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Herpes Simplex Virus Glycoprotein D/Host Cell Surface Interaction Stimulates *Chlamydia trachomatis* Persistence via a Novel Pathway.

Jennifer Vanover  
*East Tennessee State University*

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Herpes Simplex Virus Glycoprotein D/Host Cell Surface Interaction Stimulates *Chlamydia trachomatis* Persistence via a Novel Pathway

A dissertation

presented to

the faculty of the Department of Microbiology

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

by

Jennifer Vanover

December 2008

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ABSTRACT

Herpes Simplex Virus Glycoprotein D/Host Cell Surface Interaction Stimulates *Chlamydia trachomatis* Persistence via a Novel Pathway

by

Jennifer Vanover

When presented with certain unfavorable environmental conditions, *C. trachomatis* reticulate bodies (RBs) enter into a viable, yet noncultivable state called persistence. Two hallmarks of persistent chlamydiae are swollen, aberrantly shaped RBs, as viewed by transmission electron microscopy and a decrease in infectious progeny. Several models of chlamydial persistence have been described, including interferon-γ (IFN-γ), IFN-α, IFN-β, and tumor necrosis factor-α-exposure and nutrient deprivation. Previously, we established an in vitro co-infection model of two of the most common sexually transmitted pathogens in the United States, *C. trachomatis* and Herpes Simplex Virus-2 (HSV). Data from this tissue culture model indicate that: i) viral co-infection stimulates the formation of persistent chlamydiae and ii) productive HSV replication is not required for persistence induction. Further studies indicate that, co-infection-induced persistence is not mediated by: i) any known anti-chlamydial cytokine; ii) activation of inducible nitric oxide synthase or indoleamine 2, 3-dioxygenase; iii) inhibition of vesicular trafficking or sphingomyelin transport to the inclusion or; iv) amino acid, iron or glucose deprivation. These data demonstrate that co-infection-induced persistence is mediated by a previously undescribed, novel mechanism. During long-term co-infection with UV-inactivated HSV-2, chlamydiae recover following an initial suppression of chlamydial infectivity. These data indicate that HSV-
induced persistence, like other persistence models, is reversible. Co-incubation of fixed, HSV-2-infected inducer cells with viable, *C. trachomatis* infected responder cells suppresses production of infectious chlamydial progeny and stimulates the formation of swollen, aberrantly shaped RBs. Antibody neutralization of HSV glycoprotein D (gD), which prevents viral attachment to one of four known HSV co-receptors on the host cell surface, also prevents co-infection-induced persistence, suggesting that HSV gD interaction with host cell surface receptors can provide the necessary stimulus to alter *C. trachomatis* development. Finally, exposure of *C. trachomatis* infected cells to soluble, recombinant HSV-2 gD:Fc fusion proteins decreases production of infectious EBs to a similar degree observed in co-infected cultures. Thus, we hypothesize that interaction of HSV gD with the host cell surface triggers a novel host anti-chlamydial pathway that restricts chlamydial development.
DEDICATION

To Mom, Dad, and Amy. Words cannot truly express how thankful I am to the three of you for always being there to support and encourage me. Thank you Mom and Dad for never letting me settle for anything but my best. Mom, thank you for helping me to “get established.” Dad, thank you for teaching everything I ever needed to know in the fourth grade. Amy thank you for being the best friend/sister anyone could ever have.
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CHAPTER 1
INTRODUCTION

Sexually transmitted diseases (STDs) continue to represent a health crisis of monumental proportions. The World Health Organization estimates that 340 million cases of curable STDs occur annually, worldwide, with 19 million infections occurring in the United States (US) alone (CDC, 2006; Weinstock et al., 2004). Even more alarming is the report that up to one half of all new STDs each year affect people between the ages of 15-24 (CDC, 2006; Weinstock et al., 2004). Two of the most common STD agents in the US are Herpes Simplex Virus (HSV) and Chlamydia trachomatis. The Centers for Disease Control and Prevention reports that there are between 200,000-500,000 primary genital HSV infections each year, with an estimated 40-60 million people currently suffering from recurrent HSV infections. C. trachomatis infections are the most commonly reported bacterial STD in the US, with 4 million new cases each year. These staggering statistics not only describe a major health concern in the US but also indicate a substantial economic burden. The direct medical costs of treating STDs in 2006 were approximately 14.7 billion dollars. Ten billion dollars were spent on treating chlamydial infections alone in 2002 (CDC, 2006; Weinstock et al., 2004).

Co-infections with multiple STD agents further increase the difficulties of treating STDs. A recent study demonstrated that 20% of men and 42% of women with confirmed Neisseria gonorrhoeae infection tested positive for C. trachomatis. The prevalence of N. gonorrhoeae and C. trachomatis co-infection is substantial enough that treatment guidelines recommend dual treatment of both pathogens when there is a diagnosis of gonorrhea (Lyss, 2003). Co-infection is a valid concern as the presence of multiple infectious agents has the ability to affect disease pathology and prevalence. For example, an active HSV-2 infection increases the risk for Human
Immunodeficiency Virus (HIV) infection by three-fold (Freeman et al., 2007). C. trachomatis infection is also considered a co-factor for the transmission of HIV (Peipert, 2003).

**Herpes Simplex Virus**

Herpes Simplex Virus Types 1 and 2 (HSV-1 and HSV-2) belong to the Alphaherpesvirinae subfamily of the viral family, Herpesviridae. HSV-2 is the primary cause of genital herpes infection. HSV-1 is predominantly associated with oral infections; however, it is a common agent of genital herpes infections as well (Whitley, 2001). Primary HSV-2 infections usually occur on the mucous membranes and skin surrounding the genitals. Herpes infection of epithelial cells causes a characteristic vesicular lesion due to the cytopathic effects of the virus on the host cell and the host’s inflammatory response to infection. After primary infection of epithelial cells, HSV infects sensory nerve endings in the surrounding tissue. The viral nucleocapsids are transported by retrograde axonal flow to the dorsal root ganglion where they establish a latent infection. Latent infections can be reactivated by numerous stimuli, such as emotional or physical stress, fever, tissue damage, and immune suppression. Upon reactivation, virions travel through the infected neurons to the site of primary infection and once again infect the epithelial cells in that area. Skin lesions usually occur during recurrent herpes infections; however, evidence suggests that virions can be shed in the absence of noticeable symptoms (Whitley, 2001; Mertz et al., 1992). Latent HSV-2 infections are reactivated at least five times each year and some studies suggest that subtle reactivation may be almost continuous (Mertz et al., 1992; Corey et al., 1983). Active herpes infections can be suppressed with drugs targeting the viral protein tyrosine kinase (TK). However, the latency-associated transcripts are the only viral genes transcribed in latently infected neurons (Jones, 2003). Because TK is not expressed,
antiviral drugs cannot eliminate the infection once latency is established (Roizman, 2001). Although most genital HSV infections are clinically mild, HSV-2 can also cause serious diseases such as keratitis, meningitis, and disseminated neonatal HSV infection (Roizman, 2001).

Structurally, Herpes Simplex Viruses have a double stranded DNA genome, contained in an icosahedral capsid. The nucleocapsid is encircled by an unstructured tegument that contains several active viral proteins including virion host shut off protein (vhs), viral protein 16 (VP16), and VP22. The tegument is surrounded by the viral envelope composed of a lipid bilayer containing numerous viral glycoproteins (Roizman, 2001).

Infection of a host cell by HSV is initiated by a series of consecutive interactions between envelope glycoproteins and the host cell membrane (Fig. 1.1A). First, glycoproteins B (gB) and gC bind to the host cell receptor, heparan sulfate. The initial binding event is followed by interaction of gD with one of several co-receptors - herpes viral entry mediator (HVEM), Nectin-1, Nectin-2, or 3-O-sulfated heparan sulfate (Spear, 2004). The co-receptor used during infection is dependant on cell type as well as on the strain of HSV. HVEM is primarily expressed on T and B lymphocytes, epithelial cells and fibroblasts and can be used by both HSV-1 and HSV-2 for entry into the host cell (Kwon et al., 2006; Spear, 2004; Hsu et al., 1997; Marsters et al., 1997; Montgomery et al., 1996). Both nectin 1 and 2 are expressed on epithelial and neuronal cells. Nectin-1 can efficiently be used as a co-receptor by both HSV-1 and HSV-2. Nectin-2 also serves as a co-receptor for both types of HSV; however, Nectin-2 is used as a co-receptor by HSV-2 at significantly higher efficiency than by HSV-1(Spear, 2004). Lastly, 3-O-sulfated heparan sulfate is expressed on numerous cell types and is primarily used as a co-receptor by HSV-1 (Shukla et al., 1999).
Interestingly, HSV co-receptors also serve as host cell signaling molecules. HVEM is a member of the tumor necrosis factor receptor family (Mauri et al., 1998). When complexed to its natural ligand, LIGHT, HVEM stimulates cellular transduction pathways involved in the activation of T-cells (Granger and Rickert, 2003; Hsu et al., 1997). Nectin-1 and Nectin-2 are members of the Immunoglobulin superfamily and are involved in the formation of cell junctional complexes (Cocchi et al., 1998). When stimulated, nectins interact with cell signaling molecules, Cdc42 and Rac small G proteins, through their cytoplasmic tails to coordinate cytoskeletal rearrangements (Nakanishi and Takai, 2004). Additionally, Hoppe, et. al., have demonstrated that Rac1 and Cdc42 are activated following infection of epithelial cell lines with HSV-1 (Hoppe et al., 2006).

Once gD interacts with the appropriate host co-receptor, a complex of gH/L promotes fusion of the viral envelope and the host cell plasma membrane following attachment to the host cell. Once inside the host cell, viral tegument proteins are released. One such protein, VP22, functions as a microtubule-associated protein and induces reorganization of microtubules in the host cell cytoplasm (Elliott, 1998). Another virion protein, the HSV-2 virion host shut off protein (VHS), immediately suppresses host transcription by inducing degradation of host mRNAs (Roizman, 2001; Kwong, 1989). VP16 is a virion protein that is transported to the nucleus and is involved in activation of both viral and host gene transcription (Roizman, 2001).

After entry (Fig. 1.1B), viral capsids interact with the molecular motor dynein and are transported along microtubules to the nucleus where capsids dock with nuclear pore complexes and translocation of the viral genome occurs (Ojala, 2000). Transcription of the viral genome occurs in three consecutive stages. First, VP16 aids in initiating transcription of the immediate early, or α genes. The gene products of the α genes then stimulate the transcription of the β
genes. At this point in viral replication, viral DNA synthesis occurs. Lastly the $\gamma$ genes, which include many of the tegument proteins and envelope glycoproteins, are transcribed. Following transcription, viral proteins are synthesized using host cell machinery. New virions are then assembled in the nucleus, bud through viral glycoprotein enriched areas of the nuclear envelope, and exit the host cell through vesicular transport (Roizman, 2001).

Figure 1.1 Replication Cycle of HSV. A. Attachment and entry of HSV virions into the host cell. B. Replication and release of HSV from the host cell.
**Chlamydia trachomatis**

The bacterial family *Chlamydiaceae* contains several human pathogens including, *C. pneumoniae*, *C. psittaci*, and *C. trachomatis*. *C. muridarum* and *C. suis* are non-human animal pathogens, infecting mice and swine respectively (Mpiga and Ravaoarinoro, 2006). *C. pneumoniae* is a causative agent of community acquired pneumonia in humans, which has been associated with cardiovascular plaque formation and Alzheimer’s disease (Hogan *et al.*, 2004). It is estimated that 90% of all adults are infected with *C. pneumoniae* during their lives and that 10% of all community acquired pneumonia and 5% of bronchitis and sinusitis cases are due to *C. pneumoniae* (Koehler *et al.*, 1997; Summersgill *et al.*, 1995). *C. psittaci* is a zooanotic pathogen that causes a respiratory disease in humans called psitticosis. Psitticosis is often observed in poultry farm workers and owners of exotic birds (Mpiga and Ravaoarinoro, 2006; Everett *et al.*, 1999).

*C. trachomatis* is separated into two biovars, the Lymphogranuloma Venereum (LGV) biovar and the trachoma biovar, both of which are broken into several serovars. There are four LGV serovars, L1, L2, L2a, and L3 and 14 trachoma serovars, designated as serovars A-K, Ba, Da, and Ia. *C. trachomatis* serovars A-C are the etiological agents of trachoma, the world’s leading cause of preventable blindness. It is estimated that 162 million people suffer from ocular infections by serovars A-C and that 6 million people are rendered completely blind because of these bacteria (Mpiga and Ravaoarinoro, 2006; Mabey and Fraser-Hurt, 2003). *C. trachomatis* serovars D-K represent the world’s most reported bacterial sexually transmitted disease agents and cause urethritis or cervicitis. Approximately 85-90% of chlamydial infections are asymptomatic and chronic, allowing the bacteria to ascend the genital tract, which can result in endometritis, salpingitis, and pelvic inflammatory disease. The silent nature of chlamydial
infections leads to prolonged inflammation, causing fibrosis and scarring in the tissues of the genital tract. Eventually, the damage sustained by the host response to *C. trachomatis* infections promotes the development of more severe disease sequelae including ectopic pregnancy, infertility and reactive arthritis. Additionally, 30-50% of infants born to chlamydiae-infected mothers will develop chlamydial conjunctivitis and nasopharyngeal infections (Peipert, 2003). *C. trachomatis* LGV infections cause a more invasive sexually transmitted disease. Following infection, the bacteria travel from the genital tract to the inguinal and femoral lymph nodes. Once the lymph nodes are infected, necrotic abscesses develop and result in draining fistulas (Schachter, 1999). Acute chlamydial infections can be successfully treated with cyclins (doxycycline and tetracycline), quinolones (ofloxacin and levofloxacin), and macrolides (erythromycin and azithromycin) (Mpga and Ravaoarinoro, 2006).

**The Chlamydial Developmental Cycle**

All chlamydiae are Gram-negative obligate intracellular bacteria and share a unique biphasic developmental cycle (Fig 1.2). Extracellularly, chlamydiae exist as small (0.3µm), infectious, metabolically inert forms of the bacteria termed elementary bodies (EBs). EBs are round, structurally rigid, and osmotically stable and contain a highly compacted bacterial nucleoid. Chlamydiae have an interesting bacterial cell wall that contains little or no peptidoglycan. Therefore, the rigidity of EBs is maintained by crosslinking between outer membrane proteins. EBs attach to the apical surface of columnar epithelial cells and enter via receptor mediated endocytosis (Abdelrahman and Belland, 2005; Wyrick, 2000). Several surface exposed bacterial proteins present on EBs have been purposed to be involved in chlamydial attachment to the host cell, including: the major outer membrane protein (MOMP), heat shock
protein 70, outer membrane protein A, outer membrane complex protein B, members of the polymorphic membrane protein family, and heparan sulfate-like glycosaminoglycans. Likewise, several chlamydial receptor candidates are present on the surface of host cells including the heperan sulfate receptor and the estrogen receptor (Dautry-Varsat et al., 2004; Fields and Hackstadt, 2002; Bavoil et al., 2000; Wyrick, 2000). Once inside the host cell, chlamydiae-containing vesicles escape lysosomal fusion and are transported to the perinuclear region. Here they differentiate within a modified vacuole, called an inclusion, into the larger (1µm), non-infectious form of the bacterium, called the reticulate body (RB). Reticulate bodies are metabolically active and will undergo 8-12 rounds of replication within the inclusion. Following replication, RBs condense to form EBs that are released and can infect new host cells. The infectious cycle takes anywhere from 30-72 hours depending upon the chlamydial species/serovar (Wyrick, 2000). In addition to growth in the normal developmental cycle, chlamydiae have evolved a mechanism to sustain viability during adverse conditions. When developing chlamydiae are exposed to certain environmental insults, they deviate from the normal developmental cycle into a state called persistence (Hogan et al., 2004).
Chlamydial Persistence

Persistent chlamydiae are traditionally defined as a viable but non-cultivatable form of chlamydiae (Beatty et al., 1994d). It appears that chlamydiae use persistence as a general stress response to maintain viability until growth conditions improve, therefore, resulting in a prolonged existence within the host cell (Mpiga and Ravaoarinoro, 2006; Harper et al., 2000). Persistent chlamydial forms appear as swollen, diffuse, and aberrantly shaped RBs upon examination by transmission electron microscopy (TEM). While in the persistent state, RBs continue to enlarge and accumulate chromosomes, but they fail to divide, resulting in the
persistent morphology. The abnormal RBs do not differentiate into EBs, thus, causing a decrease in progeny EB infectivity (Beatty et al., 1994d; Beatty et al., 1993; Byrne et al., 1986; Johnson and Hobson, 1977; Matsumoto and Manire, 1970). Several models of persistence have been examined in culture including: antibiotic exposure, monocyte infection, nutritional deficiencies, and cytokine exposure (Gerard et al., 2001; Darville et al., 2000; Raulston, 1997; Beatty et al., 1994d).

Antibiotic-induced persistence has been most extensively examined in chlamydiae-infected cultures exposed to Penicillin G, which inhibits bacterial cell wall synthesis (Nicholson et al., 2003). Although the presence of peptidoglycan in the chlamydial cell wall remains unproven, C. trachomatis does have the genes required to produce it (McCoy et al., 2003; Stephens et al., 1998). In fact, recent studies report that chlamydiae possess functional enzymes to synthesize meso-diaminopimelate, a key component of peptidoglycan (McCoy et al., 2006). It has been suggested that peptidoglycan is produced in RBs and aids in cell division (McCoy and Maurelli, 2006). Therefore, it is possible that by interfering with cell wall synthesis, Penicillin G is able to induce persistence. When antibiotics are removed from the culture medium, RBs resume normal development and production of infectious progeny is restored (Matsumoto and Manire, 1970). Additionally, other antibiotics, including erythromycin and ampicillin have been shown to induce persistence under certain experimental conditions (Wolf et al., 2000; Clark et al., 1982).

Deficiencies in amino acids, glucose, or iron have all been shown to induce chlamydial persistence. Growth of C. trachomatis serovar E under iron-limiting conditions produces inclusions that contain numerous membrane blebs and RBs with loose, wavy outer membranes (Raulston, 1997). Likewise, removal of glucose or amino acids from the culture medium leads to
the formation of abnormally shaped RBs and a loss of chlamydial infectivity (Harper et al., 2000; Coles et al., 1993). In each case, replacement of the deficient/depleted nutrients allows the chlamydiae to recover infectivity (Harper et al., 2000; Raulston, 1997; Coles et al., 1993).

Cytokine exposure, especially interferon-γ (IFN-γ), often induces chlamydial persistence by stimulating an indirect depletion of nutrients. In human cells, IFN-γ-exposure increases host cell indoleamine 2, 3-dioxygenase (IDO) expression, leading to depletion of host cellular tryptophan. Without this essential amino acid, developing chlamydiae enter into persistence and cease productive replication (Beatty et al., 1994d). In murine cells, IFN-γ-exposure increases synthesis/activity of the p47 GTPase, Iigp1. Iigp1 inhibits chlamydial development by restricting vesicular trafficking to developing inclusions (Nelson, 2005). Other cytokines [tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α) and IL-1β] can synergize with IFN-γ to enhance its negative effect on chlamydial development at lower concentrations (Carlin and Weller, 1995; Summersgill et al., 1995; Shemer-Avni et al., 1988). Lymphotoxin-α (LT-α), IFN-α, IFN-β, and TNF-α also interfere with chlamydial development by induction of a host anti-chlamydial pathway involving inducible nitric oxide synthase (iNOS) and/or induction of iron restriction-mediated persistence (Hogan et al., 2004; Matsushima et al., 1999). When iNOS is up-regulated, the host cell produces nitric oxide (NO) that kills developing chlamydiae. Interferon-γ-induced persistence is also reversible with the addition of exogenous tryptophan to the culture medium (Beatty et al., 1994d). In the case of IFN-α, both tryptophan and iron must be added to the medium for full recovery of chlamydial infectivity (Ishihara et al., 2005).

Gerard et al. (2001) demonstrated that C. trachomatis serovar K enters persistence spontaneously, in the absence of any exogenous inducer when infecting human monocytes. Interestingly, neither the addition of tryptophan nor antibodies to IFN-γ or TNF-α allowed
recovery of chlamydial infectivity, suggesting a cytokine-independent mechanism of persistence induction (Airenne et al., 1999; Koehler et al., 1997). *C. trachomatis* serovar E has also been shown to enter spontaneous persistence when cultured in non-permissive synovial cells (Hanada et al., 2003).

A number of experimental parameters have been used as indicators of chlamydial persistence; these include: altered inclusion size, abnormal RB morphology, decreased production of progeny EBs, and recovery of infectivity following the removal of the persistence inducer (Hogan et al., 2004; Darville et al., 2000; Beatty et al., 1994d). Chlamydial MOMP expression is often decreased during persistence while expression of the chlamydial pro-inflammatory antigen, heat shock protein 60 (HSP60), is stable or increased (Hogan et al., 2004; Beatty et al., 1994a; Beatty et al., 1993). Because DNA synthesis continues in persistence, accumulation of chlamydial DNA in persistently-infected cultures is not significantly different from that in productively infected cultures (Hogan et al., 2004; Gerard et al., 2001). Continued expression of unprocessed 16S rRNA transcripts is also a key marker of persistence. Processing of 16S rRNA transcripts occurs almost immediately following transcription; thus, isolation of unprocessed transcripts in the absence of infectivity demonstrates viability of the organisms (Gerard et al., 1997).

While many characteristics of chlamydial persistence are reiterated among the various *in vitro* models, some markers of persistence vary depending on the experimental conditions used. For instance, expression of the cell separation genes, *ftsK* and *ftsW*, is reduced in *C. trachomatis* serovar K-infected monocytes as well as in IFN-γ-exposed *C. pneumoniae* (Byrne et al., 2001; Gerard et al., 2001). Conversely, Stevens et al. demonstrated that in penicillin-exposed, *C. trachomatis* serovar D infected cells, *ftsW* remains unchanged while *ftsK* is elevated (Nicholson,
2002). The timing of persistence induction also varies among persistence models. The addition of IFN-γ to chlamydiae-infected cultures must be performed prior to or early during infection for the maximal effects to be observed (Nelson et al., 2005). However, Penicillin G can be added at 24hr post infection and still induce abnormal chlamydial development (Nicholson, 2002). Though recovery of infectious progeny following the removal of the persistence inducing agent or replenishment of deficient nutrients is a key feature of most persistence models (Gerard et al., 2001; Darville et al., 2000; Raulston, 1997; Beatty et al., 1994d), recovery was not demonstrated in spontaneously persistent cultures even after the addition of tryptophan (Airenne et al., 1999; Koehler et al., 1997). The differences between persistence models serve as a reminder that experimental conditions, persistence inducer, chlamydial species/serovar, and choice of host cell employed in a study all play a role in the development of persistence. Taken together these observations indicate that although the outcome of persistence may appear similar in various models, the underlying mechanisms could be very different.

Although most known information about chlamydial persistence has been revealed by cell culture, in vivo persistence is a tangible scenario. Improper or inadequate antibiotic therapies could allow persistence to occur in vivo (Mpiga and Ravaoarinoro, 2006). Natural fluctuations in iron levels in the female genital tract could cause chlamydiae to experience iron limitations (Raulston, 1997). Release of IFN-γ by the immune system is a part of the host response to intracellular pathogens, including C. trachomatis (Morrison et al., 2000; Rank and Sanders, 1992; Ramsey et al., 1988). In fact, evidence from several studies strongly suggests that chlamydiae do become persistent in vivo (Gerard et al., 2002; Gerard et al., 2001; Dean et al., 2000; Fortenberry et al., 1999; Patton et al., 1994). An investigation of recurrent genital chlamydial infections indicated that several study participants were infected with the same
serovar and genotype of *C. trachomatis* 3-10 times in a 2-5 year time span despite appropriate antibiotic treatment. Based upon the principle that anti-chlamydial immunity is genotype specific, these results indicate that the women in this study were persistently-infected rather than becoming re-infected with a different strain of *C. trachomatis* (Dean et al., 2000). The presence of atypical RBs has been demonstrated by electron microscopy examination of fibroblasts isolated from patients suffering from reactive arthritis (Nanagara et al., 1995). Unprocessed chlamydial 16S rRNA transcripts have been amplified from snyovial tissue of arthritis patients (Gerard et al., 1997). A recent *in vitro* study demonstrated that persistent chlamydiae become refractory to killing by azithromycin (Wyrick and Knight, 2004). Finally, abnormal RBs similar to the persistent forms observed in culture have been visualized in urethral and cervical tissues from *C. trachomatis* infected patients despite treatment with azithromycin (Bragina et al., 2001). Taken together, these observations argue that *in vivo* chlamydial persistence is a relevant medical concern. Furthermore, increased release of inflammatory mediators, like Hsp-60, from persistently-infected cells *in vivo* may enhance disease pathology (Darville et al., 2000).

**HSV and *Chlamydia trachomatis* Co-infection**

There is evidence that HSV and *C. trachomatis* co-infections exist *in vivo*. Serologic investigations indicate that HSV-2 positive individuals are likely to be *C. trachomatis* positive as well (Silins et al., 2002; Paroli et al., 1990). Several studies have documented seropositivity rates of >50% for both pathogens, implying that some of the individuals had been exposed to both HSV-2 and *C. trachomatis* (Wagner et al., 1994; Duncan et al., 1992). Although IgG seropositivity does not prove coexisting infection, there is a high probability that some of the individuals were infected simultaneously with both pathogens. In addition to serological studies,
direct examination of cervical biopsy samples by PCR indicated that approximately 10% of women tested positive for *C. trachomatis*, HSV, and Human Papilloma Virus (Finan et al., 2006). Furthermore, both *C. trachomatis* and HSV have been isolated simultaneously in samples from women stricken by upper genital tract diseases (Paavonen et al., 1985; Tait et al., 1985). In fact, co-infections of *C. trachomatis* and HSV are so prevalent that a dual vaccine for both pathogens has been proposed (Macmillan et al., 2007). Although co-infections of *C. trachomatis* and HSV exist, the implications of these dual infections on disease pathology and pathogen/host interactions have not been investigated.

