Interaction of herpes simplex virus type 2 (HSV-2) glycoprotein D with the host cell surface is sufficient to induce *Chlamydia trachomatis* persistence

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When presented with certain unfavourable environmental conditions, *Chlamydia trachomatis* reticulate bodies (RBs) enter into a viable, yet non-cultivable state called persistence. Previously, we established an *in vitro* *C. trachomatis* and herpes simplex virus type 2 (HSV-2) co-infection model. These data indicate that (i) viral co-infection stimulates chlamydial persistence, (ii) productive HSV replication is not required for persistence induction, and (iii) HSV-induced persistence is not mediated by any currently characterized anti-chlamydial pathway or persistence inducer. In this study we demonstrated that chlamydial infectivity, though initially suppressed, recovered within 44 h of co-infection with UV-inactivated HSV-2, demonstrating that HSV-induced persistence is reversible. Co-incubation of chemically fixed, HSV-2-infected inducer cells with viable, *C. trachomatis*-infected responder cells both suppressed production of infectious chlamydial progeny and stimulated formation of swollen, aberrantly shaped RBs. In addition, pre-incubation of viral particles with viral glycoprotein D (gD)-specific neutralizing antibody prevented co-infection-induced persistence. Finally, exposure of *C. trachomatis*-infected cells to a soluble, recombinant HSV-2 gD:Fc fusion protein decreased production of infectious EBs to a degree similar to that observed in co-infected cultures. Thus, we conclude that interaction of HSV gD with the host cell surface is sufficient to trigger a novel host anti-chlamydial response that restricts chlamydial development.

Received 19 November 2009
Revised 13 January 2010
Accepted 25 January 2010

INTRODUCTION

Herpes simplex virus type 2 (HSV-2), an enveloped DNA virus, is the primary cause of genital herpes infections. HSV-2 initiates host epithelial cell infection through a sequential set of viral envelope glycoprotein–host cell surface interactions. First, HSV-2 glycoproteins B and C (gB and gC) bind to host cell heparan sulfate. Second, the viral glycoprotein D (gD) protein binds one of several host co-receptors (Spear, 2004). Third, the HSV glycoprotein H/L (gH/L) complex promotes viral envelope–host cell plasma membrane fusion, allowing capsid entry. After replication, progeny virions are assembled in the nucleus and bud through viral glycoprotein-enriched areas of the

nuclear envelope. Finally, enveloped virions and viral envelope glycoproteins are transported to the host cell surface (Roizman & Knipe, 2001).

Chlamydiae are Gram-negative obligate intracellular bacteria that share a unique biphasic developmental cycle. Extracellularly, chlamydiae exist as small, infectious, metabolically inert elementary bodies (EBs). EBs attach to host cells, enter and differentiate into larger, non-infectious, replicative, reticulate bodies (RBs) within a modified vacuole called an inclusion. Following replication, RBs condense to form new EBs, which are released from the host cell (Abdelrahman & Belland, 2005). *Chlamydia trachomatis* serovars D–K are the most reported sexually transmitted disease (STD) agents associated with urethritis or cervicitis worldwide. Approximately 85–90 % of chlamydial infections are asymptomatic and chronic, sometimes causing severe disease sequelae (Peipert, 2003).

When developing chlamydiae are stressed, they can deviate from the normal developmental cycle into a state termed persistence (Hogan *et al.*, 2004). Persistent RBs are viable (Beatty *et al.*, 1994) but are swollen and aberrantly shaped when viewed by transmission electron microscopy (TEM)

Abbreviations: Blk : Fc, soluble Fc control protein; EB, elementary body; β -gal, β -galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; gD, -B, -C, -H/L, viral glycoproteins D, B, C, H/L; gD : Fc, soluble gD fusion protein; h.p.c.i., hours post co-incubation; HPV, human papilloma virus; HSV-1, -2, herpes simplex virus types 1, 2; HSV-2_{UV}, UV-inactivated HSV-2; HVEM, herpes viral entry mediator; IFN, interferon; IFU, inclusion forming units; NuAb, neutralizing monoclonal antibody; RB, reticulate body; α -Sal, anti-*Salmonella* common antigen antibody; TEM, transmission electron microscopy.

