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MINIREVIEW

Chlamydia trachomatis: the Persistent Pathogen

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ABSTRACT *Chlamydia trachomatis* is an obligate intracellular bacterium whose only natural host is humans. Although presenting as asymptomatic in most women, genital tract chlamydial infections are a leading cause of pelvic inflammatory disease, tubal factor infertility, and ectopic pregnancy. *C. trachomatis* has evolved successful mechanisms to avoid destruction by autophagy and the host immune system and persist within host epithelial cells. The intracellular form of this organism, the reticulate body, can enter into a persistent nonreplicative but viable state under unfavorable conditions. The infectious form of the organism, the elementary body, is again generated when the immune attack subsides. In its persistent form, *C. trachomatis* ceases to produce its major structural and membrane components, but synthesis of its 60-kDa heat shock protein (hsp60) is greatly upregulated and released from the cell. The immune response to hsp60, perhaps exacerbated by repeated cycles of productive infection and persistence, may promote damage to fallopian tube epithelial cells, scar formation, and tubal occlusion. The chlamydial and human hsp60 proteins are very similar, and hsp60 is one of the first proteins produced by newly formed embryos. Thus, the development of immunity to epitopes in the chlamydial hsp60 that are also present in the corresponding human hsp60 may increase susceptibility to pregnancy failure in infected women. Delineation of host factors that increase the likelihood that *C. trachomatis* will avoid immune destruction and survive within host epithelial cells and utilization of this knowledge to design individualized preventative and treatment protocols are needed to more effectively combat infections by this persistent pathogen.

KEYWORDS *Chlamydia trachomatis*, heat shock protein, infertility, persistence, tubal occlusion

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Chlamydia trachomatis is a Gram-negative obligate intracellular bacterium. Humans are its exclusive natural host. Different chlamydial serovars are the major etiological agents of preventable blindness (serovars A to C), the most common bacterial sexually transmitted infections worldwide (serovars D to K), and lymphatic system infections (serovars L1 to L3). A distinctive feature of *C. trachomatis*, especially in the female genital tract, is that the majority of infected women remain asymptomatic and, therefore, do not seek treatment. In a subset of women, *C. trachomatis* is able to avoid destruction by the host's innate and adaptive immune systems, and by autophagy, it migrates to the upper genital tract and establishes a chronic infection. It has been suggested that without treatment, up to 50% of infected women continue to be infected for greater than 1 year (1). Prolonged exposure of the fallopian tube epithelium to *C. trachomatis*, or to antigens released by this microorganism, may lead to scarring and disruption of tubal integrity. A chlamydial upper genital tract infection is the leading cause of tubal factor infertility and, should conception occur, increased susceptibility to ectopic pregnancy, as well as premature pregnancy termination. Other

consequences of an upper genital tract infection in women include pelvic inflammatory disease, endometritis, and perihepatitis. In this review, we describe the unique attributes of *C. trachomatis* that contribute to its success as an intracellular pathogen of the female genital tract, delineate the host factors that influence consequences following an initial exposure to this organism, and highlight potential areas for future research.

C. TRACHOMATIS LIFE CYCLE

The distinct developmental cycle of *C. trachomatis* consists of two phases. The infectious extracellular form is called the elementary body (EB). Historically characterized as being metabolically inert, recent studies have identified active biosynthesis and metabolism within the EBs (2). Whether present in semen from an infected male partner or released from infected female genital tract epithelial cells, the EBs initially bind to heparin sulfate proteoglycans on epithelial cells, followed by interaction with a seemingly wide variety of cell surface receptors: mannose receptor, mannose-6-phosphate receptor, epidermal growth factor receptor, fibroblast growth factor receptor, platelet-derived growth factor receptor, ephrin receptor A2, protein disulfide isomerase, and β1 integrin (3). This is followed by chlamydia-induced actin remodeling that facilitates entry of the microorganism into the cytoplasm (4). The EBs become internalized into endocytic vacuoles, which then combine to form an intracytoplasmic inclusion (5). Within this structure, the EBs transform into the noninfectious replicative form of the organism called the reticulate body (RB). The RB utilizes nutrients within the host cytoplasm and replicates by binary fission. When the RB-filled inclusion reaches a critical volume, coinciding with a decreasing pool of nutrients and ATP, the conversion of RBs back to EBs occurs (6). The EBs are released into the extracellular milieu by one of two mechanisms, host cell lysis or extrusion of the cytoplasmic inclusion (7). The released EBs attach to adjacent epithelial cells where they initiate another round of infection (8, 9).