*In vitro* models of HSV-2/*C. trachomatis* co-infections have also been established. Transmission electron microscopy (TEM) analyses of Vero cells co-infected with *C. trachomatis* serovar L2 and HSV-2 revealed swollen inclusions with few RBs or EBs (Pontefract et al., 1989). Chiarini et al. reported that the number of cells positive for chlamydiae by immunofluorescence was reduced when HeLa cells were co-infected with HSV-2/*C. trachomatis* serovar D (Chiarini et al., 1996). The number of chlamydial inclusions and infectious EBs was also reduced in HSV-2 pre-infected HT1376 human bladder cells (Superti et al., 2001). Although these observations indicate that HSV-2 co-infection alters chlamydial development, these studies did not examine the co-infection process in detail.

A tissue culture model of HSV-2 and *C. trachomatis* serovar E co-infection was recently established in our laboratory. Findings from this model demonstrate that HSV co-infection decreases production of infectious chlamydial progeny, suggesting that viral co-infection drives developing chlamydiae to enter a persistent state. TEM analyses also show that numerous membrane blebs and vesicles are present in inclusions and RBs become swollen and aberrantly shapen during co-infection (Deka et al., 2006). Furthermore, co-infection with either UV-
inactivated, replication incompetent virus or with replication-competent HSV-2 in the presence of cyclohexamide reduced chlamydial infectivity (Deka et al., 2007). These data demonstrate that productive viral replication is not required for the induction of chlamydial persistence and suggest that HSV attachment and entry can provide the necessary stimulus to alter C. trachomatis development.

The experiments in this study will allow us to begin to unravel the mechanism by which HSV-2 causes C. trachomatis to enter persistence. The first objective in this study is to characterize co-infection-induced persistence by examining several known molecular markers of persistence. These include: i) chlamydial genome accumulation; ii) expression profiles of several persistence influenced genes (ftsK, ftsW, and gro-EL1); iii) accumulation of chlamydial Hsp60 and MOMP proteins; and iv) recovery of chlamydial infectivity following induction of persistence. The second aim in this study is to determine whether the mechanism of HSV-2-induced persistence is one of the previously characterized persistence inducers or if it is stimulated by novel anti-chlamydial pathway. The last aim of this study involves differentiating between the events of viral attachment and entry so that the role that these events (specifically HSV-2 attachment) play in triggering chlamydial persistence during co-infection can be determined. Examination of the cellular pathways that are stimulated by HSV-2 attachment and entry during co-infection will further illuminate the mechanisms that C. trachomatis uses to occupy its specific niche within the host cell as well as the host cell mechanisms that have co-evolved to limit infection.
CHAPTER 2

CHLAMYDIA TRACHOMATIS ENTERS A VIABLE BUT NON-CULTIVABLE (PERSISTENT) STATE WITHIN HERPES SIMPLEX VIRUS TYPE 2 (HSV-2) CO-INFECTED HOST CELLS.

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Abstract

Epidemiologic and clinical studies have shown that double infection with herpes simplex virus, type 2 (HSV-2) and *Chlamydia trachomatis* occurs *in vivo*. We hypothesized that co-infection would alter replication of these agents. To test this hypothesis, HeLa cells were infected with *C. trachomatis* serovar E, followed 24 hours (h) later by HSV-2 strain 333. Transmission electron microscopic (TEM) analyses indicated that, by 10 hours post-HSV addition, reticulate bodies (RBs) in co-infected cells were swollen, aberrantly shaped, and electron-lucent. In infectious titer assays, HSV-2 co-infection abrogated production of infectious chlamydial progeny. Western blot analyses indicated that accumulation of chlamydial major outer membrane protein (MOMP) was decreased by HSV co-infection while accumulation of chlamydial heat-shock protein 60-1 (HSP60-1) was increased. Polymerase chain reaction (PCR) experiments indicated that chlamydial genome copy number was unaltered by HSV-2 super-infection. Semi-quantitative, reverse transcriptase-PCR (RT-PCR) experiments demonstrated that levels of chlamydial *groEL, fisK, fisW, dnaA*, and unprocessed 16S rRNA transcripts were not changed by HSV-2 super-infection. These data indicate that HSV-2 super-infection drives chlamydia into a viable but non-cultivable state, which is the hallmark of persistence. Because chlamydial HSP60-1 has been associated with immunopathology *in vivo*, these results also suggest that disease severity might be increased in co-infected individuals.
Introduction

Sexually transmitted diseases (STD) have reached epidemic proportions worldwide. This year in the United States (US) there will be approximately 19 million new cases of STDs (Weinstock, 2004). The most commonly reported STD agents in the US include Chlamydia trachomatis serovars D-K with 4,000,000 new cases/year and herpes simplex virus (primarily HSV-2) with 200,000-500,000 new cases/year (Butler, 1997). Because both organisms establish asymptomatic or latent infections, these numbers underestimate the infectious burden.

The Chlamydia are a genus of Gram negative, obligate intracellular bacteria. Multiple and persistent chlamydial infections are strongly associated with increased pathology in vivo (Beatty et al., 1994c). Genital chlamydial infections are associated with serious complications; these include pelvic inflammatory disease, tubal factor infertility, and ectopic pregnancy in females and epididymitis and reactive arthritis in men (Darville, 2000). Extracellularly, chlamydiae exist as elementary bodies (EBs). Infection begins by contact of infectious EBs with the epithelial cell apical surface, followed by receptor-mediated endocytosis. Upon uptake, each EB is incorporated into an endocytic vesicle. Multiple EB-containing endocytic vesicles fuse to form an expanding, membrane-bound vacuole termed an inclusion. The small, spherical, metabolically-inactive EBs (0.2μm diameter) then transform into larger (0.8μm), metabolically-active, non-infectious, reticulate bodies (RBs). RBs use host metabolites and energy to synthesize macromolecules, grow, and divide by binary fission. The RBs then mature into infectious EBs and exit the host cell (Wyrick, 2000).

Chlamydiae can establish persistent infection of host cells in vitro under specific environmental conditions. These include IFN-γ, TNF-α and penicillin G exposure as well as amino acid or iron deprivation and monocyte infection (Beatty et al., 1994c; Raulston, 1997;
Darville, 2000; Gerard et al., 2001), all of which can occur in vivo as well. The persistent state is characterized by inclusion enlargement, the presence of swollen, diffuse RBs and decreased EB production (Matsumoto and Manire, 1970; Johnson and Hopson, 1977; Byrne et al., 1986; Beatty et al., 1993; Beatty et al., 1994a). Persistent chlamydiae can remain in a viable but less metabolically active, non-replicative state for weeks or months. Chlamydial MOMP expression is often decreased during persistence while expression of the chlamydial pro-inflammatory antigen, heat shock protein 60 (HSP60) is stable or increased (Cevenini et al., 1988; Beatty et al., 1993; Beatty et al., 1994b). Persistent chlamydial forms shed MOMP and lipopolysaccharide (LPS) into membrane blebs that find their way to the host cell surface and are released (Karimi et al., 1989; Wyrick et al., 1994; Wyrick et al., 1997; Wyrick et al., 1999). Although C. trachomatis persistence in vivo has been difficult to demonstrate, recent studies suggest that C. trachomatis establishes persistent infections in humans (Patton et al., 1994; Fortenberry et al., 1999; Dean et al., 2000; Gerard et al., 2001; Gerard et al., 2002). Increased release of inflammatory mediators from persistently-infected cells in vivo may enhance disease pathology (Darville, 2000).

The Herpesviridae are a family of enveloped DNA viruses. Primary human HSV-2 genital infections usually occur on genital skin or mucous membranes, where an intense inflammatory response is observed. The virus may then establish latency in the sacral ganglia. HSV-2-infected individuals experience an average of 5 reactivations/year throughout their lives, during which lesions and virions are present (Corey et al., 1983). HSV-2 may also cause several serious diseases including keratitis, meningitis, and disseminated herpes infection. At a single cell level, the HSV-2 replication cycle takes from 12 to 24 hours and is initiated by viral attachment to one of several host cell receptors. Once viral DNA enters the nucleus, the viral
genes are expressed in a specific temporal order. New virions are then assembled, enveloped, and released (Roizman and Knipe, 2001).

A number of studies have established that co-and super-infections with HSV-2 and C. trachomatis occur in the human population. HSV-2 and C. trachomatis have been simultaneously isolated from the genital tract of women suffering from endometritis and salpingitis (Paavonen et al., 1985) as well as cystitis (Tait et al., 1985). Several large serologic studies indicate that HSV-2 positive individuals are likely to be C. trachomatis positive as well (Paroli et al., 1990; Vetter, et al., 1990; Silins et al., 2002). Also, seropositivity rates of >50% for both HSV-2 and C. trachomatis have been reported, suggesting that some study participants had been exposed to both (Duncan et al., 1992; Wagner et al., 1994). Although IgG seropositivity cannot establish concurrent infection, it is likely that both organisms were present simultaneously in some individuals.

Cell culture models of HSV/chlamydial co-infection have been established. Pontefract et al. observed that inclusions in Vero cells co-infected with C. trachomatis serovar L2 and HSV-2 were swollen and contained few RBs or EBs (Pontefract et al., 1989). Chiarini and colleagues reported that in HSV-2/C. trachomatis serovar D co-infected HeLa cervical epithelial cells, the number of cells positive for chlamydiae by immunofluorescence (IFA) was reduced (Chiarini et al., 1996). HSV-2 pre-infection of HT-1376 human bladder cells also reduced both inclusion number and production of infectious C. trachomatis EBs (Superti et al., 2001). These data suggested that HSV-2 co-infection might alter the chlamydial developmental cycle in a manner similar to that observed during persistent infections. Because no previous study directly addressed this possibility, we established a cell culture HSV-2/C. trachomatis serovar E co-infection system and evaluated this hypothesis using multiple experimental indicators of
chlamydial persistence. These data demonstrate that HSV-2 co-infection alters the chlamydial developmental cycle similarly to other inducers of chlamydial persistence and illuminate potential mechanisms by which co-infection could enhance pathology.

**Results**

HeLa cells can be co-infected with *C. trachomatis* serovar E and HSV-2.

HeLa monolayers were either mock, singly, or co-infected (Fig. 2.1A). At 20 h post-HSV infection, HeLa cells were immunostained (Fig. 2.1B). Both *C. trachomatis* and HSV-2 singly-infected cells stained only with antibodies specific for either agent; control, mock-infected cells did not stain with either antibody. IFA using *C. trachomatis* MOMP-specific antibodies demonstrated that chlamydial inclusions were present in more than 60% of *C. trachomatis* infected or co-infected cells. HSV-2 ICP5 and ICP8 antibody staining showed that at least 90% of the HSV singly-infected and co-infected cells were HSV-2-infected (Fig. 2.1B). Similar results have been obtained in multiple experiments and demonstrate that co-infection did occur and that most chlamydiae-infected cells were super-infected with HSV-2.
Figure 2.1. HSV-2 co-infection increases *C. trachomatis* inclusion size. (A) HeLa cells were plated and divided into 4 groups: Mock (uninfected negative control), Ct-25 (infected only with *C. trachomatis*), HSV (infected only with HSV-2) and Ct-25/HSV (infected first with *C. trachomatis* and 24 hours later with HSV-2). Each group was mock-infected with 2SPG alone or *C. trachomatis* serovar E at 80% infectivity at T-25 and with HSV-2 at 10 PFU/cell or culture medium at T-1 as indicated. All cells were incubated at 35°C from T-25 until the end of the experiment. Following a 1 h attachment/entry period, all cells were refed with fresh culture medium following both *C. trachomatis* (T-25) and HSV-2 (T0) and mock (T-25 and/or T0) infection. Samples for IFA (immunofluorescence assay), TEM (transmission electron microscopy), plaque assays, EB (elementary body) titer, and Western blot experiments were collected at various times after T0. (B) Mock, HSV-2, *C. trachomatis* and co-infected cells were fixed and stained using FITC conjugated anti-*C. trachomatis* major outer membrane protein (Anti-*C. trach*) or using antibodies against HSV-2 (Anti-HSV-2) as described in the methods. Photomicrographs are 320X magnification. (C) Average relative inclusion size was determined from fluorescence photomicrographs. Images were coded and analyzed in double blind fashion as described. Means were compared using a 2-sample t-test for independent samples. Data are presented as average relative inclusion size ± the SEM. The Ct-25 and Ct-25/HSV inclusion size values are significantly different (p < 0.05)*
HSV-2 super-infection increases chlamydial inclusion size.

Because inclusion enlargement has been observed in several in vitro models of chlamydial persistence (Matsumoto and Manire, 1970; Johnson and Hobson, 1977; Byrne et al., 1986; Beatty et al., 1993; Raulston, 1997), image analysis was used to determine the average inclusion size in singly and co-infected cells at 20 hours post-HSV infection (Fig. 2.1C). Comparison of inclusion size indicated that inclusions were 28% larger in co-infected compared to singly-infected cells (448.6 ± 27 vs 624.9 ± 49). Statistical analysis indicated that average inclusion areas in these two samples were significantly different (p< 0.05).

Super-infection with HSV-2 induces morphological changes in C. trachomatis.

Persistent forms of C. trachomatis have a characteristic electron microscopic appearance (Matsumoto and Manire, 1970; Johnson and Hobson, 1977; Byrne et al., 1986; Beatty et al., 1993; Raulston, 1997). Thus, chlamydial morphology in singly and co-infected cells was compared by TEM (Fig. 2.2). At 30 hours post-chlamydial infection chlamydial inclusions in C. trachomatis singly-infected cells contained morphologically normal reticulate bodies (RB, black arrow, Fig. 2.2A); by 45 hours post infection (hpi) these inclusions contained chlamydiae progressing through the standard developmental cycle with normally sized RBs (Fig. 2.2B, black arrow) as well as intermediate and elementary bodies (IBs and EBs, black arrow with star). The C. trachomatis singly-infected control cells in Figure 2.2A and B are labeled 5h and 20h (post-HSV infection) for ease of comparison to the appropriate co-infected samples. In co-infected cells, the RBs appeared slightly swollen as early as 5 h post-HSV-2 infection (Fig. 2.2D, black arrow). At 10 h (Fig. 2.2E), 15 h (Fig. 2.2F) and 20 h (Fig. 2.2G and H) post-HSV-2 infection, the inclusions contained enlarged, irregular, and diffuse RBs (Fig. 2.2E-H, black arrows). At 20 h
after viral infection very few IBs and no EBs were observed in co-infected cells (Fig. 2.2G and H). Increased accumulation of membrane blebs was also apparent in co-infected cells at both 15 h (Fig. 2.2F, black boxes) and 20 h (Fig. 2.2G and H, black boxes) post-virus infection. HSV-2 virions were visible within nearly all co-infected cells containing aberrant chlamydial forms, beginning at 10 h post-HSV-2 infection (Fig. 2.2E, F, G and I, black circles). Neither chlamydial inclusions nor HSV-2 virions were visible in mock-infected cells (Fig. 2.2C). Similar data were obtained in multiple experiments and indicated that HSV-2 super-infection induced readily apparent alterations in *C. trachomatis* serovar E ultrastructure.
Figure 2.2. HSV-2 co-infection induces a “persistent-like” chlamydial morphology. HeLa cells were mock-infected (Mock, panel C), *C. trachomatis* infected (Ct-25, panels A and B) and *C. trachomatis*/HSV-2 co-infected (Ct-25/HSV, panels D-I) as described in Figure 2.1A. At 5 (panels A and D), 10 (panel E), 15 (panel F), or 20 h (panels B, C, G-I) post-HSV infection, cells were pre-fixed with glutaraldehyde-paraformaldehyde, processed, and then subjected to high contrast transmission electron microscopy. Panels A through H are at 12,101 X magnification. Panel I is at 29,388 X magnification.
HSV-2 super-infection abolishes production of infectious chlamydial EBs.

Previous studies have demonstrated that chlamydial persistent forms are non-infectious (Johnson and Hobson, 1977; Beatty et al., 1993). The dearth of EBs in co-infected cells observed by TEM suggested that HSV-2 co-infection interfered with RB maturation to EB. Therefore, subpassage was used to determine whether infectious chlamydiae were produced by co-infected cells (Table 2.1). Neither mock nor HSV-2 singly-infected cells produced detectable infectious EBs, whereas *C. trachomatis* singly-infected cells produced abundant EBs. Co-infected cells produced few, or no, infectious EBs, demonstrating that production of infectious chlamydial progeny was essentially abolished by HSV-2 co-infection.

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<td>&lt; 5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt; 5&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>Ct.25</td>
<td>5,869</td>
<td>18,000</td>
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<tr>
<td>Ct.25/HSV</td>
<td>&lt; 5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>120</td>
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</table>

Table 2.1. Co-infected cells do not produce infectious chlamydial EBs. <sup>a</sup>Inclusion counts from triplicate wells were averaged and used to calculate # inclusion forming units (IFU)/10<sup>6</sup> cells. <sup>b</sup>HeLa cells were mock-infected (Mock), *C. trachomatis* infected (Ct.25), HSV-2-infected (HSV) and *C. trachomatis*/HSV-2 co-infected (Ct.25/HSV) as described in Figure 2.1A. <sup>c</sup>Results from two independent co-infection experiments are shown. <sup>d</sup>Because the lowest dilution of cell lysate tested was 1/5, inclusion counts of “0” cannot be used to calculate the exact number of IFU present in undiluted cell lysates and only indicate that the number of IFU present must be less than 5.
Co-infection alters neither chlamydial nor HSV-2 genome copy number.

One characteristic of persistent chlamydiae is that they do not divide but continue to replicate their DNA (Gerard et al., 2001). Total DNA from co-infected and singly-infected cells (Fig. 2-3) was subjected to PCR using primers specific for the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, the chlamydial 16S rRNA gene and the HSV-2 G2 glycoprotein gene. All amplimers were the expected size (Fig. 2.3A) and the identity of each was confirmed by DNA sequencing (data not shown). The quantity of chlamydia DNA specific product is similar in both co-infected and C. trachomatis singly-infected samples (Fig. 2.3A, lanes 4 and 5). Likewise, the amount of HSV-2 specific amplimer appears unchanged by C. trachomatis co-infection (Fig. 2.3A, lanes 3 and 5). Amplification was never observed in negative (no template) controls (Fig. 2.3A, lane 1). GAPDH amplification products were observed in all samples at similar amounts (data not shown). HSV and chlamydia-specific products were observed only in the appropriate samples, confirming amplification specificity. The experiment shown in Figure 2.3A was repeated 3 times; 6 log dilution series of known DNA template controls were also amplified and used to generate amplification standard curves. All experimental samples quantified fell within the linear range of the PCR amplification as determined from the appropriate, co-amplified, standard curve. Chlamydial 16S rRNA and HSV G2 amplifier quantities were then normalized to host genome copy number as ascertained by PCR with human GAPDH specific primers and plotted in Figure 2.3B. Statistical analyses indicated that there was no difference in either chlamydial or HSV genome copy number in co-infected versus singly-infected cells (Fig. 2.3B).
Co-infection does not alter accumulation of chlamydial dnaA, ftsW, ftsK, groEL-1, or unprocessed 16S rRNA transcripts.

Several investigators have demonstrated that expression of one or both of the chlamydial cell division genes, ftsK and ftsW, changes after persistence induction. In contrast, expression of the DNA replication gene dnaA, remains unchanged (Byrne et al., 2001; Gerard et al., 2001;
Nicholson and Stephens, 2002). Also, by definition, persistent chlamydiae are viable but non-infectious. As a result, transcription of unprocessed 16S rRNA continues during persistence and, thus, would be expected to remain unchanged compared to actively dividing, viable chlamydiae. Co-infected and singly-infected cell cDNAs (Fig. 2.4) were subjected to RT-PCR using primers specific for the chlamydial dnaA, fisW, fisK, groEL-1 and unprocessed 16S rRNA transcripts. All amplimers were the expected size (Fig. 2.4A) and the identity of each was confirmed by DNA sequencing (data not shown). The quantity of each specific product was similar in both co-infected and C. trachomatis singly-infected samples (Fig. 2.4A, lanes 4 and 5). Amplification was not observed in negative (no template) controls (Fig. 2.4A, lane 1) or in RT(-) controls (data not shown). Specific amplimers were observed only in C. trachomatis singly and co-infected cells, confirming amplification specificity. Amplimers were quantified, compared to amplification standard curves, and then normalized to relative chlamydial genome copy number from that same sample as ascertained by PCR in Figure 2.3. Statistical analyses indicated that there was no significant difference in accumulation of any of the chlamydial transcripts in co-infected versus C. trachomatis singly-infected cells (Fig. 2.4B).
Figure 2.4. Co-infection does not alter accumulation of chlamydial dnaA, ftsW, ftsK, groEL-1, or unprocessed 16S rRNA transcripts. (A) HeLa cells were mock-infected (Mock), C. trachomatis infected (Ct), HSV-2-infected (HSV), and C. trachomatis/HSV-2 co-infected (Ct/HSV) as described in Figure 2.1A. Total cellular RNA was collected from each group of cells at 20 h post-HSV-2 infection and subjected to RT-PCR analyses using transcript specific oligonucleotide probes. Chlamydial dnaA (501 bp), ftsW (274 bp), ftsK (351 bp), groEL-1 (223 bp), and 16S rRNA primary transcript (466 bp) amplimers were electrophoresed and photographed; representative photographs are shown. The position of DNA size markers are shown to the left of each gel image in base pairs (bp). (B) Specific DNA amplimers were quantified as described. Amplimer quantities were normalized to chlamydial genome copy number (Fig. 2.3B). The values plotted are averages of three independent experiments; the value obtained for each independent experiment is the average obtained from three biological replicates. The average integrated intensities for dnaA (white bars), ftsW (grey bars), ftsK (black bars), groEL-1 (vertically striped bars) and 16S rRNA primary transcript (diagonally striped bars) were plotted versus sample identity (X-axis). Mock and HSV singly-infected reactions were negative for these amplimers; therefore, only data for chlamydiae singly-infected and co-infected cells are shown.
HSV-2 super-infection reduces accumulation of chlamydial MOMP and increases accumulation of HSP60.

Accumulation of chlamydial MOMP is often decreased when RBs enter a persistent state; in contrast, HSP60-1 levels are usually stable or increased (Cevenini et al., 1988; Beatty, et al., 1993; Beatty et al., 1994b). To determine whether HSV-2 super-infection altered MOMP or HSP60-1 accumulation, Western blot analyses were performed at 0, 5, 10, 15, and 20 h post-HSV infection. Single bands of the correct size for MOMP (40 kD) and HSP60-1 (60kD), were observed in C. trachomatis singly-infected and co-infected cell lysates but not in mock or in HSV-singly-infected cell lysates (Fig. 2.5A). The HSV-2 ICP-8 and ICP-5 proteins were observed in only HSV singly and co-infected samples (Fig. 2.5A). Bands were quantified, normalized to focal adhesion kinase (FAK-a host cellular control protein) and plotted in Figure 2.5B. Statistical analyses have demonstrated that FAK accumulation does not change over the course of the experiment (data not shown). MOMP accumulation was clearly decreased at 5, 10, 15, and 20 h post-HSV infection in co-infected cells. In contrast, HSP60-1 accumulation was increased in co-infected cells, particularly at 10 h post-HSV infection. Accumulation of the HSV-2 proteins ICP8 and ICP5 was either unchanged or diminished by chlamydial co-infection (Fig. 2.5B). Similar results were obtained in two other independent time-course experiments.
Figure 2.5. Chlamydial major outer membrane protein (MOMP) accumulation is reduced in co-infected cells. (A) HeLa cells were mock-infected (Mock), *C. trachomatis* infected (Ct-25), HSV-2-infected (HSV), and *C. trachomatis*/HSV-2 co-infected (Ct-25/HSV) as described in Figure 2.1A. Cell lysates were collected from each group of cells at 0, 5, 10, 15, and 20 h post-HSV-2 infection and Western blotted using anti-*C. trachomatis* MOMP, anti-*C. trachomatis* HSP60-1, anti-HSV-2 ICP-5, anti-HSV-2 ICP-8, or anti-human FAK sera. Three independent experiments were performed; a representative autoradiogram is shown. (B) MOMP, HSP60-1, ICP-5, and ICP-8 proteins present in cell lysates from the experiment in panel A were quantified by phosphorimage analysis. The integrated intensities were normalized to the internal control protein (FAK) and plotted in panel B as integrated intensity versus time post-HSV-2 infection.
C. trachomatis co-infection does not significantly alter HSV-2 progeny virion release or viral ultrastructure.

To determine whether chlamydial pre-infection altered subsequent viral replication, HSV-2 production/release from singly and co-infected cells was quantified using plaque assays (Fig. 2.6A). Mock-infected and C. trachomatis singly-infected cells produced no virions (Fig. 2.6A). While co-infected cells appeared to release a greater quantity of HSV-2 progeny virions at the later time intervals (22-30 h post viral infection), at no time was there greater than 10% difference between the two groups. These data suggested that co-infection of HSV-infected HeLa cells with chlamydiae did not significantly affect HSV-2 replication kinetics or release of infectious HSV-2 progeny virions.

