Protein Antigens of *Chlamydia psittaci* Present in Infected Cells but Not Detected in the Infectious Elementary Body

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Ocular infection of guinea pigs with the guinea pig inclusion conjunctivitis (GPIC) strain of *Chlamydia psittaci* produces a clinical condition representative of acute chlamydial conjunctivitis in humans. Guinea pigs which had recovered from two challenges with GPIC were used as a source of sera for the identification of antigens present in GPIC-infected tissue culture cells but absent in the infectious elementary body (EB).

Immunoblots of lysates of infected HeLa cells probed with the convalescent-phase sera identified protein antigens of 22, 34, and 52 kDa (p22, p34, and p52, respectively) that were not detected in lysates of purified EB or in uninfected HeLa cells. Protein p22 was also not detected in lysates of purified reticulate bodies. Immunoblotting of lysates of HeLa cells infected with other chlamydiae demonstrated that the antigenicity of p22 and p34 was subspecies specific. Immunoblotting was also used to detect p22 and p34 in lysates of the conjunctivae of infected guinea pigs. Adsorption of convalescent-phase sera with GPIC EB produced a reagent with dominant reactivity toward p22, p34, and a 28-kDa EB protein. Immunofluorescent staining of GPIC-infected HeLa cells demonstrated that these adsorbed sera labeled the inclusion and inclusion membrane, with no apparent reactivity toward EB or reticulate bodies. Collectively, these data identify non-EB chlamydial components which may be released into the inclusion during intracellular growth.

Chlamydiae are obligate intracellular pathogens with a life cycle unique among prokaryotes (19). Infection of target cells is mediated through a nonmetabolic life stage, the elementary body (EB), while intracellular multiplication progresses through the reticulate body (RB), a metabolically active, noninfectious form. After EB attachment and entry, chlamydiae remain separated from the cytoplasm in a membrane-bound vesicle, which grows in size until cell lysis and bacterial release. Fusion of the vesicle with host cell lysosomes is specifically inhibited during infection by chlamydiae (9, 10). The inhibition of phagolysosomal fusion protects the invading bacterium from intracellular bacteriocidal mechanisms, but organisms sequestered within the vacuole are physically separated from cytoplasmic energy sources and anaobic precursors. Therefore, the inclusion membrane must be modified for the inhibition of phagolysosomal fusion and be selectively permeable to allow the intake of nutrients required for growth. No mechanisms for the directed modification of the inclusion membrane have been put forth, nor have any chlamydial components which function in this process been identified.

Many diverse facultative intracellular bacteria have recently been shown to synthesize proteins which facilitate the entry into and survival and multiplication inside target host cells. In some model systems, including *Listeria, Salmonella*, and *Yersinia* spp., the production of these components is upregulated under conditions which model the intracellular environment (16, 25). Investigations directed at these environmentally regulated proteins have led to a better understanding of host-pathogen interactions in these systems, and have identified novel vaccine candidate antigens (4, 12). *Chlamydia* spp. may also produce proteins which are present only within infected cells. One approach to identifying such proteins is to examine the spectrum of chlamydial antigens present within infected cells and compare this pattern with those only present in purified EBs. We used sera from guinea pigs that have recovered from acute conjunctivitis caused by the guinea pig inclusion conjunctivitis (GPIC) strain of *Chlamydia psittaci* to demonstrate antigenic chlamydial proteins which are produced during in vitro and in vivo infections and are absent in the purified EB. Three such molecules were identified, one of which was detected only in the infected cell, and not in the EB or RB. Additionally, these sera were adsorbed with EBs and then used to demonstrate that chlamydial antigens can be localized to the inclusion membrane of infected cells.

**MATERIALS AND METHODS**

*Chlamydial strains and culture conditions.* EBs of *C. psittaci* GPIC, feline pneumonitis (Fel), ornithosis CP-3 (Orn), and meningoencephalitis (Men); *Chlamydia trachomatis* serovars A/Har-13, D/UW-3, F/IC-Cal-3, and L2-434; and *C. trachomatis* MoPn/Weiss (MoPn) were purified from infected HeLa cell monolayers with serial Renografin (Squibb Diagnostics, New Brunswick, N.J.) gradients, and stored at −70°C (6). RBs of GPIC were purified with identical Renografin gradients, but infected monolayers were harvested 16 to 18 h postinfection.