AUTOPHAGY

Autophagy is a physiological process operative within most cells that has two basic interrelated functions. It maintains a sufficient pool of nutrients within a cell to maintain physiological activities, and it sequesters and removes from the cytoplasm compounds and structures that interfere with the maintenance of cell homeostasis: defective mitochondria, inflammasomes, protein aggregates, and intracellular bacteria or viruses. The unwanted components are sequestered within a structure called an autophagosome, which then fuses with a lysosome, and the contents are degraded by lysosomal enzymes. The amino acid, carbohydrate, nucleic acid, and lipid components are returned to the cytoplasm for reutilization by the cell (10). Under terminally adverse conditions, autophagy may also initiate a form of programmed cell death (11).

The relationship between *C. trachomatis* and autophagy is complex. Discordant results have been reported by investigators utilizing different experimental conditions, chlamydial serovars, and cell lines (12–16). The chlamydial inclusion in epithelial cells is not sequestered within autophagosomes, nor does it fuse with lysosomes, and so the microorganisms evade autophagy-mediated destruction (12). However, since the replicating RBs require a constant supply of host nutrients, it is reasonable to assume that autophagy must be maintained in the infected epithelial cell to ensure the continued availability of precursor components. The *in vitro* replication of chlamydial serovar L2 was shown to be blocked by the addition of autophagy inhibitors (12). In a subsequent investigation, the infection of mouse embryo fibroblasts by serovar L2 was associated with an upregulation in the production of autophagy-related proteins (13). We have evaluated the association between a functional single nucleotide polymorphism (rs2241880) in a gene (*ATG16L1*) that codes for an essential autophagosome component and the detection of a *C. trachomatis* infection in pregnant women. Carriage of the variant allele that is associated with a significantly reduced capacity for autophagy was identified in 30.9% of 188 women who were negative for IgG antibody to *C. trachomatis*, as opposed to 20.4% of 28 women who were chlamydial antibody positive (A.

Jayaram, S. Inglis, and S. S. Witkin, unpublished data). This is consistent with the above-mentioned *in vitro* data that an increased capacity for autophagy (i.e., absence of the polymorphic allele) is associated with an elevated occurrence of a productive chlamydial infection. It remains to be determined whether a genetically determined reduced capacity for autophagy at other loci also lowers susceptibility to contracting a chlamydial infection. It would also be of interest to determine if the introduction of autophagy inhibitors may be a useful addition to traditional antichlamydial antibiotic therapy.

IMMUNE RESPONSE TO INFECTION

The presence of chlamydial EBs in the extracellular environment is readily recognized by components of the innate immune system (17). Toll-like receptors (TLRs) on all components of the innate immune system, including phagocytic cells and epithelial cells, especially TLR 2 and TLR 4, bind to pathogen-associated molecular patterns (PAMPs) on the surface of EBs and initiate the release of proinflammatory cytokines, as well as chemokines that attract immune cells to the site of infection (18). Following its invasion of the host cell cytoplasm, PAMPs on the newly incorporated EB are also recognized by the cytoplasmic pattern recognition receptor, nucleotide-binding oligomerization domain protein 1 (NOD1), resulting in additional proinflammatory gene activation (19). The phagocytosis of *C. trachomatis* and the subsequent expression of discrete antigens on the cell surface lead to T and B lymphocyte activation and the generation of chlamydial antigen-specific cell-mediated and humoral immunity (20). In a proportion of infected women, some organisms migrate to the uterus and fallopian tubes, where they utilize their unique characteristics to initiate a chronic infection. It should be noted that the immune responses that protect women against a *C. trachomatis* genital tract infection and prevent migration to the upper genital tract may not parallel findings from animal models and, thus, remain incompletely elucidated (21).

The contributing factors to chlamydial evasion of immune destruction are beginning to be defined. One major mechanism appears to be the chlamydia-directed production of multiple proteases. The best studied is a novel serine protease, chlamydial proteasome/protease-like activity factor (CPAF) (22). Multiple activities have been described for this enzyme that, *in toto*, inhibit antichlamydial immunity (23). CPAF degrades nuclear factor-kappa B (NF-κB), as well as other transcription factors that initiate production of multiple proinflammatory mediators. Degradation of the transcription factors RFX5 and USF-1 by CPAF inhibits the expression of major histocompatibility complex (MHC) class I and class II molecules, which are necessary for immune recognition of chlamydial antigens. *C. trachomatis*-infected cells may also inhibit MHC expression by induction of beta interferon (IFN-β), an inhibitor of IFN-γ-inducible MHC class II production (24). The inhibition of apoptosis in chlamydia-infected cells is another mechanism that promotes the survival of this pathogen (25). Recent research suggests the participation of additional factors in chlamydial evasion of immune-mediated destruction (26).

