomatis*-EB antibody titre as measured by the $A_{405}$ (y-axis) is shown following primary and secondary infection.

The chromosomal fragment in pCt4 contained two complete ORFs and one partial ORF that, collectively, resemble the *tro* operon of *Treponema pallidum* (Hardham et al., 1997). The two complete ORFs encode predicted proteins, each of 32 kDa, that display 25% and 45% identity with *T. pallidum* *TroA* and *TroB*, respectively. The incomplete ORF in pCt4 displays some similarity with *troC*, the third gene in the *T. pallidum* *tro* operon. *C. trachomatis* *troA* and *troB* are ORFs D067 and D068, respectively, in the *C. trachomatis* genome database. Comparisons of *TroA*, *TroB* and p242 sequences from the LGV serotype L2 with the ocugential serovar D genome sequence database (Stephens et al., 1998) show that these proteins are 100% conserved between the two *C. trachomatis* serovars.

**Production of fusion proteins and immunoblot analysis**

Because both *TroA* and *TroB* were encoded in their entirety on pCt4, it was not clear which protein was recognized by the convalescent antisera. To address this and other questions, MBP fusions of *TroA*, *TroB* and p242 were constructed and expressed in *E. coli*. Lysates containing each fusion protein were prepared and analysed by immunoblotting with selected antibodies (Fig. 3). Each purified protein was detected with antibody that recognizes the MBP portion of the fusion protein (Fig. 3a), and none were recognized by the x-killed EB sera (Fig. 3c), consistent with their initial characterization as infection-specific antigens. Immunoblots of uninduced and IPTG-induced *E. coli* lysates of each MBP fusion were then probed with primate convalescent sera (Fig. 3b). This experiment showed that the convalescent sera from both primates recognized *TroA* and p242, but not *TroB*.

**Fig. 2.** Four *C. trachomatis* library clones expressed in *E. coli* are recognized by convalescent antisera but not x-EB sera. Identical immunoblots were probed with serum from monkey no. 894 (a) and guinea pig x-EB sera (b). Lanes: 1, *E. coli* SOLR; 2, *E. coli* SOLR/pCt1; 3, *E. coli* SOLR/pCt2; 4, *E. coli* SOLR/pCt3; 5, *E. coli* SOLR/pCt4; 6, *C. trachomatis*-infected HeLa cells; 7, *C. trachomatis* EBs. Antigens showing differential reactivity are indicated with an asterisk immediately right of the band. Molecular mass standards are indicated.
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**Fig. 3.** Immunoblots of TroA-, TroB- and p242-MBP fusion proteins probed with antisera against MBP (a), monkey convalescent sera (b) or guinea pig antisera against fixed EBs (c). Lanes: 1, E. coli/MBP-TroA; 2, E. coli/MBP-TroB; 3, E. coli/MBP-p242. +, IPTG-induced culture.

**Fig. 4.** Sera from C. trachomatis-infected patients detect TroA and p242. Preparative immunoblot analysis was performed on purified antigens with sera from several different patients infected with C. trachomatis or C. pneumoniae. The location of the purified antigen is indicated in the left margin. Sera from 9 of 11 patients infected with C. trachomatis recognize TroA and 10 of 11 react with p242. The C. pneumoniae-infected patients showed no reactivity with TroA and one patient showed reactivity with p242. The MBP portion of these fusions was not detected or was weakly detected by the patient sera. Lanes: 1 and 2, negative control sera; 3-13, sera from 11 patients that have been infected with C. trachomatis; 14-15, sera from two patients that have been infected with C. pneumoniae.

**Fig. 5.** TroA and p242 are present in higher relative abundance in infected cells versus purified EBs. Shown are three identical immunoblots with the following lane assignments: 1, uninfected ChoK1 cells; 2, C. trachomatis-infected ChoK1 cells; 3, C. trachomatis EBs. Each immunoblot was probed using antisera with specificity for p242 (b) and TroA (c). Note the increased amounts of each antigen present in C. trachomatis-infected ChoK1 cells as compared to C. trachomatis EB. The relative amounts of chlamydial in each lane are shown using anti-Hsp60 mAb B9 (a).

The TroA and p242 MBP fusions were then used as antigens in immunoblot analysis of sera from patients with high-titre anti-C. trachomatis serum antibodies (Fig. 4). These patients had histories of infection with a variety of the oculogenital chlamydial serovars. These sera were compared to sera from patients with evidence of C. pneumoniae infection (Fig. 4, lanes 14 and 15), and to sera from patients with no evidence of serum antibodies against Chlamydia (Fig. 4, lanes 1 and 2). TroA was recognized by sera from 8 of 11 C. trachomatis-infected patients, and 10 of 11 C. trachomatis-infected patients showed specific reactivity with p242 (Fig. 4, lanes 3–13). Serum from one C. pneumoniae-infected patient recognized p242, but that same serum showed relatively high background against MBP alone. Neither of the C. pneumoniae patient sera detected TroA.