It was possible that co-infection might have decreased HSV-2 virion production and increased release such that the supernatant virus levels would have appeared unaffected. Therefore, cell pellets and culture supernatants were collected at 20 h post-virus infection and used for virus titer assays. Total PFUs (plaque forming units) produced in each culture was determined by adding the cell-associated and supernatant virus titers; the data obtained from four independent experiments are plotted in Figure 2.6B. Again, the total amount of virus obtained from co-infected cells tended to be slightly higher than that obtained from HSV singly-infected cells; however, the difference was not statistically significant. These data indicated that chlamydial co-infection had little, if any, effect on HSV-2 replication.

Viral morphology was also studied by TEM. In HSV-2 singly and co-infected cells viral capsids could be observed as early as 10 h post-infection (Fig. 2.2E). By 20 h post-infection, arrays of capsids were readily apparent in the nucleus of HSV-2 singly-infected and co-infected cells (Fig. 2.2I). Comparison of viral particles in singly and co-infected cells indicated that virion
morphology was unaltered by chlamydial co-infection (data not shown). Considered together, these data indicated that chlamydial co-infection did not significantly alter HSV-2 replication.

Figure 2.6. Chlamydial pre-infection does not significantly effect HSV-2 replication or release. (A) HeLa cells were mock-infected (Mock), HSV-2-infected (HSV), *C. trachomatis* infected and *C. trachomatis*/HSV-2 co-infected (Ct-25/HSV) as described (Fig. 2.1A). Supernatants (sup) were collected from each group of cells 2, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30 hours post-HSV-2 infection. Plaque assays were performed as described. Plaques were counted and the number of PFU per ml supernatant at the end of each time interval was calculated. These values were plotted in panel A. *C. trachomatis* singly-infected supernatants were identical to the mock values and are not shown. (B) HeLa cells were infected as described (Fig. 2.1A). Culture supernatants and cell pellets were collected from each group of cells at 20 h post-HSV-2 infection. Plaque assays were performed and the total number of PFU per culture was calculated by adding the values obtained for cell associated and supernatant virus. Average PFU/culture from 4 independent experiments ± the SEM were plotted on the histogram in panel B.

Discussion

While previous studies (Pontefract et al., 1989; Chiarini et al., 1996; Superti et al., 2001) suggested that HSV-2 co-infection alters the chlamydial developmental cycle, none of the previous studies determined whether a persistent chlamydial phenotype was induced. A number of experimental parameters have been used as “markers” of chlamydial persistence. These include: altered inclusion size, abnormal RB morphology, decreased progeny EB infectivity,
decreased MOMP and stable/increased HSP60 accumulation, unchanged DNA copy number, and decreased *ftsK* and/or *ftsW* expression (Beatty *et al*., 1994c; Darville, 2000; Hogan *et al*., 2004). However, down-regulation of *ftsK* and *ftsW* tend to be variable in the persistent state. For example, accumulation of *ftsK* and *ftsW* RNA is reduced in *C. trachomatis* serovar K infected monocytes as well as in IFN-γ exposed *C. pneumoniae* (Byrne *et al*., 2001; Gerard *et al*., 2001). In contrast, Nicholson and Stephens demonstrated that in penicillin-exposed, *C. trachomatis* serovar D infected cells, *ftsW* remains unchanged while *ftsK* is elevated (Nicholson and Stephens, 2002). Finally, in *C. pneumoniae* continuous infection, *ftsK* expression is unchanged (Hogan *et al*., 2003). It is likely that many “markers” of persistence vary according to the inducer, host cell, chlamydial species, and timing used (Hogan *et al*., 2004), which increases the importance of examining as many of the different markers as is feasible.

Because most of the previously mentioned markers were observed during HSV co-infection, it seems likely that HSV super-infection of *C. trachomatis* infected HeLa cells induces development of a persistent chlamydial state. By definition, persistent chlamydiae are viable but non-infectious. As a result, transcription of unprocessed 16S rRNA continues during persistence and, thus, would be expected to remain unchanged compared to that in actively dividing, viable chlamydiae. The observation that accumulation of unprocessed 16S rRNA is unaltered but chlamydial infectivity is abrogated, thus, indicated that HSV co-infection renders the chlamydae viable but non-infectious, the hallmark of the persistent state. Because co-infected cells quickly die in culture (approximately 30 h post-HSV addition), this is not long-term persistence in the classical sense. However, because it is similar in many respects, we shall continue to refer to HSV-induced, chlamydial developmental changes as persistence.
An alternate interpretation of the observation that co-infection reduces chlamydial infectivity and MOMP expression is that HSV super-infection simply delays the chlamydial developmental cycle. If HSV causes a chlamydial developmental cycle delay, one would expect chlamydial DNA replication to be slowed or halted. In contrast, persistent chlamydiae continue to replicate their chromosome (Gerard et al., 2001). Thus, the observation that chlamydial genome copy number was similar in co-infected and singly-infected cells strongly supports the conclusion that HSV super-infection of \textit{C. trachomatis} infected HeLa cells induces development of chlamydial persistence rather than simply delaying the chlamydial developmental cycle. Additionally, delayed chlamydial development would not be expected to induce the alterations in EB morphology observed in co-infected cells.

There are a number of mechanisms by which HSV-2 super-infection could induce chlamydial persistence. One simple explanation is that HSV-2-induced cell death aborts the chlamydial developmental cycle. Chlamydial morphologic alterations are observed as early as 5 h post-HSV infection, much earlier than HSV-induced cell death usually occurs (Roizman and Knipe, 2001). Trypan blue staining experiments indicate that >99% of co-infected HeLa cells are viable at 20 h post-HSV infection (R.V. Schoborg, unpublished data). Also, TEM analysis of co-infected cells at 20 hours post-HSV infection does not reveal any ultrastructural alterations associated with cell death. These observations argue that cell death is not inducing this effect.

Amino acid starvation is a well-studied inducer of \textit{C. trachomatis} persistence (Beatty et al., 1994c; Darville, 2000), suggesting that HSV-2 might “out-compete” the chlamydiae for cellular amino acid pools. However, persistent-like chlamydial morphological changes are observed at 5 and 10 h post-HSV addition, well before the bulk of HSV-2 protein synthesis occurs. Additionally, the HSV-2 VHS (virion host shut off) and ICP27 proteins act early in the
replication cycle to diminish host gene expression (Roizman and Knipe, 2001). Thus, at early
times post-HSV infection, it seems likely that amino acids would be more readily available to
support both HSV-2 and chlamydial replication. These observations reduce the likelihood that
HSV-induced chlamydial persistence is simply an effect of amino acid depletion.

A more interesting possibility is that a specific viral protein(s) could induce chlamydial
.persistence. If so, this protein is most likely a virion component or an early gene product because
the effect is evident by 5 hours post-HSV infection. The HSV-2 VHS and ICP27 proteins are
known to profoundly influence host cell gene expression. VHS is a virion component that
suppresses host transcription beginning immediately after entry (Kwong et al., 1988). ICP27 is a
viral inhibitor of host cell mRNA splicing/transport that is expressed early in the replication
cycle (Smith et al., 1992; Sandri-Goldin and Mendoza, 1992; Hardy and Sandri-Goldin, 1994).
Although either protein could induce chlamydial persistence, it is not likely to be due to their
effects on host gene expression because cyclohexamide-exposure is known to enhance
chlamydial development (Ripa and Mardh, 1977).

A previously published study suggested that pre-infection of host cells with *C.
trachomatis* increases HSV-2 replication by up to 40%, as quantified by IFA (Chiarini et al.,
1996). In contrast, we did not observe any difference in staining intensity or frequency in co-
infectected compared to HSV singly-infected cells. Also, plaque assays show no significant
difference in total virus production from singly and co-infected cells. Because any major effect
of co-infection on viral replication would be readily visible as an increase or decrease in viral
progeny production, these data indicate that chlamydial pre-infection does not significantly affect
HSV-2 replication.
Induction of chlamydial persistence by HSV-2 co-infection has significant implications for pathogenesis. The presence of membrane blebs similar to those observed in chlamydia/HSV co-infected cells has been associated with release of chlamydial LPS from persistently-infected cells (Karimi et al., 1989; Wyrick et al., 1994; Wyrick et al., 1999). Because complications of chlamydial infection are thought to be due, at least in part, to release of inflammatory mediators from infected cells, any stimulus that increases release of these compounds might enhance disease pathology. Thus, it is possible that co-infection will result in increased release of inflammatory molecules and more severe immunopathology compared to that in an individual infected with either agent alone.

Co-infection in vivo could also influence transmission or immunity to either organism. Both *C. trachomatis* and HSV-2 have been shown to modulate immune cell function. For example, HSV infection suppresses dendritic cell function (Pollara et al., 2003). *C. trachomatis* infection increases IL-11 production, which may suppress local immune responses and aid in establishment of infection (Dessus-Babus et al., 2000). It is easy to envision how an organism that manipulates the local immune response in the genital tract might also suppress or alter the host response to other pathogens that are present at the same time. Also, super- or co-infection could increase genital tract susceptibility or increase shedding of either organism by induction of inflammatory lesions in a manner analogous to that proposed in co-infections with HIV (Cameron et al., 1989; Plummer et al., 1991; Laga et al., 1993; McClelland et al., 2001; McClelland et al., 2002). Determination of the effect of co-infection on disease severity and transmission is dependent upon determination of the exact mechanism by which HSV interferes with the chlamydial developmental cycle and development of an animal co-infection model, studies that are currently ongoing in our laboratory.
Experimental Procedures

*Chlamydia*, HSV-2, and host cells.

A human urogenital isolate of *C. trachomatis* E/UW-5/CX was originally obtained from S.P. Wang and C. C Kuo (University of Washington, Seattle, WA). The same standardized inoculum of *C. trachomatis* serovar E elementary bodies, propagated in McCoy cells, was used for all experiments (Wyrick *et al.*, 1996). Herpes simplex type 2 strain 333 stock was prepared in monolayers of CV-1 simian kidney cells using standard techniques (Duff and Rapp, 1971).

Co-Infection experimental design.

HeLa cells, a cervical adenocarcinoma epithelial cell line (ATCC #CCL2), were used for all infection experiments. They were propagated in MEM with Earle’s salts containing L-glutamine, 10% fetal calf serum (Atlanta Biologicals), and 1 µg/ml gentamycin at 37°C in an atmosphere of 5% CO₂. HeLa cells were divided into four groups, 1 x 10⁶ cells per 60 mm culture dish, for mock infection, chlamydial infection, HSV-2 infection, and both *C. trachomatis* and HSV-2 double infection (Figure 2.1A). Host cells were infected with a titer of crude EB stock (200 µl) calculated to infect 80% of the HeLa cells and with HSV-2 at a multiplicity of infection (MOI) of 10 PFU/cell; mock-infected cells were treated similarly except they were exposed to 200 µl of either 2SPG (0.2 M sucrose, 6mM NaH₂PO₄, 15mM Na₂HPO₄, 5mM L-glutamine, pH 7.2; mock *C. trachomatis* infected) or growth medium (mock viral infection).
Microscopy and image analysis.

For fluorescence analyses, infected cells were fixed and permeabilized as described (Saltarelli et al., 1994). A pool of FITC-conjugated monoclonal antibodies generated against *C. trachomatis* MOMP (SYVA MicroTrak, Wampole Laboratories) was used to visualize chlamydial inclusions. Monoclonal antibodies against HSV-2 ICP5 (the major capsid protein; Virusys Corp.) and ICP8 (the viral DNA binding protein; Virusys Corp.) were combined, diluted 1/200 in PBT buffer (1X PBS, 1% BSA, 0.05% Tween 20) and used to immunostain for HSV-2 for 1 h at 37°C. After washing, HSV-2 antigens were visualized by staining with a Texas Red-conjugated secondary anti-murine IgG (Jackson Immuno Research) diluted 1/100 in PBT buffer for 1 h at 37°C. Stained monolayers were washed, mounted, and photographed at 320X using a Zeiss Axiovert S100 inverted microscope and Axiocam camera. FITC-labeled cells were photographed at an exposure of 8 s using a FITC-band pass filter (excitation 470 nm and emission 540 nm); Texas Red-labeled cells were photographed at an exposure of 2 s using a Rhodamine-long pass filter (excitation 546 nm and emission 590 nm).

Selected photomicrographs were subjected to image analysis using the Quantity One software package (Version 4.2.1, Biorad). The relative area of each fluorescent focus was determined using the software volume analysis function. Ten random fields (40-50 inclusions/field) from each experimental group (mock, HSV-2-infected, *C. trachomatis* infected and co-infected cells) were analyzed blind and the average inclusion size for each set was determined.

Duplicate samples of infected HeLa cells were processed at 5, 10, 15, and 20 h for high contrast transmission electron microscopy as described (Wyrick et al., 1994). Counterstained
gold thin sections were examined using a Tecnai 10 (FEI) transmission electron microscope operating at 60-80 kV.

**Chlamydial titrations by subpassage.**

At 20 hours post-HSV-2 infection, monolayers were scraped into 1 ml cold growth medium, pelleted, and resuspended in 1 ml fresh medium. Infected host cells were lysed by freeze/thaw and sonication and centrifuged (500 X g for 5 min) to pellet cell debris. Supernatants were centrifuged twice (8000 X g for 30 min). Control experiments demonstrated that >95% of the “contaminating” HSV-2 was removed by discarding supernatants from the two high speed centrifugation steps, while >75% of the input chlamydial EBs were recovered in the pellet (data not shown). Final EB pellets were resuspended in 200µl 2SPG, diluted (1/5 to 1/200) and used to infect HeLa cells plated on glass coverslips in triplicate. After infection, the HeLa monolayers were refed with medium containing 1µg/ml cyclohexamide and 400µM phosphonoformate (Cheng et al., 1981). Neither drug interferes with chlamydial inclusion development while completely abrogating productive replication of HSV-2 (data not shown). HeLa monolayers were incubated at 35°C for 48 h, fixed, and stained with SYVA stain as described above. The number of inclusions in 10 random microscopic fields per sample was determined using a Zeiss Axiovert S100 Microscope at 320X. Triplicate coverslips were counted and the counts averaged. The number of inclusion forming units (IFU) in the undiluted inoculum was then calculated and expressed as IFU/10⁶ cells.
RNA and DNA isolation.

Total DNA and RNA were simultaneously isolated from the same plate of cells without manipulation of the cells before the lysis step using modifications to the RNeasy Mini (Qiagen) and QIAmp DNA Blood Mini (Qiagen) kits. After aspiration of the culture medium, 750 μl of RLT (lysis) buffer (RNeasy Mini Kit) was immediately added to each monolayer. The plates were scraped and the lysate passed 10 times through a 20-gauge needle using a 1cc syringe. Six hundred μl of cell lysate was used for total RNA isolation and DNase treatment using the Qiagen RNase-free DNase Kit following the manufacturer’s protocol. One-hundred fifty μl of each cell lysate was diluted with 50 μl of 1XPBS and used for total DNA isolation as per the kit instructions. Control experiments with known quantities of RNA and DNA demonstrated that this modification did not alter recovery efficiency or quality of isolated host cellular, chlamydial or HSV nucleic acids (data not shown). Total RNA and DNA preparations were quantified using optical density (OD) at 260 and 280 nm; all samples had OD260/280 ratios > 1.9. The concentration and integrity of each RNA sample was further confirmed by formaldehyde-agarose gel electrophoresis and ethidium bromide (EtBr) staining (data not shown).

Reverse transcription, PCR and RT-PCR.

One μg of total RNA was subjected to reverse transcription using SuperScript II RNase H minus reverse transcriptase (RT; Invitrogen) and random hexamer primers (Pharmacia) as per the manufacturer’s suggested protocol. Each reaction also contained 20U of Super RNase Inhibitor (Ambion). Duplicate reactions containing ddH2O substituted for RT (RT – reactions) were performed in parallel. PCR was performed using total cellular DNA or cDNA for RT+ and RT-reactions as appropriate. Experimental template DNAs were used at dilutions ranging from 1/10
to 1/1000 (in ddH₂O) such that each reaction was in the linear amplification range. Amplification was performed with MasterTaq (Eppendorf) under the manufacturer’s recommended reaction conditions. Human GAPDH, chlamydial dnaA and HSVG2 reactions were run without MasterTaq enhancer (Eppendorf); all other reactions contained enhancer at 1X concentration. Published primer sets included chlamydial dnaA, fisK, fisW (Gerard et al., 2001), chlamydial 16S rRNA primary transcript (Gerard et al., 1997), and HSV-2 glycoprotein gene 2 (HSV G2, Filen et al., 2004). We designed specific primers for chlamydial CT-110/groEL-1 (forward: 5′-GAG TTA AGA CTT TAG CTG AAG CTG T-3′, reverse: 5′-GTA GCT GTT GTA GTT CCG TCT CCA-3′), chlamydial 16S rRNA (forward: 5′-GGA CGG AAG TCT GAC GAA-3′, reverse: 5′-TCA AATCCA GCG GGT ATT-3′) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH- forward: 5′-GTC CAC CAC TGA CAC GTT G-3′, reverse: 5′-GGG AAA CTG TGG CGT GAT-3′). Most PCR reactions were heated to 94°C for 1 min and then cycled under the following conditions 30 times: 94°C, 1 min; 58°C, 1 min; 72°C, 1 min. Human GAPDH reactions were performed under identical conditions but were cycled 35 times; chlamydial dnaA PCR reactions were performed for 30 cycles but annealed at 54°C. After PCR, all reactions were electrophoresed on 1.5% agarose/TBE gels. Gels were stained with EtBr and visualized using a BioRad Chemi Doc XRS Image Capture System. Amplimers were quantified on the image analysis system using Quantity One V4.5.0 software (BioRad).

**SDS-PAGE and western blotting.** At various times post-HSV-2 infection, monolayers were lysed in 1 ml 1X PBS + 0.1% SDS + 0.1% Nonidet NP-40. Equal cell equivalents of each lysate (2x10⁴ cells) were electrophoresed; gels were then Coomassie stained, fixed, and dried. Densitometric analysis
indicated that the variation in protein concentrations between samples was <5%. Total protein concentrations for each set of lysates were also determined using the Micro-BCA method (Pierce), which confirmed the results obtained by Coomassie stains (data not shown). For determination of relative protein accumulation, 1 x 10⁴ cell equivalents of each lysate were electrophoresed on 4-12% NuPage precast gels using the MES-SDS buffer system (Invitrogen). Gels were electrophoresed at 150V for 25 min and blotted to PVDF membranes (Pall, Inc.) for 1 hour at 200mA. Blots were blocked in 1X PBS + 0.1% Tween 20 + 5% nonfat dried milk (BLOTTO) for 1 h at 25°C. Replicate blocked blots were incubated with a 1/1000 to 1/5000 dilutions the following primary antibodies in BLOTTO: polyclonal goat anti-MOMP (OEM Concepts); polyclonal, monospecific rabbit anti-HSP60-1 (a kind gift from J. Raulston); polyclonal rabbit anti-human FAKC20 (SantaCruz Biologicals) or monoclonal antibodies against HSV-2 ICP5 or ICP8 (Virusys Corp.). Blots were incubated for 1 h, washed 5 times in 1X PBS + 0.1% Tween 20 (PBS-T), and reprobed with peroxidase-conjugated rabbit anti-goat, rabbit anti-mouse, or goat anti-rabbit secondary antibodies (Cappel) at 1/20,000 dilution. After 5 additional washes, blots were subjected to chemiluminescent detection using SuperSignalWest Pico reagent (Pierce). Specific bands were quantified using an FX phosphorimager and Quantity One V2.5.0 software (BioRad).

**HSV-2 plaque assay.**

At various times post-HSV-2 infection, culture supernatants or frozen/thawed cell pellets were centrifuged at 4000 X g for 5 min and 4°C to remove cell debris. Plaque assays were carried out on the resulting supernatants as described (Duff and Rapp, 1971). Duplicate, infected cultures were incubated for 72 h at 37°C, fixed, stained, and counted in a blind fashion. Average
plaque counts from each set of duplicate plates were used to calculate the PFU/ml present in the original supernatant.

**Statistical analyses.**

Statistical analyses were performed using MiniTab (Version 9). Comparison of means was done by using a 2-sample t-test for independent samples. P values of $\leq 0.05$ were considered significant.

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References


CHAPTER 3

AN EARLY EVENT IN THE HERPES SIMPLEX VIRUS TYPE-2 REPLICATION CYCLE IS SUFFICIENT TO INDUCE CHLAMYDIA TRACHOMATIS PERSISTENCE.

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Key words: Chlamydial persistence, Chlamydia trachomatis, herpes simplex virus, sexually transmitted infection.

Abstract

Epidemiological studies have demonstrated that co-infections of herpes simplex virus type 2 (HSV-2) and Chlamydia trachomatis occur in vivo. Data from a tissue culture model of C. trachomatis/HSV-2 co-infection indicate that viral co-infection stimulates the formation of persistent chlamydiae. Transmission electron microscopic (TEM) analyses demonstrated that in both HeLa and HEC-1B cells co-infection caused developing chlamydiae to exhibit swollen, aberrantly shaped reticulate bodies (RBs), characteristically observed in persistence. Additionally, HSV-2 co-infection suppressed production of infectious chlamydial elementary bodies (EBs) in both host cell types. Co-infection with HSV type 1 (HSV-1) produced similar morphologic alterations and abrogated infectious EB production. These data indicate that virus-induced chlamydial persistence was neither host cell nor virus strain specific. Purification of crude HSV-2 stocks demonstrated that viral particles were required for co-infection-induced chlamydial persistence to occur. Finally, co-infection with either UV-inactivated, replication incompetent virus or with replication-competent HSV-2 in the presence of cyclohexamide reduced chlamydial infectivity without altering chlamydial genomic DNA accumulation. These data demonstrate that productive viral replication is not required for the induction of chlamydial persistence and suggest that HSV attachment and entry can provide the necessary stimulus to alter C. trachomatis development.
Introduction

Treatment of sexually transmitted diseases and their sequelae cost the United States billions of dollars each year. Approximately 19 million people in the US were infected with a STD in 2000. Nearly half of those individuals were between the ages of 15 and 24 (Weinstock et al., 2004). Given the fact that several STD agents can establish chronic or latent infections, clinical management of STDs remains a challenge of epidemic proportions. Two of the most common sexually transmitted disease agents are Herpes Simplex Virus Type 2 (HSV-2) causing 200,000-500,000 new infections annually and Chlamydia trachomatis serovars D-K causing 4 million new infections annually (Weinstock et al., 2004; Butler, 1997).

Herpes Simplex Virus Type 1 (HSV-1) and HSV-2 are enveloped DNA viruses of the viral family Herpesviridae. Upon infection, herpes simplex viruses bind to the host cell receptor heparin sulfate. The initial binding event is followed by interaction of viral envelope glycoproteins with one of four known co-receptors that facilitate viral entry (Spear, 2004). Once inside the host cell, viral tegument proteins are released into the host cell and the capsid is transported to the nucleus, where viral genome replication occurs. The viral genome is transcribed and viral proteins are synthesized using host cell machinery. New virions are then assembled and exit the host cell (Roizman, 2001).

Herpes Simplex Virus Type-2 is the primary cause of genital herpes infection. HSV-2 infection usually occurs on the mucous membranes and skin surrounding the genitals causing a characteristic lesion. After primary infection, HSV-2 can establish a latent, life-long infection in the neurons of the sacral ganglia. On average, latent HSV-2 infections are reactivated five times each year. Upon reactivation, the virus travels through the neurons to the site of initial infection where it can be shed from skin lesions (Corey et al., 1983). Although most genital HSV
infections are clinically mild, HSV-2 can also be responsible for serious diseases such as keratitis and meningitis (Roizman, 2001).

*Chlamydia trachomatis* is a Gram-negative, obligate intracellular bacterium that has a biphasic developmental cycle. During the infectious phase of the cycle, chlamydiae exist as small, infectious, metabolically inert, elementary bodies (EBs). The EBs attach and enter a host cell, where they differentiate within an inclusion into the non-infectious, vegetative form of the bacterium called the reticulate body (RB). Once the RBs have completed several rounds of replication, they condense to form EBs. The EBs are then released and can infect new host cells (Wyrick, 2000). Genital chlamydial infections are often chronic and asymptomatic leading to severe complications including pelvic inflammatory disease, ectopic pregnancy, and infertility (Darville, 2000).

When developing chlamydiae are exposed to certain environmental factors, they deviate from the normal developmental cycle into a state called persistence (Hogan *et al.*, 2004). While in a persistent state, chlamydiae form large, abnormally shaped RBs. Although they remain viable, persistent chlamydiae are no longer infectious. Some of the most well studied inducers of chlamydial persistence include Interferon-γ (IFN-γ) exposure, amino acid and iron deficiencies, and exposure to penicillin G (Gerard *et al.*, 2001; Darville, 2000; Raulston, 1997; Beatty *et al.*, 1994a).

Several studies have shown that *C. trachomatis* and HSV-2 co-infections occur *in vivo*. Both pathogens have been simultaneously isolated from women afflicted with endometritis, salpingitis, and cystitis (Paavonen *et al.*, 1985; Tait *et al.*, 1985). Serologic investigations indicate that HSV-2 positive individuals are likely to be *C. trachomatis* positive as well (Silins *et al.*, 2002; Paroli *et al.*, 1990). Several studies have documented seropositivity rates of >50% for
both pathogens, implying that some of the individuals had been exposed to both HSV-2 and *C. trachomatis* (Wagner *et al.*, 1994; Duncan *et al.*, 1992). Although IgG seropositivity does not prove coexisting infection, there is a high probability that some of the individuals were infected simultaneously with both pathogens.