HeLa cells were grown in minimal essential medium supplemented with gentamicin (20 μg/ml) and 10% fetal calf serum (MEM-10) before infection. After infection with *Chlamydia* spp., cells were incubated with MEM-10 plus 0.7 μg of cycloheximide per ml (MEM-10 C). All HeLa cell cultures were grown at 37°C in 5% CO₂. Prior to inoculation, chlamydiae were diluted in SPG (0.25 M sucrose, 10 mM sodium phosphate, 5 mM l-glutamic acid, pH 7.2) and kept on ice. Forty-eight-well tissue culture plates were used for small-scale production of infected HeLa cells, and monolayers were infected at a multiplicity of infection of 0.5 to 1.0. Plates were centrifuged for 1 h at 1,000 × g; the inocula were

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removed, and 500 μl of MEM-10 C was added. Large-scale production of infected HeLa cells was accomplished in 150-cm² tissue culture flasks with a multiplicity of infection of approximately 1. Flasks were rocked for 2 h at 37°C prior to addition of 50 ml of MEM-10 C. Monolayers used for fluorescence microscopy were grown on sterile glass coverslips in 24-well microculture plates and inoculated at a multiplicity of infection of approximately 0.05. The inoculated cells were rocked for 2 h at 37°C prior to addition of 1 ml of MEM-10 C and subsequent culture.

Preparation of HeLa cell lysates for electrophoresis. Cells infected with GPIC or mock-infected control cells were harvested at 24, 30, or 40 h as indicated. Lysates of other chlamydial strains were prepared from infected cells incubated for 20 h (Fel, MoFn), 30 h (Orn, Mn, F/IC-Cal-3, L2-434), or 48 h (A/Har-13, D/UW-3). After the appropriate incubation, monolayers were washed twice with phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM NaPO₄, pH 7.2) and then lysed with 200 μl (for each well of a 48-well plate) or 2 ml (for 150-cm² flasks) of 1× electrophoresis sample buffer (1% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 6.8], 1% 2-mercaptoethanol, 10% glycerol). Lysates were mixed and removed, incubated in a boiling water bath for 2 min, aliquoted, and stored at −20°C.

Preparation of antisera. Convalescent-phase sera (convalescent sera) were collected from guinea pigs that had recovered from two serial cases of experimental acute chlamydial conjunctivitis. Two thousand inclusion-forming units (IFU) of GPIC diluted in SPG were inoculated onto the lower conjunctivae of anesthetized female Hartley strain guinea pigs (500 to 600 g). Infections were monitored by culture as described by Watkins et al. (31). Infections were repeated 100 to 120 days post-primary challenge and again monitored by culture. Sera were collected 30 to 35 days post-secondary challenge and were stored at −20°C.

Polyclonal antisera to live and formalin-fixed GPIC EBs were also produced in Hartley strain guinea pigs. For immunization with killed EBs, bacteria were fixed in 1% formalin–PBS overnight at 4°C and then washed twice in PBS. Equal numbers of live or fixed EBs (10⁶ IFU) were used in each of two serial immunizations, with the Ribi Adjuvant System (Ribi Immunocóm, Hamilton, Mont.). Animals were bled prior to the initial immunization and 30 days after each subsequent set of injections.

Electrophoresis and immunoblotting. Polyacrylamide gel electrophoresis was conducted with 12% running gels and 4% stacking gels. Electrophoretic transfer of proteins to nitrocellulose was performed with the Bio-Rad Trans Blot Cell (Bio-Rad Laboratories, Hercules, Calif.) with sodium phosphate blot buffer (25 mM, pH 7.8) at 0.9 A for 90 min. After transfer, blots were blocked with PBS plus 0.1% Tween 20 (TBPS) and 2% bovine serum albumin (BSA) (BSA-TBPS). Antisera were diluted 1/100 in BSA-TBPS and incubated on the blot at room temperature for between 2 and 16 h. After three washes in TBPS, blots were incubated for 1 h in ¹²⁵I-labeled staphylococcal protein A (approximately 124 nCi/ml in 2% BSA-TBPS; New England Nuclear, Boston, Mass.). Blots were washed three times in TBPS, rinsed quickly in water, and dried. Autoradiography film was placed directly on the blot and exposed at room temperature for 16 h.