The three purified MBP fusions were also used to immunize mice and/or rabbits for the production of monospecific antisera. These sera were used to probe immunoblots of mock-infected HeLa cells, C. trachomatis-infected HeLa cells and purified EBs (Fig. 5). Similar amounts of C. trachomatis antigen are present in the infected cells and purified EB lysates, as shown in the immunoblot probed with anti-Hsp60 mAb (Fig. 5a). However, both TroA and p242 are present in higher amounts in the infected cells as compared to the purified EBs (Fig. 5b, c).
Immuno-fluorescence microscopy

The location of TroA and the p242 gene product within infected HeLa cells was determined by indirect immuno-fluorescence microscopy (Fig. 6). A virtually identical staining pattern was observed for TroA (Fig. 6a) and p242 (Fig. 6c). Inclusion membrane staining with anti-C. trachomatis IncA (Bannantine et al., 1998b) is also shown for comparative illustration purposes (Fig. 6b, d). Both TroA and p242 were localized to the chlamydial developmental forms adjacent to the inclusion membrane within the infected cell and appeared to localize to the cell wall of chlamydial developmental forms.

DISCUSSION

Previous studies have suggested that prior chlamydial infection of animals or humans protects against a subsequent homologous challenge (Morrison et al., 1992). While antigens presented in the context of infection appear to elicit a protective immune response, immunization with purified, killed Chlamydia may lead to more severe sequelae following subsequent infection. Therefore, a driving force behind the current studies was to identify antigenic proteins that are recognized uniquely during C. trachomatis infection, as they may be uniquely associated with a protective immune response. A urethral challenge of primates with C. trachomatis serovar L2 was used as a challenge system representative of primary human infection with a lymphogranuloma serovar. This type of challenge was used, as opposed to challenge with a classic oculogenital serovar, because of the ability of LGV infection to stimulate higher serum antibody titres (Perine & Stamm, 1999). Clinical disease was monitored following infection, but the only apparent signs were transiently enlarged inguinal lymph nodes in each infected animal. Such swelling is a hallmark of C. trachomatis LGV infections in humans. In untreated cases the swelling and subsequent symptomology can be severe, but no such problems were observed in the experimentally infected primates. Antibody titres in these animals rapidly increased following primary challenge. One animal experienced
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Little information exists about the ability of individual antigens that do not stimulate strong antibody production but may be critically important in host-cell-mediated immune responses. Optimally we would have used monkeys to produce the antisera against killed EBs as the control in these experiments. Because of resource and animal limitations, we chose instead to produce the anti-EB antisera in guinea pigs. The guinea pig sera were carefully tested, but the difference in host species introduces additional variables that cannot be controlled for. For example, guinea pigs immunized parenterally with killed EBs may respond differently to similarly immunized monkeys. Such inherent problems with these controls may lead to improper assignment of antigens as infection-specific. In the present experiments, however, our conclusion that TroA and p242 are not present in EBs is also supported by immunoblot analysis with monospecific antisera, which showed each protein to be under-represented in EBs (Fig. 5). Therefore the guinea pig antisera were appropriate as a control for identification of antigens present uniquely in the context of infection.

Further research will address kinetics of protein accumulation and decay during the chlamydial developmental cycle. Convalescent sera from animals experimentally infected with chlamydiae have been used successfully in this study and others to identify a collection of antigenic chlamydial proteins recognized predominantly in the context of infection. In each case, identified antigens are relatively under-represented in EBs. The present study uses a primate model system of chlamydial genital infection to identify TroA and p242, proteins present in infected cells but under-represented in EBs. Similar experimentation in a guinea pig model of chlamydial conjunctivitis led to the identification of proteins uniquely present in the chlamydial inclusion membrane within infected cells (Bannantine et al., 1998a, b; Rockey et al., 1995). Each of these proteins, IncA, IncB and IncC, was also found in RBs but none was detected in EBs.

While each system has been useful in identifying novel antigenic proteins, the approach is somewhat limited in cases where proteins do not elicit strong antibody responses. This was particularly evident with the guinea pig system, where the inclusion membrane protein IncC was identified because incC was adjacent to and cotranscribed with incB (Bannantine et al., 1998a). IncC would not have been identified as a protein in the inclusion membrane if incC had not been adjacent to incB in the genome. An example of this from the current study is TroB, encoded by the second gene in the C. trachomatis tro operon. TroB was not recognized by the convalescent sera and was not directly identified in this system. While this is problematic for uncovering unique proteins such as IncC and TroB, it is seriously limiting for the identification of potential infection-specific antigens that do not stimulate strong antibody production. Our current ability to identify these antigens limits their usefulness in the development of a vaccine.
C. trachomatis proteins to induce protective immunity. While the importance of TpOA and p242 in immunity against chlamydial disease is currently unknown, it is apparent that each of these novel proteins is antigenic in the context of infection of both humans and monkeys. We are currently using a vaccinia-based system to investigate the protective ability of these and other infection-specific proteins, and we are hopeful that such investigations can identify candidate proteins for use in subunit vaccines against chlamydial infection.

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REFERENCES


Thomas, W. R., Callow, M. G., Dilworth, R. J. & Audesho, A. A.