*In vitro* models of HSV-2/*C. trachomatis* co-infections have also been established. Transmission electron microscopy (TEM) analyses of Vero cells co-infected with *C. trachomatis* serovar L2 and HSV-2 revealed swollen inclusions with few RBs or EBs (Pontefract *et al.*, 1989). Chiarini *et al.* reported that the number of cells positive for chlamydiae by immunofluorescence was reduced when HeLa cells were co-infected with HSV-2/*C. trachomatis* serovar D (Chiarini *et al.*, 1996). The number of chlamydial inclusions and infectious EBs were also reduced in HSV-2 pre-infected HT1376 human bladder cells (Superti *et al.*, 2001). Although these observations indicate that HSV-2 co-infection alters chlamydial development, these studies did not examine the co-infection process in detail.

Previously, we developed a tissue culture model of *C. trachomatis* and HSV-2 co-infection. Data from these studies indicate that during *C. trachomatis* serovar E and HSV-2 co-infection, chlamydiae become persistent. Such data include abnormal ultrastructure and decreased production of infectious EBs. Although less infectious, chlamydiae within HSV-2 co-infected cells remain viable as demonstrated by continued accumulation of precursor 16S rRNA and chlamydial genomes (Deka *et al.*, 2006). However, the mechanism by which HSV-2 induces chlamydial persistence is currently unknown. As a first step in defining how viral-induced persistence occurs, we have determined whether productive viral replication is necessary for induction of chlamydial persistence. The present study will allow us to begin to unravel the mechanism by which HSV-2 causes *C. trachomatis* to enter persistence.
Results

HSV-2 induction of chlamydial persistence is not host cell type specific.

Previous studies have shown that host cell interaction with chlamydiae can be a determining factor in the induction of chlamydial persistence. For example, IFN-γ induction of chlamydial persistence has been shown to occur in several host cell types, including HeLa cells (Hogan et al., 2003). However, developing chlamydiae within HEC-1B cells are resistant to IFN-γ because these host cells do not produce indoleamine 2,3-dioxgenase in response to IFN-γ (Wyrick and Knight, 2004; Kane and Byrne, 1998). To be certain that induction of chlamydial persistence during HSV-2 co-infection is not a specific property of HeLa cells, HEC-1B cells were also used as host cells for co-infection. Duplicate monolayers of HeLa and HEC-1B cells were either mock, singly, or co-infected. Cells were collected 20 hours post-HSV-2 infection and processed for either TEM or chlamydial EB titration. Electron micrographs demonstrated that chlamydiae within both HeLa and HEC-1B co-infected cells exhibited abnormal RB morphology characteristic of persistence (Figure 3.1, B and E). EBs were absent from chlamydial inclusions within HSV-2 co-infected HeLa and HEC-1B cells (Figure 3.1, B and E). In contrast, in both C. trachomatis singly-infected cell types, RBs appeared normal and EBs were present (Figure 3.1, A and D). Furthermore, chlamydial EB titer assays indicated a significant decrease in the production of infectious EBs during HSV-2 co-infection in both host cell types (Figure 3.2, A and C) when compared to chlamydia singly-infected controls. Interestingly, singly-infected HEC-1B cells consistently produced higher titers of chlamydiae than did HeLa cells. These data indicate that HSV-2 co-infection-induced persistence is not limited to a specific host cell type.
Figure 3.1: HSV co-infection induces persistent chlamydial morphology. Cultures of HeLa (Panels A, B, C, and F) or HEC-1B (Panels D and E) cells were *C. trachomatis* infected or co-infected with *C. trachomatis* and HSV-2 strain 333 (Panels A, B, D, E, and F) or HSV-1 strain KOS (Panel C) at 10 MOI (Panels A, B, C, D, and E) or 1 MOI (Panel F). Cells were harvested for TEM analyses. Black arrows on electron micrographs indicate EBs and white arrows indicate RBs.

**HSV induction of chlamydial persistence is not virus specific.**

Although HSV-2 is the primary agent of genital herpes infection, Herpes Simplex Virus Type 1 (HSV-1) infections also account for many genital infections *in vivo* (Whitley, 2001). Therefore, the effect of HSV-1 co-infection on *C. trachomatis* development was examined. HeLa cell monolayers were co-infected with *C. trachomatis* serovar E and HSV-1 strain KOS as described. *C. trachomatis* singly-infected cells contained abundant EBs and RBs of normal morphology (Figure 3.1A). However, in HSV-1 co-infected cells, RBs were enlarged and
misshapen and few or no EBs were observed (Figure 3.1C). Chlamydial infectivity was also significantly reduced by HSV-1 co-infection compared to that in *C. trachomatis* singly-infected controls (Figure 3.2B). These data were indistinguishable from the results observed with HSV-2 in HeLa cells (Fig 3.1B and Fig 3.2A), thus indicating that both HSV-2 and HSV-1 co-infection can interfere with normal chlamydial development.

![Graphs A, B, C, D](image.png)

**Figure 3.2:** HSV co-infection decreases chlamydial infectivity. Cultures of HeLa or HEC-1B cells were mock, *C. trachomatis*, or HSV singly-infected and *C. trachomatis*/HSV co-infected. Cells were harvested for EB titration assays. A. HeLa cells were co-infected with HSV-2 strain 333 at 10 MOI. B. HeLa cells were co-infected with HSV-1 strain KOS at 10 MOI. C. HEC-1B cells were co-infected with HSV-2 strain 333 at 10 MOI. D. HeLa cells were co-infected with HSV-2 strain 333 at 0.1, 1, or 10 MOI. EB titers are expressed as IFU/ml sample ± SEM; n=3. Asterisks (*) indicate titers that are significantly different (by t-test) compared to those from *C. trachomatis* singly-infected cells (p<0.05). The data shown are representative of three independent experiments.
HSV-2 induction of chlamydial persistence is viral dose dependent.

In order to ensure that every chlamydiae-infected cell was also infected with HSV-2, co-infections were originally conducted using a viral MOI of 10 PFU/cell. To examine whether chlamydial persistence could be induced with lower concentrations of virus, co-infection experiments were performed using various MOIs of HSV-2. Triplicate HeLa cell monolayers were either mock, singly, or co-infected with *C. trachomatis* and HSV-2 at 10, 1, or 0.1 MOI. The cells were collected at 20 h post-HSV-2 infection and processed for TEM and chlamydial titration. Inclusions in *C. trachomatis* singly-infected cells contained normal developing EBs and RBs similar to those shown in Figure 3.1A. In cells co-infected with *C. trachomatis* and HSV-2 at 1 MOI, chlamydiae exhibited morphological characteristics of persistence (Figure 3.1F). Subpassage experiments revealed that HSV-2 co-infection at 10, 1, and 0.1 MOI significantly suppressed production of infectious EBs compared to that in *C. trachomatis* singly-infected cells (Figure 3.2D). Furthermore, the degree to which EB production was reduced correlated directly to increased viral MOI.

Purification of HSV-2 does not diminish its capacity to induce chlamydial persistence during co-infection.

It is well known that cellular host factors, especially the cytokines IFN-γ, IFN-α, TNF-α, can induce or enhance chlamydial persistence (Hogan *et al.*, 2004). Because the viral inocula used in this study were crude stocks, it is possible that these cytokines might be present in the HSV-2 stocks and could be responsible for the observed alterations in chlamydial development during co-infection. To test this possibility, crude lysates from either HSV-2 or mock-infected cells were subjected to centrifugal purification; the resultant purified components were used in
co-infection experiments with *C. trachomatis*. Four separate viral stocks were prepared: HSV-2 crude, mock crude, purified HSV-2, and purified mock. Luminex assays for IFN-γ, IFN-α, TNF-α, and IL-6 were performed on both crude and purified stocks to determine; 1) whether these cytokines were present and 2) whether these cytokines were removed by this purification method. Although IL-6 has not been associated with chlamydial persistence, HSV infection is known to induce this cytokine, thus it serves as a positive control (Kanangat S, 1996). Of the four cytokines examined, only IL-6 was detected in viral stocks. The HSV-2 crude and mock crude stocks contained IL-6 at 1,121 pg/ml and 2,835 pg/ml, respectively. IL-6 concentrations were reduced at least 43-fold by centrifugal purification (purified HSV-2, 26 pg/ml and purified mock, 38 pg/ml). These data demonstrate that the purification method used efficiently removed cytokines from the stocks and that IFN-γ, IFN-α, TNF-α cytokines known to alter chlamydial development were not present in any of the viral inocula used in this study.

Co-infection with crude and purified viral stocks was performed as described. Data from chlamydial titer assays indicated that only HSV-2 crude and purified HSV-2 stocks significantly decreased infectious EB yield during co-infection (Figure 3.3). Neither the mock crude nor purified mock stock controls reduced chlamydial infectivity in “co-infected” cells compared to *C. trachomatis* singly-infected cells (Figure 3.3). These data indicate that co-infection-induced chlamydial persistence is not mediated by IFN-γ, IFN-α, or TNF-α present in viral stocks and suggest that it is dependant on the presence of viral particles.
Figure 3.3: Purified HSV-2 co-infection decreases chlamydial infectivity. Cultures of HeLa cells were infected with *C. trachomatis*, or co-infected with chlamydia and HSV-2 crude stock, purified stock, mock crude stock, or mock purified stock at 10 MOI or the equivalent volume of mock stock. Cells were harvested for EB titration analyses. EB titers are expressed as IFU/ml sample ± SEM; n=3. Asterisks (*) indicate titers that are significantly different (by t-test) compared to those from *C. trachomatis* singly-infected cells (p<0.05). The data shown are representative of three independent experiments.

**Cyclohexamide exposure does not abrogate HSV-induced *C. trachomatis* persistence.**

Productive replication of HSV-2 within a host cell is dependant on host cell enzymes and machinery (Roizman, 2001). Cyclohexamide is a well known inhibitor of eukaryotic protein synthesis. By preventing host cell protein synthesis, cyclohexamide inhibits synthesis of viral proteins and prevents productive replication of HSV-2 (Vasquez, 1979; Swanstrom *et al.*, 1975). In contrast, cyclohexamide actually enhances *C. trachomatis* development (Ripa KT, 1977).

Therefore, cyclohexamide exposure was used to investigate whether productive viral replication is required to induce chlamydial persistence during co-infection with HSV-2. Monolayers of HeLa cells were either mock or chlamydiae infected, exposed to cyclohexamide, and HSV-2-infected as described in the methods. Control experiments indicated that productive viral replication and viral protein synthesis was inhibited by >95% under these exposure conditions, as
ascertained by plaque assays and S\textsuperscript{35}-methionine incorporation (data not shown). Co-infected cells exposed to either diluent (ddH\textsubscript{2}O) or cyclohexamide contained chlamydiae of abnormal morphology (Figure 3.4, B and E) in electron micrographs. Chlamydial inclusions in singly-infected cells contained normal RBs and developing EBs regardless of cyclohexamide exposure (Figure 3.4, A and D). As previously observed, the quantity of infectious EBs recovered from co-infected cells was significantly lower than that recovered from singly-infected cells (Figure 3.4C). Cyclohexamide exposure during co-infection did not reverse this result (Figure 3.4F).

**Figure 3.4:** Co-infection with HSV-2 in the presence of cyclohexamide induces chlamydial persistence. Cultures of HeLa cells were infected with \textit{C. trachomatis}. At 23 hours post-chlamydial infection the cells were refed with medium containing either diluent (ddH\textsubscript{2}O) (Panels A, B, and C) or 1 µg/ml cyclohexamide (Panels D, E, and F) for 1 h. The cells were then infected with HSV-2 inoculum containing cyclohexamide or ddH\textsubscript{2}O. The cells remained under cyclohexamide exposure until they were harvested for TEM and EB titration analyses. EBs on electron micrographs are indicated by black arrows; RBs by white arrows. EB titers are expressed as IFU/ml sample ± SEM; n=3. Asterisks (*) indicate titers that are significantly different (by t-test) compared to those from \textit{C. trachomatis} singly-infected cells (p<0.05). The data shown are representative of three independent experiments.
Co-infection with replication incompetent HSV-2 induces chlamydial persistence.

Although cyclohexamide greatly reduces productive viral replication, it does not completely block replication (Sanfilippo, 2004; Vasquez, 1979; Swanstrom et al., 1975). Previous studies have demonstrated that UV-inactivation of HSV-2 renders the virus completely replication incompetent (Moxley, 2002). Replication incompetent HSV-2 (HSV-2_{UV}) was generated using ultraviolet irradiation. A UV dose of 2.5 J/cm² sufficiently inactivated the virus so that no infectious HSV-2 virions were detectable in plaque assays (data not shown). However, UV irradiation did not affect PCR amplification of HSV-2 DNA (data not shown).

Duplicate HeLa cell monolayers were either mock, singly, or co-infected with \textit{C. trachomatis} and HSV-2 or HSV-2_{UV}. Infections with HSV-2_{UV} were performed using a volume of stock equivalent to 10 MOI of replication-competent HSV-2. Infected cell monolayers were collected and assayed for chlamydial titer, TEM, and PCR analyses at 0 and 20 h post-HSV infection. Supernatants were also collected from each sample for plaque assay analyses of viral replication. Infectious viral particles were only recovered from cultures infected with replication-competent HSV-2 (data not shown). Electron micrographs from chlamydia singly-infected cells depicted inclusions containing RBs of normal morphology and several developing EBs (Figure 3.5A). Alternatively, chlamydiae in HSV-2_{UV} co-infected cells exhibited the same swollen, abnormally shaped RBs that were observed in cells co-infected with replication-competent virus (Figure 3.5, B and C). Additionally, co-infection with HSV-2_{UV} decreased production of infectious EBs similarly to that observed with replication-competent HSV-2 (Figure 3.5D).

Total cellular DNA was isolated immediately following (T₀) and at 20 h (T₂₀) post-HSV-2 infection from singly-and co-infected cells from triplicate experiments and the accumulation of human, chlamydial, and HSV-2 DNA was determined by PCR. In each experiment, a six log
dilution series of known DNA template controls was used to generate amplification standard curves. Experimental samples were only quantified if they fell within the linear range of the PCR. Template negative samples were also included in every experiment; amplification in template-negative samples was never observed. Accumulation of host cell DNA as ascertained by human GAPDH amplification was similar in all samples (data not shown). Chlamydial 16S rRNA was only amplified in those samples infected with *C. trachomatis*. Likewise, HSV-2 gG DNA was amplified only in virus-infected samples (data not shown). Both chlamydial and HSV-2 genomic DNA was normalized to host cell DNA after quantification. The amount of HSV-2 DNA increased from T₀ to T₂₀ in all the samples infected with replication-competent HSV-2 (Figure 3.5E). However, the amount of HSV-2 DNA did not increase from T₀ to T₂₀ in those samples infected with HSV-2<sub>UV</sub>, indicating that there was no viral DNA replication during the infection (Figure 3.5E). It is important to note that the quantity of cell-associated HSV DNA at T₀ is similar in HSV-2- and HSV-2<sub>UV</sub> -infected cultures (Figure 3.5E), demonstrating that UV exposure does not inhibit virus/host cell attachment. Therefore, HSV-2<sub>UV</sub> viral particles were capable of attaching to the host cells but were unable to productively replicate. These data indicate that chlamydiae become persistent in the presence of replication-incompetent HSV-2.
Figure 3.5: Co-infection with HSV-2UV induces chlamydial persistence. Cultures of HeLa cells were C. trachomatis-infected followed by infection with replication-competent HSV-2 at 10 MOI or an equal amount of HSV-2UV. Cells were harvested for TEM (panels A, B, and C), C. trachomatis titer analyses (Panel D) and total DNA isolation (panel E) at 0 or 20 h post-HSV infection. HSV-2 viral stocks were UV-inactivated by exposure to 2.5 J/cm² UV irradiation at 4°C; plaque assays demonstrate that HSV-2UV stocks contain no replication-competent virions (data not shown). EBs on electron micrographs are indicated by black arrows; RBs by white arrows. EB titers are expressed as IFU/ml sample ± SEM; n=3. Asterisks (*) indicate titers that are significantly different (by t-test) compared to those from C. trachomatis singly-infected cells (p<0.05). The data shown are representative of three independent experiments.

Co-infection in the presence of cyclohexamide or with HSV-2UV does not alter accumulation of chlamydial DNA.

Chlamydiae in a persistent state remain viable and continue to replicate their DNA although they do not divide (Gerard et al., 2001). Therefore, the amount of chlamydial DNA should not differ between persistent and normal chlamydial infections. To ensure that
Chlamydiae remain viable and that replication of chlamydial DNA is not halted by co-infection, HeLa cells were co-infected with HSV-2/C. trachomatis and the cells were harvested at both T₀ and T₂₀ for PCR analyses. The quantity of chlamydial DNA amplified at T₂₀ is significantly higher than that at T₀, demonstrating that continued accumulation of chlamydial DNA occurred during HSV-2 co-infection (Figure 3.6A). These data show that both the analysis method is able to detect an increase in chlamydial DNA and that the chlamydiae remain viable.

Chlamydial DNA accumulation at T₂₀ was also measured in co-infected cultures performed in the presence of cyclohexamide or with HSV-2UV. PCR analyses demonstrated that there were similar amounts of chlamydial DNA in the singly and co-infected cells despite cyclohexamide exposure or HSV-2UV infection (Figure 3.6B). HSV-2 genome accumulation was also similar in HSV-2 singly and co-infected cells as previously observed (Deka et al., 2006). As expected, levels of HSV-2 DNA in co-infected cells exposed to cyclohexamide or infected with HSV-2UV were significantly decreased compared those in diluent-exposed/HSV-2 co-infected cells (data not shown). These results demonstrate that the chlamydiae within co-infected cells continue to accumulate genomic DNA in the presence of cyclohexamide or replication incompetent virus and, thus, are viable.
Figure 3.6: Co-infection does not alter accumulation of chlamydial DNA. Total DNA was purified and subjected to PCR analyses using host genome (GAPDH), and chlamydial genome (16S rRNA gene) specific DNA oligonucleotides. Amplimers were electrophoresed and quantified as described. The data in panel A were quantified after conversion from Macintosh to Personal Computer format where as the data in panel B were quantified using the Macintosh format, thus causing differences in the intensity scales. Chlamydial DNA quantities were normalized to host DNA as ascertained by PCR with human GAPDH-specific primers (data not shown). These data are representative of three independent experiments and are averages of three biological replicates. The average integrated intensity for each amplimer was plotted ± SEM. A. HeLa cells were co-infected with HSV-2/C. trachomatis. Total cellular DNA was collected at 0 (T₀) and 20 (T₂₀) h post-HSV-2 infection. B. HeLa cells were C. trachomatis infected, or co-infected with HSV-2, HSV-2UV, or HSV-2 in the presence of cyclohexamide. Total cellular DNA was collected at 20 h post-HSV infection.

Discussion

Chlamydiae that have deviated from the normal developmental cycle and exist in a viable, yet non-cultivable state are described as being persistent. Studies of chlamydial persistence have revealed several mechanisms by which developing chlamydiae are stimulated to enter this state. The most thoroughly investigated models of persistence include amino acid and iron deprivation, INF-γ exposure, and β-lactam antibiotic exposure (Gerard et al., 2001; Darville, 2000; Raulston, 1997; Beatty et al., 1994a). In general, it seems that when chlamydiae are presented with an environmental factor that is unfavorable for their growth, they respond by
entering persistence. Previous data from our laboratory indicate that viral co-infection also stimulates the formation of persistent chlamydiae as evidenced by abnormal RB morphology, and decreased chlamydial titer during co-infection (Deka et al., 2006). In this study, we have further characterized HSV-2 co-infection-induced persistence. In particular, we have demonstrated that this phenomenon is not dependent on a specific host cell type or HSV strain. Additionally, our data indicate that productive viral replication is not required for the induction of chlamydial persistence.

HSV-2/C. trachomatis co-infection has the potential to elicit several unfavorable environmental conditions for chlamydial growth, including amino acid deprivation and induction of IFN-γ. One of the most obvious potential mechanisms for co-infection-induced persistence is that the virus simply exhausts cellular amino acids to synthesize viral proteins, thereby, depriving the chlamydiae of nutrients. UV-inactivation of HSV-2 prevents viral DNA replication and viral protein synthesis (Moxley, 2002). Consequently, HSV-2UV would not be expected to deprive chlamydiae of amino acids by using them to manufacture viral proteins. Cyclohexamide also strongly inhibits HSV protein synthesis and enhances C. trachomatis development by inhibiting host cell protein synthesis (Ripa KT, 1977). However, cyclohexamide exposure during co-infection also did not prevent chlamydial persistence. Therefore, it does not appear that HSV-2 co-infection-induced chlamydial persistence is based upon simple amino acid deprivation.

A second possible mechanism for co-infection-induced persistence is that HSV-2 infection stimulates IFN-γ synthesis/release that, in turn, up-regulates indoleamine 2, 3-dioxygenase and depletes cellular tryptophan. Without this source of tryptophan, chlamydiae would be expected to enter a persistent state (Hogan et al., 2004; Beatty et al., 1994b). However, chlamydiae exhibit characteristics of persistence within co-infected HEC-1B cells. Previous
studies have demonstrated that chlamydiae do not respond to IFN-γ exposure in HEC-1B cells (Wyrick and Knight, 2004; Kane and Byrne, 1998). Therefore, it is unlikely that IFN-γ-induced IDO activity is responsible for co-infection-induced persistence.

In our studies, the reduction in *C. trachomatis* infectivity due to HSV co-infection was dose dependant. Despite the fact that HSV infection at 1 or 0.1 MOI does not productively infect all chlamydiae-infected host cells, chlamydial infectivity in these cultures was significantly reduced. These experiments might suggest the involvement of a factor secreted from HSV-infected cells. However, HSV produces 50-200 defective viral particles for every replication-competent virion (Roizman, 2001). Given the results obtained with UV-inactivated virions and cyclohexamide exposed co-infected cells, a more likely explanation is that defective virions in the HSV-2 inocula can alter chlamydial development in a manner similar to replication-competent virions.

Several studies have demonstrated that while UV-irradiation of HSV prevents productive replication, it does not inhibit virion associated functions such as attachment and entry (Sanfilippo and Blaho, 2006; Moxley, 2002). Plaque assays and PCR from co-infection experiments confirm these observations. Interestingly, co-infection with UV inactivated, replication incompetent HSV-2 alters chlamydial development similarly to that observed during co-infections with replication-competent virus. Likewise, cyclohexamide exposure decreases HSV-2 productive replication >95% but does not abrogate HSV-2-induced *C. trachomatis* persistence. These data indicate that productive HSV-2 replication is not required to stimulate chlamydial persistence. Rather, these results suggest that an early event in the viral replication cycle, most likely host cell attachment and entry is sufficient to force chlamydiae into a persistent state.
Both cyclohexamide exposure and UV-inactivation strongly inhibit de novo HSV protein synthesis, particularly those of the delayed-early and late kinetic classes. Although a small amount of immediate-early viral protein synthesis occurs in both cases, the magnitude is considerably diminished, particularly in the case of UV-inactivated virus. In contrast, viral attachment and entry are unaffected (Sanfilippo and Blaho, 2006; Sanfilippo, 2004; Moxley, 2002; Vasquez, 1979; Swanstrom et al., 1975). However, co-infection with HSV-2UV induced chlamydial persistence, suggesting that de novo viral protein synthesis is not required. Data obtained from cyclohexamide exposure experiments also support this interpretation. These experimental results suggest that chlamydial persistence in co-infected cells is induced by: 1) a virus/host cell binding event, 2) introduction of a virion associated protein into the host cell or, less likely, 3) expression of a viral immediately-early transcript.

During viral attachment and entry, HSV binds to one of several co-receptors using the viral gD envelope glycoprotein (Spear, 2004). These co-receptors include herpes viral entry mediator (HVEM), Nectin-1, Nectin-2, and 3-O-Sulfated Heparan Sulfate (Spear, 2004). HVEM is a member of the tumor necrosis factor receptor family (Mauri et al., 1998). When complexed to its natural ligand, LIGHT, HVEM has been shown to stimulate cellular transduction pathways involved in the activation of T-cells (Granger and Rickert, 2003; Hsu et al., 1997). Nectin-1 and nectin-2 are members of the Immunoglobulin superfamily and are involved in the formation of cell junctional complexes (Cocchi et al., 1998). When stimulated, nectins interact with cell signaling molecules, Cdc42 and Rac small G proteins, through their cytoplasmic tails to coordinate cytoskeletal rearrangements (Nakanishi and Takai, 2004). Given these observations, it is feasible that stimulation of viral co-receptors by attachment of HSV-2 could transmit a cellular signal that has downstream effects on developing chlamydiae.
HSV has also been shown to trigger cellular defense mechanisms by interacting with toll-like receptors (TLR). TLR-2 is host cell surface exposed and appears to recognize herpes virion envelope glycoproteins. For example, the gB envelope glycoprotein of human cytomegalovirus, a betaherpesvirus, provides sufficient stimulus to activate anti-viral responses through TLR-2 (Compton et al., 2003). Infection with varicella zoster virus, HSV-1, and HSV-2 can all stimulate IL-6 production in culture; this induction is TLR-2 dependant and does not require productive viral replication (Aravalli, 2005; Kurt-Jones, 2005; Wang, 2005). TLR-2 knockout mice challenged with HSV-1 exhibit reduced production of inflammatory cytokines and diminished neuropathogenesis when compared to wildtype control animals, suggesting that HSV-mediated stimulation of the inflammatory response through TLR-2 occurs in vivo as well (Kurt-Jones et al., 2004). Additionally, the intracellular toll-like receptor, TLR-9, is stimulated by unmethylated CpG motifs commonly found in HSV genomic DNA (Pyles, 2002). Although not definitive, these studies suggest the possibility that HSV-mediated stimulation of TLR-linked anti-viral or anti-chlamydial cascades could affect developing chlamydiae within co-infected cells.

