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Antigen Trafficking within *Chlamydia trachomatis*-Infected Polarized Human Endometrial Epithelial Cells.

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by
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ABSTRACT

Antigen Trafficking within *Chlamydia trachomatis*-Infected
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Chlamydia trachomatis serovars D-K are the leading cause of bacterially-acquired sexually transmitted infections in the United States. As an obligate intracellular pathogen, *C. trachomatis* infects columnar epithelial cells of the genital mucosae and can cause deleterious sequelae such as pelvic inflammatory disease, infertility, and ectopic pregnancy. Several chlamydial antigens reach the host cell cytosol prior to the natural release of chlamydiae at the end of the developmental cycle. While some of these extra-inclusion antigens traffic to the host cell surface, others remain intracellular where they are proposed to influence vital host cell functions and antigen trafficking and presentation. The research herein examines the escape and trafficking of the immunodominant chlamydial antigens MOMP, LPS, and cHsp60 within *C. trachomatis* serovar E-infected polarized human endometrial epithelial cells. Studies using high-resolution transmission electron microscopy (TEM) and immuno-TEM report the novel escape mechanism of chlamydial antigens via vesicles everted/pinched off from the inclusion membrane, an occurrence observed both in the presence and absence of the antibiotic azithromycin. These extra-inclusion vesicles were differentiated from Golgi vesicles and were shown to deliver chlamydial heat shock protein 60 (cHsp60)-homologs 2 and 3, but not homolog 1, to the infected cell surface. Examination of the iron-responsiveness of the three cHsp60 homologs by immuno-TEM revealed a

significant increase in cHsp60-2 following iron deprivation. Further investigation of the trafficking of chlamydial MOMP and LPS antigens enveloped within the protective everted inclusion membrane vesicles within host cells involved density gradient centrifugation for the separation of epithelial secretory pathway components followed by SDS-PAGE and Western blot to determine whether the chlamydial antigen-containing vesicles could fuse with and deliver the antigens to host cell organelles. Coupled with immuno-TEM, these data confirmed the presence of major chlamydial antigens within the endoplasmic reticulum of infected host cells. Additionally, chlamydial lipopolysaccharide (LPS) was co-localized with CD1d, a lipid antigen-presenting molecule. Collectively, these studies (i) establish a novel escape mechanism for chlamydial antigens, (ii) identify cHsp60-2 as a marker of iron stress response in *C. trachomatis,* and (iii) define for the first time the host cell ER as a destination for selected chlamydial antigens during infection.

DEDICATION

This dissertation is dedicated to the memory of Jane Elizabeth Raulston, Ph.D.

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Grateful acknowledegments are made to my friends and colleagues with whom I have worked in the laboratory: Sophie Dessus-Babus, Melanie Sal, Cheryl Moore, and Maria Schell. Particular recognition is given to Judy Whittimore, who trained me in electron microscopy, and to Brian Dill, who befriended me throughout our academic experience at ETSU. Dr. Priscilla Wyrick deserves special recognition and gratitude for her instruction and guidance in my program of study.

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CHAPTER 1

INTRODUCTION

The family *Chlamydiaceae* consists of two genera and three species that are important human pathogens. The genus *Chlamydophila* comprises *C. pneumoniae* and *C. psittaci. C. pneumoniae*, transmitted human to human, causes respiratory tract infections and has been linked to atherosclerotic coronary disease. *Chlamydophila psittaci*, common in birds and mammals, is responsible for enteric and respiratory infections. The genus *Chlamydia* comprises the third major human species *C. trachomatis*, of which there are two human biovars—trachoma and lymphogranuloma venereum (LGV). *C. trachomatis* is further subdivided into serovars A-K, with serovars A-C causing the eye disease trachoma and serovars D-K causing sexually transmitted diseases (STD). The second human biovar, *C. trachomatis* LGV, instigates an invasive STD by infecting subepithelial and lymphatic tissues.

C. trachomatis serovars D-K are the most common agents of bacterially-acquired STD in the United States and worldwide (CDC 2006). The hallmark of genital infections by serovars D-K is the bacterium's extended survival and gradual pathogenesis inside target epithelial cells, resulting in an ability to establish chronic latent or persistent infections. After causing a typically asymptomatic urethritis or cervicitis, chlamydiae ascend the genital tract to cause chronic infections involving serious sequelae such as prostatitis, epididymitis, pelvic inflammatory disease (PID), ectopic pregnancy, and infertility (Schachter 1999). Furthermore, the *Chlamydia*-induced cell damage and local

immune response may be risk factors for acquiring HIV infection (Ho and others 1995; Rottingen and others 2001).

C. trachomatis is a Gram-negative obligate intracellular pathogen that infects columnar epithelial cells of the mucosae. There are two biological and morphological forms of *Chlamydia*; the metabolically inactive, environmentally stable elementary body (EB) and the metabolically active, intracellularly dividing reticulate body (RB). For growth and propagation, *Chlamydia* undergoes a unique developmental cycle (Fig. 1.1) that uses both extracellular (EB) and intracellular (RB) chlamydial forms. First, EB encounter the epithelial apical surface, where electrostatic interactions occur to bring EB in close proximity to receptors on the host cell surface (Kuo and others 1972, 1973, 1976). Several chlamydial ligands [heparan sulfate-like glycosaminoclycan (GAG), major outer membrane protein (MOMP), 60-kDa cysteine rich outer membrane complex protein OmcB, and heat shock protein 70] and host cell receptors (heparan sulfate receptor, mannose and mannose-6-phosphate receptors, and the estrogen receptor) have been implicated in attachment and entry of *Chlamydia* (Zhang and Stephens 1992; Chen and Stephens 1994; Su and others 1996; Davis and Wyrick 1997; Rostand and Esko 1997; Stephens and others 2000; Stephens and others 2001; Davis and others 2002; Kuo and others 2002; Raulston and others 2002; Puolakkainen and others, 2005). Once internalized, the EB-containing endosome bypasses lysosomal fusion, joins the exocytic pathway and is rapidly redistributed to the perinuclear region. The EB soon differentiate into RB, which multiply by binary fission within a vacuole termed an inclusion. The chlamydial inclusion is modified with host cell-acquired lipids (Hackstadt and others 1996; Wylie and others 1997) and chlamydial proteins to secure an

unobtrusive intracellular niche (Hackstadt and others 1997). During chlamydial development, the introduction of stress, such as antibiotic treatment (Tamura and Manire 1968; Kramer and Gordon 1971; Tribby and others 1973; Johnson and Hobson 1977; Dreses-Werringloer and others 2000), interferon-γ exposure (Kazar and others 1971; Beatty and others 1993), amino acid deprivation (Allan and others 1985; Coles and others 1993), and iron restriction (Raulston 1997; Al Younes and others 2001), causes chlamydiae to enter persistence, defined as the long-term interaction between chlamydiae and their host cell wherein decreased growth or replication results in a viable, yet culture-negative, chlamydial form. Chlamydial persistence is becoming increasingly recognized as a significant phase of development that allows survival under duress (Beatty and others 1994) and is hypothesized to be the predominant chlamydial form in infected humans *in vivo*. Absence or removal of stress allows RB maturation back into infectious EB to prepare for release by host cell lysis or inclusion extrusion (Hybiske and Stephens 2007).

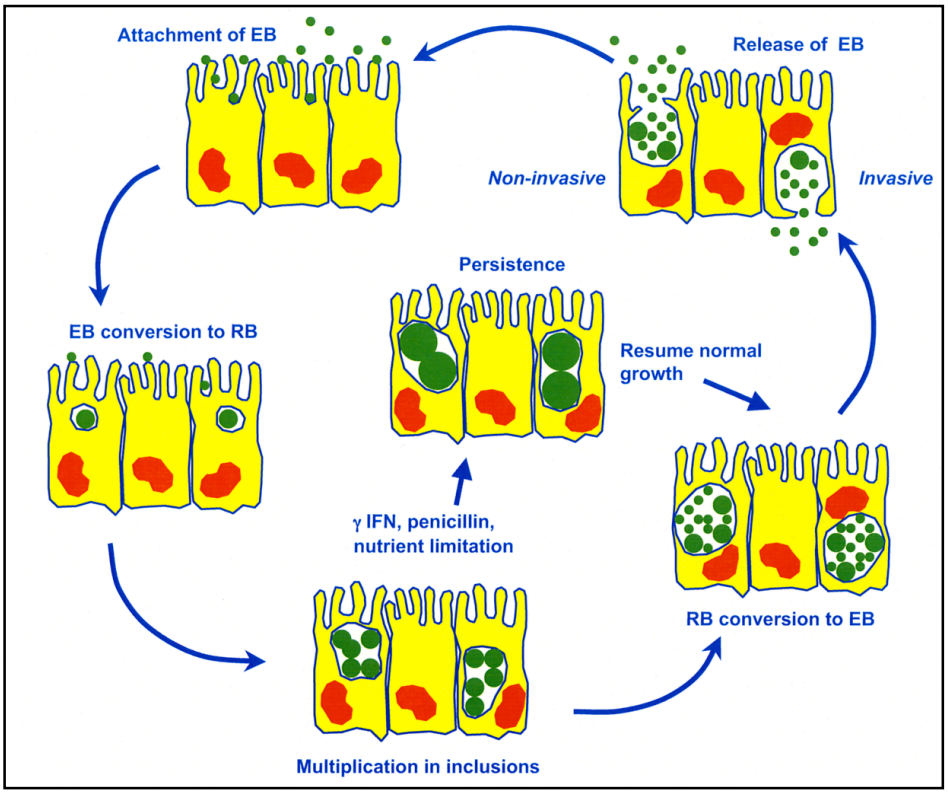

Figure 1.1 The chlamydial developmental cycle, including persistence. (used with permission from Jane E. Raulston).

Host immune response to *C. trachomatis* infection begins with the innate defenses present at the site of infection. In the genital tract, cervical and endometrial epithelial cells initiate anti-chlamydial immune mechanisms such as cytokine and chemokine production (Rasmussen and others 1997; Dessus-Babus and others 2000). Endometrial epithelia also express natural antimicrobials (beta-defensins) and toll-like receptors and are capable of MHC-I and MHC-II expression and antigen presentation (Van Eijkeren and others 1991; King and others 2003; Wira and others 2005). The recognition of chlamydiae elicits activation and recruitment of innate effector cells, such as polymorphonuclear nuetrophils (PMNs), macrophages, and natural killer (NK) cells (La Verda and Byrne 1994; Wyrick and others 1999; Hook and others 2004). Therefore,

to perpetuate infection, chlamydial EB must penetrate epithelial barriers, resist secreted antibacterial molecules, and avoid phagocytic uptake.

Chlamydial infection induces both an antibody response and a cell-mediated immune response, although the relative contribution of each of these adaptive immune arms to the clearance of infection is a matter of debate. Animal models have clearly indicated that CD4 cells, particularly the Th1 subpopulation, are critical for protective immune response to genital chlamydial infection (Landers and others 1991; Igietseme and others 1993). The antibody production from CD4-activated B cells also plays a role in both the resolution of infection and resistance to reinfection (Ramsey and others 1988; Igietseme and Rank 1991; Kelly and Rank 1997). However, Morrison and colleagues (1992) demonstrated that antibodies generated against *Chlamydia* confer only limited, serovar specific, protection against reinfection.

Because epithelial cells predominantly present antigens to $CD8^+$ T cells, there has been an increased focus on the $CD8^+$ T cell response to *Chlamydia*. $CD8^+$ T cells not only appear during *C. trachomatis* infection but also participate in the clearance of the disease, especially in humans and in other primates (Brunham and others 1985; Beatty and Stephens 1994; Starnbach and others 1994; Van Voorhis and others 1997; Kim and others 2000). Such a response implies accessibility of chlamydial proteins to the cytosol of infected cells, an occurence gaining acceptance due to identification of chlamydial proteins, including Type III secretion effectors, within the host cell cytosol (Peters and others 2007). The effort by *Chlamydia* to evade intracellular immune detection further substantiates involvement of $CD8^+$ T cells. *C. trachomatis* can downregulate MHC-I and MHC-II molecules and produces proteases that may protect

chlamydial proteins from antigen processing and T cell recognition (Zhong and others 2001; Misaghi and others 2006).

Among the chlamydial antigens that have been shown to play a role in immunopathogenesis, some of the best studied are the major outer membrane protein (MOMP), lipopolysaccharide (LPS), chlamydial heat shock protein 60 (cHsp60), and inclusion membrane protein A (IncA). MOMP, which makes up 60% of total cell envelope protein, is a protective antigen and a leading vaccine candidate, whereas LPS, a strong immunogen with unusually long fatty acyl chains, harbors some vaccine potential despite its low endotoxic activity. Numerous studies have localized chlamydial LPS exterior to the inclusion yet within the host cell, as well as on the host cell surface and released extracellularly (Campbell and others 1994; Wyrick and others 1999). As speculated by Heine and colleagues (2003), such accumulation of LPS in the infected microenvironment might contribute to the inflammatory response; however, the demonstrated altered host plasma membrane fluidity, as a result of LPS incorporation, could also protect the chlamydiae-infected cells from damage by natural killer cell granzymes and perforin (Wilde and others 1986). Like LPS, cHsp60 can interact with TLR2, TLR4, and CD14, likely resulting in induction of the inflammatory response (Ingalls and others 1995; Bulut and others 2002; Darville and others 2003). The *Chlamydia* genome contains three cHsp60 homologs, any or all of which may play a role in establishing chronic chlamydial infection. Recently, IncA, a protein with SNARE-like properties and involved in homotypic vesicle fusion (Hackstadt and others 1999; Delevoye and others 2004), was also identified as an important immunogenic antigen in human *C. trachomatis* infection (Tsai and others 2007).

The recommended treatment for chlamydial infections is the antibiotic azithromycin, a protein synthesis inhibitor that can access chlamydiae by both spontaneous cellular uptake and delivery by azithromycin-loaded (in bloodstream) chemotactic PMN (Raulston 1994; Paul and others 1997). Electron microscopy revealed that the azithromycin-mediated killing of chlamydiae within infected polarized epithelial cells *in vitro* involves an increase in chlamydial outer membrane blebbing (Figure 1.2A, arrowheads) as well as the appearance of extra-inclusion vesicles (Figure 1.2B, arrowheads) containing the chlamydial envelope antigens LPS and MOMP (Wyrick and others 1999). These vesicles, also observed during normal infection, traffic to and release LPS and MOMP onto the infected cell surface and into the extracellular milieu. This mid-developmental cycle release of chlamydial antigens, as opposed to natural release of chlamydiae and antigens at the end of the developmental cycle, may influence antigen trafficking and presentation by the host cell and contribute to the prolonged inflammatory response typical of chlamydial infections.

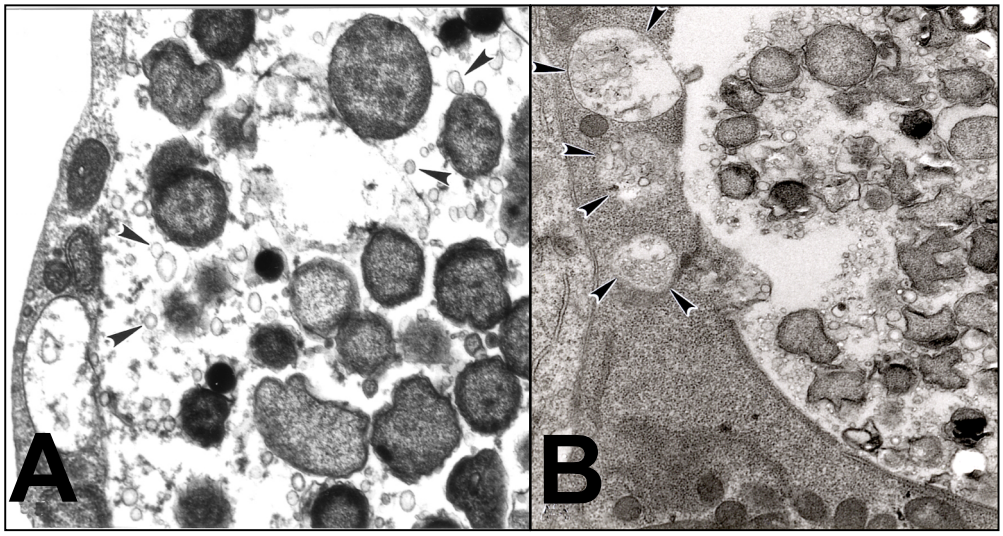

Figure 1.2 The azithromycin-mediated killing of chlamydiae involves the appearance of chlamydial outer membrane blebs (A) and vesicles exterior to the inclusion (B). (adapted from Wyrick and others 1999).

Specific Aims

The overall goal of this study is to examine the trafficking of chlamydial antigens within *C. trachomatis* serovar E-infected polarized human endometrial epithelial cells. *C. trachomatis* serovar E is the most common STD isolate in the United States. Initial experiments focus on elucidating the origin and antigen content of the extra-inclusion vesicles shown in Figure 1.2B. Of special interest is the destination(s) of these vesicles and their antigens, particularly whether or not the vesicles intersect host cellular organelles, such as the endoplasmic reticulum and/or Golgi, leading to the novel possibility of MHC-I and MHC-II antigen loading and presentation by epithelial cells. Secondly, specific antisera to the three cHsp60 homolog proteins will be used to probe *C. trachomatis*-infected polarized endometrial epithelial cells during normal infection and iron-deprived infection. The localization and relative expression of the three cHsp60 homologs may provide insight into both their response following stress and participation in the destructive inflammatory sequelae associated with chlamydial infection.

The Specific Aims of this study are: 1) to determine the origin and antigen content of the extra-inclusion vesicles observed during chlamydial infection of polarized endometrial epithelial cells; 2) to explore the destination(s) and immunological consequences of these extra-inclusion vesicles; and (3) to determine, by immunoelectron microscopy, the localization and iron-responsiveness of the three cHsp60 homologs during infection of polarized endometrial epithelial cells.

CHAPTER 2

ULTRASTRUCTURAL ANALYSIS OF CHLAMYDIAL ANTIGEN-CONTAINING VESICLES EVERTING FROM THE *CHLAMYDIA TRACHOMATIS* INCLUSION

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Keywords: Chlamydia trachomatis, Inclusion membrane proteins (Inc), Azithromycin, Antigen escape, vesicle formation

Abstract

Several chlamydial antigens have been detected in the infected epithelial cell cytosol and on the host cell surface prior to their presumed natural release at the end of the 72-96 h developmental cycle. These extra-inclusion antigens are proposed to influence vital host cell functions, antigen trafficking and presentation and, ultimately, contribute to a prolonged inflammatory response. To begin to dissect the mechanisms for escape of these antigens from the chlamydial inclusion, which are enhanced on exposure to antibiotics, polarized endometrial epithelial cells (HEC-1B) were infected with *Chlamydia trachomatis* serovar E for 36 h or 48 h. Infected cells were then exposed to chemotactic human polymorphonuclear neutrophils not loaded or pre-loaded *in vitro* with the antibiotic azithromycin. Viewed by electron microscopy, the azithromycin-mediated killing of chlamydiae involved an increase in chlamydial outer membrane blebbing followed by the appearance of the blebs in larger vesicles (i) everting from but still associated with the inclusion as well as (ii) external to the inclusion. Evidence that the vesicles originated from the chlamydial inclusion membrane was shown by immuno-localization of inclusion membrane proteins A, F, and G on the vesicular membranes. Chlamydial heat shock protein 60 (chsp60) copies 2 and 3, but not copy 1, were released from RB and incorporated into the everted inclusion membrane vesicles and delivered to the infected cell surface. These data represent direct evidence for one mechanism of early antigen delivery, albeit membrane-bound, beyond the confines of the chlamydial inclusion.

1. Introduction

The obligate intracellular pathogen *Chlamydia trachomatis* continues to be the leading cause of ocular and bacterially-acquired sexually transmitted infections worldwide. The genital serovariants (serovars D-K) target columnar epithelial cells of the genital mucosae. Initial chlamydial infection of the lower genital tract typically induces cervicitis or urethritis that often remain undetected. Without antibacterial therapy, more serious sequelae can result from ascending infection to the upper genital tract causing complications such as prostatitis, epididymitis, pelvic inflammatory disease (PID), ectopic pregnancy, and infertility [1].

The recommended treatment for chlamydial infections is the antibiotic azithromycin [2], a protein synthesis inhibitor that can access chlamydiae in their intracellular inclusions by pinocytosis following delivery by azithromycin-loaded polymorphonuclear neutrophils (PMN) or exogenous addition of the antibiotic to tissue culture medium [3,4]. Although it is not directly cidal to infectious, metabolically-inactive chlamydial elementary bodies (EB), azithromycin rapidly inhibits chlamydial protein synthesis in both intracellular reticulate bodies (RB) and purified host-free RB [5]. *In vitro* pharmacokinetic analyses revealed both higher cellular uptake and slower release of azithromycin versus erythromycin especially in polarized versus non-polarized genital epithelial cells [4,6]. This three-fold uptake of azithromycin in polarized cells is believed to contribute to the long pharmacological half-life *in vivo*. Studies with human phagocytic cells have not only indicated significant accumulation of azithromycin within monocytes, PMN, and alveolar macrophages, but they have also documented the transport and delivery of azithromycin by chemotactic PMN to infected cells [3,7]. *In*

vitro susceptibility studies have shown the advantages of azithromycin over erythromycin, doxycycline, and tetracycline [8,9], and clinical studies have reported single-dose effectiveness and good patient tolerance and compliance [10-12].