C. TRACHOMATIS PERSISTENCE

A major component of the antichlamydial immune response, and the factor that has received the most research attention, is gamma interferon (IFN-γ) (27, 28). *C. trachomatis* is unable to synthesize tryptophan and must obtain this essential amino acid from its host (29). IFN-γ induces the production of indoleamine-2,3-dioxygenase 1 (IDO1), the enzyme that degrades tryptophan; thereby, the presence of this enzyme inhibits the growth of chlamydial RBs (30). The consequences of tryptophan starvation for *C. trachomatis* are 2-fold: the RBs may die and the infection is cleared, or the RBs may substantially alter their gene transcription and metabolism and enter into what is known as a persistent state (31, 32). The RBs cease to divide but remain viable. The synthesis of structural and membrane proteins and lipopolysaccharide ceases while production of the stress-inducible chlamydial 60-kDa heat shock protein (hsp60) becomes greatly upregulated. When the extracellular chlamydial infection is cleared and IFN-γ is no longer being induced, the host pool of tryptophan increases, and this amino

acid again becomes available to the RB. The persistent state is reversed, and RB replication resumes (33, 34). Thus, *C. trachomatis* that has entered into its intracellular RB developmental stage has developed a survival mechanism that promotes its persistence in the face of immune attack. Individual variations in the ease of IFN-γ induction and the extent of its production in response to specific infections may determine which of the two mechanisms predominates. We have demonstrated that women positive for a single nucleotide polymorphism associated with elevated IFN-γ production (rs2430561) had a lower likelihood of having a *C. trachomatis* cervical infection than did women who were negative for this variant allele (35). Thus, the extent of IFN-γ production in individual women most likely also influences the likelihood of chlamydial survival.

A second IFN-γ-IDO1-related chlamydial survival mechanism has recently been elucidated (36). *C. trachomatis* serovars that infect the genital tract, but not serovars that infect epithelial cells in the eye, possess a gene, *trpBA*, that converts indole to tryptophan (37). Thus, the presence of indole in the genital tract may facilitate chlamydial survival despite IFN-γ-induced tryptophan depletion. Lactobacilli dominate the vaginal microbiome in the majority of reproductive-age women (38), but not in any other mammal, including nonhuman primates (39, 40). The evolution of *Lactobacillus* dominance in humans may have been a response to the unique sexual behavior of humans and the need to prevent genital tract infections that interfere with fertility and pregnancy (40). Lactobacilli kill vaginal pathogens, including *C. trachomatis* (41). Indole is not present in genital tract secretions when lactobacilli predominate. However, when non-lactobacilli are numerically dominant, as occurs in the common condition known as bacterial vaginosis (42), indole can be readily detected (28, 43). In addition, recent evidence has reported the existence of microorganisms in the uterus and fallopian tubes of apparently healthy asymptomatic women (44, 45). Thus, the dogma that the upper genital tract is sterile may be incorrect, and variations in the composition of the microbiota at these sites in individual women may also influence the ability of *C. trachomatis* to survive host immune system activation and persist. However, the validity of reports that detected bacteria in presumably "sterile" regions of the female genital tract has very recently been questioned (46). The likelihood of bacterial colonization and its relationship to the consequences of a chlamydial infection clearly need further investigation.

Additional factors contribute to chlamydial survival and persistence. For example, concurrent infections involving *C. trachomatis* and herpes simplex virus are not uncommon. There is evidence suggesting that in the presence of a herpesvirus infection, *C. trachomatis* RBs are induced to enter into a persistent state (47). Thus, any alteration in the host that leads to transient immunosuppression and reactivation of a latent herpesvirus infection will also influence the course and consequences of a chlamydial infection.