Comparisons of the antibody responses to denatured EBs, RBs, and infected cells were conducted using preparations standardized for the relative amounts of the 39-kDa major outer membrane protein (MOMP). These samples were electrophoresed in adjacent lanes, transferred to nitrocellulose, and probed with convalescent sera.

Detection of chlamydial antigen proteins in vivo. The conjunctivae of female Hartley strain guinea pigs were infected with 10,000 IFU of GPIC and sacrificed on day 7 postinfection. Control guinea pigs were mock infected with SPG. Conjunctival epithelia were removed from sacrificed guinea pigs by pipetting a total of 50 μl of 1× electrophoresis sample buffer into the upper and lower conjunctival sac. This lysate was removed and stored frozen at −20°C. Samples were thawed, incubated in a boiling water bath for 2 min, and centrifuged (8,000 × g, 10 min). The supernatant was removed and used as antigen for immunoblots.

Proteinase K treatment. Small-scale cultures of GPIC-infected HeLa cells were lysed 30 h postinfection with 200 μl of proteinase K buffer (20 mM Tris [pH 7.4], 20 mM EDTA [pH 8], 0.5% SDS). The lysates were removed from the well and stored at −20°C prior to analysis. One-hundred-microliter aliquots were incubated either with or without proteinase K (Life Technologies, Grand Island, N.Y.; 200 μg/ml) at 37°C for 90 min. These samples were mixed with 2× electrophoresis sample buffer, electrophoresed, blotted, and probed with convalescent sera.

Preparation of adsorbed antisera. EBs (10⁶ IFU/ml) were incubated in sterile water–0.5% 2-mercaptoethanol overnight at 4°C. After incubation, the extracted EB were dialyzed twice into PBS at 4°C and mixed with an equal volume of convalescent sera previously diluted 1:10 with 2% BSA in PBS (BSA-PBS). Control antisera were produced with homologous convalescent serum mock adsorbed with 2% BSA-PBS. Antisera were adsorbed for 6 h at room temperature on a platform rocker, and insoluble particulates were removed from solution by centrifugation at 12,000 × g for 20 min. The recovered supernatants were used as adsorbed sera in immunoblotts and fluorescence microscopy and were stored at 4°C in 0.02% sodium azide.

Microscopic analysis of infected cells. Both phase-contrast microscopy and indirect fluorescent antibody microscopy (FA) were performed on the same infected cell with a Zeiss Photo-Microscope III (Carl Zeiss Inc., Thornwood, N.Y.). Infected and mock-infected monolayers were washed with PBS and fixed with 100% methanol between 30 and 35 h postinfection. Fixed cells were washed with PBS and stored at 4°C in PBS prior to staining.

Monolayers to be examined by FA were first incubated in 2% BSA-PBS for 20 min. A primary antibody solution was then added at a 1:100 dilution in 2% BSA-PBS. Plates were incubated on a rocker platform at room temperature for 1 h. Wells were washed three times with PBS, and fluorescein-labeled goat anti-guinea pig immunoglobulin G (Cappel, West Chester, Pa.) was added at a dilution of 1:250 in 2% BSA-PBS. Monolayers were then incubated with secondary antibody in the dark for 1 h, and the wells were washed three times with PBS.

RESULTS

General characteristics of chlamydial infection. Infection of guinea pigs with GPIC resulted in acute chlamydial conjunctivitis which resolved by day 20 postinfection. Animals were culture positive through day 28, with bacterial titers approaching 10⁶ IFU per infected eye on day 7. Secondary infections resulted in a subacute clinical condition, with lower numbers of culturable bacteria at the tested time points (day 7 bacterial titers between 10² and 10³ IFU per eye). Culture data and clinical spectra in these experiments
Characterization of infection-specific chlamydial proteins produced in vivo and in vitro. Immunoblots of lysates of GPIC EBs and GPIC-infected cells demonstrated that convalescent sera recognized a spectrum of chlamydial components common to both EBs and infected HeLa cells, including the 60-kDa protein antigens (HSP 60, OMP 2), MOMP, a 28-kDa EB protein (p28), and lipopolysaccharide (LPS). However, antigenic bands which were not detected in the EBs were present in the infected cells (Fig. 1A). Lysates of infected cells had three additional components, at relative molecular masses of 22, 34, and 52 kDa (p22, p34, and p52, respectively). Approximately 90% of guinea pigs which had recovered from two episodes of chlamydial conjunctivitis produced detectable antibodies reacting with p22 and p34 in immunoblots, while approximately 50% of the convalescent sera reacted with p52 (not shown). Immunoblots of lysates of uninfected cells were uniformly negative (Fig. 1A). Protein p22 was absent in lysates of purified GPIC RBs while p34 was detected at relatively reduced concentrations (Fig. 1B). Each of these antigens was shown to be proteinaceous, as their antigenicity was eliminated with proteinase K (Fig. 1C).