Viewed by transmission electron microscopy, the azithromycin-mediated killing of chlamydiae within infected, polarized human endometrial epithelial cells (HEC-1B) cultured *in vitro* reveals a dramatic increase in chlamydial outer membrane blebbing prior to destruction of metabolically-active RB. In addition, a notable finding is the appearance during mid-developmental cycle of extra-inclusion vesicles containing chlamydial envelope antigens [3,13]. These extra-inclusion vesicles can traffic to the infected cell surface to release chlamydial major outer membrane protein (MOMP) and lipopolysaccharide (LPS) into the extracellular milieu much prior to release of infectious progeny, possibly via apoptotic bodies [14], at the end of the developmental cycle. Furthermore, residual chlamydial envelopes and MOMP and LPS antigens also persist within inclusions in azithromycin-exposed cells for up to 4 weeks, and PMN chemotaxis to these antigen-containing epithelial cells can still be triggered *in vitro* [13]. Such persistence and slow release of chlamydial antigens, whether antibiotic-induced or not, may contribute to the hallmark prolonged inflammatory response considered accountable for the damage and sequelae in chlamydial infections. Importantly, the premature escape of chlamydial antigens from the membrane-bound inclusion is not restricted to stressful conditions, induced by antibiotics or immune modulators, but has been observed to occur during the normal developmental cycle [15-17]. A recent study by Patton *et al.* [18] yielded encouraging findings when azithromycin was used as the treatment in a macaque model of chlamydial pelvic inflammatory disease; azithromycin

treatment resulted in dramatic reduction of both immunopathologic damage and inflammatory response as evidenced by the absence of MOMP and chsp60 antigens in the monkey tissues following hysterectomy.

Native chlamydial MOMP elicits a protective response in chlamydiae-challenged mice and is a leading vaccine candidate [19,20]. While the unusually long fatty acyl chains of chlamydial LPS significantly reduce its endotoxic activity, it is speculated that local accumulation of LPS in the microenvironment of an infected niche may still be sufficient to promote an inflammatory response [21]. In contrast, several studies have identified an association between chlamydial heat shock protein 60 (chsp60) and severe ocular inflammation [22] as well as the deleterious sequelae of genital chlamydial infection, including tubal infertility, PID, and salpingitis [23-28], although the mechanism is somewhat controversial [29]. More recently, women with either PID or multiple *C. trachomatis* infections were shown to have reduced production of interferon-gamma in response to chsp60, supporting a role for chsp60 in establishing chronic chlamydial infections [30]. Whole genome sequencing of *C. trachomatis* serovar D [31] revealed two additional hsp60 homolog proteins, chsp60 copies 2 and 3 (chsp60-2 and chsp60-3) in addition to *groEL* (chsp60-1). Based on previous data published by the Hudson laboratory, the expression of these genes is independent of one another and differential expression has been observed in active versus persistent infection [32].

The purpose of this study was to further investigate the azithromycin-enhanced extra-inclusion vesicles in chlamydiae-infected cells to determine their origin and to probe them for the presence of chsp60.

2. Materials and Methods

2.1. Growth of Chlamydia and eukaryotic host cell

A human urogenital isolate *C. trachomatis* E/UW-5/CX, originally obtained from S.P. Wang and C.-C. Kuo (University of Washington, Seattle, WA, USA), has been maintained in our laboratory for several years. Standardized inocula of *C. trachomatis* infectious EB were prepared from McCoy cells grown on Cytodex 3 beads (Sigma) as described previously [33]. Progeny EB were harvested, titrated for infectivity, and stored as described previously [34].

The human endometrial epithelial cell line HEC-1B (HTB-113; American Type Culture Collection, Rockville, Md.), originally derived from a patient with endometrial carcinoma, was the cell line used in these studies. The HEC-1B cells, grown in a polarized manner [35] on commercial inserts (BioCoat Matrigel invasive chambers, 0.3 cm^2, Becton Dickinson Labware), were cultivated in Dulbecco's modified Eagle MEM (Life Technologies GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and 10 mM HEPES, pH 7.3 (DMEM) and maintained at 35°C in an atmosphere of 5% carbon dioxide and 95% humidified air.

2.2. Azithromycin

The antibiotic azithromycin, obtained from Pfizer Laboratories (Groton, CT, USA), was first solubilized in ethanol before prepararation of a 1 g/L stock solution in sterile water. Dilutions were made in DMEM and the antibiotic solutions were stored at 4°C in the dark.

2.3. Bacterial infection of cells and exposure to azithromycin

Polarized HEC-1B cells were inoculated with *C. trachomatis* EB by passive adsorption with a titre of crude stock diluted to a concentration demonstrated to yield at least 50% infected cells. In order to simulate the *in vivo* delivery of azithromycin to chlamydiae-infected tissues, the antibiotic was delivered to infected cells *in vitro* by chemotactic, azithromycin-loaded human polymorphonuclear neutrophils (PMN). Briefly, Histopaque-purified human peripheral blood PMN, obtained from chlamydiae-negative healthy volunteers via an Institutional Review Board approved protocol, were either not loaded or pre-loaded with azithromycin (25 mg/L) *in vitro*. The PMN (10^6 PMN per 10 μL) were then added to an agarose well underneath the extracellular matrix (ECM) layer supporting the polarized HEC-1B cells infected for 36 or 48 hours. The PMN were co-cultured with the chlamydiae-infected cells for 3, 6, or 12 hrs to allow for PMN activation and basal-to-apical chemotaxis, as detailed before [3,13]. Quantitation of fluorescence in calcein-AM-loaded PMN revealed 60-120-fold increase in chemotactic PMNs reaching the ECM layer in 36 h versus 24 h infected monolayers [36].

Alternatively, azithromycin was delivered exogenously to chlamydiae-infected cells as a dilution in the growth medium. Azithromycin was added at either 24 or 36 hours post-infection (hpi) and samples were taken every 12 h until the end of the developmental cycle.

2.4. Transmission electron microscopy and antibodies

Samples were processed and embedded in Epon-araldite for high contrast or in Lowicryl (Polysciences, Inc.) for immunoelectron microscopy, as described previously [15].

The primary antibodies used in this study to detect Golgi markers were (1) goat polyclonal antibodies directed against AP1G1 (Abcam); (2) monoclonal antibodies directed against Golgi Complex (Abcam); and (3) rabbit polyclonal antibodies directed against Giantin (Covance).

The primary antibodies used in this study to detect chlamydial antigens were (1) monoclonal antibodies directed against inclusion membrane protein A (IncA) generously donated by Dr. Dan Rockey (Oregon State University, Corvallis, OR); (2) rabbit polyclonal antibodies directed against IncA (via Dr. Rockey), and IncF and IncG, generously donated by Dr. Ted Hackstadt (Rocky Mountain Laboratories, Hamilton, MT); (3) monoclonal antibodies directed against the genus-specific epitope of *C. trachomatis* L2 lipopolysaccharide, provided by S. J. Richmond and S. Campbell (University of Manchester, UK); and (4) monospecific polyclonal antibodies targeted against three custom-designed 17-21-amino acid peptides in *C. trachomatis* serovar E heat shock protein 60kDa (hsp60) homologs, generated commercially in New Zealand White female rabbits by Sigma-GenoSys. The specific hsp60 peptide domains include MSRSANEGYDALRDAYT for chsp60-1, IPQEEIGYITSSIRAMTESLR for chsp60-2, and SQRSGSTLHLVKGIQTQKGY for chsp60-3. Preimmune serum was also provided. Peptide purity (>95%) was determined by high pressure liquid chromatography (HPLC) and mass spectral analysis. Enzyme immunoassays to determine the peptide

reactivities of the preimmune and first bleed antisera were conducted by Sigma-GenoSys as part of their quality control. After arrival in our laboratory, the immunoglobulin G (IgG) fraction of each serum was purified using ImmunoPure Protein A IgG purification kits (Pierce). Fractions containing 0.5 mg/ml, as assessed using UV spectrophotometry (280nm), were combined and dialyzed overnight against 10mM phosphate buffer (pH 7.0, 4°C) prior to storage at -20°C. Aliquots of each serum were pre-adsorbed against HEC-1B cells and recombinant *E. coli* LMG194 expressing the other two chlamydial hsp60 homologs engineered in the arabinose-inducible vector pBAD(HisA). Western blotting was done to confirm the absence of cross-reactivity to HEC-1B cell proteins as well as specific reactivity to all chlamydial hsp60 proteins. Primary antibodies were diluted 1:250 (AP1G1), 1:1 (Golgi Complex), 1:800 (Giantin), 1:30 (IncA polyclonal), 1:10 (IncA monoclonal and IncF polyclonal), 1:50 (chsp60-1), and 1:20 (chsp60-2) and visualized with a 1:200 dilution of 15-nm gold-conjugated second-affinity antibodies (Amersham Biosciences).

3. Results

3.1. Extra-inclusion vesicles in azithromycin-exposed and -unexposed chlamydiae-infected polarized human endometrial epithelial cells (HEC-1B)

Transmission electron microscopy examination of numerous azithromycin-exposed chlamydiae-infected HEC-1B samples at 60 hpi confirmed that azithromycin-mediated killing of chlamydiae involved an increase in chlamydial outer membrane blebbing as well as the appearance of extra-inclusion vesicles (Fig. 2.1B-E). Similar results were observed in samples infected for 24 and 36 h followed by 12 h exposure to azithromycin (data not shown). These vesicles were observed in many forms, ranging from seemingly empty vesicles of various sizes to vesicles containing membranous blebs and vesicular material, and they have been located all along the circumference of the chlamydial inclusion. The extra-inclusion vesicles have also been identified in polarized infected cells not exposed to azithromycin (Fig. 2.1A), but they are considerably reduced in number. Further, these extra-inclusion vesicles are not observed in uninfected cells (data not shown).

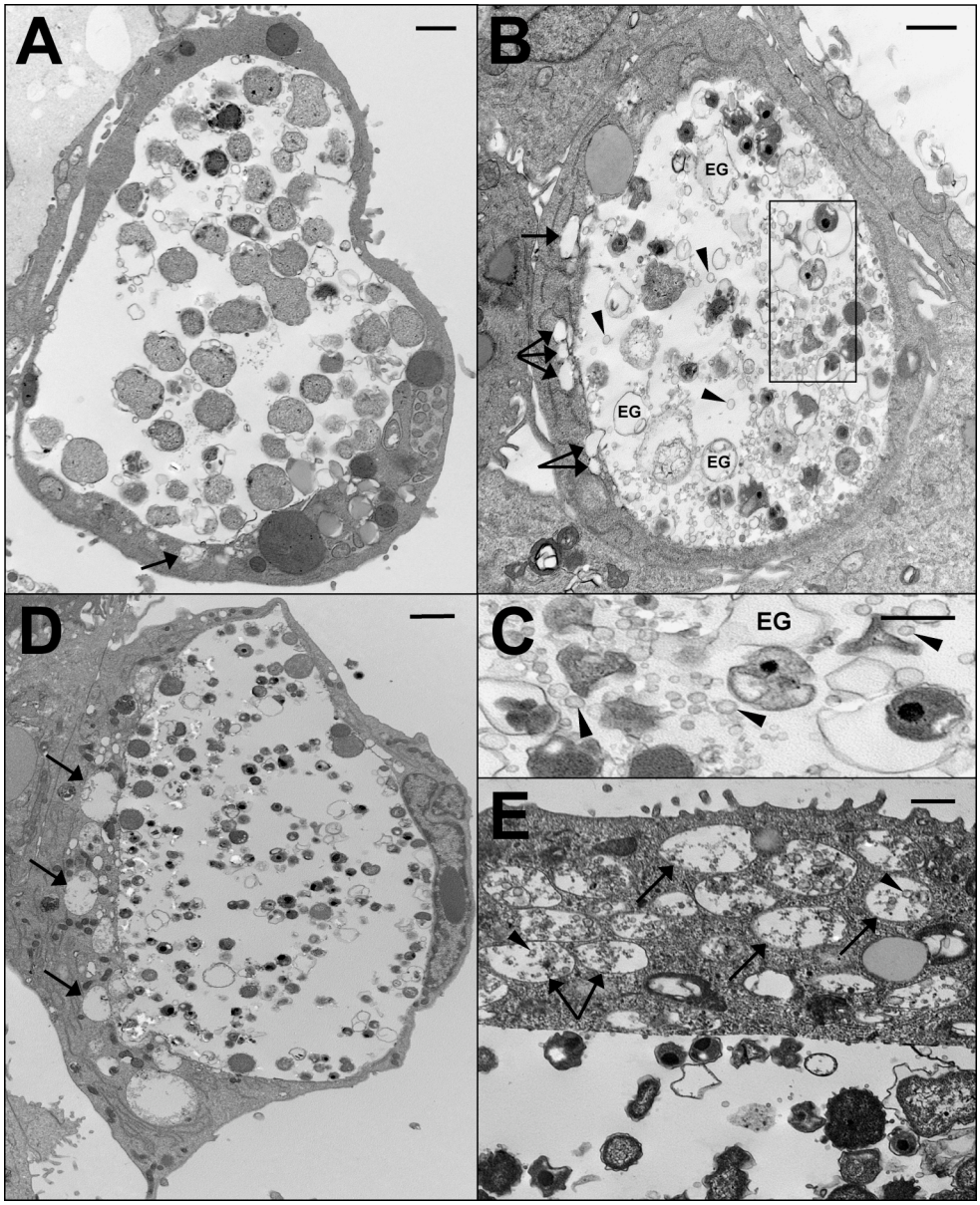

Figure 2.1 Identification by transmission electron microscopy of extra-inclusion vesicles in *C. trachomatis* serovar E-infected polarized human endometrial epithelial cells (HEC-1B) in the presence or absence of azithromycin. (A) Extra-inclusion vesicles (arrow) are present but reduced in number in infected cells cultured in the absence of azithromycin. (B-E) Numerous extra-inclusion vesicles (arrows) are visible in chlamydiae-infected cells incubated for 48 hrs, followed by exposure for 12 h to chemotactic PMNs pre-loaded with azithromycin. When the transepithelial PMNs contact infected cells, degranulation ensues, releasing bioreactive azithromycin which, in turn, is delivered to the chlamydial inclusion. (B) The initial effect on metabolically-active RB is excessive outer membrane blebbing (arrowheads) and the subsequent appearance of outer membrane-derived envelope ghosts (EG) (enlarged in C). (D,E) Eventually, numerous vesicles exterior to the inclusion appear (arrows), often containing blebs (arrowheads) and debris. Bars = A,B) 1 μm, C) 500 nm, D,E) 2 μm.

3.2. Ultrastructural evidence for vesicles originating from the chlamydial inclusion

Extensive high contrast ultrastructural analysis of azithromycin-unexposed (Fig. 2.2A) and azithromycin-exposed (Fig. 2.2B-E) chlamydiae-infected epithelial cells provided evidence for a direct link between the extra-inclusion vesicles and the chlamydial inclusion in that vesicles could be visualized actually everting/budding from the inclusion. A closer examination of the interior of the vesicles revealed the presence of material that resembled chlamydial remnants from inside the inclusion, i.e. chlamydial membrane blebs, envelope ghosts, and other debris following azithromycin exposure (compare Fig. 2.2D, arrowheads and Fig. 2.2E, arrows). Because RB are osmotically fragile, the environment within the inclusion must be somewhat hypertonic; however, the turgor pressures involved in vesicle eversion are unclear.

Figures 2.1 and 2.2A-E are representative electron photomicrographs derived from delivery of azithromycin to the chlamydiae-infected HEC-1B cells via the natural *in vivo* mechanism of azithromycin-loaded chemotactic PMNs. Similar morphological results are obtained when the antibiotic is added exogenously to the tissue culture medium at 24-48 hpi (Fig. 2.2F).

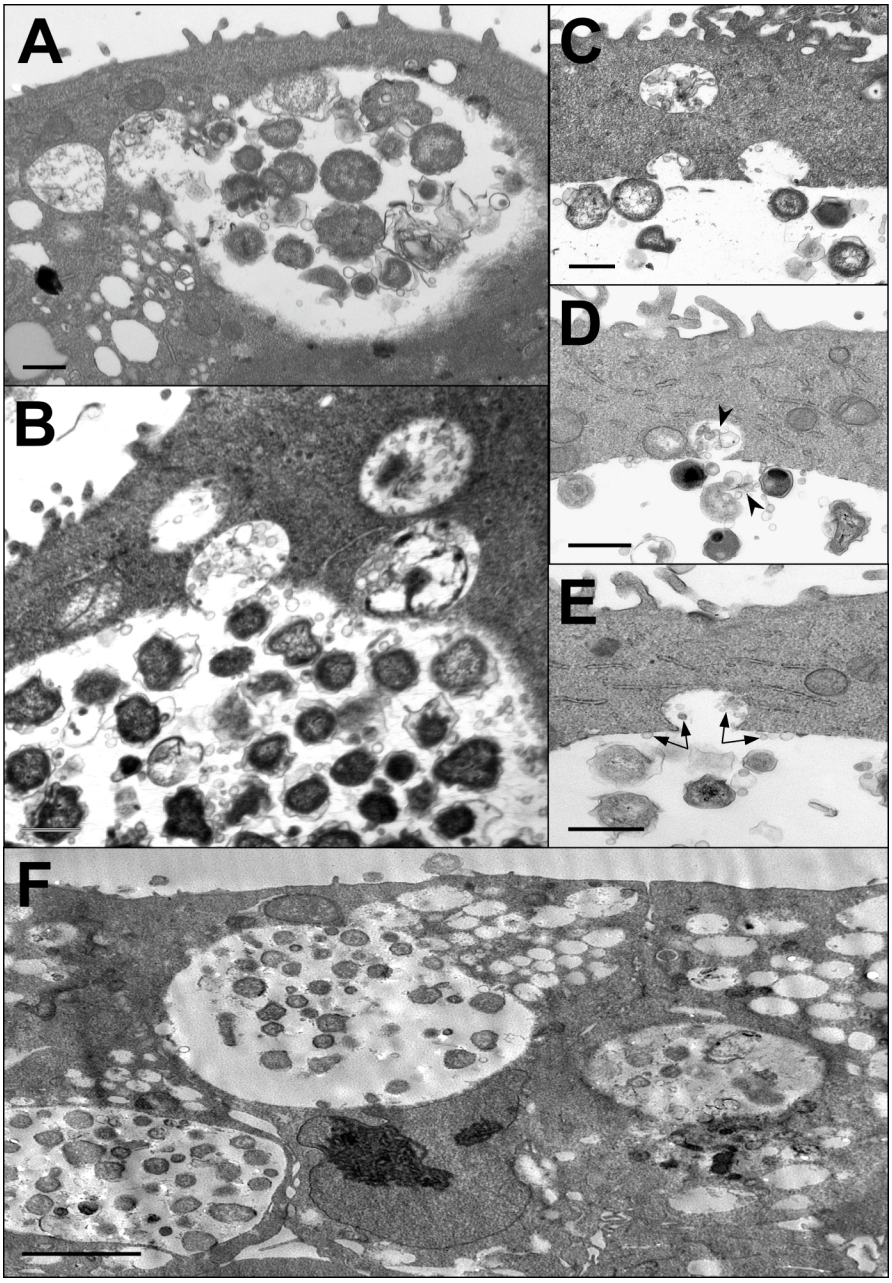

Figure 2.2 Ultrastructural identification of the formation of vesicles everting from inclusions of *C. trachomatis* serovar E-infected epithelial cells in the presence and absence of azithromycin. (A) A vesicle everting from an inclusion in a HEC-1B cell infected with *C. trachomatis* for 60 h in the absence of azithromycin. (B-E) Vesicles everting from inclusions in epithelial cells infected with *C. trachomatis* for 48 h and exposed to chemotactic PMNs pre-loaded with azithromycin for 12 h. Note the vesicle contents resemble outer membrane blebs like those found inside the inclusion (compare arrowheads in D and arrows in E). (F) The appearance of vesicles within polarized HEC-1B cells infected with *C. trachomatis* serovar E and exposed to azithromycin added exogenously to the tissue culture medium at 24 hpi and processed for TEM at 48 hpi. Bars = A) 500 nm, B) 2 μm, C-E) 500 nm, F) 4 μm.

Having identified vesicles in the process of eversion from the inclusion, we sought confirmation that they were, indeed, chlamydial inclusion membrane in origin.

3.3. Chlamydia-specific inclusion membrane proteins A (IncA) and F (IncF) and G (IncG) localize to the membrane of extra-inclusion vesicles

In the process of establishing their intracellular niche, chlamydiae produce proteins that are inserted into and modify the inclusion membrane. These proteins, called inclusion membrane proteins (Incs), contain a characteristic bi-lobed hydrophobic domain and have been localized to the inclusion membrane by fluorescence microscopy [37-41]. Primary polyclonal and monoclonal antibodies generated against IncA and polyclonal antibodies generated against IncF and IncG were used in the present study for post-embedding labeling immuno-electron microscopy analysis of the inclusion everting vesicles; the primary antibodies were detected by colloidal gold-conjugated second-affinity antibodies.

IncA was located in RB, particularly the outer membrane, as well as on the inclusion membrane (Fig. 2.3A and B). Both IncA and IncF were observed on areas of the inclusion membrane appearing to be in the process of everting/budding outward into the host cell cytosol (Fig. 2.3C, D, and E, circles). Further, IncA was found to be present on the membranous material within the inclusion membrane vesicles (Fig. 2.3E and F). In addition to localizing to chlamydial RB and the inclusion membrane, IncA, IncG (data not shown) and IncF were found on the membranes of vesicles exterior to the inclusion (Fig. 2.3D, arrows and 2.3E, circle). The immuno-localization of chlamydia-specific inclusion membrane proteins A, F, and G supports the eversion of these vesicles from

the inclusion membrane. Controls always included post-exposure of duplicate Lowicryl sections to (i) gold-conjugated second-affinity antibody alone and (ii) an irrelevant primary antibody plus gold-conjugated second-affinity antibody (data not shown) to confirm the specificity of the signal in the test samples.