Another possibility is that viral transport within the cell, activity of a virion-associated protein or nuclear entry of viral DNA is responsible for inducing chlamydial persistence. After fusion with the cellular membrane, viral capsids interact with the molecular motor dynein and are transported along microtubules to the nucleus where capsids dock with nuclear pore complexes and translocation of the viral genome occurs (Ojala, 2000). Upon viral entry, active viral tegument proteins are released into the cytoplasm. One such protein, VP22, functions as a microtubule associated protein and induces reorganization of microtubules in the host cell.
cytoplasm (Elliott, 1998), suggesting the possibility that HSV co-infection might alter or inhibit vesicular transport to the developing inclusion. However, inclusion size analyses revealed that chlamydial inclusions in co-infected cells are actually larger than those in *C. trachomatis* singly-infected cells (Deka *et al.*, 2006). These data suggest that viral-induced changes in host cell trafficking diminish neither vesicular transport to nor development of the inclusion. Another virion protein, the HSV-2 virion host shut off protein (VHS), immediately suppresses host transcription by inducing degradation of host mRNAs (Roizman, 2001; Kwong, 1989). VP16 is a virion protein that is transported to the nucleus and is involved in activation of both viral and host gene transcription (Roizman, 2001). Thus, it is also possible that the activity of one or more virion-associated proteins such as VHS and VP22 could either activate a cellular anti-chlamydial response or disrupt a cellular pathway that is required for normal chlamydial development, leading to persistence. As stated above, another intriguing possibility is that viral nuclear entry or other viral nuclear functions that occur prior to immediate-early gene expression might alter chlamydial development in co-infected cells. Unfortunately, the limited information available regarding host nuclear functions required for normal chlamydial development makes it difficult to speculate upon which virus/host nuclear interactions might be required for this effect.

Previous studies have confirmed the important role that chlamydia/host cell interactions play in the proper development of infectious chlamydial progeny. When these crucial host cell interactions are disrupted, chlamydiae often survive by becoming persistent (Gerard *et al.*, 2001; Darville, 2000; Raulston, 1997; Beatty *et al.*, 1994a). Additionally, it appears that the host has evolved “anti-chlamydial” pathways such as the IFN-γ/IDO system that when stimulated interfere with chlamydial development. The developing chlamydiae may then respond by becoming persistent (Hogan *et al.*, 2004). This study suggests that attachment and entry of HSV-
2 may: 1) disrupt host cellular function, such that chlamydiae cannot complete the developmental cycle and/or 2) trigger a novel host anti-chlamydial pathway that restricts chlamydial development. Examination of the cellular pathways that are stimulated by HSV-2 attachment and entry during co-infection will further illuminate the mechanisms that *C. trachomatis* uses to exist within its host cell and the methods by which host cells combat this bacterial invader.
Experimental Procedures

Chlamydia, HSV-2, and host cells.

A human urogenital isolate of C. trachomatis E/UW-5/CX was originally obtained from S.P. Wang and C. C Kuo (University of Washington, Seattle, WA). The same standardized inoculum of C. trachomatis serovar E elementary bodies, propagated in McCoy cells, was used for all experiments (Wyrick, 1996). Herpes simplex virus type 2 strain 333 and HSV-1 strain KOS stocks were obtained from Mary K. Howett and Udayasankar Kumaraguru. Viral stocks were prepared in monolayers of Vero cells (African green monkey kidney cells ATCC#CCL-81) using standard techniques (Duff and Rapp, 1971).

Co-infection experimental design.

HeLa cells, a cervical adenocarcinoma epithelial cell line (ATCC #CCL2), or HEC-1B cells, an endometrial epithelial cell line (ATCC#HTB-113), were used for all infection experiments. In each experiment the appropriate host cells were divided into four groups, 1 x 10^6 cells per 60 mm culture dish for mock infection, chlamydial infection, HSV infection, and both C. trachomatis and HSV double infection. In some experiments, HEC-1B or HeLa cells were polarized on 3.0 micron, Collagen IV coated chamber inserts (Biocoat 4544, Becton Dickinson) as previously described (Wyrick, 1989). The results obtained from co-infection in polarized host cells were identical to those obtained using non-polarized cells. Host cells were infected with a dilution of crude EB stock (200 µl) calculated to infect >80% of the cells. For most experiments the cells were infected with HSV-2, HSV-1, or an amount of replication incompetent HSV-2 (HSV-2_{uv}) equivalent to a multiplicity of infection (MOI) of 10 PFU/cell. In some experiments, HSV-2 infections were also conducted at 1 or 0.1 MOI. Mock-infected cells were treated
similarly except they were exposed to 200 µl of either 2SPG (0.2 M sucrose, 6mM NaH₂PO₄, 15mM Na₂HPO₄, 5mM L-glutamine, pH 7.2; mock *C. trachomatis*-infected) or growth medium (mock viral infection).

**Cyclohexamide exposure during co-infection.**

In a selected group of co-infections, the infected host cells were exposed to cyclohexamide. Monolayers of HeLa cells were either mock or chlamydiae-infected. Twenty-three hours post-chlamydial infection, 1µg/ml cyclohexamide or an equivalent volume of ddH₂O (diluent exposed controls) was added to the growth medium. Cells were incubated at 37°C for another hour, at which time they were either mock or HSV-2-infected. The viral inoculum also contained 1 µg/ml cyclohexamide or diluent (ddH₂O). Following HSV-2 infection the cells were refed with medium again containing either 1 µg/ml cyclohexamide or ddH₂O. The cells remained in the presence of cyclohexamide until they were collected at 20 h post-HSV-2 infection.

**Generation of replication incompetent HSV-2 (HSV-2<sub>UV</sub>).**

A UV Crosslinker (Spectroline Microprocessor Controlled UV-Crosslinker Spectrolinker XL1500, Spectronics Corporation, New York) was used to generate stocks of UV-inactivated, replication incompetent HSV-2. Stock HSV-2 was thawed and 200 µl was aliquoted into each well of a 24 well plate. The plates were placed on a 4°C heat sink during UV exposure to prevent heat inactivation of the samples. HSV-2 inactivation was assayed by performing plaque assays with the UV inactivated virus using the same protocol as detailed for replication-competent
HSV-2. A UV dose of 2.5 J/cm² was found to be sufficient to completely inactivate $10^9$ PFU of HSV-2.

Purification of HSV-2 and mock-infected crude stocks.

Viral purification was performed as described previously with the following modifications (Sathananthan, 1997). Duplicate flasks of Vero cells were either mock or HSV-2-infected. The cells were monitored and collected when the virally infected cells began to exhibit signs of viral cytopathic effect. Each stock (mock and HSV-2-infected) was split into duplicate aliquots. The stocks were frozen/thawed to lyse the cells and centrifuged for 10 min (1000xg, at 4°C) to remove large cellular debris. The supernatant from one aliquot of the mock and HSV-2 stocks was subaliquoted and stored at -80°C. The supernatant from the second aliquot of each stock was centrifuged again for 1 hr at 18,000 rpm (39,2000xg) in a Beckman JA-20 rotor. After centrifugation, the supernatants were removed and the pellets were resuspended overnight in 4ml growth medium without agitation. The purified mock and HSV-2 stocks were subaliquoted and stored at -80°C. Plaque assays were performed to determine the titer of each stock and to ensure that both the crude mock and purified mock stocks were free of contaminating HSV-2.

Luminex assay.

Aliquots of crude, mock crude, pure, and mock pure HSV-2 stocks were examined for the cytokines IFN-γ, IFN-α, TNF-α, and IL-6 using the BioSource Multiplex Bead Immunoassay (BioSource International, Camarillo, CA) according to the manufacturer’s instructions. Samples were UV-irradiated as previously described before the assay was performed such that no infectious virions were present in the samples. A Luminex 100 instrument (Luminex
Corporation, Austin, TX) was used to measure the quantity of each cytokine bound to antibody coupled beads.

**Fluorescent and transmission electron microscopy.**

Fluorescence analyses were performed as described previously (Deka et al., 2006) except FITC-conjugated monoclonal antibodies generated against *C. trachomatis* MOMP (Pathfinder *Chlamydia trachomatis* monoclonal antibody 30702, BioRad) were used to stain chlamydial inclusions. Duplicate samples of infected HeLa and HEC-1B cells were processed at 20 h post-HSV infection for high contrast transmission electron microscopy as described (Wyrick, 1994). Counterstained gold thin sections were examined using a Tecnai 10 (FEI) transmission electron microscope operating at 60-80 kV.

**Chlamydial titrations by subpassage.**

Chlamydial titrations were performed as previously described (Deka et al., 2006) except that Pathfinder anti-chlamydial stain was used to stain chlamydial inclusions formed from subpassaged EBs. Inclusions present on triplicate HeLa cell monolayers were counted and averaged. The number of inclusion forming units (IFU) in the undiluted inoculum was then calculated and expressed as IFU/ml.

**DNA isolation.**

Infected HeLa cells were collected at 0 h (T₀) and/or 20 h (T₂₀) post-HSV infection in 200 µl medium and lysed by freeze/thaw. Total DNA from the resulting cell lysates was isolated using the QIAmp DNA Blood Mini Kit (Qiagen) according to the manufacture’s instructions.
Total DNA preparations were quantified using optical density (OD) at 260 and 280 nm; all samples had OD260/280 ratios > 1.9.

**PCR.**

PCR was performed using purified total cellular DNA as template. Experimental template DNAs were used at dilutions ranging from 1/10 to 1/1000 (in ddH2O) such that each reaction was in the linear amplification range. PCR was performed using identical conditions to those previously described (Deka et al., 2006). Published primer sets included HSV-2 Glycoprotein G (Filen et al., 2004) as well as chlamydial 16S rRNA and human glyceraldehyde-3-phosphate dehydrogenase (Deka et al., 2006). After PCR, all reactions were electrophoresed on 1.5% agarose/TBE gels stained with Ethidium Bromide. A BioRad Chemi Doc XRS Image Capture System with Quantity One V4.5.0 software (BioRad) was used to visualize and quantify amplimers.

**HSV plaque assay.**

At various times post-HSV infection, culture supernatants were centrifuged at 4000 X g for 5 min and 4°C to remove cell debris. Plaque assays were carried out on the resulting supernatants as described (Duff and Rapp, 1971). Quadruplicate, infected cultures were incubated for 72 h at 37°C, fixed, stained, and counted. Average plaque counts from each set of plates were used to calculate the PFU/ml present in the original supernatant.

**Statistical analyses.**
Statistical analyses were performed using Microsoft Excel. Comparison of means was
done by using a 2-sample t-test for independent samples. P values of \( \leq 0.05 \) were considered
significant.

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References


CHAPTER 4
HERPES SIMPLEX VIRUS CO-INFECTION-INDUCED CHLAMYDIA TRACHOMATIS
PERSISTENCE IS NOT MEDIATED BY ANY KNOWN PERSISTENCE INDUCER OR
ANTI-CHLAMYDIAL PATHWAY.

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Key words: Chlamydial persistence, Chlamydia trachomatis, herpes simplex virus, sexually
transmitted infection.

Abbreviations: HSV-1, 2; Herpes Simplex Virus Type 1, 2, IFN; Interferon, IL; Interleukin, LT-
α; lymphotoxin-α, TNF-α; Tumor necrosis factor α, GM-CSF; Granulocyte-macrophage colony-
stimulating factor, IDO; Indoleamine 2, 3-dioxygenase, iNOS; Inducible nitric oxide synthase,
NO; Nitric oxide, HVEM; Herpes viral entry mediator, EB; Elementary Body, RB; Reticulate
body, MOMP; Major outer membrane protein, VHS; Virion Host Shut-off.

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Summary

Several inducers of chlamydial persistence have been described including Interferon-γ (IFN-γ), IFN-α, IFN-β, and tumor necrosis factor-α (TNF-α) exposure and iron, amino acid, or glucose deprivation. A tissue culture model of *C. trachomatis*/*Herpes simplex virus type-2* (HSV-2) co-infection indicates that viral co-infection stimulates the formation of persistent chlamydiae. This study was designed to ascertain whether co-infection-induced persistence is mediated by a previously characterized mechanism. Luminex assays indicate that IFN-γ, IFN-α, and TNF-α are not released from co-infected cells. Semi-quantitative RT-PCR studies demonstrate that IFN-β, IFN-γ, indoleamine 2, 3-dioxygenase, lymphotoxin-α, and inducible nitric oxide synthase are not expressed during co-infection. These data indicate that viral-induced persistence is not stimulated by any persistence-associated cytokine. Supplementation of co-infected cells with excess amino acids, iron-saturated holotransferrin, glucose, or a combination of amino acids + iron does not restore chlamydial infectivity, demonstrating that HSV-2-induced persistence is not mediated by depletion of these nutrients. Finally, inclusions within co-infected cells continue to enlarge and incorporate C₆-NBD-ceramide, indicating that HSV-2 co-infection does not inhibit vesicular transport to the developing inclusion. Collectively these data demonstrate that co-infection-induced persistence is not mediated by any currently characterized persistence inducer or anti-chlamydial pathway. Previous studies indicate that HSV-2 attachment and/or entry into the host cell is sufficient for stimulating chlamydial persistence, suggesting that viral attachment and/or entry may trigger a novel host pathway which restricts chlamydial development.
Introduction

Herpes Simplex Virus Types 1 and 2 (HSV-1 and HSV-2) are enveloped DNA viruses of the viral family, *Herpesviridae*. HSV-2 is the primary cause of genital herpes infection, causing 200,000-500,000 new infections annually. HSV-2 infection usually occurs on the mucous membranes and skin surrounding the genitals, causing a characteristic lesion. After primary infection, HSV-2 can establish a life-long latent infection in the neurons of the sacral ganglia that is reactivated, on average, five times each year. Although most genital HSV infections are clinically mild, HSV-2 can cause serious diseases including keratitis and meningitis (Roizman, 2001).

Herpes simplex viruses begin their infection of epithelial cells by binding to the host cell receptor, heparan sulfate. After this initial binding, viral envelope glycoproteins interact with one of four known co-receptors (herpes viral entry mediator [HVEM], Nectin-1, 2, and 3-O-sulfated heparan sulfate), facilitating viral entry (Spear, 2004). Following fusion of the viral envelope with the host cell, viral tegument proteins are released into the cytoplasm and the capsid is transported to the nucleus, where viral genome replication occurs. The viral genome is transcribed and viral proteins are synthesized using host cell machinery. New virions are then assembled and exit the host cell (Roizman, 2001).

*Chlamydia trachomatis* is a Gram-negative, obligate intracellular bacterium that is responsible for 4 million sexually transmitted infections each year in the US (Butler, 1997; Weinstock *et al.*, 2004). Genital chlamydial infections are often chronic and asymptomatic, leading to severe complications including pelvic inflammatory disease, ectopic pregnancy, and infertility (Darville, 2000). *In vivo* and in culture, chlamydiae carry out a characteristic biphasic developmental cycle. Extracellularly, chlamydiae exist as infectious elementary bodies (EBs).
EBs attach to and enter a host cell where they differentiate within an inclusion into non-infectious reticulate bodies (RBs). The infectious cycle spans 30-72 hours (depending upon the chlamydial species/serovar). Near the end of the cycle, RBs develop back into EBs and are released from the infected host cell. When developing chlamydiae are exposed to certain adverse environmental factors (such as interferon-γ [IFN-γ] or penicillin G) or are deprived of nutrients (such as amino acids, iron, or glucose), they deviate from the normal developmental cycle into a state termed persistence (Hogan et al., 2004). Once in the persistent state, RBs continue to grow but do not divide, resulting in the formation of enlarged, abnormally shaped RBs. Though they remain viable and continue genomic DNA replication and rRNA synthesis, persistent chlamydiae are no longer infectious. Interestingly, persistent chlamydiae can reenter the normal developmental cycle and recover infectivity if the environmental stressor is removed. For example, nutrient deprived, persistent chlamydiae resume development into infectious EBs if the appropriate nutrients are replenished (Harper et al., 2000; Matsushima et al., 1999; Raulston, 1997).

Several host cell pathways have evolved to limit the spread of chlamydial infections. In human cells, IFN-γ exposure increases host cell indoleamine 2, 3-dioxygenase (IDO) expression, leading to depletion of host cellular tryptophan. Without this essential amino acid, developing chlamydiae enter into persistence and cease productive replication (Beatty et al., 1994). In murine cells, IFN-γ exposure increases synthesis/activity of the p47 GTPase, Iigp1. Iigp1 inhibits chlamydial development by restricting vesicular trafficking to developing inclusions (Nelson, 2005). IFN-α, IFN-β, and tumor necrosis factor-α (TNF-α) interfere with chlamydial development by induction of IDO, induction of inducible nitric oxide synthase (iNOS), and/or restriction of intracellular iron stores (Hogan et al., 2004; Matsushima et al., 1999). When iNOS
is up-regulated, the host cell produces nitric oxide (NO) that kills developing chlamydiae. Although these pathways are intended to eliminate the bacterial infection, chlamydiae are often able to survive the onslaught of the host cell’s arsenal by entering into a persistent state until conditions become favorable for their continued development.

Several studies have shown that *C. trachomatis* and HSV-2 co-infections occur *in vivo* (Paavonen et al., 1985; Tait et al., 1985). *In vitro* models of HSV-2/*C. trachomatis* co-infections have indicated that HSV-2 co-infection alters chlamydial development; however, previous studies did not examine the co-infection process in detail (Chiarini et al., 1996; Pontefract et al., 1989; Superti et al., 2001). Data from a tissue culture model of *C. trachomatis* and HSV-2 co-infection established in our laboratory indicate that during *C. trachomatis* serovar E and HSV-2 co-infection HSV attachment and or entry transmits a signal that interrupts the normal chlamydial developmental cycle and induces persistence (Deka et al., 2007). These, and other data, have lead us to hypothesize that HSV attachment to and/or entry activates a novel anti-chlamydial defense pathway in mucosal epithelial cells.

**Methods**

*Chlamydia*, HSV-2, and host cells.

A human urogenital isolate of *C. trachomatis* E/UW-5/CX was originally obtained from S.P. Wang and C. C Kuo (University of Washington, Seattle, WA). The same standardized inoculum of *C. trachomatis* serovar E, propagated in McCoy cells, was used for all experiments (Wyrick et al., 1996). Herpes simplex virus type 2 strain 333 and HSV-1 strain KOS stocks were obtained from Mary K. Howett and Udayasankar Kumaraguru, respectively. Viral stocks were
prepared in monolayers of Vero cells (African green monkey kidney cells ATCC#CCL-81) as described (Duff & Rapp, 1971).

Co-infection experimental design and nutrient supplementation.

HeLa cells, a human cervical adenocarcinoma epithelial cell line (ATCC #CCL2), or HEC-1B cells, a human endometrial epithelial cell line (ATCC No. HTB-113), were used for all infection experiments. In each experiment, the appropriate host cells were divided into four groups for mock infection, chlamydial infection, HSV infection, and \( C. trachomatis/\)HSV double infection. Host cells were incubated with a dilution of crude EB stock calculated to infect >80% of the cells. Following an hour of adsorption, monolayers were refed with Minimal Essential Medium (MEM; Gibco) and incubated at 35 °C for either 4 or 24 h. Cultures were then infected with either HSV-2 or HSV-1 at a multiplicity of infection (MOI) of 10 PFU/cell. Mock-infected HeLa or HEC-1B cells were treated similarly except they were exposed to either 2SPG (0.2 M sucrose, 6mM NaH$_2$PO$_4$, 15mM Na$_2$HPO$_4$, 5mM L-glutamine, pH 7.2; mock \( C. trachomatis \) infection) or growth medium (mock viral infection). In most experiments, monolayers were refed with MEM following HSV adsorption. For nutrient supplementation studies, cultures were either refed with MEM or MEM supplemented with excess essential and non-essential amino acids (Gibco), iron saturated holotransferrin (hTF; Sigma), glucose or a combination of amino acids and hTF as described in the legend to Supplementary Fig. 4.3.

Luminex assay.

Aliquots of culture supernatants were examined for IFN-\( \gamma \), IFN-\( \alpha \), TNF-\( \alpha \), Granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-6 (IL-6) using the BioSource
Multiplex Bead Immunoassay (BioSource International) according to the manufacturer’s instructions (Deka et al, 2007).

**RNA and DNA isolation.**

Total RNA and DNA were isolated simultaneously from experimental samples using the RNeasy Mini (Qiagen) and QIAmp DNA Blood Mini (Qiagen) kits as described (Deka et al., 2006). Total RNA and DNA preparations were quantified using optical density (OD) at 260 and 280 nm; all samples had OD260/280 ratios >1.9.

**Reverse transcription, PCR and RT-PCR.**

Reverse transcription of total RNA, PCR, and RT-PCR was performed using identical conditions to those previously described (Deka et al., 2006). PCR was performed using purified total cellular DNA or cDNA as a template. Control and experimental template DNAs were diluted from 1/10 to 1/1000 (in ddH2O) and synthetic control DNA targets diluted from 10 to 0.1 pg ml⁻¹, ensuring that each reaction was quantified in the linear amplification range. Published primer sets included chlamydial 16S rRNA, human glyceraldehyde-3-phosphate dehydrogenase and IDO (Deka et al., 2006; Nelson, 2005). We designed specific primer sets and, in some cases, synthetic DNA targets for chlamydial *trpA*, human *18S rRNA*, *ifn-β*, *inf-γ*, *lymphotoxin-a*, and *inos* (see Supplementary table 4-1 for sequences). All synthetic DNA targets and primer sets were designed using Ensembl and Vector NTI Advance V10 (Invitrogen). After PCR, all reactions were electrophoresed and quantified as described (Deka et al., 2006).
Griess reaction.

Aliquots of select culture supernatants (50 µl) were examined for nitrite using the Griess Reagent System (Promega) according to the manufacturer’s instructions.

Fluorescent microscopy and image analysis.

Fluorescence analyses were performed as described previously (Deka et al., 2006) except FITC-conjugated monoclonal antibodies generated against *C. trachomatis* major outer membrane protein (Pathfinder *Chlamydia trachomatis* monoclonal MOMP antibody #30702, BioRad) were used to stain chlamydial inclusions. Images were captured using a Zeiss Axiovert S100 microscope and Axiocam camera. Measurement of inclusion sizes was performed as described by Deka et al. (2006) except that selected photomicrographs were converted to grayscale using Adobe Photoshop V5.0. The relative area of each inclusion was determined using Dymension II Imaging Software V2.06 (SynGene).

Cer$_6$-NBD-ceramide labeling.

HeLa cells were plated in 24 well plates (1.5x10$^5$ cells/well), and mock, singly, or co-infected as described above. At 16.5 h post-HSV-2 infection, cells were stained with 200 µg ml$^{-1}$ BSA pre-conjugated $C_6$-NBD-ceramide stain (Molecular Probes) for 30 min at 4 °C. The stain was then back-extracted for 3 h at 37 °C until 20 post-HSV-2 infection (Alzhanov et al., 2007; Hackstadt et al., 1995). Cells were mounted with GelMount (Fisher) and examined by fluorescence microscopy using FITC filters. Images were captured as described above with a 4 s exposure and 320x magnification.
Chlamydial titrations by subpassage.

Chlamydial titrations were performed as previously described (Deka et al., 2006) using Pathfinder anti-chlamydial stain (BioRad) to stain chlamydial inclusions formed from subpassaged EBs. The number of inclusion forming units (IFU) in the undiluted inoculum was then derived from triplicate counts and expressed as IFU ml⁻¹.

Spectro ferritin ELISA.

Intracellular ferritin in cell lysates collected from mock-, chlamydiae-singly-infected +/- 50 uM Desferal and C. trachomatis/HSV-2 co-infected cells was measured using the Spectro Ferritin ELISA kit (Ramco) as described in the legend to Supplementary Fig. 4.3. Addition of the iron chelating agent Desferal (Sigma) to chlamydiae-infected cultures was used as a positive control for iron depletion (Raulston, 1997).

Statistical analyses.

Statistical analyses were performed using Microsoft Excel. Comparison of means was done by using a 2-sample t-test for independent samples. P values of ≤ 0.05 were considered significant. All plotted values are averages of either eight or nine biological replicates divided between 3 individual experiments ± SEM.
Results and Discussion

*C. trachomatis/*HSV co-infected cells do not produce cytokines known to alter chlamydial development.

Cytokine exposure, in particular IFN-γ, is one of the most extensively studied mechanisms of inducing chlamydial persistence. However, other cytokines can also negatively affect *C. trachomatis* development. TNF-α, IL-1α and IL-1β can synergize with IFN-γ, lowering the concentration at which IFN-γ exposure affects developing chlamydiae (Carlin & Weller, 1995; Shemer-Avni *et al.*, 1988; Summersgill *et al.*, 1995). Lymphotixin-α (LT-α), TNF-α, and IFN-α/β exposure can also up-regulate iNOS expression and cellular NO production, inhibiting chlamydial development (Devitt *et al.*, 1996; Matsushima *et al.*, 1999). Cycloheximide exposure experiments (Deka, 2007) suggest that HSV-induced persistence occurs in the absence of *de novo* host protein synthesis. However, the observation that cycloheximide exposure did not completely abrogate host protein synthesis (Deka *et al.*, 2007) made it important to confirm that “anti-chlamydial” cytokines were not released from co-infected cells. HSV-infected, human corneal epithelial cells produce IFN-β, as well as the cytokines TNF-α, interleukin-8 (IL-8), and IL-6 (Li *et al.*, 2005). However, co-infected epithelial cells may produce cytokines that are not elicited by infection with either pathogen alone (or by other commonly used experimental stimuli, such as cytokine exposure). Therefore, production of all cytokines known to elicit an anti-chlamydial response was examined in triplicate mock, singly, or co-infected cultures as described in the methods. Twenty h post-viral infection, supernatants were collected and assayed for IFN-γ, IFN-α, TNF-α, GM-CSF, IL-1α/β and IL-6. IFN-γ, IFN-α, TNF-α, and GM-CSF were not detected in HeLa supernatants, regardless of infection status. IL-1α (11.1-13.4 pg ml⁻¹), IL-1β (34.7-46.9 pg ml⁻¹), and IL-8 (1.7-3.6 pg ml⁻¹) were detected, but at similar levels in *C.
*trachomatis* singly-infected and co-infected HeLa cultures, indicating that they were unlikely to be involved in HSV-2-induced persistence. As expected, IL-6 was strongly induced by HSV-2 infection and was increased in HSV singly and co-infected HeLa cultures compared to that in mock and *C. trachomatis* singly-infected cultures (Fig. 4.1a). However, in duplicate singly and co-infected HEC-1B cultures, similar quantities of IL-6 were detected in all experimental samples (Fig. 4.1b). Because HSV-induced persistence also occurs in HEC-1B cells, it is unlikely that IL-6 production stimulates HSV-induced persistence (Deka et al., 2007). Co-infection experiments using HSV-1 yielded similar results (data not shown).