(Matsumoto & Manire, 1970). Because persistent RBs fail to differentiate into EBs, they are non-infectious (Beatty *et al.*, 1993, 1994; Byrne *et al.*, 1986; Johnson & Hobson, 1977). Evidence from several studies suggests that chlamydiae also enter persistence *in vivo* (Bragina *et al.*, 2001; Dean *et al.*, 2000; Fortenberry *et al.*, 1999; Gerard *et al.*, 2001, 2002; Nanagara *et al.*, 1995; Patton *et al.*, 1994). Several models of persistence have been characterized in culture, including penicillin exposure, monocyte infection, nutrient starvation and interferon (IFN)- γ exposure (Hogan *et al.*, 2004).

Our previously published data demonstrate that HSV co-infection of *C. trachomatis*-infected epithelial cells induces chlamydial persistence (Deka *et al.*, 2006). This effect is not mediated by any previously characterized anti-chlamydial pathway or persistence inducer (Vanover *et al.*, 2008). Data from this study indicate that contact between the HSV-2 gD protein and the host cell surface is sufficient to induce chlamydial persistence. We hypothesize that interaction between gD and an unknown host cell surface receptor activates a novel anti-chlamydial defence pathway in epithelial cells that alters the chlamydial developmental cycle.

METHODS

Chlamydia, HSV and host cells. The human urogenital isolate *C. trachomatis* E/UW-5/CX was propagated in McCoy cells (Wyrick *et al.*, 1996). HSV-2 strain 333 and HSV-1 strain tk12 (Montgomery *et al.*, 1996) were obtained from Mary K. Howett (Drexel University) and Patricia Spear (Northwestern University), respectively. Viral stocks were prepared in Vero cells (ATCC CCL-81) as described by Duff & Rapp (1971). HeLa cells, a human cervical adenocarcinoma epithelial cell line (ATCC CCL2), were grown in Minimal Essential Medium (MEM; Gibco) and used for all co-infection/co-incubation experiments. Chinese hamster ovary-K1 cells (CHO-K1; ATCC CCL-61) were obtained from Russell Hayman (Quillen College of Medicine, East Tennessee State University).

Co-infection experimental design. HeLa monolayers were mock-, *C. trachomatis*-, HSV- and co-infected essentially as described by Deka *et al.* (2006, 2007). Host monolayers were either mock-infected [using 2SPG (0.02 M phosphate buffer, 0.2 M sucrose, 5 mM glutamine, pH 7.2)] or incubated for 1 h with a dilution of crude *C. trachomatis* EB stock calculated to infect >80% of the cells, refed and incubated at 35 °C for 24 h. Subsequently, some cultures were either mock-infected (using MEM) or infected with HSV-2, UV-inactivated HSV-2 (HSV-2_{UV}), HSV-1 tk12 or antibody-pre-incubated HSV-1 tk12 at an m.o.i. of 10 p.f.u. per cell (or the equivalent) for 1 h at 35 °C. In some experiments, virus was replaced by 300 ng per well of purified Fc fusion protein. Finally, monolayers were refed and incubated for 20 h at 35 °C. For long-term co-infection and co-incubation studies, cultures were refed immediately after HSV infection (or co-incubation) and every 48 h thereafter with MEM + 1 μ g cycloheximide ml⁻¹. Cycloheximide exposure does not interfere with chlamydial development or HSV-induced persistence (Deka *et al.*, 2007).

Co-incubation experiments. Co-incubation experiments were performed as described by Savage *et al.* (1991), with modifications. HeLa monolayers were mock- or HSV-2-infected, incubated at 37 °C for 20 h, and fixed for 15 min at 37 °C in 1% paraformaldehyde, 2%

fetal bovine serum (FBS) and 1 $\times$ PBS. Fixed monolayers were washed, incubated in MEM overnight, resuspended in fresh MEM and overlaid onto viable mock- or chlamydiae-infected responder cell monolayers at a ratio of five fixed inducer cells to one viable responder cell. Co-cultures were incubated at 35 °C for 20, 44, 68 or 120 h.