While Lowicryl is a high contrast resin for immuno-electron microscopy, it is a lower contrast resin compared with Epon resin; post-fixation staining with osmium tetroxide and heat curing are omitted to avoid accelerated exothermic reactions that could lead to antigenic destruction. Therefore, the membranous bleb content of the extra-inclusion vesicles is not always apparent (compare Figs. 2.3D and 2.5B [Lowicryl] with Figs. 2.2B and D [Epon]). However, this membranous bleb content has been observed with definition in Lowicryl (Fig. 2.3F), although to a lesser extent. Because Lowicryl is a low viscosity, low temperature, photopolymerized resin, all processing was carried out at 4°C and -20°C, reducing the possibility that these everting inclusion membrane vesicles were artifacts of processing. Finding the same amount and pattern of everting inclusion membrane vesicles in duplicates of Lowicryl-processed compared with Epon-processed samples strengthens their existence.

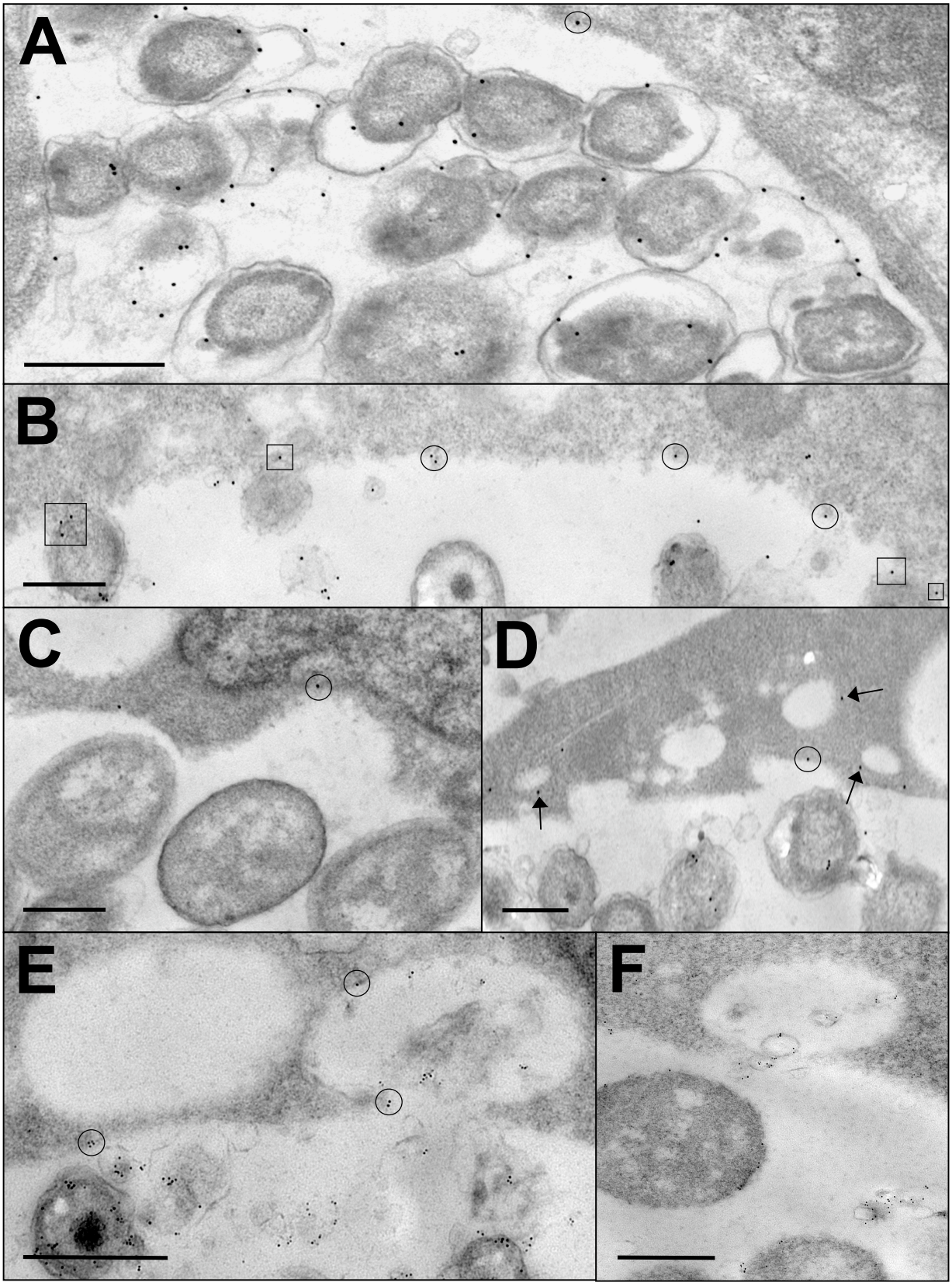
A
B
C
D
E
F

Figure 2.3 Ultrastructural localization of IncA and IncF. (A) Detection of IncA (polyclonal antibodies) in RB, associated with the RB outer membrane, and on the inclusion membrane (circle) at 48 hpi in the absence of azithromycin. (B) Detection of IncA (polyclonal antibodies) on the inclusion membrane (circles) and associated with RB outer membranes juxtaposed with the inclusion membrane (squares) during azithromycin exposure. (C) Detection of IncA (monoclonal antibodies) on an area of the inclusion membrane appearing to protrude outward into the host cell cytosol (circle) at 48 hpi minus azithromycin. (D) Detection of IncF (polyclonal antibodies) on the surface of extra-inclusion vesicles (arrows) and on an area of the inclusion membrane appearing to pinch outward into the host cell cytosol (circle). (E,F) Detection of IncA (polyclonal antibodies) on membranous material within the inclusion membrane vesicles. Again, IncA was localized to the inclusion membrane and on the surface of everting inclusion membrane vesicles (circles). Bars = 500 nm.

3.4. Differentiation between Golgi vesicles and inclusion membrane-derived vesicles

It has been well-established, via numerous elegant studies from the Hackstadt laboratory [42,43], that exocytic vesicles from the Golgi are intercepted by and fuse with the early chlamydial inclusion, resulting in acquisition of spingomyelin and cholesterol [44] by EB and RB envelopes. Thus, immuno-exposure of Lowicryl thin sections of azithromycin-exposed, serovar E-infected HEC-1B cells to primary antibodies generated against the cytoplasmic domain of the membrane-inserted Golgi marker Giantin, followed by gold-conjugated second-affinity antibodies, did label some chlamydiae and a few chlamydial envelope blebs within the inclusions and in bleb-containing extra-inclusion vesicles. However, the immuno-marker was not detected on the inclusion membrane nor the membrane of the extra-inclusion vesicles but did label numerous Golgi vesicles (Fig. 2.4D). Even though *C. trachomatis* infection distorts the Golgi complex and Golgi components are difficult to find among the numerous extra-inclusion vesicles, they could also be detected by additional Golgi markers, including the clathrin-associated adapter protein complex 1 (AP1G1) and the Golgi zone cytoplasmic marker

(Fig. 2.4A-C). In contrast, these markers were not detected on the inclusion membrane nor on the extra-inclusion vesicle membranes.

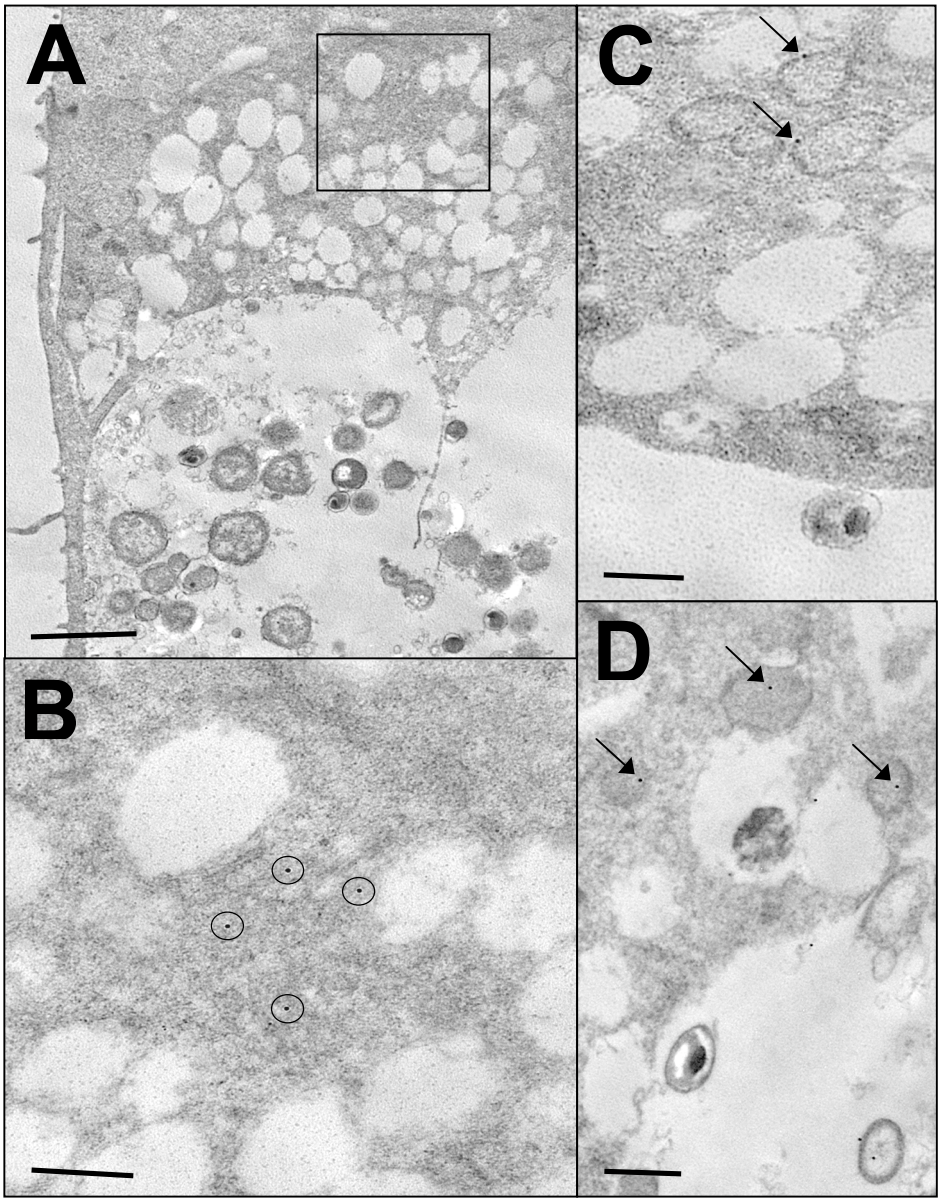

Figure 2.4 Ultrastructural localization by post-embedding immuno-electron microscopy using Golgi markers in chlamydiae-infected epithelial cells exposed to exogenous azithromycin at 36 hpi for 12 h. (A,B) Detection of the polyclonal Golgi marker AP1G1 in an area devoid of extra-inclusion vesicles. The low magnification in A illustrates the abundance of vesicles, while magnification of the box (B) depicts the region labeled by AP1G1 (circles). (C) Detection of the monoclonal Golgi marker Golgi Complex on vesicles morphologically distinct from the everting inclusion membrane vesicles. (D) Detection of the polyclonal Golgi marker Giantin associated with Golgi vesicles (arrows). Bars = A) 2 μm, B-D) 500 nm.

From these data, it can be reasoned that (i) azithromycin does not effect Golgi trafficking but does likely effect chlamydial inclusion membrane composition, (ii) extra-inclusion vesicles containing chlamydial outer membrane blebs appear and become numerous in azithromycin-exposed, *C. trachomatis* serovar E-infected epithelial cells, and (iii) the extra-inclusion vesicles routinely and reproducibly label with antibodies to Inc proteins but rarely with Golgi-specific antibodies whereas Golgi-specific vesicles are devoid of chlamydial envelope blebs and do not label with antibodies to Inc proteins. Such findings strongly suggest that the bleb-containing extra-inclusion vesicles are arising from eversion of the chlamydial inclusion membrane versus representing a Golgi vesicle “kiss and run” scenario.

3.5. Detection of chlamydial heat shock protein 60 (chsp60) within extra-inclusion vesicles

Previous studies had demonstrated by immuno-electron microscopy that the outer membrane blebs in the extra-inclusion vesicles contained the major outer membrane protein (MOMP) and lipopolysaccharide (LPS) [13] and these findings were confirmed in the present study (data not shown). Polyclonal monospecific antibodies generated against specific peptide domains of chsp60-1,2,3 were used for immuno-electron microscopic analysis to determine if these antigens could also be detected in the everted inclusion vesicles. Chsp60-1 remained within the chlamydial inclusion confined to RB (Fig. 2.5A), confirming previous studies [45]. Chsp60-2 was detected in RB, loose inside the inclusion, within everted inclusion membrane vesicles, on the surface of host cells, and released extracellularly (Fig. 2.5B). A similar pattern of labeling was

observed for chsp60-3 (data not shown). The significance of escape from the inclusion of chsp60-copies 2 and 3 versus copy 1 is unknown at this time. However, the fact that there is selective escape of chlamydial antigens from the inclusion is supported by the retention in RB in the inclusion of chlamydial hsp70 and histone protein 1 [45].

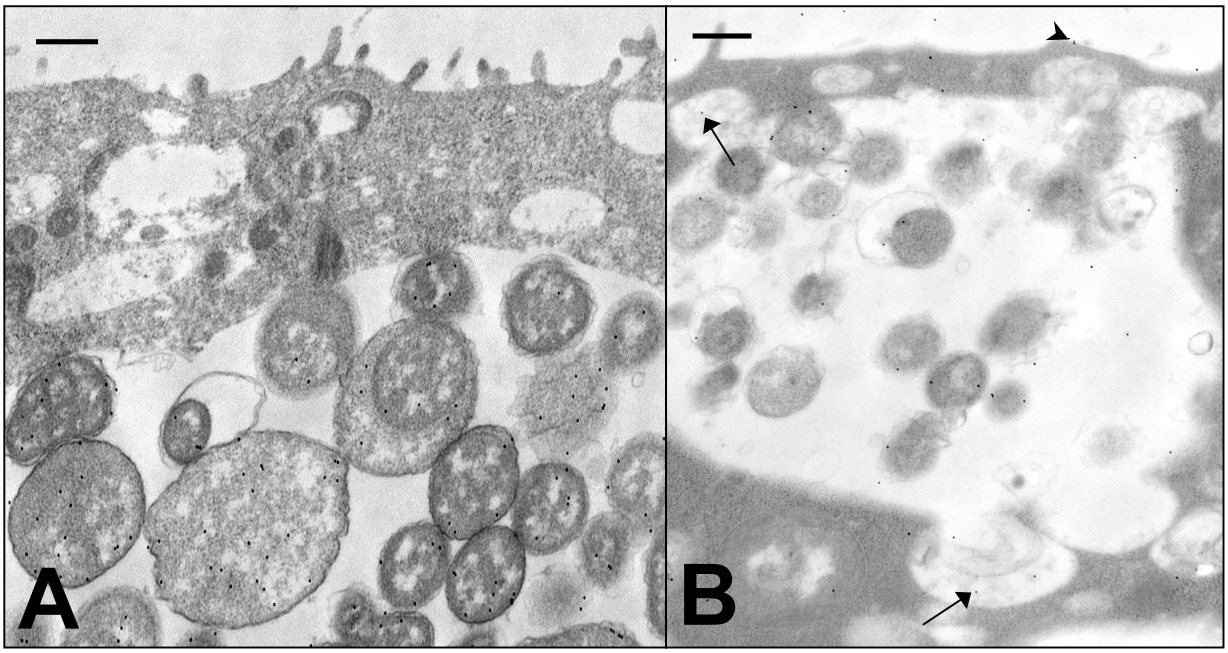

Figure 2.5 Ultrastructural localization by post-embedding immuno-electron microscopy of chlamydial heat shock protein 60 (chsp60) copies 1 and 2 in chlamydiae-infected epithelial cells at 48 hpi. (A) Chsp60-1 is confined to RB. (B) Detection of chsp60-2 inside RB, in the chlamydial inclusion, within vesicles everting from the inclusion (arrows), and on the host cell surface (arrowhead). Bars = 500 nm.

4. Discussion

Twenty-five years ago, Richmond and Stirling [46] were the first to report the excretion of *C. trachomatis* serovar E LPS from the inclusion, concomitant with RB replication and blebbing; the LPS was subsequently detected on the surfaces of infected cells and spread to adjacent uninfected cells. These surprising findings were confirmed in an elegant study by Karimi et al. [47]. *C. trachomatis* F- or L2-infected cells were superinfected at 20 hpi with vesicular stomatitis virus; progeny virus virions budded from the infected cells 8 hr later were enriched with chlamydial LPS. Wilde and Karimi et al. [48] then employed spin probe electron spin resonance spectroscopy to show that the fluidity of the LPS-containing infected cell plasma membrane versus mock-infected plasma membrane was significantly decreased. The authors speculated that the altered biophysical properties of the chlamydiae-infected host cell surface could protect the chlamydiae within from immune-mediated destruction, i.e., cytolysis from a complement-mediated mechanism or from natural killer or T cells.

That chlamydial LPS-containing outer membrane blebs escaped from the inclusion mid-developmental cycle was surprising. It was generally assumed chlamydial antigen escape occurred at the end of the developmental cycle when the inclusion membrane fused with the infected cell apical plasma membrane to release infectious progeny (Fig. 2.6A). However, additional support also existed in the early transmission electron microscopic studies of Matsumoto et al. [49].

When these authors added penicillin exogenously to infected cells to induce a chlamydial persistent state, there was a period of excessive RB outer membrane

blebbing during the progressive stages of disruption of cell wall biosynthesis. These blebs, containing LPS and MOMP, appear to “melt” into the inclusion membrane and emerge on the cytosolic side (Fig. 2.6D and E); perhaps by trafficking on Inc fibers extending from the inclusion membrane into the cytosol [50]. LPS and MOMP can subsequently be detected at 48 hpi by immuno-electron microscopy on the surface of the infected cells and in the extracellular milieu [15]. Proof that Gram-negative bacterial outer membrane blebbed vesicles can fuse with the plasma membrane of target host cells, at least from an external direction, as a mechanism to deliver bacterial virulence factors, DNA, and toxins has been shown in many studies [51-55].

If, however, the prokaryotic protein synthesis-inhibiting antibiotic azithromycin is exposed to chlamydiae-infected epithelial cells, a different mechanism of chlamydial outer membrane bleb antigen escape occurs – via vesicles everting from the inclusion membrane, as revealed in the present study. Because these inclusion membrane vesicles occur more frequently in the presence of azithromycin, one clever hypothesis first suggested by Hackstadt was that the inhibition of protein synthesis in metabolically-active RB by azithromycin was reducing the production of Inc proteins; thus, reduced Inc incorporation into the inclusion membrane might result in more fluid inclusion membrane domains. New studies by the Dautry-Varsat group [56] have provided additional insight. These investigators have proposed that IncA, previously reported to be involved in membrane fusion [46,57], can assemble into stable multimeric structures resembling a SNARE-like membrane fusion complex. Interestingly, heterologous expression of IncA in HeLa cells resulted in the localization of IncA in the endoplasmic reticulum (ER). Thus, it is tempting to hypothesize that a potential interaction between

the small, IncA-positive, everted inclusion membrane vesicles and the ER is possible for delivery of chlamydial antigens into the lumen of the ER. Further evidence that the inclusion membrane and, possibly, the membrane of the everted vesicles might participate in vesicle fusion events with the ER and other host organelles stems from the extensive studies of Rzomp et al. [58], in which several Rab-GTPases, important regulators of membrane trafficking, were localized to chlamydial inclusions. Rab1 and Rab6, which function, respectively, in ER-to-Golgi trafficking and the reverse, were specifically recruited to *C. trachomatis* inclusions. The authors also co-localized GFP-Rab11, involved in receptor recycling to the plasma membrane, with *C. trachomatis* IncG; thus, they proposed that interactions between the inclusion membrane and the host cell may occur via pairings of Inc proteins with Rab-GTPases. Each pairing, either by direct mediation or following recruitment of effector molecules, is responsible for fusion and/or motility events with the target membrane. Based on their functions in eukaryotic cells, Rab-GTPases localized to the chlamydial inclusion could mediate tethering and fusion with the ER, Golgi and plasma membrane. Taken together, these studies imply that if the everted membrane vesicles have incorporated the tethering/fusion machinery, multiple routes of antigen trafficking are likely possible.

It is accepted that CD8+ T cells are primed during chlamydial infection in both mice and humans [59-61]. To explain how antigens from intracellular pathogens could be presented to MHC Class I molecules and trigger a CD8+ T cell response, the concept of cross-presentation has been proposed [62,63]. This model is better suited to intracellular pathogen antigens, i.e., from *Salmonella*, *Legionella*, and *Brucella*, in macrophage phagosomes where endoplasmic reticulum organelle dynamic

convergence occurs [64-66]. Bacterial peptides are proposed to be retro-translocated to the cytoplasmic side of the phagosome via sec61 for ubiquination and proteosomal degradation and then translocated back into the phagosome lumen for loading into MHC Class I molecules. Whether or not the chlamydial inclusion might function similarly would be considered highly controversial at this time because organelle dynamic convergence and cross-presentation are not functions of epithelial cells. However, Wylie et al. [67] have shown that the inclusion membrane acquires some ER composition. Clearly, the issue of antigen release from the inclusion, and the implications thereof, for antigen function in the host cell and trafficking are important.