Expression of IFN-γ, IFN-β, and LT-α mRNAs during co-infection was measured by semi-quantitative RT-PCR. As shown in Fig. 4.1(c), *ifn-γ*, *ifn-β*, and *lt-α* were not expressed in mock, singly, or co-infected cells. Amplification of diluted synthetic DNA targets specific to the *ifn-γ*, *ifn-β*, and *lt-α* genes demonstrated that PCR reactions were sensitive and specific. Human 18S rRNA targets were also amplified to ensure that the cDNAs were not degraded (Supplementary Fig. 4.1b). Amplification of a dilution series of control HeLa cell genomic DNA ensured that all 18S rRNA amplification reactions were quantified within the linear range of the PCR (Deka et al., 2006). All template negative (Fig. 4.1c) and RT(-) (data not shown) samples were negative, as expected. Finally, all amplimers were the expected size and sequence (data not shown). Taken together, these data indicate that HSV/*C. trachomatis* co-infection does not stimulate host epithelial cells to produce any known “anti-chlamydial” cytokine.
The iNOS/NO pathway is another an important component of the anti-chlamydial defense (Devitt et al., 1996; Matsushima et al., 1999). Although iNOS activity is not stimulated by either IFN-γ or chlamydial infection in cultured human epithelial cells (Roshick et al., 2006), the possibility that the additional stimulus provided by viral co-infection might activate iNOS/NO production in human cells is untested and should be evaluated. Therefore, *C. trachomatis*/HSV-2 co-infected cells were examined for iNOS mRNA up-regulation by semi-
quantitative RT-PCR and for nitrite accumulation in supernatants using the Griess reaction. Neither iNOS mRNA nor nitrite was detected in any of experimental cell cultures (Supplementary Fig. 4.1a and data not shown), confirming that HSV-2-induced chlamydial persistence is not mediated by iNOS induction or NO production.

Inclusions within co-infected cells continue to enlarge and incorporate C₆-NBD-ceramide.

In murine cells, IFN-γ exposure activates the p47 GTPase, Iigp1 that halts *C. trachomatis* development by interrupting sphingomyelin transport and vesicular trafficking to the inclusion. If *C. trachomatis*-infected murine cells are exposed to IFN-γ early in the developmental cycle, inclusions remain small (Nelson *et al.*, 2005). Recent studies indicate that Iigp1 is up-regulated in HSV-1-infected murine cells at 3 hpi (Pasieka *et al.*, 2006). Although a human Iigp1 homologue has not been identified, it is possible that HSV/host cell interaction activates human p47 GTPases that function similarly. Therefore, *C. trachomatis*-infected HeLa cells were co-infected with HSV-2 at either 4 or 24 h post-chlamydial infection. Replicate cultures were harvested either immediately after HSV adsorption or 20 h post-HSV-2 infection. Monolayers were fixed, immunostained with anti-MOMP antibodies, and photographed and relative inclusion size was determined (Deka *et al.*, 2006). Average inclusion size was similarly increased in both singly and co-infected cells over the course of the experiment when HSV-2 was added 4 h post-chlamydial infection (Fig. 4.2a). In cells co-infected with HSV-2 at 24 h post-*C. trachomatis* infection, the starting inclusion size was identical in both cultures. However, inclusions in co-infected cells were significantly larger than those in singly-infected controls after the 20 h HSV-2 infection period, as previously reported (Deka *et al.*, 2006). Additional cultures were infected as described above, labeled with BSA-conjugated, C₆-NBD-ceramide stain, and visualized by fluorescence...
microscopy (Alzhanov et al., 2007; Hackstadt et al., 1995). No obvious difference was observed in the intensity or distribution of ceramide staining between chlamydial inclusions in singly and co-infected cells at either time interval tested (Fig. 4.2b and data not shown). Although it is possible that vesicular trafficking to the inclusion is subtly altered by HSV infection, these data demonstrate that neither gross vesicular trafficking nor sphingomyelin transport to the inclusion are disrupted by viral co-infection.

![Figure 4.2: Inclusions within co-infected cells continue to enlarge and incorporate C6-NBD-ceramide.](image)

a. Cultures of mock-, singly- and co-infected HeLa cells were harvested immediately after HSV adsorption or 20 h later, fixed, subjected to IFA, and photographed at 320X. Relative inclusion size from 25 random fields was determined. Values are averages of triplicate determinations. Asterisks (*) indicate values that are significantly different (by t-test) compared to those from C. trachomatis singly-infected cells immediately post-HSV adsorption or a significant difference (**) between C. trachomatis singly-infected and co-infected cells at 20 h post-HSV infection (p<0.05). b. Cells were C. trachomatis-infected, HSV-2 co-infected at 24 h post-chlamydial infection and C6-NBD-ceramide stained, as described. Cells were photographed (320X) at 20 h post-HSV infection. All data shown are representative of three independent experiments.
HSV-2 induction of chlamydial persistence is not mediated by IDO activity or global nutrient deficiencies.

Degradation of the essential amino acid tryptophan by the host enzyme IDO strongly induces chlamydial persistence (Hogan et al., 2004). In response to tryptophan deprivation, chlamydiae up-regulate expression of the tryptophan synthase gene, trpA (Belland et al., 2003). To determine whether or not our HeLa cell clone can express IDO, mock- and C. trachomatis-infected HeLa monolayers were exposed to IFN-γ (50 U ml⁻¹) for 48 h followed by RNA isolation. Expression of ido and trpA mRNA in IFN-γ-exposed control cultures was then evaluated by semi-quantitative RT-PCR. IDO expression was significantly increased in both mock- (data not shown) and C. trachomatis-infected HeLa cells in response to IFN-γ, compared to that in unexposed cells (Supplementary Fig. 4.2a). A significant increase in trpA expression was also observed in C. trachomatis-infected cells following IFN-γ exposure versus unexposed, chlamydiae-infected cultures (data not shown).

To determine if co-infection similarly elevates ido or trpA levels, total RNA was isolated from triplicate sets of mock, singly, or co-infected HeLa cells and subjected to reverse transcription and semi-quantitative RT-PCR with primers specific for the human ido and chlamydial trpA genes. Again, amplification of a dilution series of cDNA from C. trachomatis-infected, IFN-γ exposed HeLa cells indicated that the IDO RT-PCR was specific and sensitive. IDO mRNA was not detected in any of the experimental samples tested, regardless of infection status (Supplementary Fig. 4.2b). Various dilutions of chlamydial genomic DNA were amplified to ensure that all trpA amplification reactions were quantified within the linear range of the PCR (data not shown). Additionally, chlamydial genome copy number in each sample was determined
by amplification of the chlamydial 16S rRNA gene (data not shown). All trpA RT-PCR values were normalized to the chlamydial genome copy number (Deka et al., 2006). In contrast to trpA expression during IFN-γ exposure, no statistical difference in trpA expression was observed between C. trachomatis singly-infected and co-infected samples (Supplementary Fig. 4.2c). These data confirm that C. trachomatis/HSV-2 co-infected cells are not deprived of tryptophan by the action of IDO.

Global deprivation of amino acids and other nutrients, especially iron and glucose, can also cause developing chlamydiae to enter persistence (Harper et al., 2000; Raulston, 1997). To investigate the possibility that HSV-2 co-infection induces chlamydial persistence by limiting iron availability, we first determined whether co-infected cells were iron-deprived. Intracellular ferritin levels can be used as an indirect measure of host intracellular iron levels (Dill & Raulston, 2007). As a positive control for iron depletion, duplicate C. trachomatis-infected HeLa cultures were exposed to the iron chelating agent, Desferal, following chlamydial infection. Intracellular ferritin levels were not significantly reduced in chlamydiae singly-infected or co-infected cells, compared to that in mock-infected controls (Supplementary Fig. 4.3a), indicating that co-infected cells were not iron-deprived. In contrast, Desferal exposure significantly reduced intracellular ferritin levels, as expected (Supplementary Fig. 4.3a).

During HSV-2 replication, cellular amino acids are depleted through the synthesis of viral proteins (Roizman, 2001). While it is possible that co-infection induces persistence by depriving the chlamydiae of amino acids, it remains unlikely, given previous data, that UV-inactivated, replication-incompetent HSV-2 stimulates chlamydial persistence (Deka et al, 2007). However, several incoming HSV virion proteins such as the virion host shut off protein (VHS) produce significant host cellular physiologic alterations in the absence of viral replication
Thus, the possibility that viral infection induces nutrient deprivation via an indirect pathway cannot be excluded. Notably, supplementation of nutrient-starved chlamydiae with amino acids, iron, or glucose reverses the negative effect of the nutrient deficiency (Hogan et al., 2004; Raulston, 1997). Additionally, the anti-chlamydial effect stimulated by IFN-α can only be completely reversed by addition of both excess amino acids and iron (Devitt et al., 1996). Based upon this information, we performed co-infection supplementation studies with excess amino acids, iron, glucose, or a combination of amino acids + iron. Immediately following HSV-2 infection, mock, singly, or co-infected cells were refed with MEM or MEM supplemented with a 5x concentration of both essential and non-essential amino acids, 6 mg ml⁻¹ iron-saturated holotransferrin (hTF), 450 mg ml⁻¹ glucose, or a combination of 5X amino acids + 6 mg ml⁻¹ hTF. This concentration of hTF has been previously shown to rescue chlamydial infectivity in iron-deprived, infected epithelial cells (Raulston, 1997). Twenty hours after HSV-2 infection, the monolayers were collected and processed for chlamydial titration. The titer of infectious EBs was significantly reduced in epithelial cells co-infected with HSV-2 compared to that from cultures singly-infected with C. trachomatis (Supplementary Fig. 4.3b). Supplementation of co-infected host cells with hTF did not rescue chlamydial infectivity (Supplementary Fig. 4.3b), indicating that supplementation with excess iron was not able to restore production of infectious chlamydial progeny during co-infection. Similar results were obtained with excess amino acids, glucose (Supplementary Fig. 4.3c), and a combination of amino acids + iron supplementation (data not shown). These data demonstrate that co-infection-induced chlamydial persistence is not mediated by limitation of amino acids, iron, glucose, or the combination of amino acids + iron within the host cell. Because the anti-chlamydial effect of
IFN-α is mediated by restriction of both tryptophan and iron, these results support previous data indicating that co-infection does not induce persistence through an IFN-α-dependent mechanism.

Collectively, these data demonstrate that co-infection-induced persistence is not mediated by: i) any known anti-chlamydial cytokine; ii) activation of iNOS or IDO; iii) inhibition of vesicular trafficking or sphingomyelin transport to the inclusion or; iv) amino acid, iron, or glucose deprivation. The reduction in chlamydial infectivity stimulated by HSV-2 co-infection is of smaller magnitude than that observed in other models of persistence (Beatty et al., 1994; Pantoja et al., 2001). Additionally, the negative effects of HSV-2 co-infection on chlamydial development are observed when the stimulus is added at 24h post-C. trachomatis infection, where as IFN-γ must be added to chlamydiae-infected cultures either before infection or very early during the developmental cycle for maximal effect (Nelson et al., 2005). Taken together, these observations support the hypothesis that HSV-2 co-infection induces C. trachomatis persistence through an undescribed and, therefore, novel mechanism.

Previously, we reported that both cycloheximide-exposure during co-infection with replication-competent HSV-2 and co-infection with UV-inactivated HSV-2 can stimulate chlamydial persistence, indicating that early events during HSV-2 infection are sufficient for this effect (Deka, 2007). During the host cell entry process, HSV binds to one of several co-receptors: HVEM, Nectin-1, Nectin-2, or 3-O-sulfated heparin sulfate (Spear, 2004). Previous studies have demonstrated that each of these co-receptors can alter cellular signaling pathways when bound to their natural ligands (Granger & Rickert, 2003; Hsu et al., 1997; Mauri et al., 1998; Nakanishi & Takai, 2004). Therefore, HSV-2 attachment to the host cell during co-infection could alter cellular signaling and activate a novel host anti-chlamydial response, leading to persistence. Another possibility is that HSV stimulates Toll-like receptors, initiating
such an anti-chlamydial pathway (Aravalli, 2005; Compton et al., 2003; Kurt-Jones, 2005; Pyles, 2002; Wang et al., 2005). Conversely, there is the potential that viral tegument proteins, released into the cell during entry, could stimulate chlamydial persistence by altering the host cytoskeleton, degrading host mRNAs or altering host gene transcription (Elliott & O'Hare, 1998; Kwong & Frenkel, 1989; Roizman, 2001). Investigations of anti-chlamydial host cell responses have provided valuable information regarding *C. trachomatis* biology, pathogenesis, and its interactions with the host cell. Illumination of the mechanism behind HSV-2-induced persistence will allow us to evaluate the contribution of this novel anti-chlamydial response to *C. trachomatis* pathogenesis and host defense.

**Acknowledgments**

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### Supplementary Table 4.1: Semi-quantitative RT-PCR Primer and Synthetic DNA Target Sequences

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Supplementary Figure 4.1: *C. trachomatis/HSV co-infected cells do not produce nitric oxide synthase.* Total cellular RNA from mock-, *C. trachomatis* and HSV-2 singly- and co-infected HeLa cells was used for semi-quantitative RT-PCR with primers specific to human *inos* (a) and *18S rRNA* (b). A dilution series of synthetic DNA targets or host genomic DNA served as an amplification control for each gene. N=8.

Supplementary Figure 4.2: *HSV-2 co-infection does not stimulate ido or trpA expression.* *C. trachomatis*-infected HeLa cultures were either exposed to diluent (-IFN-γ) or 50U ml⁻¹ of IFN-γ (+IFN-γ) beginning immediately after infection. Total RNA was isolated at 48 h PI as described and subjected to RT-PCR using primers specific for human *ido* (a). Total cellular RNA was isolated from mock-, *C. trachomatis* and HSV-2 singly- and co-infected HeLa cultures for semi-quantitative RT-PCR using primers specific to human *ido* (b) and chlamydial *trpA* (c). A dilution series of synthetic DNA targets (*ido*) or chlamydial genomic DNA (*trpA*) served as an amplification control for each gene. Chlamydial *trpA* and human *ido* amplimers were quantified as described and normalized to chlamydial (*trpA*) or host (*ido*) genome copy number (data not shown). N=8.
Supplementary Figure 4.3: HSV-2 induction of chlamydial persistence is not mediated by nutrient deprivation.  

*a. Cultures of HeLa cells were mock-, *C. trachomatis* singly-infected +/- Desferal or co-infected. Protein concentrations were standardized using the MicroBCA kit (Pierce) prior to analysis by ELISA. Ferritin concentration is expressed as ng ml⁻¹ sample ± SEM; n=3. Asterisks (*) indicate ferritin concentrations that are significantly different (by t-test) compared to mock-infected cells (p<0.05).

*b and c. Cultures of mock-, *C. trachomatis* and HSV-2 singly- and co-infected HeLa cells were refed with either MEM (controls) or MEM + 6mg ml⁻¹ hTF, 5x (1.4 mM) essential and non-essential amino acids or 450mg ml⁻¹ glucose following HSV-2 adsorption and harvested for EB titration at 20 h post-HSV-2 infection. EB titers are expressed as IFU/ml sample ± SEM; n=3. Asterisks (*) indicate titers that are significantly different (by t-test) compared to those from *C. trachomatis* singly-infected cells (p<0.05). The data shown are representative of three independent experiments.
CHAPTER 5

INTERACTION OF HSV-2 GLYCOPROTEIN D WITH THE HOST CELL SURFACE IS SUFFICIENT TO STIMULATE CHLAMYDIA TRACHOMATIS PERSISTENCE

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Running title: HSV-2 gD induces C. trachomatis persistence.

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Key words: Chlamydial persistence, Chlamydia trachomatis, herpes simplex virus, glycoprotein D sexually transmitted infection.
**Summary**

When presented with certain unfavorable environmental conditions, *C. trachomatis* reticulate bodies (RBs) enter into a viable, yet non-cultivable state called persistence. Several models of chlamydial persistence have been described, including IFN-γ, IFN-α, IFN-β, and TNF-α-exposure and nutrient deprivation. Previously, we established an *in vitro* co-infection model of two common sexually transmitted pathogens, *C. trachomatis* and Herpes Simplex Virus-2. Data from this model indicate that: i) viral co-infection stimulates the formation of persistent chlamydiae and ii) productive HSV replication is not required for persistence induction. Other data indicate that co-infection-induced persistence is not mediated by any currently characterized anti-chlamydial pathway or persistence inducer. The present study demonstrates that co-incubation of fixed, HSV-2-infected inducer cells with viable, *C. trachomatis*-infected responder cells suppresses production of infectious chlamydial progeny and stimulates the formation of swollen, aberrantly shaped RBs. Though chlamydial infectivity is initially suppressed, infectivity recovered within 44hr during long-term co-infection with UV-inactivated HSV-2, demonstrating that HSV-induced persistence is reversible. Pre-incubation of viral particles with gD specific neutralizing antibody prevents co-infection-induced persistence, suggesting that HSV gD interaction with host cell surface receptors provides the necessary stimulus to alter *C. trachomatis* development. Finally, exposure of *C. trachomatis* infected cells to a soluble, recombinant HSV-2 gD:Fc fusion protein decreases production of infectious EBs to a similar degree observed in co-infected cultures. Thus, we conclude that interaction of HSV gD with the host cell surface triggers a novel host anti-chlamydial response that restricts chlamydial development.

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Introduction

Herpes simplex virus type 2 (HSV-2) and Chlamydia trachomatis are two of the leading sexually transmitted disease agents in the United States (Weinstock et al., 2004; Butler, 1997). Several studies have shown that C. trachomatis and HSV-2 co-infections occur in vivo (Paavonen et al., 1985; Tait et al., 1985). In one study, examination of cervical biopsy samples indicated that approximately 10% of women were positive for C. trachomatis, HSV and HPV (Finan et al., 2006). In vitro models of HSV-2/C. trachomatis co-infections have indicated that HSV-2 co-infection alters chlamydial development; however, the mechanism by which this occurred was not defined (Superti et al., 2001; Chiarini et al., 1996; Pontefract et al., 1989).

Herpes Simplex Virus Types 1 and 2 (HSV-1 and HSV-2) are enveloped DNA viruses of the viral family Herpesviridae. HSV-2 is the primary cause of genital herpes infection, causing 200,000-500,000 new infections annually. Although predominantly associated with oral infections, HSV-1 is a common agent of genital herpes infections as well (Whitley, 2001). Primary genital herpes infections usually occur on genital mucous membranes or the surrounding skin, causing a characteristic vesicular lesion. After primary infection of epithelial cells, HSV infects nearby sensory nerve endings and establishes latent infection in the sacral ganglia. Latent infections can be reactivated by numerous stimuli such as emotional or physical stress, fever, tissue damage, and immune suppression. Upon reactivation, virions are transported by infected neurons to the site of primary infection where they re-infect the epithelial cells in that area. Skin lesions often occur during recurrent infections; however, virions can also be shed in the absence of noticeable symptoms (Whitley, 2001; Mertz et al., 1992). On average, reactivations occur at least five times each year (Corey et al., 1983). Most genital HSV infections are not severe;
however, HSV-2 infection can cause keratitis, meningitis, and disseminated neonatal HSV infection (Roizman, 2001).

Infection of a host cell by HSV is initiated by a series of interactions between envelope glycoproteins and the host cell surface. First, glycoproteins B (gB) and gC bind to heparan sulfate. Second, the viral gD binds one of several host co-receptors (Spear, 2004). Once gD and the appropriate co-receptor interact, the viral gH/L complex mediates fusion of the viral envelope and the host cell plasma membrane. Once the viral capsid enters the host cell, viral tegument proteins are released. Viral transcription and genome replication occur in the nucleus and viral proteins are synthesized using host cell machinery. New virions are assembled in the nucleus bud through viral glycoprotein-enriched areas of the nuclear envelope and exit the host cell through vesicular transport (Roizman, 2001).

All chlamydiae are Gram-negative obligate intracellular bacteria and share a unique biphasic developmental cycle. Extracellularly, chlamydiae exist as small (0.3µm), infectious, metabolically inert forms of the bacteria termed elementary bodies (EBs). EBs attach to host cells and enter via receptor mediated endocytosis (Abdelrahman and Belland, 2005; Wyrick, 2000). Once inside the host cell, chlamydiae containing vesicles escape lysosomal fusion and are transported to the perinuclear region. Here the EB differentiate within a modified vacuole called an inclusion into larger (1µm), non-infectious reticulate bodies (RBs). Reticulate bodies are metabolically active and will undergo 8-12 rounds of replication. Following replication, RBs condense to form EBs, which are released and can infect new host cells (Wyrick, 2000). C. trachomatis serovars D-K represent the world’s most reported sexually transmitted disease agents causing urethritis or cervicitis. Approximately 85-90% of chlamydial infections are asymptomatic and chronic, resulting in endometritis, salpingitis, and pelvic inflammatory
disease. The damage sustained by the host response to *C. trachomatis* infections promotes the development of more severe disease sequelae including ectopic pregnancy, infertility, and reactive arthritis (Peipert, 2003).

In addition to growth in the normal developmental cycle, chlamydiae have evolved a mechanism to sustain life during adverse conditions (Harper *et al.*, 2000). When developing chlamydiae are exposed to certain environmental insults, they deviate from the normal developmental cycle into a state called persistence (Hogan *et al.*, 2004). Persistence is traditionally defined as a viable but non-cultivable form of chlamydiae (Beatty *et al.*, 1994). Persistent chlamydial forms appear as swollen, diffuse, and aberrantly shaped RBs upon examination by transmission electron microscopy (TEM). While in the persistent state, RBs continue to grow and replicate chromosomes but fail to divide, resulting in the persistent morphology. The abnormal RBs do not differentiate into EBs; thus, causing a decrease in progeny EB infectivity (Beatty *et al.*, 1994; Beatty *et al.*, 1993; Byrne *et al.*, 1986; Johnson and Hobson, 1977; Matsumoto and Manire, 1970). Evidence from several studies strongly suggests that chlamydiae do enter the persistent state *in vivo* (Gerard *et al.*, 2002; Bragina *et al.*, 2001; Gerard *et al.*, 2001; Dean *et al.*, 2000; Fortenberry *et al.*, 1999; Nanagara *et al.*, 1995; Patton *et al.*, 1994). Several models of persistence have been examined in culture, including: antibiotic exposure, monocyte infection, nutritional deficiencies, and cytokine (IFN-γ) exposure (Gerard *et al.*, 2001; Darville *et al.*, 2000; Raulston, 1997; Beatty *et al.*, 1994)

Characterization of a tissue-culture model of HSV and *C. trachomatis* co-infection indicates that co-infection stimulates chlamydiae to become persistent as evidenced by: i) decreased production of infectious EBs; ii) abnormal RB morphology; and iii) continued accumulation of chlamydial chromosomes and the expression of chlamydial 16S rRNA primary
transcripts (Deka et al., 2006). In addition, co-infection-induced persistence is not mediated by any previously characterized anti-chlamydial pathway or persistence inducer (Vanover et al., 2008). In this study we will present data that suggest that interaction of the viral glycoprotein gD with host co-receptors activates a novel anti-chlamydial defense pathway in mucosal epithelial cells resulting in development of chlamydial persistence.

Results

Chlamydial infectivity recovers following long-term co-infection with UV-inactivated HSV-2 (HSV-2UV)

Under constant stimulus, persistent chlamydiae can be sustained in culture for long periods of time (Byrne et al., 1989; Matsumoto and Manire, 1970). Additionally, chlamydiae recover infectivity following removal of the persistence inducer. For example, when Penicillin G is removed from the culture medium, RBs resume normal development and production of infectious progeny is restored (Matsumoto and Manire, 1970). Replacement of deficient/depleted nutrients also allows recovery of chlamydial infectivity (Harper et al., 2000; Raulston, 1997; Coles et al., 1993). Finally, IFN-γ-induced persistence is also reversible with the addition of exogenous tryptophan to the culture medium (Belland et al., 2003; Beatty et al., 1994). Previous data indicate that co-infection with UV-inactivated HSV-2 (HSV-2UV) induces persistence (Deka et al., 2007). HSV-2UV is both replication incompetent and nonlethal to infected cells (Moxley et al., 2002). This property of HSV-2UV was exploited to study the long-term effects of co-infection on C. trachomatis serovar E development.