Host genetic variation in is another factor that influences the consequences of a *C. trachomatis* genital tract infection. Mannose-binding lectin (MBL) is a component of the innate immune system present in female genital tract secretions. It is a lectin and binds to carbohydrate residues on microbial surfaces. Microorganisms with surface-bound MBL are destroyed by either complement-mediated lysis or phagocytosis by cells possessing cell surface MBL receptors (48, 49). Glycoproteins on the *C. trachomatis* surface bind MBL, and this interaction inhibits infectivity *in vitro* (50). We have shown that a polymorphism at codon 54 in the *mbl2* gene (rs17287498) is associated with reduced MBL levels and an increased prevalence of *C. trachomatis*-mediated fallopian tube damage in Hungarian women who were positive for chlamydial antibodies (51). We have now verified this association in a Brazilian population (I. M. Linhares and S. S. Witkin, unpublished data).

The persistence of *Chlamydia* in *in vitro* culture has also been documented to be induced by the restriction of iron (52), as well as in the presence of several antibiotics (53). The involvement of these factors in persistence in the female genital tract remains to be established.

Many questions remain as to why some women are more vulnerable to the consequences of a *C. trachomatis* infection than are other women. The rates of development of pelvic inflammatory disease in women in the general population following a chlamydial infection appear to be low (54). An area for future studies could be the identification of additional gene polymorphisms that increase susceptibility to chlamydial persistence in some women and increased attention to the prevention of infection and early detection and treatment in this subgroup.

THE *C. TRACHOMATIS* 60-kDa HEAT SHOCK PROTEIN

When a cell, either a prokaryote or a eukaryote, is under physiological stress, the synthesis of a class of proteins known as heat shock proteins is rapidly upregulated. These proteins, highly conserved throughout evolution, aid survival by preventing protein misfolding or denaturation and facilitating the removal of terminally denatured proteins (55). As mentioned above, *C. trachomatis is* able to survive despite host humoral and cellular immune responses due, in part, to its ability to enter into a viable nonreplicative persistent state (32, 34). These persistent forms of RBs are under physiological stress, upregulate their expression of hsp60, and release this protein into the extracellular milieu (31). hsp60 is a highly conserved protein, and the eukaryote and prokaryote proteins share numerous amino acid sequences (56, 57). A consequence of shared antigenic epitopes is that an immune response to the bacterial hsp60 can result in the induction of autoantibodies to eukaryotic hsp60 (58).

Specific regions of homology with the chlamydial and human hsp60 proteins have been described (59). Sera from eight women who had an ectopic pregnancy and were positive for antibodies to *C. trachomatis* were evaluated for reactivity to 12-mer synthetic peptides that spanned the entire chlamydial hsp60 sequence. Reactivity to 13 epitopes was detected and the sequences of the epitopes compared to regions of the human hsp60; seven cross-reactive epitopes were identified. This established that sera from women with chlamydia-associated tubal damage were positive for antibodies that recognized the human hsp60. A subsequent study evaluated the lymphocyte proliferative response to five epitopes (amino acids 49 to 58, 85 to 96, 144 to 153, 275 to 283, and 291 to 298) that were conserved between the chlamydial and human hsp60 (60). Proliferative responses to two of these epitopes, regions 275 to 283 and 291 to 298, were identified in five of 10 women with recurrent pelvic inflammatory disease, one of nine women with a first episode of this disorder, and in 0 of 32 healthy control women. Interestingly, amino acid sequences homologous to these two epitopes are also present in hsp60 produced by *Escherichia coli* (61) and species of *Mycobacterium* (62). Thus, following sensitization of a woman's lymphocytes to the chlamydial hsp60, the subsequent presence of other hsp60-producing bacteria in her fallopian tubes may trigger a reactivation of hsp60-sensitized lymphocytes and further exacerbate tissue destruction. This mechanism offers an explanation for the observation that no bacterial pathogen is detected in 30% of women with pelvic inflammatory disease (63). Additional studies (summarized in reference 64) highlighted the presence of immunity to the conserved chlamydial hsp60 epitope 260 to 271 in women from Hungary, Sweden, and France who had blocked fallopian tubes or an ectopic pregnancy. However, the precise variables that facilitate the induction of cross-reactive immunity to the human hsp60 in individual women as a consequence of chlamydial persistence still remain incompletely determined.