More recently, a chlamydia-specific protein, the Chlamydial Protease-like Activity Factor (CPAF), has been localized to the cytoplasm of the infected host cells late in chlamydial infection stages [16,17,68]. This protease degrades both RFX5 and USF-1, transcription factors essential for expression of MHC Class I and Class II antigens, respectively. Subsequently, Stenner-Liewen et al. [69] described another late protein CADD, for Chlamydia protein Associating with Death Domains, found in the host cytoplasm co-localized with Fas, which may modulate induction of apoptosis until late in chlamydial development. Finally, Fling and Starnbach and colleagues [60,61] generated CD8+ T cells cytotoxic for *C. trachomatis*-infected cells; interestingly, the antigens used to generate the CD 8+ T cells were an Inc protein and an Inc-associated protein, Cap1. Thus, chlamydiae are clearly sending signals to the eukaryotic host cytoplasm, some of which are directed at the immune response. How are those signals getting from the inclusion?

It has been postulated that some of these components/antigens may be secreted into the inclusion membrane and into the infected host cell by the chlamydial type III secretion system, in this case operating from “inside out” [70]. Support for this idea comes from the clever strategy of using *Yersinia* [71-73], *Shigella* [74], and *Salmonella* [75] as heterologous systems for identifying type III secreted effectors, including CopN and IncC, Tarp, IncA, B, and C from *C. pneumoniae*, and CopD and Pkn5. The hollow projections on EB and RB (Fig. 2.6B and C, arrowheads), which originate in the chlamydial cytoplasm, extend through the envelope, and can pierce the inclusion membrane [76], were originally proposed by Matsumoto to serve as a conduit for uptake of nutrients from the host cell cytoplasm for the growing RB. Speculation is popular that these projection structures are the type III secretion apparatus-equivalent but there is no information yet on the actual composition of the isolated projections. In any case, these structures may provide yet another mechanism for communication between this obligate intracellular vacuolar pathogen and the infected host cytoplasm and for possible modulation of vital host cell functions. Whether or not this route can be used to deliver $CD8^+$ T-cell protein antigens to the eukaryotic cytosol for access and classical presentation to the MHC Class 1 pathway is not known but is being pursued in various novel approaches.

Finally, the RB outer membrane is associated, at certain times, with the inclusion membrane (Fig. 2.6C, arrows); this is believed to be a tight association which leaves chlamydial outer membrane material adhered to/intercalated with the inclusion membrane after RB dissociation, as evidenced by the retention of darker staining areas (Fig. 2.6C inset, arrows). Genomic and bioinformatic analyses have suggested that

some members of the large family of *Chlamydia* polymorphic outer membrane proteins (Pmps) are surface exposed, antigenic and phase variable, can form the B-barrel translocating unit in the outer membrane, and may serve as the type 5 autotransporter secretion pathway [77-80]. It is unknown if, during RB feeding at the inclusion membrane, incorporation into the inclusion membrane of bi-directional functioning Pmp autotransporters might occur for uptake of nutrients as well as secretion of molecules for modulation of apoptosis and the immune response.

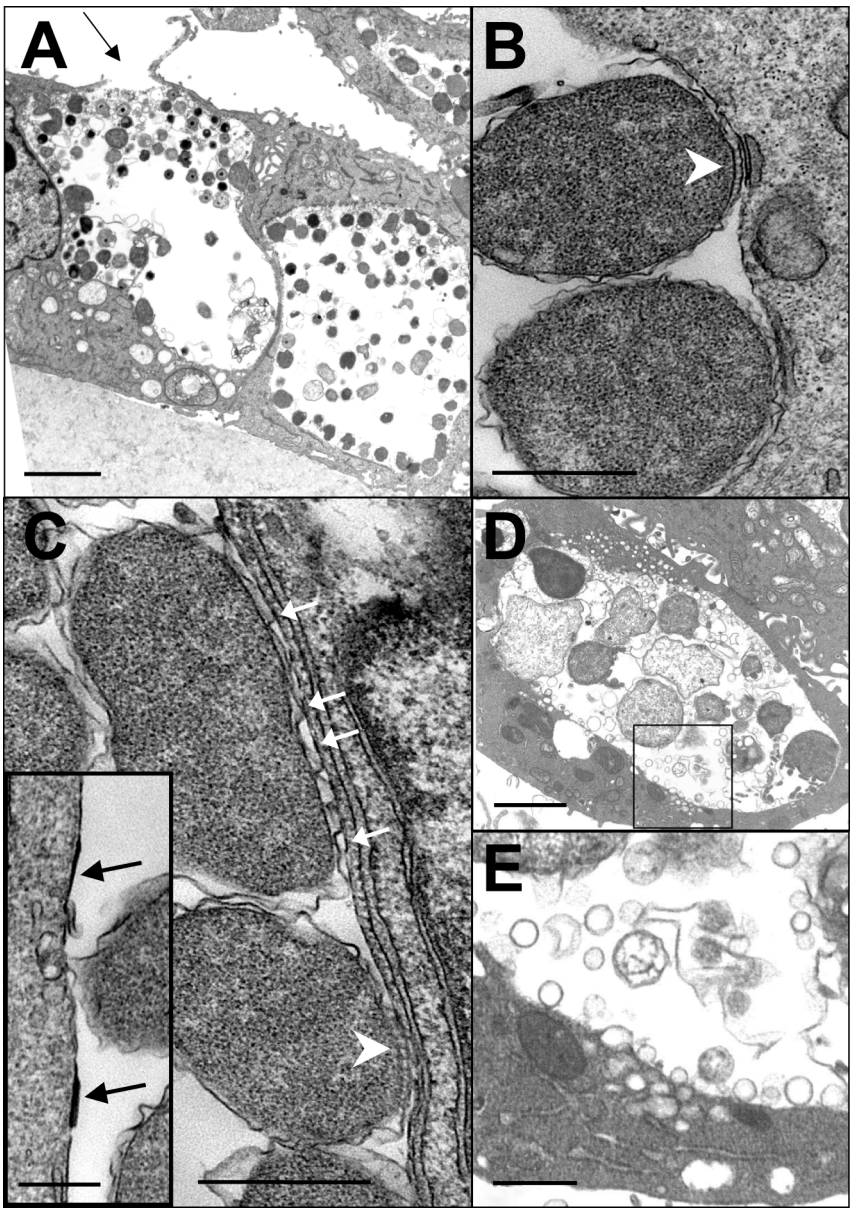

Figure 2.6 Summary composite illustrating known and suspected methods for escape of chlamydial antigens from the inclusions in infected human epithelial cells. (A) Apical release (arrow) of chlamydiae and chlamydial antigens from polarized epithelial cells at the end of the developmental cycle. (B,C) Intimate association between RB and their outer membranes and projections and the chlamydial inclusion membrane. Arrows indicate presence of surface projections extending from the RB outward to the inclusion membrane. Arrowheads point to the darker staining areas where the RB outer membrane makes contact with the inclusion membrane. Inset – some darkly-stained material remains on the inclusion membrane after dissociation of RB (arrowheads). (D,E) Chlamydial outer membrane blebs, generated in the process of inducing enlarged, aberrant persistent RB following exposure of infected cells to penicillin G, emerge from the interior of the inclusion onto the cytosolic side. Bars = A) 10 μm, B) 2 μm, C-E) 500 nm, E,inset) 250 nm.

In summary, multiple mechanisms apparently exist for escape of chlamydial antigens from the protected inclusion niche in mucosal epithelial cells, which are beginning to be re-analyzed in newer contexts. These components likely interact with host cell components to modulate several vital host functions—probably for the benefit of chlamydiae. Some antigens, however, may be the initial trigger for an inflammatory response. That such critical signals originate in non-immune cells prompted Stephens [35] to propose the "cellular paradigm of chlamydial pathogenesis" wherein the processes occurring at the infected epithelial cell are necessary and sufficient to orchestrate the resultant inflammation and fibrosis of disease and chronic sequelae.

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CHAPTER 3

CHLAMYDIAL HSP60-2 IS IRON RESPONSIVE IN *CHLAMYDIA TRACHOMATIS* SEROVAR E-INFECTED HUMAN ENDOMETRIAL EPITHELIAL CELLS *IN VITRO*

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Abstract

Chlamydial heat shock proteins 60kDa (cHsp60s) are known to play a prominent role in the immunopathogenesis of disease. It is also known that several stress-inducing growth conditions, such as heat, iron deprivation, or exposure to gamma interferon, result in the development of persistent chlamydial forms that often exhibit enhanced expression of cHsp60. We have shown previously that the expression of cHsp60 is greatly enhanced in *Chlamydia trachomatis* serovar E propagated in an iron-deficient medium. The objective of this work was to determine which single cHsp60 or combination of the three cHsp60 homologs encoded by this organism responds to iron limitation. Using monospecific polyclonal peptide antisera that recognize only cHsp60-1, cHsp60-2, or cHsp60-3, we found that expression of cHsp60-2 is responsive to iron deprivation. Overall, our studies suggest that the expression of cHsp60 homologs differs among the mechanisms currently known to induce persistence.

Keywords: *Chlamydia trachomatis*; 60kDa heat shock protein (Hsp60); Iron; Persistence

Introduction

Studies have consistently shown that there is a correlation between the production of chlamydial 60-kDa heat shock protein (cHsp60) antibodies in chlamydiae-infected patients and adverse disease consequences. These observations appear to be universal for *Chlamydia* species and their disease presentations. Early studies of whether cHsp60 plays a role in immunopathogenesis involved analyses of serum antibodies from female patients presenting with *Chlamydia trachomatis*-associated tubal infertility (7, 52); elevated levels of anti-cHsp60 in the sera of these patients were significantly associated with disease. A separate group of investigators described the contribution of cHsp60 in a guinea pig model of trachoma; an intense mononuclear cell inflammatory response was observed after conjunctival inoculation of cHsp60 following resolution of a primary ocular infection with *Chlamydia psittaci* GPIC (37). Patients with coronary artery disease often have serological evidence of previous infection by *Chlamydia pneumoniae* (45), and cHsp60 has been directly identified in human atheromatous tissue (31). Most recently, *C. pneumoniae* and *Chlamydia pecorum* cHsp60s have been implicated in urogenital tract disease in koalas (*Phascolarctos cinereus*), leading to infertility and death; chlamydiae are the most commonly recognized disease agents in the threatened koala population (23, 24).

Studies to determine the role of cHsp60 and immunopathogenesis are still being performed. Recently, workers have examined the initial interactions of cHsp60 with host cells that induce an inflammatory response. For example, cHsp60 interacts with Toll-like receptor 4, which stimulates the proliferation of human vascular smooth muscle cells (47), activates macrophages, and activates endothelial cells (8). The interaction

between cHsp60 and Toll-like receptor 4 also leads to apoptosis in primary human trophoblasts, placental fibroblasts, and a trophoblast cell line by both caspase-dependent and -independent pathways (18). cHsp60 and other microbial ligands can also activate mononuclear cells by binding to CD14, the monocyte receptor for lipopolysacharide (32). Although cHsp60 clearly plays a prominent role in chlamydial pathogenesis, it is not the only molecule involved. The genetically linked cHsp10, encoded by the *groES* gene upstream of *groEL-1*, is also associated with disease complications (6, 23, 27, 33). Moreover, several studies have demonstrated that genetic predisposition plays a significant role in chronic chlamydial disease (10, 12, 38). Perhaps most interesting is the fact that cHsp60 has been used in a human trial involving women at high risk for *C. trachomatis* infection; cHsp60 was used to stimulate the patients' peripheral blood mononuclear cells to produce gamma interferon (IFN-γ), and the results indicated that a protective response against incidental infection developed (11).

Our laboratory is involved in identifying and analyzing *C. trachomatis* proteins that respond to iron restriction, as well as the mechanisms involved (42, 43, 55); cHsp60 is one of several proteins whose expression increases significantly during iron limitation *in vitro* (43). It is known that iron sources and the availability of iron fluctuate in menstruating women due to the cyclic pressures of estrogen and progesterone (1, 29); active or persistent *C. trachomatis* organisms in the reproductive tract are therefore likely to respond to this dynamic environment using transcriptional, translational, or posttranslational mechanisms to alter the production of specific chlamydial proteins. While we are not involved in direct studies of persistent chlamydiae, which have been

defined as viable but non-culturable organisms (3), iron deprivation is one of several modes for induction of persistent chlamydiae (22, 39, 43). In women with tubal factor subfertility, cHsp60 is a serological marker for persistence (15) along with chlamydial proteasome/protease-like activity factor (48). However, the result of recent studies with *C. psittaci* (22) and *C. pneumoniae* (39) indicate that cHsp60 is not a general marker for persistence.

When the complete sequence of the *C. trachomatis* serovar D chromosome became available, one of many surprises was that there are three open reading frames (ORF) that code for *groEL*-related proteins (49). These ORF are positioned in separate regions of the chromosome and designated as follows: CT110 or *groEL-1,* encoding cHsp60-1; CT604 or *groEL-2,* encoding cHsp60-2; and CT755 or *groEL-3,* encoding cHsp60-3. Only *groEL-1* is preceded by *groES*. Matching cHsp60s in different *Chlamydia* species appears to be conserved in the sequences that are currently available. For example, the predicted level of amino acid sequence identity between cHsp60-1 in *C. trachomatis* serovar D and cHsp60-1 in *C. pneumoniae* AR39 is 91%. However, there are considerable differences between cHsp60-1, cHsp60-2, and cHsp60-3 in a given species or serovariant. In *C. trachomatis serovar* D, the levels of amino acid identity and similarity between cHsp60-1 and cHsp60-2 are 23% and 19%, respectively; the levels of amino acid identity and similarity between cHsp60-1 and cHsp60-3 are 18% and 20%, respectively; and the levels of amino acid identity and similarity between cHsp60-2 and cHsp60-3 are 17% and 15%, respectively (28, 35, 49).

Although the majority of previous studies clearly involved cHsp60-1, as confirmed by sequence analysis, certain studies, including our study (43), generated new questions

concerning the extent to which each cHsp60 responds to a given microenvironment, especially a microenvironment leading to chlamydial persistence. Thus, the purpose of this study was to determine which cHsp60 is iron responsive in *C. trachomatis* serovar E.

Materials and methods

Bacterial strains, eukaryotic host cells, and growth

Stock inocula of *C. trachomatis* serovar E/UW-5CX EB were generated in McCoy cell fibroblasts and titrated to determine their infectivity. Polarized human endometrial epithelial cells (HEC-1B) were used as host cells in iron deprivation experiments and were maintained in Eagle's minimal essential medium containing 2 mM glutamine and 5% (vol/vol) heat-inactivated fetal bovine serum at 37°C. For induction of iron deprivation, chlamydiae-infected cultures were allowed to grow to 36 hrs post-inoculation (hpi) and one-half of the samples were exposed to 500 μM Desferal for 30 min and 1 hr and 2 hrs.

Escherichia coli LMG194 (pBAD/HisA) was used to engineer and overexpress each cHsp60. The recombinants expressing cHsp60-1, cHsp60-2, and cHsp60-3 were designated as *E. coli* LMG194 (pJER516), *E. coli* LMG194 (pJER517), and *E. coli* LMG194 (pJER518), respectively. Each recombinant *E. coli* was grown in reduced medium (Invitrogen, Carlsbad, CA) containing 0.2% (wt/vol) glucose and 100 μg/ml ampicillin (Sigma Genosys, The Woodlands, TX) to the mid-log phase (A_{600}, 0.4 to 0.6) at 37°C. Cultures were subsequently centrifuged, washed, and resuspended in prewarmed glucose-free medium. Arabinose was then added to each culture for 4 h of

induction. Maximum expression of cHsp60-1and cHsp60-2 in *E. coli* LMG194 (pJER516) and *E. coli* LMG194 (pJER517) required 0.002% (wt/vol) arabinose, whereas maximum expression of cHsp60-3 in *E. coli* LMG194 (pJER518) required 20% (wt/vol) arabinose.

DNA amplification, cloning, and sequence analysis

The primers used for the PCR amplification of the chlamydial *groEL* genes were designed using the genome sequence of *C. trachomatis* serovar D (49). The reactions were carried out with an Expand High Fidelity PCR system kit (Roche, Nutley, NJ) in the presence of 0.5 pmol of forward, 0.5 pmol of reverse primer, and 10-fold (1:10-1:1,000) dilutions of *C. trachomatis* serovar E DNA template. After 35 cycles of amplification, the PCR products were cleaned-up using a QIAquick PCR purification kit (Qiagen, Germantown, MD); the sizes and concentrations of the purified products were monitored by agarose gel electrophoresis in the presence of ethidium bromide and by determining the optical density, respectively. All PCRs were done in duplicate to reduce introduction of errant nucleotides. The PCR products were then directionally cloned into the pBAD/HisA vector (Invitrogen, Carlsbad, CA), under the control of the araC promoter with a N-terminal six-histidine tag for recombinant protein detection and used to transform *E. coli* LMG194 by the traditional $CaCl_2$ method (46). For each *groEL* gene, the recombinant plasmids from three clones were purified using the Concert nucleic acid purification kit (Invitrogen) and then sequenced to verify in-frame cloning and to determine the complete nucleic acid sequences.

Peptide antibodies

The predictive amino acid sequences of *C. trachomatis* serovar E Hsp60-1, Hsp60-2, and Hsp60-3 were aligned using the EditSeq and MegAlign software from DNAStar, Inc. (Madison, WI). Peptides that were 17 to 21 residues long, detailed by Giles *et al.* (21), were commercially synthesized, the purity was assessed by analytical high-pressure liquid chromatography and mass spectroscopy, and each peptide was subsequently used to immunize two female New Zealand White rabbits (Sigma-Genosys, The Woodlands, TX). The results of enzyme-linked immunosorbent assays were provided by the manufacturer to ensure reactivity.

The majority of antiserum from each bleed was immediately stored at -20°C upon receipt. One milliliter of antiserum from each bleed was kept at 4°C to determine to determine the Western blot reactivities of the crude preimmune and immune sera against total protein from HEC-1B cells and *E. coli* LMG194 as controls and arabinose-induced recombinant *E. coli* LMG194 (pJER516), *E. coli* LMG194 (pJER517), and *E. coli* LMG194 (pJER518). Immune sera exhibiting the most selective reactivity with the intended Hsp60 homolog were then placed on protein A columns (ImmunoPure immobilized protein A, Pierce, Rockford, IL) to purify immunoglobulin G, and Western blotting was performed to determine the degree of monospecificity and the reduction in the cross-reactivity with other *E. coli* proteins. Antiserum against the peptide from cHsp60-3 required a further step of adsorption against whole cells of arabinose-induced *E. coli* LMG194 (pJER516) expressing cHsp60-1. A monoclonal antibody reagent (Sigma-Genosys, The Woodlands, TX) against the polyhistidine tag was also used in this study.

Protein quantitation, sodium dodecyl sulfate-polyacrilamide gel electrophoresis, Western blotting, and chemiluminescence

The total protein concentrations of samples were determined using the Micro BCA Assay (Pierce, Rockford, IL). Cell pellets were resuspended in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 mg/ml lysozyme, 0.1 mg/ml DNase I, and 10 mM $CaCl_2$) and subjected to three freeze-thaw cycles. After the final thaw, samples were centrifuged at 8,000 X *g* for 10 min, and each supernatant was combined with denaturing sample buffer and heated at 100°C for 5 min. The proteins were resolved in small-format 4-12% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes for Western blotting. Preliminary separations were conducted in large-format 12.5% polyacrylamide gels loaded with 1 mg of protein to accommodate multiple blots for screening and titrating antisera.

For Western blotting, membranes were blocked with Blotto-plus (5% [wt/vol] dry non-fat milk in phosphate-buffered saline, 0.1% [vol/vol] Tween 20, and 10% [vol/vol] heat-inactivated fetal bovine serum), and washing was performed with phosphate-buffered saline containing 0.1% (vol/vol) Tween 20. Various dilutions were examined for the polyclonal peptide antisera generated against each of the cHsp60s, and a monoclonal antibody against the poly-His tag (Sigma-Genosys, The Woodlands, TX) was also used as a control. Specific signals were then detected either (i) by a colorimetric assay with an anti-rabbit alkaline phosphatase-conjugated secondary antibody and Western Blue substrate (Promega, Madison, WI), or (ii) by chemiluminescence using an anti-rabbit horseradish peroxidase conjugate, the SuperSignal West (Pierce) solution, and Kodak X-OMAT AR film.

Electron microscopy

Samples of *C. trachomatis*-infected polarized HEC-1B cells at 36 hpi were exposed to 500μM Desferal for 30 min and 1 h and 2 h; mock-exposed samples were used as controls. Each sample was immediately washed, fixed, processed, and embedded in Epon-araldite and Lowicryl (Polysciences, Inc.) for high-contrast electron microscopy and immunoelectron microscopy, respectively, as described by Giles *et al.* (21).

Visualization and image capture were done using a Philips Tecnai 10 transmission electron microscope (FEI Company, Hillsboro, Oregon) operating at 80 kV.

Nucleotide sequence accession numbers

The sequences determined in this study have been deposited in the GenBank database under the following accession numbers: AY447001 for *C. trachomatis* serovar E *groEL-1*, AY447002 for *C. trachomatis* serovar E *groEL-2*, and AY447003 for *C. trachomatis* serovar E *groEL-3*.