Triplicate HeLa cell monolayers were either mock, singly, or co-infected with C. trachomatis and HSV-2UV. Cultures were refed with fresh medium containing 1µg/ml
cyclohexamide (Cx) following HSV-2UV infection and every 48hpi thereafter. Cyclohexamide was added to the medium to prevent cell overgrowth and does not interfere with HSV-induced persistence (Deka et al., 2007). Replicate samples were harvested at 20h (Day1), 68h (Day 3), and 140h (Day 6) post-HSV-2UV infection and processed for total DNA isolation and chlamydial titration as described in the Experimental Procedures. HSV and chlamydial genome accumulation was determined by semi-quantitative PCR; human GAPDH was amplified as an internal control (Deka et al., 2006). Dilution series of HSV-2, chlamydial, and HeLa cell DNA were amplified as positive controls to insure that results were quantified within the linear range of the PCR (Deka et al., 2006). HSV DNA was not amplified in any of the HSV-infected samples (Fig. 5.1A), indicating that viral replication was inhibited during co-infection with HSV-2UV. Plaques assays also indicated that no viral replication occurred during the course of the co-infection with HSV-2UV (data not shown). Also, the amount of chlamydial DNA in chlamydia singly-infected and co-infected cultures was equivalent (Fig. 5.1A). However, the amount of infectious EBs produced in the co-infected cultures on Day 1 was significantly lower than that in the C. trachomatis singly-infected cultures (Fig. 5.1B) as previously observed (Deka et al., 2007). In contrast, EB production in HSV-2UV/C. trachomatis samples collected on Days 3 and 6 was not significantly different from those in chlamydia singly-infected samples. These data indicate that chlamydiae can recover infectivity if incubated for more than 24h following a single round of HSV co-infection. Thus, like other persistence inducers, HSV-induced persistence is reversible.
Figure 5.1: Chlamydial infectivity recovers during long-term co-infection with UV-inactivated HSV-2 (HSV-2_{UV}). Hela cell monolayers were either mock, singly or co-infected with *C. trachomatis* and an amount of HSV-2_{UV} equivalent to 10 MOI replication competent HSV-2. Replicate samples were harvested at 20h (Day 1), 68h (Day 3), and 140h (Day 6) post-HSV-2_{UV} infection and processed for DNA isolation (Panel A) and chlamydial titration (Panel B). A. Total DNA was used to determine relative HSV (HSV G2), chlamydial (Ct 16S rRNA gene) and host (GAPDH) genome accumulation in co-infected cells by PCR. A dilution series of purified HSV-2, *C. trachomatis* or HeLa DNA was amplified to insure that results were quantified within the linear range of the PCR (not shown). Results shown are representative of three biological replicates. B. Cells were harvested for EB titration analyses. EB titers are expressed as IFU/ml sample ± SEM; n=3. Asterisks (*) indicate titers that are significantly different (by t-test) compared to those from *C. trachomatis* singly-infected cells (p<0.05) collected at the same time.
HSV-2-induced chlamydial persistence may be triggered by interaction of viral glycoproteins with host cell surface receptors.

Co-infection with either HSV-2UV or with replication-competent HSV-2 in the presence of cyclohexamide triggers chlamydial persistence, indicating that productive viral replication is not required for this phenomenon (Deka et al., 2007). To determine whether viral glycoprotein/host receptor interactions alone induce persistence we performed co-incubation studies. These experiments are based on the observation that molecules present on the surface of fixed cells can interact with molecules on the surface of live responder cells causing signal transduction pathways to be activated (Savage et al., 1991). HeLa cell monolayers were either mock- or HSV-2-infected for 20h; at which time HSV envelope glycoproteins are expressed on the infected host cell surface (Roizman, 2001 and Fig. 5.2A). These monolayers were paraformaldehyde fixed using conditions which preserve protein structures on the host cell surface (Savage et al., 1991) but inactivate all infectious virions present in these cultures. These fixed cells were used as either mock (MI) or HSV-infected (HI) inducer cells in subsequent co-incubation experiments. To confirm the presence of viral glycoproteins on the surface of fixed inducer cells, replicate MI and HI cultures were immunostained with α-HSV glycoprotein antibodies + AlexFlor 488 conjugated secondary antibody. As an irrelevant antibody control, cells were also immunostained with α-Salmonella Common Antigen (α-Sal; Millipore). Immunofluorescent photomicrographs demonstrate that HSV gB, gC, gD, and gH are all present on the surface of the paraformaldehyde fixed HI (Fig. 5.2A). In contrast, no glycoprotein staining was observed on the surface of MI (Fig 5.2A), nor was staining observed in HI and MI cultures stained with secondary antibody alone (data not shown).
Duplicate viable HeLa cell monolayers were either mock (not shown) or chlamydiae-infected. At 24hpi viable mock or chlamydiae-infected responder cells were overlayed with MI or HI cells at a ratio of 5 fixed inducer cells/1 viable responder cell. The cells were co-incubated for 20h (Day 1) and then harvested for EB titration analysis or transmission electron microscopy (TEM). *C. trachomatis* singly-infected and co-infected HeLa cultures were also prepared as positive controls. Replicate co-incubated cultures were also harvested at 44h (Day 2), 68h (Day 3), and 140h (Day 6) and processed for chlamydial titration. As expected, HSV-2 co-infection significantly reduced infectious EB production compared to that from singly-infected cells (Fig. 5.2B). Co-incubation of fixed, HI cells with viable *C. trachomatis*-infected responder cells for 20h similarly reduced chlamydial infectivity (Fig. 5.2B). However, EB production in HI co-incubated samples recovered by Day 2 (44h) and was no longer significantly different from MI co-incubated samples by Day 3 (Fig. 5.2B). Electron microscopic studies (Fig. 5.2C) indicate that RB within *C. trachomatis*-infected responder cells co-incubated with HI cells for 20h became swollen and misshapen, typical of persistent chlamydiae (Fig. 5.2C, gray arrow). Conversely, co-incubation of MI cells with chlamydiae-infected responder cells did not alter the morphology of developing RB (Fig. 5.2C, White arrow) or prevent EB development (Fig. 5.2C, Black arrow). Importantly, both viral plaque assays and viral DNA PCR studies indicate that: i) no viable HSV was present and ii) no HSV replication occurred in co-incubated cultures (data not shown). These data suggest that interaction between HSV-2 virion glycoproteins and their cognate host cell receptors is sufficient to stimulate chlamydial persistence and confirm that HSV-induced persistence is reversible.
Figure 5.2: Co-Incubation of HSV-2-infected fixed inducer cells with *C. trachomatis*-infected responder cells. A. Mock (MI) or HSV-2-infected (HI) inducer cells were paraformaldehyde fixed and immunostained with α-Sal, α-gB, α-gC, α-gD, or α-gH NuAb + AlexFlor 488 conjugated secondary antibody. Photomicrographs are 320X magnification. B. MI or HI inducer cells were co-incubated with mock or chlamydiae-infected responder cells for 20h (Day 1), 44h (Day 2), 68h (Day 3), and 140h (Day 6) and then harvested for EB titration analysis. *C. trachomatis* singly-infected and co-infected HeLa cultures were prepared as positive controls. Asterisks (*) indicate titers that are significantly different (by t-test) compared to those from either *C. trachomatis* singly-infected cultures (for Ct/HSV controls) or *C. trachomatis* + mock inducer cultures (for HI+Ct samples) (p<0.05). C. Co-incubated cultures were harvested at 20h, fixed with glutaraldehyde-paraformaldehyde and processed for TEM. Sections were examined using a Tecnai Philips Transmission Electron Microscope. Each photomicrograph is 7,000 x magnification.
Neutralization of virions with HSV glycoprotein D specific antibody prior to co-infection prevents induction of chlamydial persistence.

HSV attachment studies have demonstrated that interaction of gD or gH with neutralizing antibodies (NuAbs) inhibits viral entry and replication. In contrast, anti-gC NuAbs have no effect on HSV entry or infectivity (Parry et al., 2005; Fuller et al., 1989; Fuller and Spear, 1987). Based upon this information, antibody neutralization of individual HSV glycoproteins during C. trachomatis/HSV co-infection was used to determine which glycoprotein(s) are required for altering chlamydial development during co-infection. A strain of HSV-1 expressing the β-galactosidase gene (HSV-1/β-gal) was used in these experiments so that viral entry into the host cell could be examined. For each replicate sample, α-gB, α-gC, or α-gD NuAb was added to HSV-1/β-gal before infection. An equal quantity of α-Sal or α-Human Papilloma Virus 18 (α-HPV: OEM Concepts) was used as irrelevant Ab controls. HSV host cell entry is not affected by α-HPV (data not shown) or by α-Sal (Fuller and Spear, 1987). In duplicate experiments, spin concentrated α-gH hybridoma supernatant was used to neutralize gH. To control for effects caused by concentration of serum components in the α-gH hybridoma supernatant, concentrated medium+10% serum (cMEM) and medium+10% serum+α-HPV (cMEM/α-HPV) were used as negative controls. In these experiments, HeLa cell monolayers were co-infected with C. trachomatis and neutralized HSV-1/β-gal. Replicate cultures were processed for β-gal assays (Fig. 5.3A), chlamydial titration (Fig. 5.3B) and plaque assay analyses (data not shown). As expected, β-galactosidase activity was present in the HSV-1/β-gal infected monolayers as well as in cells infected with cMEM, cMEM/α-HPV, α-Sal, α-gB, or α-gC neutralized HSV-1/β-gal (Fig. 5.3A). Only α-gD and α-gH inhibited HSV-1/β-gal entry into the host cells, as evidenced by the lack of β-galactosidase activity (Fig. 5.3A). Chlamydial titer analyses indicate that co-infection
with non-neutralized HSV-1/β-gal or α-Sal cMEM, cMEM/α-HPV, α-gB α-gC, and α-gH Ab neutralized virus, significantly decreased infectious EB production compared to C. trachomatis singly-infected samples (Fig. 5.3B and C). Notably, chlamydial titer was not decreased when cells were co-infected with α-gD neutralized HSV-1/β-gal virus (Fig. 5.4B). Plaque assays indicated that viral replication and release was decreased by > 90% when HSV-1/β-gal was neutralized with α-gD or α-gH NuAbs (data not shown). Anti-Sal, cMEM, cMEM/α-HPV, α-gB, or α-gC had no effect on virion replication and release (data not shown). These data indicate that HSV gD/host co-receptor interaction is required, and is likely sufficient, for induction of co-infection-induced chlamydial persistence.
Figure 5.3: Co-infection of *C. trachomatis* with antibody neutralized HSV-1/β-gal. A. Cultures of Hela cells were either mock, singly or co-infected with *C. trachomatis* and HSV-1/β-gal or HSV-1/β-gal neutralized by α-Sal, α-gB, α-gC, α-gD or α-gH NuAbs, cMEM or cMEM/α-HPV, incubated for 6h and assayed for β-galactosidase activity. Monolayers in 24 well tissue plates were photographed with an Epson Perfection 3200 Photo Scanner. Replicate cultures of Hela cells were either mock, singly, or co-infected with *C. trachomatis* and HSV-1/β-gal or HSV-1/β-gal neutralized by α-Sal, α-gB, α-gC, α-gD (Panel B) or α-gH NuAbs, cMEM or cMEM/α-HPV (Panel C) and processed for chlamydial titration. EB titers are expressed as IFU/ml sample ± SEM; n=3. Asterisks (*) indicate titers that are significantly different (by t-test) compared to those from *C. trachomatis* singly-infected cells (p<0.05).
HSV gD/host cell co-receptor interaction is sufficient to stimulate chlamydial persistence.

Because gD was implicated as the major virion component necessary for stimulating HSV-induced persistence, the effects of soluble, recombinant HSV-2 gD protein on chlamydial infectivity were examined. Plasmids expressing a soluble HSV-2 gD/rabbit IgG Fc (gD:Fc) fusion protein and the vector control (Blk:Fc) were obtained from Dr. Patricia Spear (Yoon et al., 2003). The gD:Fc expression plasmid was constructed such that the transmembrane portion of gD was replaced with the Fc portion of rabbit IgG, which allows the fusion protein to be secreted from expressing cells and purified by protein A chromatography. The Blk:Fc control plasmid expresses only rabbit IgG Fc (Yoon et al., 2003). Culture supernatants from stable CHO cell lines containing each plasmid were collected and secreted gD:Fc fusion proteins were purified and quantified as described in the Experimental Procedures. Binding of purified gD:Fc to HeLa cells was confirmed by immunofluorescent staining with Texas Red conjugated α-Rabbit IgG Fc antibodies (Figure 5.4A).

Many cell surface receptors must aggregate to initiate signal transduction. For example, Nectin molecules must dimerize before initiating associated cell signaling cascades (Ogita and Takai, 2006). If the same is true for the gD initiated signaling pathway responsible for persistence induction, then signaling (and chlamydial persistence induction) would require multimerization of the gD:Fc protein. Such ligand clustering can be achieved by adding anti-rabbit IgG (Fig. 5.4B, right). Replicate C. trachomatis singly-infected samples were incubated with α-Rabbit IgG, gD:Fc, Blk:Fc, mixtures of α-Rabbit IgG + gD:Fc (αIgG/gD:Fc) or αIgG/Blk:Fc at 1:4 αIgG:fusion protein ratios for 1h. Samples were then refed with culture medium, harvested at 20h post-fusion protein exposure, and processed for chlamydial titration. As a positive control, HeLa cell monolayers were co-infected with C. trachomatis and HSV-2
(Fig. 5.4C; Ct/HSV). As expected, chlamydial infectivity was decreased by HSV-2 co-infection. No significant difference in EB production was observed when chlamydiae-infected samples were exposed to αIgG, gD:Fc, or Blk:Fc alone or to the αIgG/Blk:Fc negative control (Fig. 5.4C). However, when chlamydiae-infected cells were exposed to αIgG/gD:Fc, EB production was significantly decreased compared to the C. trachomatis singly-infected control. Data from preliminary experiments indicated chlamydial infectivity was reduced in a dose dependent manner; lower αIgG to fusion protein ratios (1:4 and 1:8), which would be expected to produce maximal ligand cross-linking, have the greatest effect on chlamydial infectivity (data not shown). In duplicate experiments, C. trachomatis singly-infected samples were incubated with αIgG/gD:Fc or αIgG/gD:Fc that had been previously incubated with α-gD NuAbs (αIgG/gD:Fc + αgD). Chlamydial titer analyses demonstrated that neutralization with gD specific antibody prevented the decrease in EB production observed when chlamydiae-infected cells were exposed to αIgG/gD:Fc (Fig. 5.4D). Overall, these data indicate that: i) HSV-2 gD interaction with host cell surface receptors is sufficient to induce chlamydial persistence and ii) ligand (and likely receptor) clustering is required for the inhibitory effect (Fig. 5.4B).
Figure 5.4: Interaction of HSV-2 gD:Fc fusion proteins with *C. trachomatis* infected cultures decreases chlamydial infectivity. A. HeLa cell cultures were immunostained with 1xPBS (Mock), Blk:Fc or gD:Fc +/- Texas Red conjugated α-Rabbit IgG Fc antibodies. Cells were photographed at 320x magnification. B. Model for activation of signaling from HSV co-receptors by antibody/ligand cross-linking and receptor clustering. Because many cell surface receptors must aggregate to initiate signal transduction, HSV gD may require multimerization to initiate chlamydial persistence. Clustering of gD:Fc can be achieved by adding α-IgG FcAb. C. Replicate *C. trachomatis* singly-infected samples were incubated with α-IgG, gD:Fc, Blk:Fc or mixtures of α-IgG + gD:Fc (αIgG/gD:Fc) or αIgG/Blk:Fc at a 1:4 ratio and harvested for chlamydial EB titration. As a positive control, Hela cultures were co-infected with both *C. trachomatis* and HSV-2 (Ct/HSV). D. *C. trachomatis* singly-infected samples were incubated with αIgG/gD:Fc or αIgG/gD:Fc neutralized with α-gD NuAbs (αIgG/gD:Fc + αgD) and harvested for EB titer analysis at 20h post-fusion protein exposure. Asterisks (*) indicate titers that are significantly different (by t-test) compared to those from either *C. trachomatis* singly-infected cultures.
Discussion

The studies presented here further characterize HSV-2 co-infection-induced, C. trachomatis serovar E persistence. The results demonstrate that chlamydiae can recover infectivity after undergoing HSV-induced persistence. However, co-infection-induced persistence was not prolonged following HSV-2\textsubscript{UV} infection or co-incubation of chlamydiae-infected responder cells with HI. These results most likely reflect the fact that chlamydial recovery was examined following a single stimulation event. In contrast, other models demonstrate that persistent chlamydiae were maintained for several days as long as the exogenous persistence inducer was regularly added to the culture medium (Byrne \textit{et al.}, 1989).

Co-incubation of fixed, HSV-infected inducer cells with viable C. trachomatis-infected responder cells reduces production of infectious chlamydiae, suggesting that interaction between HSV-2 glycoproteins and their associated host cell receptors is sufficient to stimulate chlamydial persistence. Viral attachment to the host cell is a dynamic and active process that stimulates cellular signaling cascades. For example, TLR-2 is host cell surface exposed and appears to recognize the herpes virion protein, gB. This interaction stimulates TLR-linked anti-viral cascades in host cells (Aravalli \textit{et al.}, 2007; Kurt-Jones \textit{et al.}, 2005; Compton \textit{et al.}, 2003). Likewise, human cytomegalovirus gB provides sufficient stimulus to activate anti-viral responses through TLR-2 (Compton \textit{et al.}, 2003). Infection with varicella zoster virus, HSV-1, and HSV-2 can all stimulate IL-6 production in culture; this induction is TLR-2 dependant and does not require productive viral replication (Aravalli \textit{et al.}, 2005; Kurt-Jones \textit{et al.}, 2005; Wang \textit{et al.}, 2005). However, neutralization of gB, gC, or gH was not able to circumvent the decrease in infectious chlamydial progeny caused by HSV co-infection. In addition, recombinant gD:Fc fusion protein induces persistence. Finally, IFN-β is one of the first genes activated by HSV-
induced TLR signaling (Schneider et al., 2004). Previously we demonstrated that IFN-β mRNA is not expressed in HSV-2/C. trachomatis co-infected cells (Vanover et al., 2008). Therefore, HSV gB/TLR receptor interaction is unlikely to be the mechanism behind co-infection-induced chlamydial persistence.

Antibody neutralization experiments indicated that the viral gD, but not gC, gB, or gH/L, was required for stimulation of co-infection-induced persistence. Addition of clustered, soluble gD:Fc fusion proteins to chlamydiae-infected cells also decreased infectivity. Taken together these data indicate that HSV-2 gD interaction with host cell surface receptors is sufficient to stimulate developing chlamydiae to enter persistence. The primary role of gD during viral attachment is to bind to at least one of several co-receptors, herpes viral entry mediator (HVEM), Nectin-1, Nectin-2, or 3-O-Sulfated Heparan Sulfate, present on the surface of the host cell (Spear, 2004).

HVEM is a member of the tumor necrosis factor receptor family (Mauri et al., 1998). When complexed to its natural ligand, LIGHT, HVEM interacts with TNF-associated factors that trigger NF-κB signaling pathways. Activation of these cascades by HVEM has been shown to be involved in the activation of T-cells (Granger and Rickert, 2003; Hsu et al., 1997). HVEM is primarily expressed on T and B lymphocytes, epithelial cells, and fibroblasts and can be used by both HSV-1 and HSV-2 for entry into the host cell (Kwon et al., 2006; Spear, 2004; Hsu et al., 1997; Marsters et al., 1997; Montgomery et al., 1996). Nectin-1 and Nectin-2 are members of the Immunoglobulin superfamily and are involved in the formation of cell junctional complexes (Cocchi et al., 1998). When stimulated, nectins activate cell signaling molecules Cdc42 and Rac small G proteins via c-Scr that leads to reorganization of the actin cytoskeleton and formation of cell-cell adherens junctions (Nakanishi and Takai, 2004; Shimizu and Takai, 2003). Activation
of Cdc42 via Nectin also leads to changes in gene expression by triggering the c-JunN-terminal kinase (JNK) pathway (Nakanishi and Takai, 2004). Nectin-activated pathways are involved in important cellular events such as apoptosis and cell growth regulation (Sakisaka and Takai, 2004; Lin, 2003; Johnson and Lapadat, 2002). Both Nectin 1 and 2 are expressed on epithelial and neuronal cells (Spear, 2004). However, Nectin-2 is primarily a co-receptor for HSV-2, whereas, Nectin-1 is used with equal efficiency by both HSV-1 and HSV-2. Lastly, 3-O-sulfated heparan sulfate is expressed on numerous cell types, although it is only used as a co-receptor by HSV-1 (Shukla et al., 1999).

Interestingly, gD/co-receptor interaction has been shown to trigger several signaling cascades associated with HVEM and Nectin-1/2. UV-inactivated HSV-1 and HSV-2 can stimulate cytokine production in human cells, specifically TNF-α production from IFN-γ primed macrophages and IL-15 production from monocytes (Ahmad et al., 2007; Paludan, 2001). Both HSV-1 gD and UV-inactivated virions can stimulate NF-κB activity at 1-3 hpi (Teresa Sciortino et al., 2007; Amici et al., 2006). Activation of Rac1 and Cdc42 has been shown early during HSV-1 infection of MDCK canine kidney cells (Hoppe et al., 2006). HSV attachment/entry also stimulates calcium-signaling pathways leading to intracellular calcium uptake (Cheshenko et al., 2003). Given these observations, it is feasible that stimulation of viral co-receptors by attachment of HSV-2 could transmit a cellular signal that has downstream effects on developing chlamydiae.

These observations lead to the following question. If gD/co-receptor interaction initiates a signal resulting in persistence, which co-receptor is involved? Several lines of evidence point to the involvement of Nectin-1 in co-infection-induced persistence. First, both HSV-1 and HSV-2 induce chlamydial persistence. Because both HVEM and Nectin-1 serve equally well as co-receptors for the two types of HSV, both of these receptors are candidates for initiating this
response. However, Montgomery et al. demonstrated that anti-HVEM serum only had marginal effects on the entry of HSV-1 into HeLa cells, suggesting that HVEM is probably not the principle receptor for HSV entry into HeLa cells (Montgomery et al., 1996). Secondly, exposure to non-clustered, soluble gD:Fc fusion proteins had little effect on chlamydial infectivity. In contrast, IgG-preclustered gD:Fc significantly reduced chlamydial infectivity. Interestingly, aggregation of Nectin molecules by ligand clustering is also required for initiation of cell signaling cascades (Ogita and Takai, 2006). We hypothesize that preclustering of gD:Fc fusion proteins increases receptor multimerization and, thus, increases the efficiency of downstream cell signaling. Collectively, the observations that: i) both HSV-1 and HSV-2 induce persistence and use Nectin-1 and HVEM as co-receptors, ii) HVEM is not considered the primary HSV co-receptor in HeLa cells, and iii) clustering of gD receptors is required for the gD:Fc fusion protein stimulated effects on chlamydial infectivity argues that HSV gD interaction with Nectin-1 is the most likely candidate for stimulation of co-infection-induced persistence.

However, caution must be exerted when eliminating potential mechanisms for co-infection-induced C. trachomatis persistence. HVEM encoding cDNA was originally isolated from a HeLa cell library (Montgomery et al., 1996). Although HVEM may not be the major co-receptor present on HeLa cells, it is possible that interaction between gD and the small amount of HVEM present provides adequate signal transduction to halt chlamydial development. Though Nectin-2 does not efficiently mediate HSV-1 entry, the low affinity of HSV-1 gD for Nectin -2 could be sufficient to stimulate anti-chlamydial signaling pathways. HSV-2 can also enter Chinese hamster ovary (CHO) cells by an unknown receptor (Spear, 2004). Thus, there is a chance that gD interaction with an unknown HSV co-receptor could trigger chlamydial
persistence. Finally, gD interaction with multiple co-receptors could activate several host pathways that work together to negatively affect developing chlamydiae.

Investigations of the underlying mechanisms that drive developing chlamydiae to enter persistence have provided valuable information about the biology and pathogenesis of this fascinating organism (Nelson et al., 2005; Fehlner-Gardiner et al., 2002). HVEM, Nectin-1, and Nectin-2 all have endogenous, natural ligands (Nakanishi and Takai, 2004; Granger and Rickert, 2003; Shimizu and Takai, 2003; Hsu et al., 1997). This observation raises the question of whether natural stimulation of these receptors could also negatively affect chlamydial development. If so, this novel host pathway could be involved in limiting the spread of chlamydiae in host epithelial cells, much like the well characterized IFN-γ-induced anti-chlamydial response. Thus, investigations of the pathways that are activated by HSV co-receptors and how these cascades interact with developing chlamydiae have the potential to reveal new and captivating aspects of the relationship that C. trachomatis fosters with its host cell.
Experimental Procedures

Chlamydia, HSV, and host cells.

A human urogenital isolate of *C. trachomatis* E/UW-5/CX was originally obtained from S.P. Wang and C. C Kuo (University of Washington, Seattle, WA). The same standardized inoculum of *C. trachomatis* serovar E, propagated in McCoy cells, was used for all experiments (Wyrick *et al.*, 1996). Herpes simplex virus type 2 strain 333 and HSV-1/β-gal (originally denoted as HSV-1 strain tk-12; Montgomery *et al.*, 1996) stocks were obtained from Mary K. Howett (Drexel University) and Patricia Spear (Northwestern University) respectively. Viral stocks were prepared in monolayers of Vero cells (African green monkey kidney cells ATCC#CCL-81) as described (Duff & Rapp, 1971). Chinese Hampster Ovary-K1 cells (CHO-K1 ATCC#CCL-61) were obtained from Russell Hayman (James H. Quillen College of Medicine).

Co-infection experimental design.