Antibody pre-incubation experiments. mAbs to HSV-1 gD (III-174), gB (II 105-1.6) and gC (II529-1) were obtained from Patricia Spear [Northwestern University (Fuller & Spear, 1987)]. One hundred microlitres (2×10^6 p.f.u.) of HSV-1 tk12 were combined with 4 μ l MEM, anti-*Salmonella* common antigen antibody (α -Sal; Millipore, MAB746), α -gB, α -gC or α -gD, incubated for 1 h at 37 °C, and used to infect HeLa monolayers (Fuller & Spear, 1987). Four microlitres of α -gD neutralizing monoclonal antibody (NuAb) completely neutralize 2×10^6 p.f.u. of HSV-1 tk12 (data not shown). α -gH (LP11) hybridoma supernatant [from Helena Browne, University of Cambridge (Parry *et al.*, 2005)], MEM + 10% FBS and MEM + 10% FBS + α -HPV (OEM Concepts, M2-V56) were concentrated using Amicon Ultra-15 centrifugal filters. Complete neutralization of 2×10^6 p.f.u. of HSV-1 tk12 required 25 μ l concentrated α -gH (data not shown). In control samples, 25 μ l concentrated MEM (cMEM) or cMEM/ α -HPV was added to viral inocula.

Preparation of HSV-2 gD:Fc fusion protein. Plasmids expressing soluble HSV-2 gD-rabbit IgG Fc (gD:Fc) fusion protein and the Fc-expressing vector control (Blk:Fc) were obtained from Patricia Spear (Yoon *et al.*, 2003). After sequencing, plasmids were transfected into CHO-K1 cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Stable, plasmid-containing cell populations were selected in medium + geneticin (250 μ g ml⁻¹). Secreted gD:Fc and Blk:Fc fusion proteins were purified from culture supernatants using Prosepa Montage protein A spin columns (Millipore) and concentrated using Amicon Ultra-15 centrifugal filters. Purified HSV-2 gD:Fc and Blk:Fc fusion proteins were separated on Nu-PAGE 4–12% BisTris gels (Invitrogen), visualized with SYPRO Ruby stain (Pierce) and quantified by comparison with known protein standards (Broad Range Standards, Pierce) using a Bio-Rad ChemiDoc XRS image capture system; the purified fusion proteins were >90% pure (data not shown). In some experiments, Fc proteins were pre-clustered by combining fusion protein and murine anti-Rabbit IgG (Jackson ImmunoResearch) at a 1:4 antibody:Fc protein molar ratio for 1 h at room temperature (Ogita & Takai, 2006).

Chlamydial titrations by subpassage. Chlamydial titrations were performed as described previously (Vanover *et al.*, 2008). Inclusion forming units (IFU) in the undiluted inoculum were derived from triplicate counts and expressed as IFU ml⁻¹.

DNA isolation and PCR. Total DNA was isolated from HeLa monolayers, quantified and PCR-amplified using primers specific for the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chlamydial 16S rRNA, and HSV-2 glycoprotein G2 genes (Deka *et al.*, 2006). Electrophoresed amplicons were visualized using a Bio-Rad ChemiDoc XRS image capture system and quantified with Quantity One v4.5.0 software (Bio-Rad) (Deka *et al.*, 2006).

β -Galactosidase (β -gal) assay for HSV entry. β -Gal activity within HSV-infected monolayers was assayed by X-Gal staining (Montgomery *et al.*, 1996). Images were captured using an Epson 3200 scanner and Photoshop Elements Software.

Fluorescence microscopy, TEM and image analysis. Fluorescence analyses were performed as described by Deka *et al.* (2007), except that, to limit staining to the exterior cell surface, cells were not

permeabilized. In Fig. 2(a), fixed inducer cells were washed with $1 \times$ PBS, blocked with 15% FBS in PBS for 45 min at room temperature, and stained for 1 h with a 1:50 dilution of mouse α -gB, α -gC, α -gD or α -gH mAb. Cells were washed and incubated for 1 h with Alexa Fluor donkey anti-mouse IgG (1:1000 dilution, Invitrogen). In Fig. 4(a), cell surface-bound HSV gD:Fc fusion proteins were visualized using Texas red-conjugated donkey anti-rabbit IgG (1:200 dilution, Jackson ImmunoResearch). Fluorescent images were captured with a Zeiss Axiovert S100 microscope and AxioCam camera. In co-incubation experiments, duplicate samples were processed at 20 h post-co-incubation for TEM (Wyrick *et al.*, 1994).