Antisera produced in animals immunized systemically with live or formalin-fixed EBs were compared with the convalescent sera. Equal amounts of bacteria and identical immunization schedules were used in the preparation of antisera to fixed and viable EBs. Immunoblotting with these sera demonstrated that each reacted with a common set of EB components, but antisera prepared against viable EBs also reacted with p22, p34, and p52 (Fig. 2). The pattern of reactivity with the antisera prepared against viable EBs was similar to that seen with the convalescent sera.

Immunoblotting of lysates of GPIC-infected conjunctivae demonstrated that the infection-specific proteins were detected on day 7 postchallenge (Fig. 3). The pattern observed in infected animals is similar to that seen in infected cells. MOMP, antigens at 60 kDa, and p28 were easily detected, and p22 and p34 were detected in infected conjunctivae with extended autoradiographic exposure times.
Immunoblotting of different chlamydiae. Antigenic diversity of p22 and p34 was examined by blotting lysates of HeLa cells infected with selected strains of C. psittaci and C. trachomatis and probing with the convalescent sera. Cross-reactivity with the HSP 60 molecule in each chlamydial strain was evident (Fig. 4), and faint reactivity with an unknown 25- to 27-kDa protein was visible in each lane of the infected cells. However, the antigenicity of p22 and p34, and the 28-kDa EB protein (p28), was limited at the subspecies level. The proteins p28 and p34 have apparent homologs in two of the three examined C. psittaci strains (Orn and Mn) but no C. trachomatis serovars, while p22 was detected only in the lysates of GPIC-infected cells.

Immunoblotting with adsorbed convalescent sera. Antibody reactivity specific for EB components was removed from convalescent sera by adsorption with EB extracts (Fig. 5). This adsorption procedure produced a reagent with minimal reactivity toward all EB proteins and LPS, with the exception of p28. Antibody reactivity directed at p22 and p34 was unaffected by these adsorptions.

Fluorescence microscopy. Adsorbed and mock-adsorbed convalescent sera and sera from animals immunized with formalin-fixed EBs were used to examine infected cells by fluorescence microscopy. Chlamydial developmental forms were the dominant feature in GPIC-infected HeLa cells which were probed with either antisera against fixed EBs (Fig. 6B) or the unadsorbed convalescent sera (not shown). However, staining with the adsorbed convalescent sera demonstrated a distinctly different pattern; the chlamydiae within the inclusion fluoresced very weakly, but other structures within the inclusion fluoresced strongly (Fig. 6D). Staining was intense in the perinuclear center of the inclusion while staining in the periphery was associated with the margins of lobed structures which surrounded groups of individual chlamydiae. These lobes, or multiple inclusions, varied in size from approximately 2 to 6 μm. The lobed structures shown in these micrographs are structurally consistent with published descriptions of the inclusion morphology observed with GPIC-infected L cells (26). These results suggest that the adsorbed convalescent sera reacted with the inclusion membrane of GPIC-infected cells. Sera from unin-
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The described antigens were not detected in lysates of uninfected cycloheximide-treated HeLa cells, nor were they found in cycloheximide-treated cells infected with Chlamydia trachomatis. These results eliminated the possibility that these antigens are immunologically cross-reactive HeLa cell proteins produced during normal growth or under the stress of chlamydial infection.

Antibodies directed against certain chlamydial antigens were not consistently produced in convalescent guinea pigs, resulting in certain discrepancies between blots probed with different pools of convalescent sera. This is demonstrated in the blots shown in Fig. 1. A protein at 45 kDa and the 52-kDa RB protein (p52) are differentially present and absent in these blots. These inconsistencies reflect differences in the sera used in the blots and not differences in the antigen preparations (not shown).