Results

Amplification and nucleotide sequence analysis of *C. trachomatis* serovar E *groEL* ORF

DNA that was the expected size was amplified with all dilutions of the *C. trachomatis* serovar E *groEL* DNA templates except the *groEL-3*/CT755 template (Fig. 3.1A, lanes 3). Figures 3.1B and 3.1C show the results of a change in primer strategy, and the data revealed that CT755 was present along with the flanking sequences. Further analysis showed that a missing cytosine residue in the initial sequence of the CT755 in serovar E at position 25 was responsible for the lack of primer hybridization and amplification (Fig. 3.1D). As determined by comparison with the published sequence of CT755 in serovar D, a frameshift placed the ORF back into frame by insertion of a cytosine residue at position 30 in serovar E. The final results were confirmed using a new set of primers (Figure 3.1E). The nucleotide sequences of *C. trachomatis* serovar E *groEL-1*, *groEL-2,* and *groEL-3* are 99.7%, 98.5%, and 99.2% identical to their counterparts in *C. trachomatis* serovar D, with only 6-, 25-, and 8-bp differences, respectively.

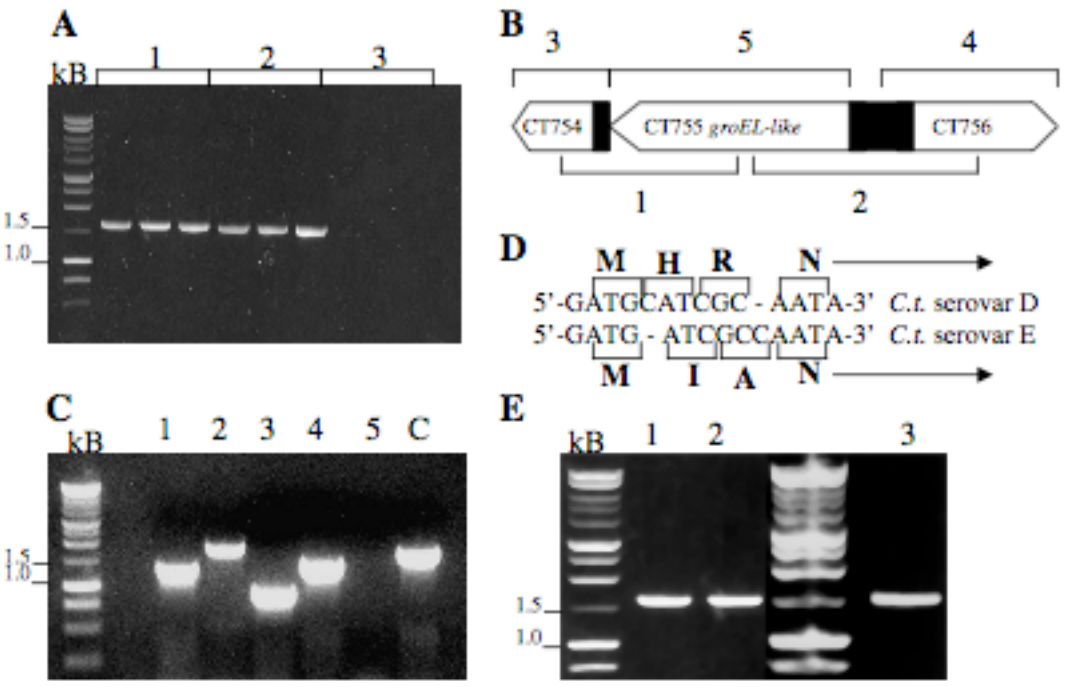

Figure 3.1 PCR amplification of *C. trachomatis* serovar E *groEL*. (A) Initial attempt to amplify *groEL-1, groEL-2,* and *groEL-3* (lanes 1, lanes 2, and lanes 3, respectively) from *C. trachomatis* serovar E DNA template and primers based on the sequence of *C. trachomatis* serovar D. (B) Strategy used to amplify *groEL-3* and flanking sequences. (C) Result of amplification of *groEL-3* and flanking sequences. Lanes 1 through 5 contained areas indicated in panel B, and amplification of *groEL-1* was used as a control. (D) Difference in the starting sequences of *C. trachomatis* serovars D and E. (E) Amplification of *C. trachomatis* serovar E *groEL-1*, *groEL-2,* and *groEL-3* with redesigned primers for *groEL-3*.

Specificity of the peptide antisera

Monospecificity was achieved for anti-cHsp60-1 and anti-cHsp60-2 with purification of immunoglobulin G alone (Fig. 3.2A). Antiserum against cHsp60-3 initially exhibited faint cross-reactivity with Hsp60-1 that was removed by cross-adsorption against whole cells of arabinose-induced *E. coli* LMG194 (pJER516) expressing cHsp60-1. A control using an anti-histidine monoclonal antibody (Sigma-Genosys, The Woodlands, TX) was included.

Iron-responsiveness of cHsp60s

Next, the peptide antibodies were used to examine *C. trachomatis*-infected cells with or without 500 μM Desferal, which were exposed for 30 min and 1 h and 2 h, beginning at 36 hpi (Fig. 3.2B). Preliminary experiments using the standard 50 μM Desferal for 96 h resulted in induction of a persistence-like state (43); these initial experiments indicated that only cHsp60-2 responds to iron deprivation beginning at 36 hpi (data not shown). Therefore, we changed the strategy in a manner analogous to application of heat, cold, or acid shock in other bacteria. Figure 3.2B confirms that cHsp60-2 is the primary cHsp60 that responds to iron limitation. Notably, cHsp60-2 is a target of proteolysis during cell lysis even in the presence of protease inhibitors (several combinations of inhibitors were tested). The data also showed that cHsp-1 is strongly expressed, but there was little or no difference between the expression in the absence of Desferal and the expression in the presence of Desferal; cHsp60-3 expression was delayed, but again, there was little difference between expression in the absence of Desferal and the expression in the presence of Desferal.

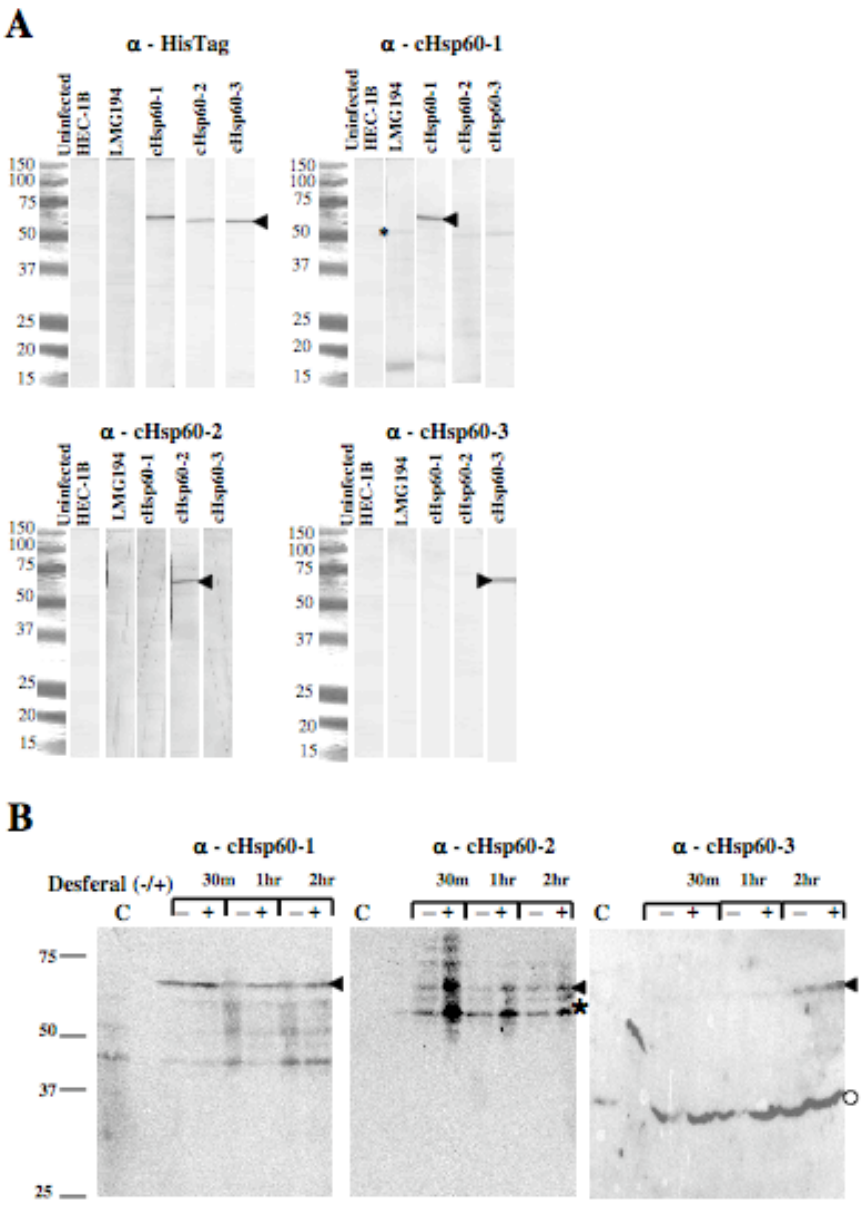

Figure 3.2 Specificity of peptide antisera and response of cHsp60-2 to iron deprivation. (A) Samples used for Western blotting included uninfected HEC-1B cells, *E. coli* LMG194 alone, and arabinose-induced recombinants *E. coli* LMG194 (pJER516), *E. coli* LMG194 (pJER517), and *E. coli* LMG194, representing cHsp60-1, cHsp60-2, and cHsp60-3, respectively. An anti-His tag monoclonal was used as a control (upper left panel). (B) Samples included uninfected HEC-1B cells (control) (lanes C), cells mock exposed for 30 min and 1 h and 2 h, and cells exposed to Desferal for 30 min and 1 h and 2h. One milligram of protein was loaded onto preparative gels (A), whereas 15 μg was loaded into each lane in panel B. Arrowheads indicate the position of cHsp60. The asterisk indicates the position of a major proteolytic product of cHsp60-2, and the circle indicates the position of a cross-reactive protein in HEC-1B cells.

Immunoelectron microscopy

To confirm that *C. trachomatis* cHsp60-2 is iron responsive, the final experiment involved examination of thin sections by immunoelectron microscopy. Multiple images were captured, saved as TIFF files, and printed; 2 μm square grids were used to enclose 10 to 15 randomly selected squares containing chlamydial reticulate bodies (RB) on prints of each sample, and the gold particles in each box were counted (Fig. 3.3).

Figure 3.3A, C, and E represent chlamydial RB in HEC-1B cells at 36 hpi (mock exposure); Figure 3.3B, D, and F illustrate RB at 36 hpi in cells that were exposed to 500μM Desferal for 1 h. Consistent with the results of the Western blot analyses, cHsp60-1 was strongly expressed, but there was not a significant difference between mock-exposed chlamydiae and chlamydiae exposed to Desferal (Fig. 3.3A and B); the number of particles in Figure 3.3A and B for cHsp60-1 were 19 (+/- 8) and 18(+/- 6), respectively. Likewise, for weakly expressed cHsp60-3 there was no difference between mock exposure and exposure to Desferal; the numbers of gold particles in both Fig. 3.3E and F were 5 (+/- 2). However, there was a significant difference ($P < 0.001$) between expression of cHsp60-2 between mock exposure and exposure to Desferal (Fig. 3.3C and D), as determined by Student's two-tailed *t* test; the numbers of gold particles in Fig. 3.3C and D were 4 (+/- 2) and 10 (+/- 3), respectively.

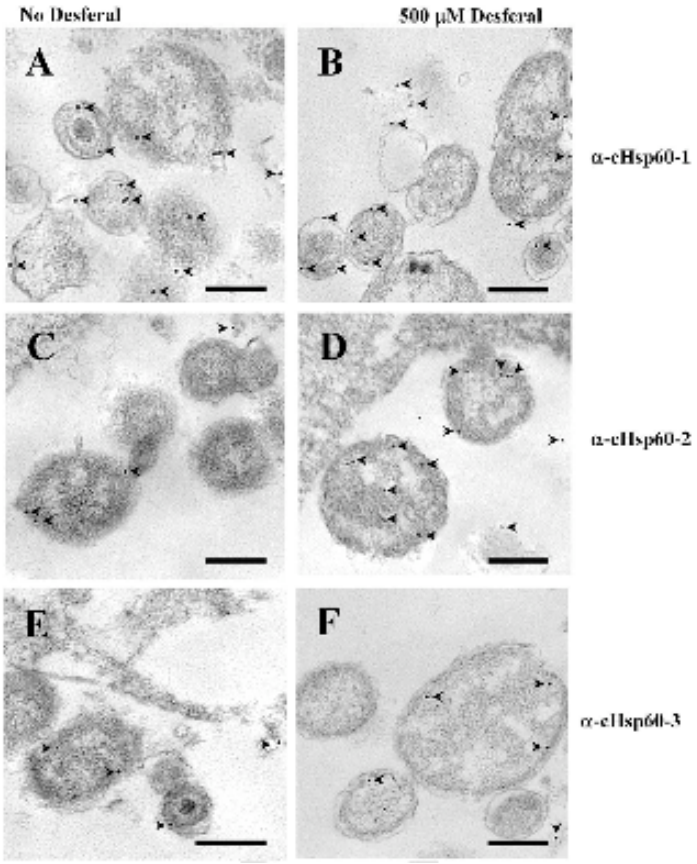

Figure 3.3 Immunolabeling transmission electron microscopy showing the response of cHsp60-2 to iron limitation. Chlamydia-infected HEC-1B cells at 36 hpi were either not exposed to Desferal (A, C, and E) or exposed to 500μM Desferal (B, D, and F) for 1 h and labeled using a 1:100 (vol/vol) dilution of anti-cHsp60-1 (A and B), a 1:20 (vol/vol) dilution of anti-cHsp60-2 (C and D), or a 1:40 (vol/vol) dilution of anti-cHsp60-3 (E and F). A 15nm gold-conjugated anti-rabbit serum (Amersham Biosciences) was used at a 1:200 (vol/vol) dilution for visualization. Bars = 0.5 μm. The arrowheads indicate gold particles.

Discussion

In this report we show conclusively that *C. trachomatis* serovar E Hsp60-2 is the primary cHsp60 that exhibits enhanced expression in response to iron restriction. In a larger context, the specificity of cHsp60-2 expression as a result of iron limitation indicates that the mechanisms for development of chlamydial persistence have unique signatures. This is an emerging concept in the study of chlamydial pathogenesis. The expression of cHsp60s, as determined by either protein expression or transcript analysis, has been examined in several models of persistence (2, 4, 17, 19, 22, 39).

Belland *et al.* (4) conducted a comprehensive microarray study of *C. trachomatis* serovar D transcription and compared standard growth and growth of IFN-γ-mediated persistent chlamydiae in HeLa 229 cells. None of the *groEL* transcripts varied significantly for the first 24 h; however, by 48 hpi, transcription of *groEL-1* increased 2.8-fold due to tryptophan depletion by IFN-γ. Tryptophan is an essential amino acid for *C. trachomatis*. A separate group of investigators examined transcription using quantitative real-time PCR for three distinct modes of persistence, exposure to IFN-γ, penicillin G, and iron depletion, in *C. psittaci* growing in HEp-2 cells (22). At 24 hpi, *groEL-1* was upregulated only in the penicillin G model of persistence; IFN-γ persistence actually showed a significant downregulation of *groEL-1*. Downregulation of *groEL-1* was also observed for 48 hpi for *C. psittaci* persistence induced by iron deprivation; *groEL-2* was not examined in this study. Using a different stress environment, Karunakaran and colleagues (28) examined transcription using a heat shock model. HeLa 229 cells were infected with *C. trachomatis* serovar D for 18 h and subsequently subjected to a 10-min heat pulse at 45°C. mRNA was quantified using a microarray

procedure, and the results showed that there was a >5-fold increase in *groEL-1* transcripts; the quantities of the *groEL-2* and *groEL-3* transcripts did not change. In studies of protein expression, expression of *C. trachomatis* serovar A cHsp60-1, as determined using Western blotting and an anti-cHsp60-1 monoclonal antibody, was enhanced in an *in vitro* model of IFN-γ-mediated persistence (2). For *C. pneumoniae* cHsp60-1, there was a twofold increase in expression at 48 hpi with the following three different models of persistence and/or stress: (i) IFN-γ exposure, (ii) iron deprivation, and (iii) heat shock (39).

Our findings are more consistent with results reported by Gerard and colleagues (20). These investigators quantified mRNA for each *groEL* homolog in *C. trachomatis* serovar K using real-time reverse transcription-PCR with the following systems: (i) active infection in HEp-2 cells, (ii) persistent infection in human monocytes, and (iii) synovial tissue from patients with *Chlamydia*-associated arthritis. In active HEp-2 cell infection, all *groEL* transcripts were present beginning at 8 hpi, and the levels increased throughout chlamydial development; *groEL-3* was transcribed at the highest levels. In the monocyte persistence model, the levels of *groEL-1* and *groEL-3* transcripts were low, whereas the level of the *groEL-2* transcripts increased threefold over 3 days as the organisms entered the persistent state. Findings for the synovial tissues also showed that the levels of *groEL-2* transcripts were high. Comparisons with this model of *C. trachomatis* serovar K persistence in monocytes may not be entirely legitimate because our model involves *C. trachomatis* serovar E, a less invasive organism, in epithelial cells, but the observations are intriguing nonetheless.

From the standpoint of immunopathogenesis, the importance of cHsp60s in disease has been the subject of several excellent reviews (9, 13, 14, 16, 26, 30, 34, 40). Our previous work, performed with the antisera generated in this study, showed that cHsp60-2 and cHsp60-3, but not cHsp60-1, escape from chlamydial inclusions via vesicle eversion, a process that is exacerbated by exposure to azithromycin (21, 44). The vesicles are thought to interact with host cell antigen presentation and to contribute to the inflammatory response. Studies of heat shock proteins, in general, are being performed because heat shock proteins carry antigens and deliver peptides to the major histocompatibility complex, thus priming the adaptive immune response by inducing specific B and T cells in the absence of adjuvants (41). Heat shock proteins also participate in the innate immune response by stimulating the production of chemokines (41). In one study, cHsp60 serum antibodies were shown to serve to be the best predicting factor for tubal factor infertility (51). Mapping of cHsp60-1 peptide epitopes in human sera has been done (50, 54); it may be worthwhile to investigate whether peptides of cHsp60-2 and/or cHsp-3 contribute to the generation of specific immunoglobulins.

GroEL proteins are essential for bacterial growth and ensure that newly synthesized proteins are functional; expression of GroEL proteins increase in response to a variety of stresses, including heat shock and nutrient deprivation (56). Structural studies of cHsp60s have shown that although the primary amino acid sequences of cHsp60s differ from the primary amino acid sequences of other organisms, amino acid residues involved in binding polypeptides are conserved (28). It is also clear that cHsp60-1 is negatively regulated by the interaction of a negative regulator, HcrA, with a CIRCE

element in the operator regions of the *groES-groEL-1* and *dnaK* operons; HrcA does not appear to regulate *groEL-2* or *groEL-3* (53). Although the results of studies of *C. trachomatis* serovar K persistence and synovial fluid support the hypothesis that there is regulation of chlamydial *groEL-2* at the level of transcription, there appears to be no Fur/DcrA binding site in upstream sequences. There is only a partial Fur box approximately 300 bp downstream in *C. trachomatis* serovar E *groEL-2*; determining whether DcrA binds to *groEL-2* sequences is part of a separate project in our laboratory. A likely alternative mechanism for enhanced expression of cHsp60-2 may involve small RNAs that regulate genes posttranscriptionally or by stabilization of mRNA; numerous iron-responsive proteins in other bacteria are known to be regulated in this fashion (25). Chlamydiae code for several small RNAs (5). Finally, the increased level of cHsp60-2 might also involve protein stability. For example, when *E. coli* GroEL is bound to an unfolded substrate *in vitro*, the complex remains stable at 25°C for more than 2 weeks; at 43°C, the half-life is 1.5 h (36).

Overall, the results of this study and our previous work (21, 43) strongly indicate that cHsp60-2 should be considered a potential mediator of immune-mediated damage, and they mechanistically indicate that not all modes of chlamydial persistence are identical.

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CHAPTER 4

THE TRAFFICKING OF CHLAMYDIAL ANTIGENS TO THE ENDOPLASMIC RETICULUM OF *CHLAMYDIA TRACHOMATIS*-INFECTED ENDOMETRIAL EPITHELIAL CELLS

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Abstract

Confinement of the obligate intracellular bacterium *Chlamydia trachomatis* to a membrane-bound vacuole, termed an inclusion, within infected epithelial cells neither prevents secretion of chlamydial antigens into the host cytosol nor protects chlamydiae from innate immune detection. However, the details leading to chlamydial antigen presentation are not clear. By immunoelectron microscopy of infected endometrial epithelial cells, chlamydial MOMP, LPS, and the inclusion membrane protein A (IncA) were localized to the endoplasmic reticulum (ER) and co-localized with multiple ER markers, but not with markers of the endosomes, lysosomes, *cis*-Golgi, nor mitochondria, in isolated cell secretory compartments. Chlamydial LPS was also co-localized with CD1d in the ER. Because the chlamydial antigens, contained in everted inclusion membrane vesicles, were likely delivered to the ER by a novel fusion process, these data raise additional implications for antigen processing by infected uterine epithelial cells for classical and non-classical T cell antigen presentation.