A number of studies have shown that both humoral and cell-mediated immune responses to the chlamydial hsp60, as well as the generation of immune responses that recognize the human hsp60, contribute to the pathogenesis of a chlamydial genital tract infection (65–68). It should be noted that when *C. trachomatis* was added to a fallopian tube organ culture, only minimal damage was noted (69). Exposure to the chlamydial hsp60 induces strong cell-mediated and humoral immune responses in animal models of chronic pelvic inflammatory disease (68). Animals that had been previously sensitized to *C. trachomatis* developed inflammation of their fallopian tubes after a subsequent exposure to recombinant chlamydial hsp60 (70). This indicates that

the chronic release of chlamydial hsp60 by RBs while in a persistent state may mediate fallopian tube inflammation. Ultimately, sufficient damage and scar formation will result in tubal occlusion and infertility. It has been suggested that detection of circulating antibodies to the *C. trachomatis* hsp60 is the most sensitive test, even better than a hysterosalpingogram, for diagnosing chlamydia-related tubal factor infertility (71). The correlation between antibody and cell-mediated immune responses to the chlamydial hsp60 and the occurrence of pelvic inflammatory disease, tubal occlusion, infertility, and ectopic pregnancy in women from a number of different countries has been reported (66, 72–76). This reinforces the probable validity of immunity to hsp60 as a major factor in chlamydial pathogenesis.

Women who are infertile due to occluded fallopian tubes now seek to become pregnant by *in vitro* fertilization and embryo transfer (IVF-ET). There is a multitude of accumulating evidence, however, that immunity to the chlamydial hsp60 and/or to epitopes shared with the human hsp60 reduces success following this procedure. hsp60 is one of the first proteins expressed by early-stage embryos (77). A study utilizing *in vitro*-cultured mouse embryos demonstrated that treatment with monoclonal antibodies to hsp60 resulted in a failure to progress (78). This established that hsp60 was present on the cell surface of early embryos and was accessible to antibody binding. The addition of chlamydial hsp60 to a trophoblast cell line was shown to induce apoptosis; this response was abrogated by the addition of monoclonal antibody to TLR 4 (79). Thus, while most women undergoing IVF-ET have no evidence of a cervical chlamydial infection by culture or gene amplification assays, this observation suggests that the presence of a persistent chlamydial infection in some of these women and the release of the chlamydial hsp60 could, by inducing trophoblast apoptosis, contribute to pregnancy failure by disrupting formation of the placenta. The presence of antibodies to the chlamydial hsp60 in the cervixes of women undergoing IVF-ET has been correlated with a failure to achieve a detectable uterine implantation or to only transient implantation that is soon lost (80). In another study, follicular fluid taken during an IVF-ET cycle that was positive for chlamydial hsp60 was associated with a diagnosis of tubal factor infertility and with subsequent low implantation rates (81). The detection of antibodies directed against a conserved hsp60 epitope shared by the chlamydial and human hsp60 has been associated with a lower spontaneous conception rate and a higher rate of adverse pregnancy outcome (67). In contrast, a recent study of women with recurrent miscarriage showed no significant difference in the prevalence of antichlamydial hsp60 antibodies between women with or without this occurrence (82). Since a spontaneous pregnancy loss may be due to many diverse factors, this failure of a significant association with hsp60 was not surprising.

There are conflicting reports on the influence of *C. trachomatis* on pregnancy outcome in women with spontaneous conceptions. In addition to individual variations in factors associated with chlamydial persistence, as mentioned above, whether or not the infection occurred during the index pregnancy is another important variable that has not always been taken into consideration. An ascending chlamydial infection during pregnancy has been associated with a number of deleterious conditions, including premature rupture of membranes, chorioamnionitis, preterm labor, neonatal infections, and postpartum endometritis (83–85). Pregnancy is associated with an upregulation of autophagy in the mother in response to the preferential draining of nutrients by the developing fetus (86). In women harboring a persistent chlamydial infection, this may increase nutrient availability and favor the resumption of RB replication and reappearance of an active infection. Elevated levels of progesterone during gestation may also impact chlamydial growth by an as-yet-undetermined mechanism. The extent to which immunity to hsp60 may contribute to complications of ongoing pregnancies in chlamydial antibody-positive women remains largely unexplored.

It is clear that the consequences of a *C. trachomatis* infection differ greatly between individual women. Despite expanded efforts to diagnose and treat chlamydial genital tract infections in young women, the number of reported cases has been increasing

(87). Efforts to develop an effective vaccine against chlamydial infections have also encountered significant roadblocks (88). While our knowledge of the factors utilized by this organism to persist and cause disease has progressed at a high rate, a more thorough elucidation of host variables that influence disease acquisition and persistence remains to be accomplished. A more complete understanding of the differences between women in terms of factors that influence the consequences of a chlamydial genital tract infection and utilization of this knowledge will lead to the development of more individualized and more focused treatments, thereby reducing the occurrence of adverse outcomes.

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