Immunoblots of selected strains of C. psittaci and C. trachomatis were performed to examine the distribution of the infection-specific antigens within members of the genus Chlamydia. There was no evidence of cross-reactivity with similar antigens in any of the tested C. trachomatis serovars, and the cross-reactivity with C. psittaci strains was limited: p34 has an apparent homolog in two of the three tested strains, while there was no evidence of antigenic cross-reactivity with a homolog of p22. These results support the conclusion that the antigenicity of p22 and p34 is subspecies specific.

To our knowledge, this is the first description of chlamydial antigens not detected in purified EBs. In the guinea pig system, several authors have identified GPIC antigens which are recognized in the context of infection, but the antigens uniformly used in immunoblots were lysates of purified GPIC EBs (3, 22, 29). Many approaches have been used to examine the antigenicity of MOMP, LPS, and the 60-kDa proteins (2, 30, 32; reviewed in reference 18), and additional EB antigens have been identified in surveys of the antibody response in infected humans and infected or immunized animals. These include a spectrum of different protein species and a genus-common glycolipid antigen, GLX A (7, 11, 13, 21, 23, 27). While some of these antigens have been reported to be released from chlamydial developmental forms (15, 27, 28), none were shown to be unique to infected cells.

Adsorption of convalescent sera with 2-mercaptoethanol-treated EBs yielded a reagent that did not react with LPS, MOMP, or other identified EB components. The adsorbed sera retained strong reactivity toward p22 and p34, and toward the 28-kDa EB protein. Because all reactivity with the dominant EB antigens was eliminated by these adsorptions, these results provide evidence that p22 and p34 are not breakdown products of previously identified higher-molecular-weight proteins. The adsorbed sera were also used in fluorescence microscopy of methanol-fixed infected HeLa cells. These sera reacted with antigens associated with the surface of the lobed inclusions and not with the developmental forms within each lobe. Essentially identical FA results were obtained with paraformaldehyde fixation of infected HeLa cells (not shown). The lobular nature of the inclusions presented in this report is similar to that described by Spears and Storz (26), who developed a biotyping system for C. psittaci based on morphological differences of inclusions from different strains. The FA images obtained with the adsorbed sera resemble their micrographs for L cells infected with GPIC (biotype 8) and cells infected with strains from biotype 4.

The FA staining pattern observed with the adsorbed...
convalescent sera contrasted with the pattern seen with antisera raised against killed EBs (Fig. 6), which predominantly recognized the developmental forms within the inclusion. Because the adsorbed sera recognized principally the three proteins of 22, 28, and 34 kDa in immunoblots, it is possible that one or more of these proteins is released from intracellular chlamydiae and is in contact with the inclusion membrane. However, it is clear from the immunoblot in Fig. 5 that other antigens (39, 60, and 100 kDa) are faintly recognized by these adsorbed sera. The production of monospecific antisera will be required to identify individual chlamydial proteins which are localized to the inclusion membrane of infected cells.

The biphasic growth cycle used by chlamydiae is unique among bacteria. However, a similar life cycle is carried out by Toxoplasma gondii, a protozoan parasite of humans and other animals (14). A collection of Toxoplasma proteins (ROP 1, GRA 1 to 5) is released from the parasite upon infection, two of which are associated with the parasitophorous vacuolar membrane (1, 5, 8, 14, 20, 24). It is proposed that these proteins are important in the modification of the intracellular environment for the benefit of the parasite. We anticipate that similar experimental approaches will facilitate the identification and characterization of individual chlamydial proteins which may function on or about the inclusion membrane.

This work was initiated to identify potentially important immunogens that may be present only during chlamydial infection. The rationale for this approach is that the most successful current technique for generating solid immunity to chlamydial infection is prior infection with a homologous strain or serovar (18). The novel proteins described in this paper are subspecies-specific antigens only identified in infected cells in vivo and in vitro, characteristics which are consistent with the requirements for protective chlamydial antigens. Research addressing the roles of these proteins in the protective immune response is dependent on the production of preparative amounts of purified candidate proteins, and such efforts are in progress.

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REFERENCES