Keywords: *Chlamydia trachomatis*; Inclusion membrane protein (Inc); LPS; endoplasmic reticulum; antigen trafficking; antigen presentation

Introduction

Chlamydia trachomatis serovars D-K, obligate intracellular bacteria that primarily infect genital mucosal epithelial cells, are the leading cause of bacterial sexually transmitted disease (STD) in the USA and worldwide (Schachter, 1999). Chlamydial infection and propagation rely upon a developmental cycle during which an extracellular, infectious form termed the elementary body (EB) gains entry into the host cell before differentiating into an intracellular, metabolically-active reticulate body (RB). Inside the host cell, RB replicate by binary fission within a membrane-bound vacuole termed an inclusion. Redifferentiation of RB back into EB prepares the infectious progeny for release, through a mechanism recently shown to involve two pathways: host cell lysis and inclusion extrusion (Hybiske and Stephens, 2007). Incomplete chlamydial egress can result in retention of bacteria within the surviving host cell, creating a unique condition for persistence of *C. trachomatis* (Beatty, 2007).

Vital to chlamydial pathogenesis is the establishment of an unobtrusive intracellular niche that avoids immune detection for the duration of host cell occupancy. To achieve this residency, the chlamydial EB-containing endosome quickly dissociates from the endocytic-lysosomal pathway and intersects the exocytic pathway, during which *Chlamydia* acquires amino acids, nucleotides, and lipids from the host cell (McClarty, 1994). Additionally, host cell sphingomyelin and cholesterol are obtained by chlamydiae following interception of *trans*-Golgi-derived vesicles intended for transport to the plasma membrane (Carabeo *et al.*, 2003; Hackstadt *et al.*, 1996). More recently, the late endocytic pathway has been implicated in the delivery of biosynthetic precursors to the chlamydial inclusion via multivesicular bodies (Beatty, 2006) and Al Younes *et al.*

(2004) have proposed an association between *Chlamydia* and the autophagic pathway. The ever-growing interactions between *Chlamydia* and host cell organelles/vesicles are clues for solving the biology by which this intracellular pathogen succeeds in masking itself, perhaps temporarily, within the host cell.

Epithelial cells in the genital tract are also gaining recognition for their innate immune capabilities (Wira *et al.*, 2005). Human endometrial cells exhibit both MHC Class I (MHC-I) and Class II (MHC-II) expression and antigen presentation (Van Eijkeren *et al.*, 1991), secrete and respond to cytokines, and express Toll-like receptors. While chlamydial infection induces adaptive immune protection that involves both humoral and cellular responses (Roan and Starnbach, 2008), *C. trachomatis* also employs several strategies to evade the immune system, including the downregulation of MHC-I and MHC-II molecules, production of deubiquitin- and deneddylate-specific proteases for possible protection of secreted proteins from antigen degradation/T cell recognition, inhibition of host cell apoptosis and entering a persistent state (Fan *et al.*, 1998; Misaghi *et al.*, 2006; Zhong *et al.*, 2001). Despite residence of the organisms in an intracellular inclusion niche during infection, several chlamydial proteins, including Type III secretion (T3S) effectors, have been identified within the host cell cytosol (Peters *et al.*, 2007). Our laboratory previously reported the formation of chlamydial antigen-containing vesicles everting from the inclusion (Giles *et al.*, 2006). These everted vesicles were identified as arising from the inclusion membrane by the presence of the *Chlamydia*-specific incorporated inclusion membrane protein A (IncA) and they contained several immunodominant chlamydial antigens, including the major outer membrane protein (MOMP) and lipopolysaccharide (LPS). While some of these extra-inclusion antigen

vesicles traffic to the host cell surface, others remain intracellular where they are conjectured to influence vital host functions and antigen trafficking and presentation. Interestingly, elegant studies in the Subtil laboratory have unlocked the possibility of fusion events between the endoplasmic reticulum (ER) and the inclusion membrane via the SNARE-like properties of IncA (Delevoye *et al.*, 2004). Because the ER is central to antigen processing and presentation and has been linked experimentally to chlamydial IncA, this organelle is an intriguing destination for chlamydial antigens.

The present study examines the host cellular localization of chlamydial antigens, particularly MOMP, LPS, and IncA within *C. trachomatis*-infected human endometrial epithelial cells. Using density gradient centrifugation for isolation of epithelial secretory pathway compartments and high resolution immunoelectron microscopy, the study associates these chlamydial antigens with the ER of infected endometrial epithelial cells, a finding that has implications for host cell antigen processing and presentation.

Results

Ultrastructural Localization of Chlamydial Antigens within Tracts of Endoplasmic Reticulum (ER)

Despite the lack of definitive evidence for contact between the chlamydial inclusion membrane and host cell membranes, numerous studies using electron and confocal microscopy have observed a close association between the inclusion and various host cell organelles (ER, Golgi, mitochondria; Hackstadt *et al.*, 1996; Matsumoto, 1981; Wylie *et al.*, 1997). In our laboratory, the intimate association between the ER and the chlamydial inclusion membrane has been noted, at least ultrastructurally, across a breadth of research projects. Until the present study, this recurring phenomenon had not been examined further. In-depth immunoelectron microscopic examination of *C. trachomatis*-infected polarized HEC-1B cells at 48 hours post-infection (hpi) revealed the presence of chlamydial antigens localizing along the exterior of the inclusion and, in some cases, within host cell cytosolic tracts morphologically distinctive of the ER. Second affinity gold-conjugated antibodies detected primary anti-IncA polyclonal antibodies, anti-LPS polyclonal antibodies and anti-MOMP monoclonal antibodies localizing their respective antigens adjacent to the chlamydial inclusion membrane, frequently appearing in tracts of ER (Fig. 4.1A-C). Similarly, the ER luminal markers BiP/GRP78, ERp57, and calnexin all displayed a propensity for labeling at or near the inclusion membrane region and were often observed in areas positive for chlamydial LPS, MOMP, and IncA (Fig. 4.2). Importantly, additional double-labeling experiments revealed no co-localization between chlamydial LPS, MOMP, and IncA with host

markers for endosomes (EEA-1), lysosomes (LAMP1), *cis*-Golgi (GM130), or mitochondria (MTC02) (data not shown).

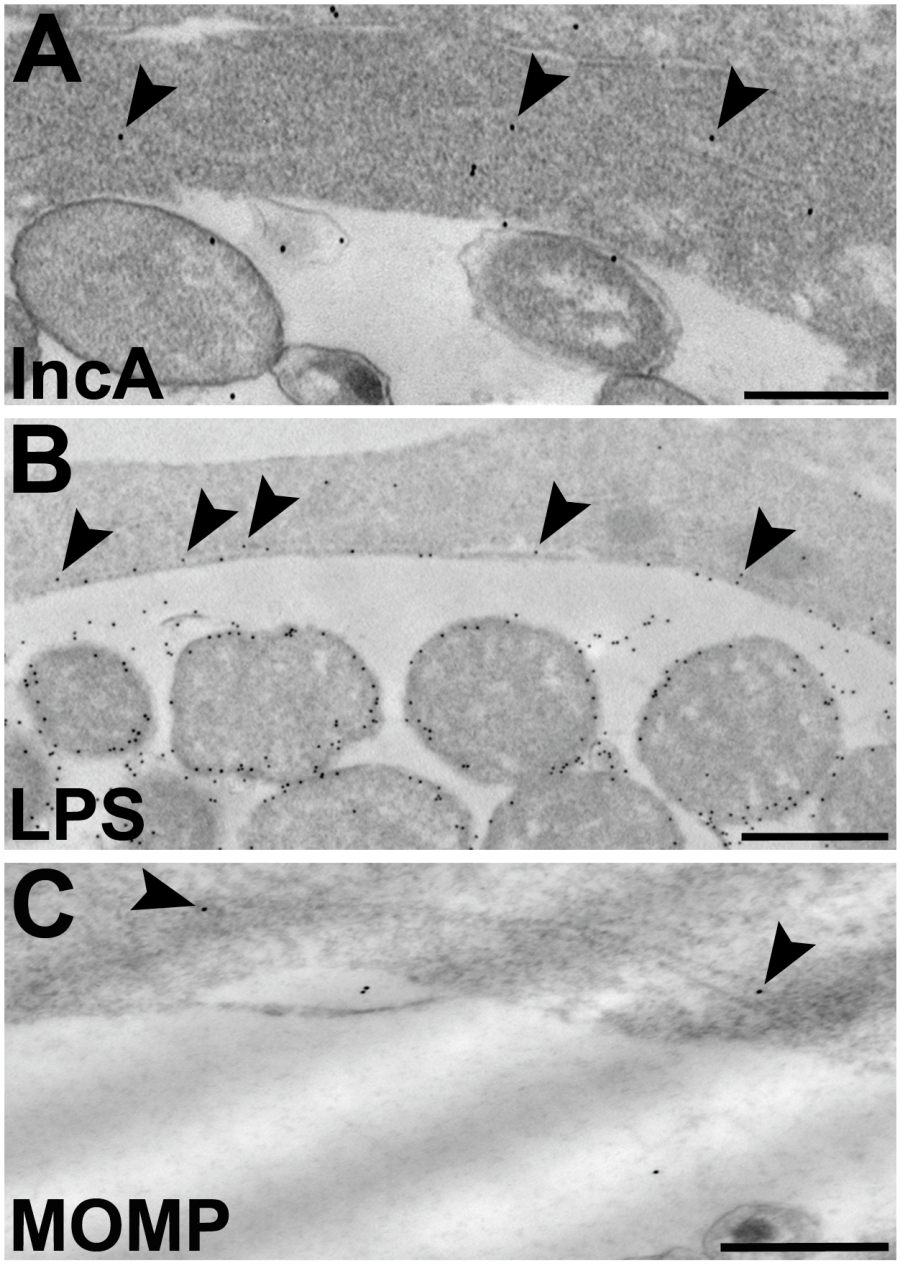

Figure 4.1 Ultrastructural Localization of Chlamydial Antigens to the Host Cell Endoplasmic Reticulum (ER). HEC-1B cells infected with *C. trachomatis* E for 48 hr were prepared for post-embedding immunoelectron microscopy. (A-C) Chlamydial IncA (A), LPS (B), and MOMP (C) were detected in host cell cytosolic tracts characteristic of ER (arrowheads). Scale bars, 500 nm.

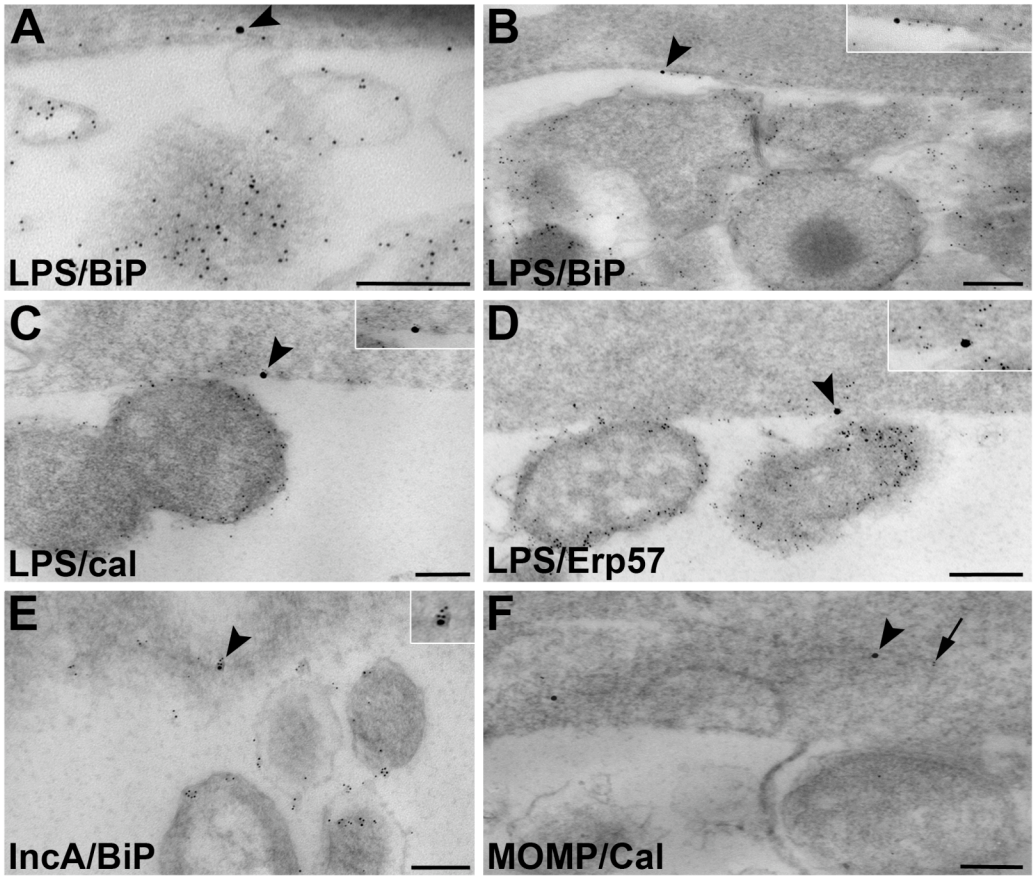

Figure 4.2 Ultrastructural Co-Localization Between Chlamydial Antigens and Host Cell ER Markers. HEC-1B cells infected with *C. trachomatis* E for 48 h were prepared for post-embedding immunoelectron microscopy. (A-D) Co-localization between chlamydial LPS (5nm gold; arrow) and the ER markers BiP/GRP78 (A and B), calnexin (C), and Erp57 (D; 15nm gold; arrowheads; magnified in insets). Scale bars, 200 nm. (E) Co-localization between chlamydial IncA (5nm gold) and the ER marker BiP/GRP78 (15nm gold; arrowhead, magnified in inset). Scale bar, 200 nm. (F) Co-localization between chlamydial MOMP (5nm gold; arrow) and the ER marker calnexin (15nm gold; arrowhead). Scale bar, 200 nm.

A Potential Mechanism of Delivery for Chlamydial Antigens to the Host Cell ER

Previous studies in our laboratory noted numerous vesicles exterior to the chlamydial inclusion within the host cell cytosol in infected HEC-1B cells; these vesicles contained chlamydial outer membrane vesicles (OMV) that labeled positive, by immunoelectron microscopy, for several chlamydial antigens including MOMP and LPS (Giles *et al.*,

2006). It was discovered that these vesicles originated by eversion or pinching off from the inclusion membrane as they contained IncA. Because these vesicles were also prevalent in the present study (Fig. 4.3), it was hypothesized these chlamydial antigen OMV-containing (Fig. 4.3B and C) everted inclusion membrane vesicles (Fig. 4.3A, arrowheads) might traffic to and fuse with the ER, thereby delivering the cytosolic protease-protected antigens directly into the ER. To test this hypothesis, cytosolic vesicles and secretory components were isolated from infected cells and examined for co-localization with chlamydial IncA, MOMP, and LPS.

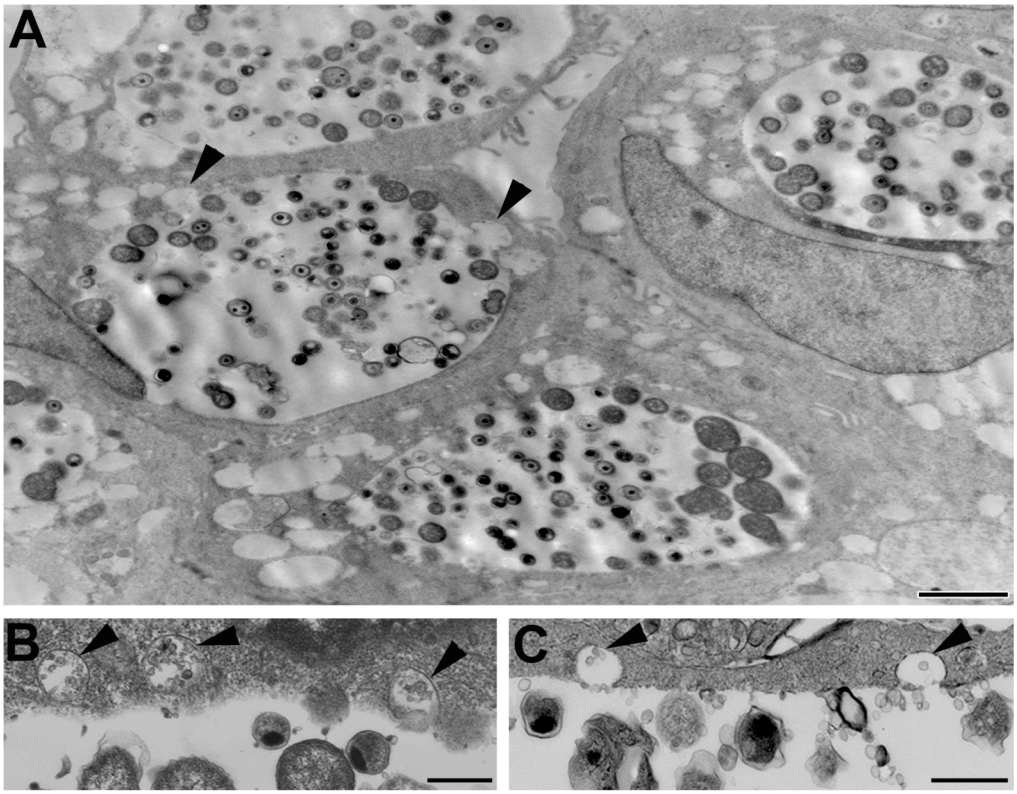

Figure 4.3 Ultrastructural Identification of Extra-Inclusion Vesicles in *C. trachomatis*-Infected Human Endometrial Epithelial Cells. (A) The appearance of extra-inclusion vesicles within the infected HEC-1B cell cytosol at 48 h. Some vesicles were observed everting from the inclusion (arrowheads). Scale bar, 2 μm. (B and C) The contents of the extra-inclusion vesicles (arrowheads) resemble outer membrane blebs found within the inclusion. Scale bars, 500 nm.

Co-localization of Chlamydial Antigen and ER Markers Following ER Isolation

Organelles of the secretory pathway were isolated from uninfected control and *C. trachomatis*-infected HEC-1B cells using Iodixanol (Optiprep) gradients and the resultant fractions were subjected to Western blot analysis by probing with antibodies to the ER and chlamydiae (Fig. 4.4). Several ER membrane (TAP-I and Sec61) and lumenal (calnexin, ERp57, PDI, and CD1d) markers positively labeled fractions 7-10, defining the ER-containing fractions. These results match those of Plonne *et al.* (1999), who found, through enzymatic assays, the ER to occupy the same area in the gradient. The bulk of chlamydial LPS, MOMP, and IncA was found in the ER-containing fractions. The positive control markers for intact EB and RB, chlamydial histone protein HC1 and heat shock protein 60-1, were detected only in the infected cell lysate (Fig. 4.4, column L), indicating that the presence of LPS, MOMP, and IncA in fractions 7-10 could not be attributed to contaminating whole chlamydial forms. Indeed, probing of the mitochondrial (12,000g) pellet yielded reactivity to all chlamydial antibodies (data not shown), suggesting that EB and RB were pelleted with the mitochondria, which is routine.

Fractions 1 and 2, which were slightly positive for chlamydial LPS, MOMP, and IncA (Fig. 4.4), were determined to be membranes incompletely removed in previous steps of the protocol, as evidenced by the presence of multiple membrane proteins (Integrin α2, TGN38, and LAMP1) from various organelles (data not shown). Chlamydial membranes from RB and/or the inclusion membrane would account for the presence of LPS, MOMP, IncA, and CT223, another inclusion membrane protein, detected in fractions 1 and 2 but absent in other fractions (data not shown). In our gradients, the

cis-Golgi was consistently found in fractions 1-4, whereas the *trans*-Golgi extended into fractions 5-7 (data not shown). Hence, there was an expected slight overlap between *trans*-Golgi and the ER/chlamydial antigen-containing fractions 7-10.

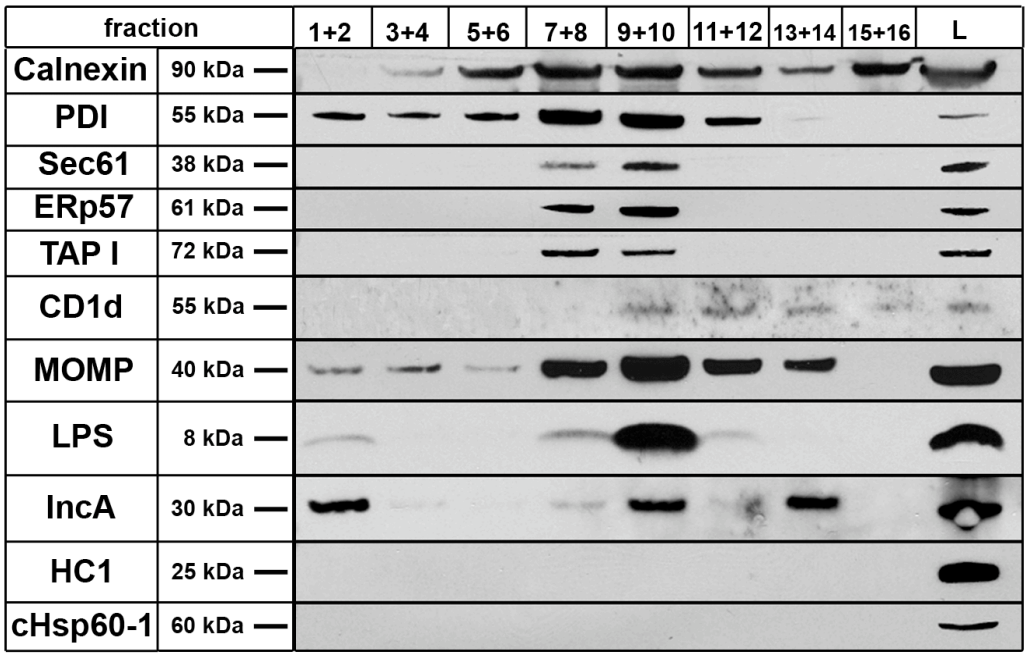

Figure 4.4 Western Blot Analysis of Gradient Fractions With Antibodies Specific for ER Components and Chlamydial Antigens From *C. trachomatis*-Infected HEC-1B Cells. Serial fractions were pooled and ten micrograms of protein was separated on 4-12% gradient gels using SDS-PAGE prior to immunoblotting. The positive control ER markers calnexin, PDI, Sec61, Erp47, and TAP I displayed strong detection in fractions 7-10, defining the ER-containing fractions. CD1d, an MHC-like glycoprotein resident of the ER, was found primarily in fractions 9-12. Chlamydial MOMP, LPS, and IncA were strongly detected in the ER-containing fractions. The positive control chlamydial markers HC1 and cHsp60-1, known to be restricted to intact chlamydial EB and RB, were only detected in the infected cell post-nuclear lysate (L).