Co-infections were performed as previously described with the following exceptions (Deka *et al.*, 2006). HeLa cells, a human cervical adenocarcinoma epithelial cell line (ATCC #CCL2), were used for all infection experiments. In most experiments, host cells were divided into four groups for mock-infection, chlamydial-infection, HSV-infection, and *C. trachomatis*/HSV-co-infection. First, HeLa cell monolayers were incubated with a dilution of crude EB stock calculated to infect >80% of the cells. Following an hour of adsorption, monolayers were refed with Minimal Essential Medium (MEM; Gibco) and incubated at 35 °C for 24h. Cultures were then infected with HSV-2, HSV-2<sup>UV</sup>, HSV-1/β-gal, or neutralized HSV-1/β-gal at a multiplicity of infection (MOI) of 10 PFU/cell. In certain studies, HSV infection was
replaced with exposure to various recombinant fusion proteins for 1h at 35°C. Mock-infected HeLa cells were treated similarly except they were exposed to either 2SPG (0.2 M sucrose, 6mM NaH$_2$PO$_4$, 15mM Na$_2$HPO$_4$, 5mM L-glutamine, pH 7.2; mock C. trachomatis infection) or growth medium (mock viral infection). Monolayers were refed with MEM following HSV adsorption. For long-term studies, cultures were refed with culture medium containing 1µg/ml cyclohexamide.

**Generation of replication incompetent HSV-2 (HSV-2$_{UV}$).**

A UV Crosslinker (Spectroline Microprocessor Controlled UV-Crosslinker Spectrolinker XL1500, Spectronics Corporation, New York) was used to generate stocks of UV-inactivated, replication incompetent HSV-2 as previously described (Deka et al., 2007). Plaque assays analyses confirmed HSV-2 inactivation (data not shown) and were performed as described previously (Deka et al., 2007).

**Co-incubation experiments.**

Co-incubation experiments were performed as described previously with modifications (Savage et al., 1991). HeLa cell monolayers were either mock or HSV-2-infected, incubated at 37°C for 20h, and fixed for 15min at 37°C in a 1% paraformaldehyde, 2% FBS, 1x PBS solution. Monolayers were washed extensively and “refed” with MEM so that the fixative could leach out of the cells overnight. The inducer cells were then washed, scraped into 5ml culture medium, and counted with a hemocytometer. The fixed mock (MI) or HSV-infected (HI) inducer cells were overlayed onto viable mock or chlamydiae-infected responder cell cultures at a ratio of 5 fixed...
inducer cells/1 viable responder cell. The cells were co-incubated at 35°C in normal growth medium for 20, 44, 68, or 120h before being harvested.

**Antibody neutralization of HSV glycoproteins.**

Neutralizing antibodies to HSV gD (III-174), gB (II 105-1.6), and gC (II529-1) were all kind gifts from Patricia Spear (Northwestern University; Fuller and Spear, 1987). For each sample 4µl of culture medium, α-Sal (Millipore, MAB746), α-gB, α-gC, or α-gD NuAb was added to 100ul of HSV-1/β-gal (10MOI). All inocula were incubated for 1h at 37°C prior to infection of mock- or chlamydiae-infected HeLa monolayers (Fuller and Spear, 1987). Plaque assay analyses demonstrate that 4 µl of α-gD NuAb sufficiently eliminated productive HSV replication (data not shown). The α-gH (LP11) mouse hybridoma supernatant was a kind gift from Helena Browne (University of Cambridge; Parry et al., 2005). Amicon Ultra Centrifugal Filter Devices were used to concentrate 10ml of α-gH hybridoma supernatant, MEM+10%FBS, and MEM+10%FBS + 40µl α-HPV Ab (OEM Concepts, M2-V56) to 1ml. Maximal neutralization of 10MOI HSV-1/β-gal required 25 µl of concentrated α-gH NuAbs (data not shown). In control samples, 25 µl of cMEM or cMEM/α-HPV was added to the viral inocula.

**Preparation of HSV-2 gD:Fc fusion proteins.**

Plasmids expressing a soluble HSV-2 gD/rabbit IgG Fc (gD:Fc) fusion protein and the vector control (Blk:Fc) were obtained from Patricia Spear (Yoon et al., 2003). The plasmids were propagated in XL1 Blue competent *Escherichia coli* under Ampicillin (50 µg/ml) selection. Plasmid DNA was harvested using the PureLink HiPure Plasmid DNA Purification Kit (Invitrogen) and the identity of each plasmid was confirmed by restriction enzyme digest (data
not shown). The plasmids were transfected into CHO-K1 cells using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacture’s recommendations. Stable, plasmid containing cell populations were isolated under geneticin selection (250 μg/ml G418). After drug selection, culture supernatants were collected and secreted gD:Fc fusion proteins were purified using PROSEPA Montage Affinity Purification Spin Columns (Millipore) and concentrated with Amicon Ultra-15 centrifugal filters according to the manufacture’s instructions.

Quantification and preclustering of HSV-2 gD:Fc fusion proteins.

Purified HSV-2 gD:Fc as well as Blk:Fc fusion proteins were separated on on Nu-PAGE 4-12% Bis-Tris gels (Invitrogen) and visualized with Sypro Ruby stain (Pierce). The amount of each fusion protein was estimated by comparison to a known quantity of protein standards (Broad Range Standards, Pierce). Gels were visualized using a BioRad Chemi Doc XRS Image Capture System. Proteins were quantified on the image analysis system using Quantity One V4.5.0 software (BioRad). Ten microliters of each fusion protein was mixed with Mouse-anti-Rabbit IgG (Jackson Immuno) at various antibody:fusion protein molar ratios and incubated for 1h at RT. Preclustering of gD:Fc fusion proteins with α-IgG was based upon the protocol published by Ogita and Takai (2006).

Chlamydial titrations by subpassage.

Chlamydial titrations were performed as previously described (Deka et al., 2006) using Pathfinder anti-chlamydial stain (BioRad) to stain chlamydial inclusions formed from
subpassaged EBs. The number of inclusion forming units (IFU) in the undiluted inoculum was then derived from triplicate counts and expressed as IFU/ml.

**DNA isolation and PCR.**

Total DNA was isolated from cells as described previously (Deka et al., 2006). Total DNA preparations were quantified using optical density (OD) at 260 and 280 nm; all samples had OD260/280 ratios > 1.9. Following DNA isolation, the human GAPDH, chlamydial 16SrRNA, and HSV-2 glycoprotein G2 genes were amplified by PCR as previously described (Deka et al., 2006). After PCR, all reactions were electrophoresed on 1.5% agarose/TBE gels. Gels were stained with EtBr and visualized using a BioRad Chemi Doc XRS Image Capture System. Amplimers were quantified on the image analysis system using Quantity One V4.5.0 software (BioRad).

**β-galactosidase assay.**

β-galactosidase activity was assayed essentially as described (Montgomery et al., 1996). Briefly, HeLa cells were infected with HSV-1/β-gal or antibody neutralized HSV-1/β-gal for 6h, washed with 1x PBS, fixed with a 2% formaldehyde/0.2% glutaraldehyde PBS solution for 10min at RT. Following fixation the cells were washed and permeablized in a 2mM MgCl₂/0.01% deoxycholate/0.02% NP40 solution for 10min at RT. The cells were stained with 0.5 mg/ml X-Gal in PBS for 1hr at 37°C. Images were captured using an Epson Perfection 3200 Photo Scanner and Photoshop Elements Software.
Fluorescent microscopy, transmission electron microscopy and image analysis.

Fluorescence analyses were performed as described previously (Deka et al., 2007). HSV gD:Fc fusion proteins bound to HeLa cells were stained using a 1:200 dilution of Texas red-conjugated donkey-anti-rabbit IgG (Jackson Immuno). The same fixation conditions used to prepare inducer cells in co-incubation experiments were used to visualize HSV glycoproteins on the surface of HeLa cells. Because the cells were not permeablized, only the surface glycoproteins are detected in this assay. Following fixation, cells were washed 3x with 1xPBS, blocked with 15%FBS in PBS for 45min at RT, and stained for 1h with 1:50 dilution of mouse-α-gB, α-gC, α-gD, or α-gH mAbs followed by staining for 1h with 1:1000 dilution of Alexa fluor donkey-anti-mouse IgG (Invitrogen). Images were captured using a Zeiss Axiovert S100 microscope and Axiocam camera. Duplicate samples of chlamydiae-infected cultures co-incubated with MI or HI were processed at 20 h post-co-incubation for high contrast transmission electron microscopy as described (Wyrick, 1994). Counterstained gold thin sections were examined using a Tecnai 10 (FEI) transmission electron microscope operating at 60-80 kV.

Statistical analyses.

Statistical analyses were performed using Microsoft Excel. Comparison of means was done by using a 2-sample t-test for independent samples. P values of ≤ 0.05 were considered significant. All plotted values are averages of either eight or nine biological replicates divided between 3 individual experiments ± SEM.
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Investigations of the underlying mechanisms that drive developing chlamydiae to enter persistence have provided valuable information about the biology and pathogenesis of this fascinating organism. Notably, studies have revealed that the mechanism of IFN-γ mediated immune control of chlamydial infection is different in mice and humans. In murine cells, IFN-γ exposure inhibits chlamydial development by restricting vesicular trafficking to developing inclusions whereas in human cells IFN-γ-exposure indirectly deprives chlamydiae of tryptophan (Nelson, 2005; Beatty, 1994d). Related studies involving IFN-γ-induced persistence have also revealed the genetic basis of differences in cell tropism between the ocular and genital serovars of C. trachomatis. Genital serovars contain a functional tryptophan synthase gene that allows them to use exogenous indole (presumably acquired from the local bacterial flora of the genital tract) to recover from IFN-γ stimulated degradation of host tryptophan. Ocular serovars do not recover from IFN-γ persistence using indole (Fehlner-Gardiner et al., 2002).

Characterization of a tissue-culture model of HSV and C. trachomatis co-infection indicates that co-infection stimulates chlamydiae to become persistent. This conclusion is evidenced by numerous observations during C. trachomatis/HSV co-infection: i) a decrease in the production of infectious EBs, ii) abnormal RB morphology, iii) increased accumulation of chlamydial Hsp60, iv) decreased accumulation of MOMP, v) continued accumulation of chlamydial chromosomes, and vi) constant expression of chlamydial 16S rRNA primary transcripts, indicating that chlamydiae remain viable although they are less infectious (Deka et al., 2006). Co-infection-induced persistence is neither cell type specific nor viral strain specific.
because it occurs in both HeLa and HEC-1B cell lines and is stimulated by both HSV-1 and HSV-2 (Deka et al., 2007). Co-infection-induced persistence is also not limited to C. trachomatis. Unpublished data from our laboratory demonstrate that C. muridarum also enters persistence following HSV-2 co-infection (data not shown). Additionally, co-infection with either UV-inactivated, replication-incompetent virus or with replication-competent HSV-2 in the presence of cyclohexamide triggers chlamydial persistence, indicating that productive viral replication is not required for this phenomenon (Deka et al., 2007). Finally, investigation of chlamydial titer following long-term exposure to HSV-2_{UV} indicates that chlamydiae recover infectivity from HSV-induced persistence in a manner similar to other tissue culture persistence models.

Because there are several characterized models of chlamydial persistence, we wanted to determine whether any of these mechanisms were activated by co-infection-induced persistence. Cytokines known to be involved in persistence induction were either not detected (IFN-γ, IFN-α, and TNF-α) or were detected in similar amounts (IL-1α, IL-1β, IL-8) in supernatants from C. trachomatis singly-infected and co-infected cultures. Additional data demonstrate that IFN-γ, IFN-β, IDO, LT-α, and iNOS mRNAs are not expressed during co-infection. Furthermore, previous studies have demonstrated that chlamydiae do not respond to IFN-γ exposure in HEC-1B cells (Wyrick and Knight, 2004; Kane and Byrne, 1998). However, developing chlamydiae exhibit characteristics of persistence within co-infected HEC-1B cells. Collectively these data eliminate cytokine activity as the inducer of persistence during co-infection. Moreover, supplementation of co-infected cells with excess amino acids, iron, glucose, or a combination of amino acids and iron does not restore chlamydial infectivity during co-infection, supporting the conclusion that virally-induced persistence is not mediated by the activities of cytokines. These
data also indicate that a global depletion of nutrients is not responsible for co-infection-induced persistence. Lastly, inclusions within co-infected cells continue to enlarge and incorporate C6-NBD-ceramide, indicating that HSV-2 co-infection does not inhibit vesicular transport to the developing inclusion. The degree of reduction in chlamydial infectivity stimulated by HSV-2 co-infection is lower than that observed in other models of persistence (Pantoja et al., 2001; Beatty et al., 1994d), suggesting that a unique mechanism could be involved. To observe the negative effects of cytokine exposure on chlamydial development, IFN-γ must be added to chlamydiae-infected cultures either before or very early during infection (Nelson et al., 2005). On the contrary, harmful effects on chlamydial development are observed with HSV-2 co-infection when the stimulus is added at 24h post-*C. trachomatis* infection (Deka et al., 2007). Overall, these observations demonstrate that HSV-2 co-infection-induced chlamydial persistence is not mediated by any known persistence inducer or anti-chlamydial pathway (Vanover et al., 2008).

As mentioned above, productive viral replication is not required for co-infection-induced persistence, indicating that events during HSV-2 attachment and/or entry are sufficient for this effect (Deka, 2007). Co-incubation of fixed, HSV-infected inducer cells with viable *C. trachomatis*-infected responder cells suggests that the events of HSV attachment, specifically interaction between HSV-2 virion glycoproteins and their cognate host cell receptors, is sufficient to stimulate chlamydial persistence. The initial stages of viral invasion are dynamic and active; thus, there are several possible avenues for stimulating persistence during virion attachment to the host cell. HSV attachment to the host cell involves co-receptors that naturally function as cell signaling molecules (Nakanishi and Takai, 2004; Granger and Rickert, 2003; Cocchi et al., 1998; Hsu et al., 1997). It is feasible that HSV attachment to co-receptors during co-infection could activate persistence by stimulating cell signaling pathways that have anti-
chlamydial effects. HSV also triggers anti-viral pathways via Toll-like receptors, thus, raising the possibility that stimulation of anti-viral cellular functions during co-infection could have a negative effect on chlamydial development (Aravalli, 2005; Kurt-Jones, 2005; Wang et al., 2005; Compton T., 2003; Pyles, 2002).

Several studies indicate that HSV gB is the major glycoprotein responsible for stimulation of TLR-linked anti-viral cascades in host cells (Aravalli et al., 2007; Kurt-Jones et al., 2005; Compton et al., 2003). Notably, in our studies, neutralization of gB was not able to restore chlamydial infectivity during co-infection. Antibody neutralization of individual HSV glycoproteins indicates that only gD interaction with co-receptors on the surface of host cells is required for stimulation of chlamydial persistence. Addition of clustered, soluble gD:Fc fusion proteins to chlamydiae-infected cells decreased production of infectious EB progeny, thus confirming the conclusion that HSV-2 gD interaction with host cell surface receptors can stimulate developing chlamydiae to enter persistence. Therefore, HSV interaction with TLR receptors is not likely the mechanism behind co-infection-induced chlamydial persistence.

The HSV envelope glycoprotein gD binds to one of several co-receptors that mediate entry of the virus into the host cell (Spear, 2004). These co-receptors include herpes viral entry mediator (HVEM), Nectin-1, Nectin-2, and 3-O-Sulfated Heparan Sulfate (Spear, 2004). HVEM is a member of the tumor necrosis factor receptor family (Mauri et al., 1998). When complexed to its natural ligand, LIGHT, HVEM interacts with TNF-associated factors that trigger NF-κB signaling pathways. Activation of these cascades by HVEM has been shown to be involved in the activation of T-cells (Granger and Rickert, 2003; Hsu et al., 1997). Nectin-1 and Nectin-2 are members of the Immunoglobulin superfamily and are involved in the formation of cell junctional complexes (Cocchi et al., 1998). When stimulated, nectins activate cell signaling molecules,
Cdc42 and Rac small G proteins, via c-Src that leads to reorganization of the actin cytoskeleton and formation of cell-cell adherens junctions. Epithelial cell polarity is based upon the maintenance of tight cell-cell junctions. Nectin stimulated, Cdc42 signaling activates downstream effectors that aid in the formation of tight junctions (Nakanishi and Takai, 2004; Shimizu and Takai, 2003). Activation of Cdc42 via nectin also leads to changes in gene expression by triggering the c-JunN-terminal kinase (JNK) pathway (Nakanishi and Takai, 2004). This pathway is involved in important cellular events such as apoptosis and cell growth (Sakisaka and Takai, 2004; Lin, 2003; Johnson and Lapadat, 2002).

Interaction of HSV-1 and HSV-2 virions or glycoproteins with host cells also triggers some of the same cell signaling cascades that are associated with HVEM and Nectin 1/2. UV-inactivated HSV-1 and HSV-2 can stimulate cytokine production in human cells, specifically TNF-α production from IFN-γ primed macrophages and IL-15 production from monocytes (Ahmad et al., 2007; Paludan, 2001). Both HSV-1 gD and UV-inactivated virions can stimulate NF-κB activity from 1-3hpi (Teresa Sciortino et al., 2007; Amici et al., 2006). Activation of Rac1 and Cdc42 has been shown at 15 to 30 minutes following HSV-1 infection of MDCK canine kidney cells (Hoppe et al., 2006). HSV attachment/entry also stimulates calcium-signally pathways leading to intracellular calcium uptake (Cheshenko et al., 2003). Conversely, HSV infection causes downregulation of the JAK/STAT signaling pathway by causing host cells to express SOCS3, suppressor of cytokine signaling 3 (Yokota et al., 2005).

Given these observations, it is feasible that stimulation of viral co-receptors by attachment of HSV-2 could transmit a cellular signal that has downstream effects on developing chlamydiae. While our studies demonstrate that the viral ligand involved in co-infection-induced persistence is gD, we are left with the question of which co-receptor is involved? As explained
below, preliminary data from our laboratory, as well as observations from other investigators, suggest that Nectin-1 has the most potential for triggering co-infection-induced persistence.

Because HSV-1 and HSV-2 are capable of inducing chlamydial persistence and both HVEM and Nectin-1 serve equally as co-receptors for the two types of HSV, these receptors are both strong candidates for triggering co-infection-induced persistence. Although HVEM cDNA was originally isolated from a HeLa cell library (Montgomery et al., 1996) studies have shown that HVEM is probably not the principle receptor for HSV entry into HeLa cells. Montgomery et al. (1996) demonstrated that anti-HVEM serum only had marginal effects on the entry of HSV-1 into HeLa cells. Additionally, both RT-PCR and western blot analyses demonstrate that HVEM is not expressed in our HeLa and HEC-1B cell line clones (data not shown).

Additional studies in our laboratory demonstrate that Nectin-1 is degraded in C. trachomatis infected HeLa cells at 36hpi (Sun et al., 2008). In our experimental model of co-infection-induced persistence, HSV infection/soluble gD exposure is performed at 24h after C. trachomatis infection before Nectin-1 degradation occurs. Western blot analysis of HeLa cells demonstrate that C. muridarum also degrades Nectin-1 and that significant degradation is observed by 24hpi (Sun et al., 2008). Co-infection of C. muridarum infected HeLa cells with HSV-2 causes a decrease in chlamydial infectivity as well. However, HSV infection of C. muridarum infected cells must be performed by 12h post-chlamydia infection for maximal effects on chlamydial infectivity to be observed. No significant decrease in EB production was seen when HSV was added to C. muridarum infected cells at 24hpi (data not shown). These data suggest that HSV co-infection cannot influence chlamydial development in the absence of Nectin-1.
Additionally, non-clustered, soluble gD:Fc fusion proteins had little to no effect on chlamydial infectivity. Exposure to gD:Fc fusion proteins decreased chlamydial infectivity only when the viral ligands were preclustered with anti-rabbit IgG. Many cell surface receptors must aggregate to initiate signal transduction; in fact, nectin molecules must dimerize before initiating cell signaling cascades (Ogita and Takai, 2006). We theorize that preclustering of gD:Fc fusion proteins increases receptor aggregation and thus, increases the efficiency of downstream cell signaling. Collectively, the observations that: i) both HSV-1 and HSV-2 induce persistence and use Nectin-1 and HVEM as co-receptors; ii) HVEM is not detected in our cell lines; iii) co-infection-induced persistence is not stimulated following Nectin-1 degradation in C. muridarum-infected cells; and iv) clustering of gD receptors is required for the gD:Fc fusion protein stimulated effects on chlamydial infectivity, suggest that HSV gD interaction with Nectin-1 is the most likely candidate for stimulation of co-infection-induced persistence.

However, without further experimental examination, we must be cautious in eliminating possible mechanisms for co-infection-induced persistence. Although our analyses indicate that HVEM is not present in our system, it is possible that only very small amounts of the protein, which are below our detection level, are required for stimulation of persistence. Nectin-2, along with Nectin-1, is expressed in our HeLa and HEC-1B cell lines (data not shown). Although Nectin-2 does not mediate HSV-1 entry, it is possible that HSV-1 gD has sufficient affinity for Nectin-2 to stimulate anti-chlamydial signaling pathways. HSV can also enter Chinese hamster ovary cells by an unknown low-affinity receptor (Spear, 2004). Thus, there is a chance that gD interaction with an unidentified HSV co-receptor is responsible for triggering chlamydial persistence. Finally, we cannot ignore the possibility that gD interaction with multiple co-
receptors could activate several host pathways that work together to negatively affect developing chlamydiae.

Induction of chlamydial persistence by HSV-2 co-infection has significant implications for pathogenesis. Release of chlamydial LPS from persistently-infected cells has been associated with membrane blebs similar to those observed in inclusions from *C. trachomatis*/HSV co-infected cultures (Wyrick *et al*., 1999; Wyrick *et al*., 1994; Karimi *et al*., 1989). Although chlamydial LPS is less potent than that of other Gram-negative bacteria, it still has the capacity to enhance the host inflammatory response (Ingalls *et al*., 1995). We have also demonstrated that Hsp60 is increased in co-infected cells. Chlamydial Hsp60 shares 48% identity with human Hsp60 and thus, increased accumulation of chlamydial Hsp60 during prolonged persistent infection has the potential to induce auto-immunity to human Hsp60 (Yi *et al*., 1997; Viale *et al*., 1994). We would predict that co-infection would increase inflammation and tissue damage by causing an increase in chlamydial LPS release or Hsp60-induced autoimmunity (Figure 6.1A). Because complications of chlamydial infection are thought to be due, at least in part, to release of inflammatory mediators from infected cells, any stimulus that increases release of these compounds might enhance disease pathology. The probability of this scenario is further enhanced by studies that suggest that virions are shed continuously even in the absence of lesions, making it is possible that viral-induced persistence could be stimulated in vivo when symptomatic viral reactivation is not obvious (Mertz *et al*., 1992). Furthermore, it is estimated that 50-200 defective virions are released for every replication competent virion produced (Roizman, 2001). Because only gD interaction with the host cell is required for persistence induction, attachment of defective virions to *C. trachomatis* infected cells could stimulate persistence. Because persistent chlamydiae have been shown to be able to withstand
bacteriocidal concentrations of antibiotics, they could potentially exist within the co-infected host for prolonged periods of time perpetuating even more tissue damage and severe disease manifestations (Wyrick and Knight, 2004).

Figure 6.1: Possible roles for gD co-receptor signaling in the development of persistent chlamydial infections. A. Immune control. B. Regulation of chlamydial development.
Given that HSV co-receptors have natural ligands, it is tempting to speculate that interaction between these molecules and their endogenous ligands could also trigger anti-chlamydidial pathways causing persistence in the absence of co-infection. In fact, one HVEM ligand, LT-α, has been demonstrated to restrict *C. pneumoniae* development (Matsushima *et al.*, 1999). Another intriguing possibility is that chlamydiae could use nectin-induced signaling to synchronize their development with the differentiation of the host cell, much like Human Papilloma Virus (HPV). During HPV infection, viral genomic replication occurs in undifferentiated basal epithelial cells; however, infectious virions are not produced from these immature cells. As the infected cells divide, the viral DNA is distributed to both daughter cells. One daughter cell will remain undifferentiated, serving as a reservoir for new epithelial cells and for viral genomic material. The other daughter cell will differentiate and move to the surface of the epithelium where infectious virions will be produced and released from the maturing cell (Longworth and Laimins, 2004). In this manner, HPV establishes both a productive, disseminating infection in the mature epithelial cells, as well as a reservoir of infection in the undifferentiated basal cells. *C. suis* has been shown to persistently infect immature, glandular epithelial cells while establishing a normal development cycle in differentiated lumenal epithelial cells (Guseva *et al.*, 2003). Because nectin-induced signaling is essential for the formation of new adherence junctions and polarity of epithelial cells (Nakanishi and Takai, 2004; Shimizu and Takai, 2003), it is feasible the nectin signaling events could be important in the replenishment of mature lumenal epithelial cells from the glands of the genital tract. Therefore, nectin-activated signal transduction might play a role in regulating chlamydial development in glandular versus lumenal epithelial cells and allow for both productive and chronic chlamydial infections to be established (Figure 6.1B).
In the absence of a genetic system, studies of chlamydial persistence have provided valuable information regarding chlamydial biology and chlamydiae/host cell interactions. Data from the investigation of HSV/\textit{C. trachomatis} co-infection have potentially uncovered yet another host-derived anti-chlamydial pathway. Examination of the cellular pathways that are activated by HSV co-receptor stimulation during co-infection has the potential to reveal new and fascinating aspects of the relationship that \textit{C. trachomatis} builds with the host cell and the mechanisms used by the host cell to rid itself of this unwelcome bacterial guest.


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Poster Presentations:


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