Supporting the hypothesis that these chlamydial antigens are creating immunological consequences is the fact that fractions 7-10 represent smooth ER, where antigens are transported into and loaded onto MHC-I molecules. It is thought that fractions 13 and 14, which were positive for MOMP, IncA, and CT223, represent a subfraction of rough ER, as shown by Plonne et al. (1999). The presence of IncA and CT223 in these

fractions may further support the idea, put forth by Luo *et al.* (2007) that inclusion membrane localization is better predicted by ER co-localization than by bi-lobed hydrophobic motifs, as was the case for *C. pneumoniae*. These fraction isolation data strongly support the presence of important chlamydial antigens in the ER of infected endometrial cells.

Examination of the Isolated ER/Chlamydial Antigen-Containing Fractions by TEM

For additional confirmation that the isolated gradient fractions 7-10 were, indeed, ER vesicles containing chlamydial antigen, pooled gradient fractions were viewed by negative stain and processed for post-embedding immunoelectron microscopy. The crude microsomal fraction, representing the sum of all gradient fractions isolated from *C. trachomatis*-infected HEC-1B cells, contained numerous vesicles ranging from 100-500 nm in size (Fig. 4.5A), representing a typical vesicle preparation previously described (Plonne *et al.*, 1999). Immunostaining of the crude microsomal fraction revealed vesicles co-labeling for chlamydial LPS and Sec61, an ER membrane marker (Fig. 4.5A, inset). Closer examination of the ER/chlamydial antigen-containing fractions 8-10 by double-label immunoelectron microscopy revealed a subset of vesicles that displayed co-localization between chlamydial LPS, MOMP, and IncA and a variety of ER markers (Fig. 4.5B-G). An LPS polyclonal antibody was detected within vesicles co-labeling for the ER luminal markers BiP/GRP78, Erp57, and calnexin (Fig. 4.5B-D). A MOMP monoclonal antibody also localized to vesicles containing calnexin (Fig. 4.5E). Chlamydial IncA was associated with vesicles positive for calnexin and Erp57 (Fig. 4.5F and G). All vesicles exhibiting co-localization were approximately 100-200nm in size, a

morphological trait characteristic of isolated microsomes containing vesicles derived from fragmented ER (Czichi and Lennarz, 1977).

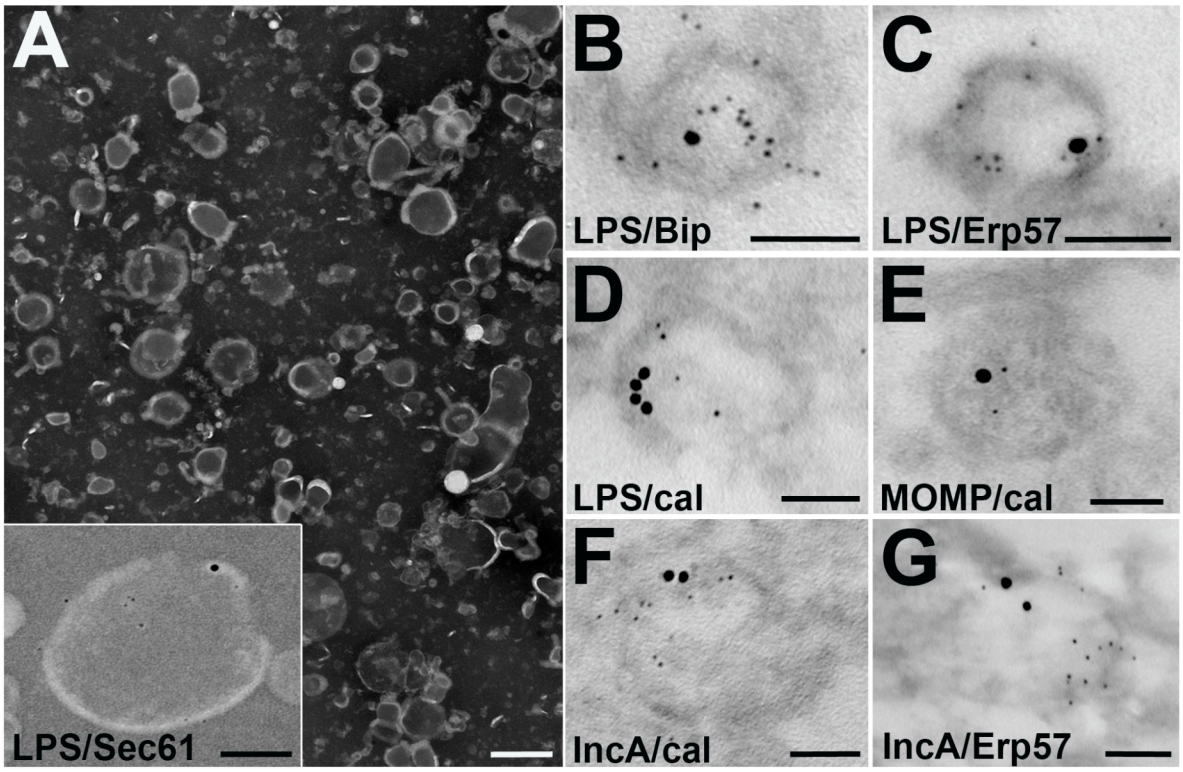

Figure 4.5 Examination of ER/Chlamydial Antigen-Containing Isolated Density Gradient Fractions by TEM. (A) Representative negative stain of the crude ER microsomal fraction from *C. trachomatis*-infected HEC-1B cells. Scale bar, 500 nm. The inset represents double-label immunogold electron microscopy co-localizing chlamydial LPS (5nm gold) and the ER membrane marker Sec61 (15nm gold) to an ER microsomal vesicle. Scale bar, 100 nm. (B-D) Double-label immunogold electron microscopy revealed co-localization between chlamydial LPS (5nm gold) and three ER markers (15nm gold): BiP/GRP78 (B), Erp57 (C), and calnexin (cal; D). Scale bars, 100 nm. (E) Co-localization between chlamydial MOMP (5nm gold) and the ER marker calnexin (15nm gold). Scale bar, 100 nm. (F and G) Co-localization between chlamydial IncA (5nm gold) and the ER markers (15nm gold) calnexin (cal; F) and Erp57 (G). Scale bar, 100 nm. Chlamydial antibodies were detected with 5nm-gold-conjugated secondary antibodies. ER antibodies were detected with 15nm-gold-conjugated secondary antibodies.

Because IncA serves as a positive control for the everted inclusion membrane vesicles containing chlamydial LPS and MOMP antigens, our results strongly support

the original findings of Delevoye *et al.* (2004) for IncA-SNARE-mediated fusion with the ER, thereby providing a conduit for deposition of chlamydial antigens.

Ultrastructural Association Between Chlamydial LPS and CD1d, a Lipid Antigen Presenting MHC-Like Glycoprotein

The localization of chlamydial LPS in the ER of infected cells was a surprising finding. Exogenous LPS binds to LPS-binding protein (LBP) prior to engaging CD14 on the host cell surface and activating the TLR4 pathway. However, the fate of intracellular LPS is limited to isolated studies with incomplete conclusions. CD1d molecules, which present lipids derived from intracellular sources, specifically signal natural killer T (NKT) cells (Van Kaer, 2007). CD1d is proposed to bind endogenous lipid, such as phosphatidylinositol, within the ER and traffic through the secretory pathway to the plasma membrane, where CD1d-restricted T cell recognition can occur. The unusually long fatty acyl chains (C22-C24 vs C12-C14; Heine *et al.*, 2003) of chlamydial LPS, coupled with the deep antigen-binding groove properties of CD1d, led to an investigation on potential interaction between chlamydial LPS and CD1d. It was first determined that HEC-1B cells do possess CD1d following Western blot of uninfected HEC-1B lysate (data not shown). Immunoelectron microscopy of *C. trachomatis*-infected HEC-1B cells revealed co-localization between chlamydial LPS and CD1d at the inclusion membrane (Fig. 4.6A-C) and in tracts and vesicular structures within the host cell cytosol (Fig. 4.6D and E). Following ER isolation, examination of ER-containing fractions by post-embedding immunoelectron microscopy indicated co-localization of chlamydial LPS and CD1d within the same vesicles (Fig. 4.6F and G).

These vesicles conformed to microsomal size (100-200nm) and matched the morphology of CD1d-positive vesicles from ER-containing fractions of uninfected HEC-1B cells (data not shown). These observations strengthen the co-localization of chlamydial LPS and CD1d to the ER, confirming the findings by Kawana *et al.* (2007, Fig. 5) and suggest an association between chlamydial LPS and CD1d during infection.

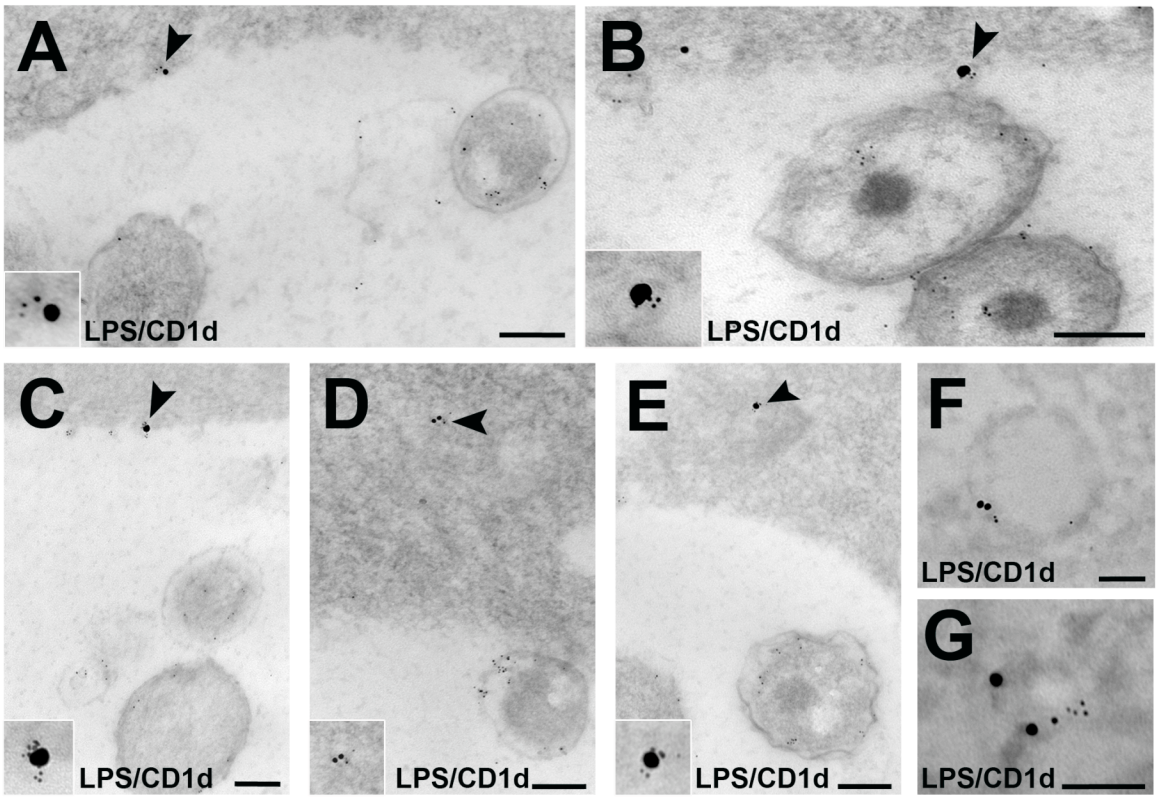

Figure 4.6 The MHC-Like Glycolipid-Binding Protein CD1d Co-Localizes with Chlamydial LPS in the ER during *C. trachomatis*-Infection of Polarized Human Endometrial Epithelial Cells. (A-C) Co-localization between CD1d (15nm gold) and chlamydial LPS (5nm gold) at the inclusion membrane (arrowheads; magnified in insets). Scale bars, 200 nm. (D and E) Co-localization between CD1d (15nm gold) and chlamydial LPS (5nm gold) within vesicles present in the infected host cell cytosol (arrowheads; magnified in insets). Scale bars, 200 nm. (F and G) Co-localization between CD1d (15nm gold) and chlamydial LPS (5nmgold) within vesicles isolated from ER-containing fractions (see Fig. 5). Scale bars, 100 nm. CD1d antibodies were detected with 15nm-gold-conjugated second-affinity antibodies. LPS antibodies were detected with 5nm-gold-conjugated secondary antibodies.

Discussion

Many studies have implicated the exocytic pathway with the host cell niche harboring *Chlamydia* (Hackstadt et al., 1997). Although most research has centered around the Golgi, there have been some intriguing observations hinting towards interaction between *Chlamydia* and the ER, an association now recognized as important for other intracellular microorganisms (Roy *et al.*, 2006). Wylie *et al.* (1997) have shown that the inclusion membrane acquires ER composition in the form of host cell phospholipids. Majeed *et al.* (1999) observed the ER proteins SERCA2 and calreticulin to be closely associated with chlamydial inclusions using confocal microscopy. Another group of investigators not only observed similar labeling of calreticulin around inclusions but also reported decreases in calreticulin labeling when infected cells were exposed to excess amino acids or the autophagic inhibitor 3-MA (Al-Younes *et al.*, 2004). Because these events can occur early-mid developmental cycle, it is tempting to speculate that the juxtaposition of the ER with the inclusion membrane is due to active recruitment by *Chlamydia* rather than the result of chlamydial inclusion expansion within the host cell resulting in organelle crowding around the inclusion. Most intriguing are the recent reports of chlamydial inclusion membrane proteins (IncA, Cpn0146 and Cpn0147) co-localizing with the ER when expressed via transgenes in HeLa cells (Delevoye *et al.*, 2004; Luo *et al.*, 2007). Our data strengthen and extend the observations of involvement of the ER during chlamydial infection by revealing that: (i) several ER markers consistently labeled proximal to the inclusion membrane; (ii) chlamydial LPS, MOMP and IncA were localized by immunoelectron microscopy to the ER of infected host cells, a finding reinforced by co-localization of chlamydial and ER markers; (iii)

Western analysis of fractions from density gradient separation of the infected host cell secretory compartment vesicles illustrated the presence of chlamydial LPS, MOMP, and IncA within ER-containing fractions; (iv) chlamydial antigens and ER markers were co-localized to identifiable ER microsomes fractions; and (v) chlamydial LPS was discovered to co-localize with CD1d, a lipid antigen-presenting molecule, both within the infected host cell and on ER microsomes from density gradient fractions. Thus, it is apparent that chlamydial antigens can access the ER of infected host cells.

From our current understanding of chlamydial pathogenesis, there are three ways by which chlamydial proteins could traverse the inclusion and reach the ER of infected epithelial cells. Zhong and colleagues (2000, 2001) were the first to show that a chlamydial protease-like activity factor (CPAF) appeared in the cytosol of infected cells and was responsible for degrading the transcription factors RFX5 and USF1, which resulted in downregulation of MHC-I and MHC-II surface expression. Subsequently, an apoptosis-modulating chlamydial protein, CADD [Chlamydial protein Associating with Death Domains], was also found in the infected cell cytosol (Stenner-Liewen *et al.*, 2002). How these proteins are transported across the inclusion membrane is unknown. Secondly, like *Salmonella* (Ochman *et al.*, 1996), intracellular chlamydiae, via T3S machinery, can inject effector proteins into the host cytosol (Fields *et al.*, 2005; Ho and Starnbach, 2005; Lugert *et al.*, 2004). Inhibition of T3S as well as heterologous expression of IncA in the ER of infected cells can, in some situations, interfere with progression of the chlamydial developmental cycle (Delevoye *et al.*, 2004; Muschiol *et al.*, 2006), underscoring the significance of protein secretion during infection. In these cases, any chlamydial protein reaching the host cytosol would be presumed subject to

classical MHC-I processing and presentation, involving chlamydial protein ubiquitination, proteasomal processing, and return of the peptides to the lumen of the ER via ATP-dependent TAP transport for MHC-I loading. Thirdly, as referred to in this study, the eversion of inclusion membrane-derived vesicles, containing chlamydial outer membrane blebbed vesicles positive for LPS, MOMP, and IncA (Giles *et al.*, 2006). Because IncA served as the inclusion membrane marker for the pinched-off vesicles, we hypothesized that these vesicles could, using the SNARE-like properties of IncA (Delevoye *et al.*, 2004), fuse with the ER and deliver chlamydial antigens into this compartment. This delivery of presumably native chlamydial protein into the ER would require intra-ER peptide trimming (Paz *et al.*, 1999) and/or Sec61/chaperone retrotranslocation into the cytosol for proteasomal degradation (Tirosh *et al.*, 2003; Wiertz *et al.*, 1996) for antigen presentation to occur.

An alternative consequence of LPS, MOMP, and IncA reaching the ER could be another cellular response involving ER stress. Known as the unfolded protein response (UPR), this phenomenon can be induced by physiological conditions known to cause protein misfolding, such as metabolite deprivation, expression of mutant proteins and viral infection (Lin et al., 2007). LPS has further been identified as an initiator of UPR (Kozlov et al., 2007). Affected host cells respond by activating ER stress pathways involving translational repression and chaperone induction (Schroder and Kaufman, 2005). A key indicator for ER stress is enhanced expression of the glucose regulated protein BiP/GRP78. Interestingly, when we compared uninfected and infected BiP/GRP78 protein expression by Western blot, there existed a noticeable increase in infected cells (data not shown). In the current study, the localization of LPS, MOMP,

and IncA to the ER, coupled with the increase of BiP/GRP78 during infection, suggests that the chlamydiae-infected cell may be subjected to ER stress.

Natural *C. trachomatis* infection occurs in genital epithelial cells, which are capable of initiating and sustaining innate immune responses (Quayle, 2002). Epithelial cells typically express high levels of MHC-I compared to MHC-II, suggesting an important role for $CD8^+$ T cells in the recognition of *C. trachomatis*-infected genital mucosa. Indeed, antigen-specific $CD8^+$ T cells are recruited to the site of chlamydial infection, can help limit infection and confer protection against *Chlamydia* challenge in mice (Igietseme *et al.*, 1994; Roan and Starnbach, 2006). Several intracellular bacteria elicit the MHC-I processing pathway, including *Salmonella*, *Mycobacteria*, *Listeria* and *Toxoplasma* (Chiplunkar *et al.*, 1986; Flynn *et al.*, 1990; Gubbels *et al.*, 2005; Zwickey and Potter, 1999). The presence of chlamydial protein either in the host cell cytosol or integrated into the inclusion membrane does not go unnoticed, as indicated by the identification of specific antigen epitopes that are T cell targets of cell-mediated immune response (Fling *et al.*, 2001; Gervassi *et al.*, 2004; Kuon *et al.*, 2001; Starnbach *et al.*, 2003). MOMP cytotoxic T lymphocyte epitope clusters have been identified and MOMP-specific $CD8^+$ T cells can be detected during *Chlamydia* infection (Kim and DeMars, 2001). IncA recently gained recognition as an important antigen as reflected by human antibody production and detection of IncA antigen in patient urine (Tsai *et al.*, 2007). Our observations of chlamydial antigens within the ER of infected cells at mid-development suggest involvement of the MHC-I pathway, the accessibility of which may be direct considering the strong localization of multiple ER markers in close proximity to the inclusion membrane.

LPS has been frequently observed on the plasma membrane of infected host cells and, in some cases, on the surface of neighboring uninfected cells (Campbell *et al.*, 1994; Karimi *et al.*, 1989; Wyrick *et al.*, 1999). It has been proposed that, as a consequence of LPS incorporation into the plasma membrane and the resultant decreased fluidity, the infected cell is refractory to cytolysis from a complement-mediated mechanism or from natural killer or T cell granzymes and perforins (Wilde *et al.*, 1986). Despite its low endotoxic potency, chlamydial LPS can stimulate proinflammatory cytokine secretion by macrophages (Ingalls *et al.*, 1995) and can signal via TLR2 and TLR4, molecules recently shown to be transcriptionally upregulated and recruited to the inclusion during *Chlamydia* infection (Erridge *et al.*, 2004; Heine *et al.*, 2003; Mackern-Oberti *et al.*, 2006; O'Connell *et al.*, 2006).

While numerous studies have examined the immunological effects of exogenous LPS, only a few studies have addressed the handling of LPS released intracellularly. *Salmonella* has been shown to release LPS from its vacuolar compartment into vesicles present in the host cell cytosol (Garcia-del Portillo *et al.*, 1997), and intracellular recognition of LPS has been reported in epithelial cells (Girardin *et al.*, 2001; Hornef *et al.*, 2003). CD1d presents endogenous lipid antigen to natural killer T (NKT) cells. Bilenki *et al.* (2005) reported evidence for the involvement of NKT cells during chlamydial infection in mice. Their data suggested that CD1d-restricted NKT cells can affect the immune response to *Chlamydia* infection and play a role in pathological outcome. CD1d was detected in our HEC-1B cells, confirming other studies demonstrating that genital epithelial cells express CD1d (Sallinen *et al.*, 2000). Furthermore, we observed by Western blot the same pattern of CD1d degradation

during *C. trachomatis* infection reported by Kawana *et al.* (2007) (data not shown). Because CD1d possesses deep independent hydrophobic pockets for anchoring long fatty acyl chains, such as the C22-C24 of chlamydial lipid A, and is able to recognize antigen intracellularly, we hypothesized that chlamydial LPS, with its unique chemical structure and proclivity to escape from the inclusion, may provide an accessible target for lipid antigen recognition by host cells. The immunoelectron microscopic localization of CD1d and LPS in the ER of host cells indicates accessibility but not recognition. If, indeed, LPS can be recognized and/or modified by CD1d, then these data suggest that host cells may use endogenous CD1d-antigen processing.

Interestingly, we also observed a few cases where CD1d and LPS were co-localized at the plasma membrane (data not shown). The extracellular release of LPS, coupled with its propensity to integrate into membranes, introduces an intriguing scenario of cross-presentation, wherein chlamydiae-infected cells regurgitate LPS extracellularly where it could be incorporated into the plasma membrane of neighboring uninfected cells and subsequently endocytosed, providing the opportunity for endosomally-acquired antigen presentation by CD1d. So although CD1d molecules are decreased on the host cell surface of *C. trachomatis*-infected cells (Kawana *et al.*, 2007), the release of LPS during infection may result in CD1d-restricted T cell recognition of uninfected epithelial cells. Depending on the level of LPS escape, such an NKT cell response could contribute to the chronic nature of inflammation in chlamydial infection, not to mention create an environment of immune mediators that induce chlamydial persistence.

In summary, our data support a greater role for the ER during chlamydial infection in polarized human endometrial epithelial cells. Considering the escapability and immunogenic properties possessed by chlamydial LPS, MOMP, and IncA, the trafficking of chlamydial lipids and proteins to the ER is likely to create ER stress, or alterations in some ER functions, and/or provide antigens for MHC-I and CD1d presentation. Whether or not the results serve a protective role or an immuno-destructive role for chlamydiae is yet to be determined.

Experimental Procedures

Cell Culture Systems and Growth of *Chlamydia*

The human endometrial carcinoma subclone 1B cell line (HEC-1B; HTB-113; ATCC, Manassas, VA) was maintained at 37°C in Minimal Essential Medium (MEM) containing Hank's salts (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and 2mM glutamine. The HEC-1B cells were grown in a polarized manner in two culture systems: (i) on extracellular-matrix (ECM) coated filters (BioCoat Matrigel Invasive Chambers, 0.3 cm^2, BD Biosciences, San Jose, CA) cultivated in Dulbecco's modified Eagle MEM (Gibco) supplemented with 10% fetal calf serum and 10 mM HEPES, pH 7.3 (DMEM) and maintained at 35°C in an atmosphere of 5% carbon dioxide, and (ii) on collagen-coated DEAE-Sephadex beads (Cytodex 3 microcarrier beads; Sigma, St. Louis, MO) in 500mL spinner flasks as described previously (Guseva et al., 2007).

A human urogenital isolate *C. trachomatis* E/UW-5/CX was used in these experiments. Standardized inocula of *C. trachomatis* infectious EB were prepared from

HEC-1B cells grown on Cytodex microcarrier beads. Progeny EB were harvested, titrated for infectivity, and stored at -80°C.

Chlamydial Infection of Polarized Cells

Polarized HEC-1B cell monolayers on ECM-coated filters were inoculated with *C. trachomatis* EB by passive adsorption with a titer of crude stock diluted to a concentration demonstrated to yield at least 80% infected cells. The same crude stock of infectious EB was used to inoculate polarized HEC-1B cells grown on beads in suspension. In both culture systems, the duration of the *C. trachomatis* developmental cycle was 52h. For experiments in the present study, host cells were infected for 48h in the absence of antibiotics.

Endoplasmic Reticulum Isolation

Major intracellular organelles of the secretory pathway, including Golgi, smooth ER, and rough ER were separated using an Endoplasmic Reticulum Isolation Kit (Sigma), based upon a protocol originally published by Plonne *et al.* (1999). Briefly, uninfected and *C. trachomatis*-infected HEC-1B cells grown in bead culture were dounce homogenized and subjected to a series of centrifugation steps to pellet the beads, cell nuclei, mitochondria, and microsomes. Careful analysis of each discarded fraction ensured removal of nuclei, mitochondria, endosomes, lysosomes, and the bulk of membranes. In infected cells, removal of intact EB and RB was confirmed by (i) Western blot antibody probing for histone protein 1 (HC1) and heat shock protein 60-1 (cHsp60-1) and (ii) the absence of chlamydial forms in the fractions processed for transmission

electron microscopy (TEM). The microsomal fraction was separated into Golgi, smooth ER and rough ER using the gradient medium Iodixanol (Optiprep). Sixteen 500μl fractions were collected from the top of the gradient downward with a syringe.

Western Immunoblotting

Fractions from the Iodixanol gradients were solubilized in Laemmli buffer with protease inhibitors (Sigma) and equal amounts of protein were determined by the RC/DC assay (Bio-Rad, Hercules, CA). Fractions were paired and proteins were separated by SDS-PAGE using 4%-12% Bis-Tris gradient gels and transferred to polyvinylidene difluoride (PVDF) membranes for Western blot analysis. The membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS) before primary antibody incubation. Horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit and rabbit anti-goat second-affinity antibodies (Pierce Biotechnology, Rockford, IL) were used prior to chemiluminescent detection (Pierce). Standard protein size markers (Bio-Rad) were used to confirm molecular weights of target proteins.

Antibodies

Chlamydial markers used in this study included (1) monoclonal antibodies directed against IncA (a kind gift from Dr. Dan Rockey, Oregon State University, Corvallis, OR), MOMP (Santa Cruz Biotechnology, Santa Cruz, CA) and LPS (Virostat, Portland, ME); (2) rabbit polyclonal antibodies directed against IncA (via Dr. Rockey) and LPS (Cortex Biochem, San Leandro, CA) and (3) goat polyclonal antibodies directed against MOMP (Biodesign International, Saco, ME). Eukaryotic host cell markers used in this study

included (1) monoclonal antibodies directed against LAMP1 (BD Biosciences), calnexin (Chemicon International, Temecula, CA), ERp57 (Novus Biologicals, Littleton, CO) and BiP/GRP78 (BD Biosciences) and (2) rabbit polyclonal antibodies directed against calnexin (a kind gift from Dr. Daniel Hebert, Yale University, New Haven, CT), Sec61 alpha (Affinity Bioreagents, Golden, CA), ERp57 (Affinity Bioreagents), TGN38 (Santa Cruz), TAP I (Novus Biologicals), and CD1d (Santa Cruz).

Transmission Electron Microscopy

The *C. trachomatis*-infected HEC-1B cell monolayers grown on filters were processed and embedded in Epon-araldite for high contrast or in Lowicryl resin (Polysciences, Warrington, PA) for immunoelectron microscopy (Giles et al., 2006). The crude microsomal pellet was resuspended in isolation buffer (Sigma) and examined by negative staining. A volume of 5μl was pipetted onto formvar-coated copper grids and allowed to adhere for 2 min before excess sample was removed by touching the grid with filter paper. The grids were washed three times with 10μl distilled water and air-dried. Then, 10μl of 2% phosphotungstic acid was pipetted onto each grid, which was allowed to stand for 30 sec before the excess was removed using filter paper. The Iodixanol gradient fractions were examined in resin enrobed and processed sectioned material. For section preparation, gradient fractions 8-10 and 12-14 were combined, mixed with 5 vol of PBS and pelleted by centrifugation at 150,000g for 90 min at 4°C in a Sorvall T-1270 rotor. The pellets were fixed for 1 h at 4°C in 2% paraformaldehyde/0.05% glutaraldehyde in 0.1M Sorenson's buffer, agar enrobed in 3% SeaKem agarose and samples were infiltrated, processed embedded in Lowicryl, and

polymerized by UV light. Ultrathin sections were prepared using a Reichert Ultracut S microtome (Leica Microsystems, Inc., Bannockburn, IL). For immunoelectron microscopy, 80-nm-sections were blocked with 1% ovalbumin/0.01M glycine in PBS for 5 min and subsequently incubated with the primary antibody for 40 min at 37°C. Sections were washed with PBS and probed with the appropriate 5 or 15nm colloidal gold-conjugated second-affinity antibody (Amersham Biosciences, Piscataway, NJ) for 30 min at 37°C. Sections were washed with PBS and distilled water prior to staining with 4% uranyl acetate prepared in 50% ethanol. For double labeling, the second primary antibody was administered following the last PBS wash and the procedure was repeated. All labeling experiments were conducted in parallel with controls using an irrelevant primary antibody or a gold-labeled second-affinity antibody alone to determine background cross-reactivity. Thin sections on grids were examined in a Phillips Tecnai-10 electron microscope (FEI) operated at 80kV.

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CHAPTER 5

CONCLUSION

The majority of patients with initial chlamydial infections are asymptomatic. This is attributed to the growth of chlamydiae in an unobtrusive intracellular vacuolar inclusion niche in infected genital epithelial cells. Moreover, the ascending sequealae from urethritis or cervicitis are immune-mediated. Thus, a major gap exists in our knowledge between chlamydial antigen trafficking and the triggers that signal the innate and subsequent adaptive immune responses.

Nearly 3 decades have passed since the first observation of a chlamydial antigen, LPS, localized exterior to the inclusion prior to the release of infectious progeny from the host epithelial cell at the end of the developmental cycle (Richmond and Stirling 1981). Only in the last 10 years have studies surfaced with further evidence of the presence of chlamydial proteins within the cytosol of infected cells (Zhong and others 2000; Stenner-Liewen and others 2002; Lugert and others 2004; Fields and others 2005; Ho and Starnbach 2005). Previous data from our laboratory identified chlamydial LPS and MOMP within vesicles exterior to the inclusion; these antigens were subsequently observed on the host cell surface and released extracellularly (Paul and others 1997; Wyrick and others 1999). Indeed, the premature escape of chlamydial antigens may influence antigen trafficking and presentation by the host cell and contribute to the prolonged inflammatory response typical of chlamydial infections. The primary goal of the studies described herein was to further characterize by high resolution immuno-

electron microscopy the vesicle-mediated route of chlamydial antigen escape and to track these antigens to potential host cellular destination(s).

The first study involved an ultrastructural examination of the origin and content of the chlamydial antigen-containing vesicles present in the host cell cytosol of *C. trachomatis*-infected polarized endometrial epithelial cells (Giles and others 2006). Extensive analysis of electron micrographs, generated from samples processed in two distinct resins (Epon for high contrastand Lowicryl for antigen detection), revealed the existence of extra-inclusion vesicles within azithromycin-exposed and -unexposed chlamydiae-infected cells (Figs 2.1 and 2.2). Closer observation uncovered the similarity between chlamydial outer membrane blebs within the inclusion and the material occupying the extra-inclusion vesicles, a finding justified when these vesicles were visualized in the act of pinching off from the chlamydial inclusion (Fig 2.2). Post-embedding labeling immunoelectron microscopy localized three chlamydial inclusion membrane proteins (IncA, IncF, and IncG) to (i) RB outer membranes, (ii) the inclusion membrane, (iii) regions of the inclusion membrane protruding into the host cell cytosol, (iv) the surface of extra-inclusion vesicles, and (v) on membranous blebs both inside the inclusion and within extra-inclusion vesicles (Fig. 2.3). The extra-inclusion vesicles were devoid of multiple Golgi-specific markers, differentiating them from Golgi vesicles (Fig. 2.4). Finally, a survey of the localization of the three chlamydial Hsp60 homolog proteins revealed confinement of cHsp60-1 to RB within the inclusion, but cHsp60-2 and cHsp60-3 were found within the extra-inclusion vesicles and on the host cell surface, suggesting a selective process governing escape of chlamydial antigens (Fig 2.5). This study added extra-inclusion vesicles to known and suspected methods of chlamydial

antigen escape, including release of chlamydiae at the end of the developmental cycle, the secretion of chlamydial effectors by Type III secretion or by autotransporters, and the appearance of small vesicles exterior to the inclusion following induction of persistence by penicillin exposure. Therefore, emphasis was placed on the potential for these escaped antigens to trigger immune mechanisms, perhaps within the non-immune host epithelial cells.

In a separate study, peptide antisera specific for each of the three chlamydial Hsp60 homologs were used for immunolabeling transmission electron microscopy to examine their iron responsiveness. For generation of a quantitative measure reflecting qualitative TEM images, multiple (>50 per condition per cHsp60 homolog) micrographs were obtained and square fields of constant size were randomly generated prior to gold particle counting. Comparison of these counts, using the Student's two-tailed *t* test, revealed an enhanced expression of cHsp60-2 in response to iron deprivation (Figure 3.3) (LaRue and others 2007). These results strengthened the notion that cHsp60-2 deserves consideration as a potential mediator of immune-mediated pathology, especially when coupled with the previous finding of cHsp60-2 escaping the inclusion via inclusion membrane-derived vesicles.

A deeper investigation of the trafficking of chlamydial antigens within infected host cells led to experiments designed to reveal the specific destination(s) of those antigens. The focus was placed on normally infected cells as opposed to azithromycin-exposed infected cells in order to mimic more accurately natural infection. Immunoelectron microscopic detection of major chlamydial antigens within *C. trachomatis*-infected polarized endometrial epithelial cells revealed localization of chlamydial MOMP, LPS,

and IncA within the host cell endoplasmic reticulum (ER) (Figure 4.1). These findings were corroborated with double-labeling experiments demonstrating co-localization between chlamydial antigens and multiple ER markers (Figure 4.2). Stressing the importance of vesicles escaping the chlamydial inclusion and serving as a delivery mechanism for antigens, an electron micrograph composite (Figure 4.3) conveyed the natural occurrence of these vesicles at mid-development of chlamydiae during infection. These ultrastructural observations prompted attempts to isolate the ER from infected cells using density gradient centrifugation. Upon separation of the organelles of the infected host cell secretory pathway, it was found that chlamydial antigens occupied fractions positive for various ER luminal and membrane markers (Figure 4.4) but not with markers specific for endosomes, lysosomes, and Golgi. When these fractions were negatively stained and/or processed for double label immunoelectron microscopy, the vesicle population resembled ER microsomes and these microsomes co-labeled for ER and chlamydial markers (Figure 4.5).

The unexpected presence of chlamydial LPS in the host cell ER led to the hypothesis that the glycolipid-binding MHC-like protein CD1d, a resident of the ER, could provide a route of LPS recognition and antigen presentation. Postembedding immunoelectron microscopy of *C. trachomatis*-infected polarized epithelial cells and isolated ER microsomes during infection revealed co-localization between chlamydial LPS and CD1d (Figure 4.6). In chlamydiae-infected cells, this association was observed at the inclusion membrane and in small vesicles within the host cell cytsol, and the isolated ER microsomes were shown to contain both chlamydial LPS and CD1d. Taken together, these studies suggest trafficking, perhaps via inclusion

membrane-derived vesicles, of chlamydial antigens to the ER of host epithelial cells. The results also hint towards a potential method of endogenous chlamydial LPS recognition and antigen presentation as evidenced by LPS and CD1d co-localization within infected epithelial cells.

Collectively, the findings from these studies emphasize the escape of chlamydial antigens from the inclusion as a contributor to the local immune response. Whether by a novel mechanism of inclusion vesicle formation or by other means such as Type III secretion or autotransportation, chlamydial antigens were shown to traffic to the host cell ER and plasma membrane and be released extracellularly. Such placement of antigens may elicit host innate and adaptive immune responses that could either limit the infection or exacerbate the inflammatory processes associated with infection. Attempts to further define the "cellular paradigm of chlamydial pathogenesis" (Stephens 2003) demand a better understanding of the infected human host epithelial cell's immune capabilities. Then, by examining intracellular molecular interactions of the human epithelial cell and chlamydiae, the nature of immune response may be determined.

The epithelial cell, as host to *C. trachomatis*, plays a crucial role in determining the outcome of infection. Human genital epithelia display a characteristic polarized structure consisting of basolateral and apical plasma membrane domains. This three-dimensional orientation provides organization for efficient intracellular trafficking as well as creating directionality for extracellular signaling. Only when polarized can epithelial cells form intercellular tight junctions that create a selective barrier defense against mucosal pathogens. In addition to serving as a physical barrier, epithelial cells

participate in other innate immune functions such as recognition of pathogen-associated molecular patterns (PAMPs), production and release of antimicrobial mediators, and secretion of cytokines and chemokines. It is these attributes that make epithelial cells a vital component in chlamydial biology, pathogenesis and infection. Previous *in vitro* studies have already emphasized the differences in chlamydial development when using non-polarized versus polarized cell culture. Among these findings are variations in EB infectivity, EB entry and exit, developmental cycle progression, chlamydial persistence, hormone responsiveness, antibiotic reactivity and population of inflammatory response cells. The distinctions between non-polarized and polarized cell culture strongly suggest that *in vitro* chlamydial infection using polarized cell culture mimics more closely *in vivo* chlamydial infection as well as the subsequent host cellular response to infection. Hence, justifying the use of polarized cells in the present studies.

It is expected that genital epithelial cells will continue to reveal recognition molecules that play a role in pathogen detection and clearance. As evaluated in Chapter 4, molecules such as CD1d could be instrumental in detecting chlamydial lipids both within the infected cell and those released extracellularly. Another recognition molecule worthy of consideration in chlamydial infections is Nod1, a member of intracellular proteins with homology to plant disease-resistant gene products. Nod1 has been shown to confer responsiveness and binding capability to microbial LPS (Inohara and others 2001). Because chlamydial LPS is often detected in the host cell cytosol, especially more frequently than other chlamydial antigens, the presence of LPS recognition molecules in epithelial cells would provide optimal detection potential against chlamydial infection. It is becoming increasingly evident that the epithelial host

cell is instrumental for initiating innate immune responses to intracellular bacteria and, even though chlamydiae secrete proteins to counteract this response in the infected cell, there may also be important consequences for chlamydial antigens that are released from the host cell to be recognized by neighboring uninfected epithelial cells via cross-presentation.

Chlamydiae are well equipped for survival within the host epithelial cell. Possessing an array of effector proteins, chlamydiae can manipulate multiple host cellular processes for the purpose of intracellular survival. Documented host-pathogen interactions instigated by chlamydiae include (i) recruitment of actin and cellular signal transduction regulators during entry, (ii) hijacking of trans-Golgi vesicles, multivesicular bodies, and lipid bodies, (iii) exploitation of the microtubular network, (iv) recruitment of host cell signaling molecules, (v) inhibition of host cell apoptosis, and (vi) degradation of host transcription factors involved in MHC-I and MHC-II induction. Clearly, *Chlamydia* employ a variety of effectors to manipulate its intracellular environment. The secretion of antigens detailed in the present study may serve, ironically, to invoke an immune response for chlamydial survival, perhaps by triggering persistence. Even an immune response capable of eliminating chlamydiae in an active infection may fail in clearance of chlamydiae present in a persistent infection. Chlamydial infection of the urogenital tract may consist of regions of epithelia subject to differing infection states, *i.e.* active infection of the cervix and persistent infection of the endometrium. On the other hand, there may exist a state of alternating active and persistent infection. Either scenario, by causing a sustained or cyclical inflammatory response, could explain the immunopathology associated with chlamydial infection.

The escape of chlamydial antigens may also have effects on the host cell that indirectly influence pathogenesis. For instance, if the accumulation of chlamydial antigens in the ER were to initiate ER stress, there could follow premature protein destruction causing loss of cellular function or the accumulation of proteins in the ER as toxic end products. Both of these defects would impair normal secretory function and likely have an adverse effect on chlamydial development. Conversely, ER dysfunction may, through an unknown mechanism, contribute to chlamydial propagation and disease progression. Further understanding of *Chlamydia*-host cell interaction will help define targets for chemotherapeutic and immunological intervention.

In summary, the findings herein introduce a novel mechanism for premature antigen escape from the chlamydial inclusion via vesicles that may possess fusion capability with the host cell ER. That chlamydial antigens were localized to the ER of infected epithelial cells increases relevance of the ER during infection, particularly hinting towards potential epithelial cell intracellular recognition and processing of antigens for classical and non-classical T cell antigen presentation. Identification of cHsp60-2 as iron-responsive emphasizes *Chlamydia*'s ability to combat stress by using multiple avenues leading to persistence and highlights cHsp60-2 as a potential mediator of the immune-mediated damage observed in chlamydial infection. As new tools emerge for studying chlamydial biology and advances are made to further understand epithelial cell immune capacity, there will follow discoveries that refine our knowledge of *Chlamydia*-host interactions and potentially lead to the development of a vaccine.

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Invited Oral Presentations:	Using Density Gradient Centrifugation to Examine Chlamydial Antigen Trafficking within *Chlamydia trachomatis* Serovar E-infected Polarized Endometrial Epithelial Cells. The 3rd biennial meeting of the *Chlamydia* Basic Research Society. Louisville, Kentucky. 2007.
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