Statistical analyses. Statistical analyses were performed using Microsoft Excel. Means were compared using a two-sample *t* test for independent samples. *P* values of ≤ 0.05 were considered significant. All values are means $\pm$ SEM of eight or nine biological replicates divided between three independent experiments.

RESULTS

Chlamydial infectivity recovers following long-term co-infection with HSV-2_{UV}

Persistent chlamydiae characteristically recover infectivity following removal of the stressor (Hogan *et al.*, 2004). Notably, co-infection with HSV-2_{UV} induces persistence

(Deka *et al.*, 2007); HSV-2_{UV} is also replication-incompetent and non-lethal to infected cells (data not shown and Moxley *et al.*, 2002). To determine whether persistent chlamydiae can recover infectivity after HSV co-infection, HSV-2_{UV} was generated (Deka *et al.*, 2007) and used to co-infect HeLa monolayers with *C. trachomatis*. Samples were harvested at 20 h (day 1), 68 h (day 3) or 140 h (day 6) post-HSV-2_{UV} infection. HSV and chlamydial genome accumulation was determined by semiquantitative PCR; GAPDH was amplified as an internal control (Deka *et al.*, 2006). Amplification of HSV-2, chlamydial and HeLa cell DNA serial dilutions demonstrated that all data were obtained within the linear range of the PCR (Deka *et al.*, 2006). Both PCR (Fig. 1a) and plaque assays (data not shown) indicated that HSV_{UV} did not replicate. The quantity of infectious EBs produced from co-infected cultures on day 1 was significantly lower than that in *C. trachomatis* singly infected cultures (Fig. 1b), while the amount of chlamydial DNA was unchanged (Fig. 1a). In contrast, EB production was unaltered at days 3 and 6 (Fig. 1b). These data indicate that chlamydiae recover infectivity if incubated for more than 24 h following a single round of HSV co-infection. Thus, like other persistence inducers, HSV-induced persistence is reversible if continuous viral replication is prevented.

Fig. 1. Chlamydial infectivity recovers during long-term co-infection with HSV-2_{UV}. HeLa cell monolayers were mock-, singly or co-infected with *C. trachomatis* and HSV-2_{UV}. Replicate samples were harvested at days 1, 3 and 6 post-HSV-2_{UV} infection and processed for DNA isolation (a) and chlamydial titration (b). (a) PCR was used to determine relative HSV (HSV G2), chlamydial (Ct 16S rRNA Gene) and host (GAPDH) genome accumulation in co-infected cells. (b) EB titres are expressed as IFU (ml sample)⁻¹ $\pm$ SEM, *n*=3. Asterisks indicate titres that were significantly different (by *t* test) from those of *C. trachomatis* singly infected cells (*P*<0.05) collected at the same time.

Fig. 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 fixed and immunostained with α -Sal, α -gB, α -gC, α -gD or α -gH mAbs and Alexa Fluor 488-conjugated secondary antibody. Photomicrographs are at $\times 320$ magnification; bar, 120 μ m. (b) MI or HI inducer cells were co-incubated with mock- or chlamydiae-infected responder cells for 20 h (day 1), 44 h (day 2), 68 h (day 3) and 140 h (day 6), and then harvested for EB titration. Singly infected and co-infected HeLa cultures were included as positive controls. Asterisks indicate titres that are significantly different (by *t* test) from those of either *C. trachomatis* singly infected cultures (for Ct/HSV controls) or *C. trachomatis*+MI cultures (for HI+Ct samples) ($P < 0.05$). (c) Co-incubated cultures were harvested at 20 h.p.c.i. (day 1), fixed with glutaraldehyde/paraformaldehyde and processed for TEM. Both photomicrographs are at $\times 7000$ magnification; bars, 2 μ m.

HSV-2-induced chlamydial persistence may be triggered by interaction of viral glycoproteins with host cell surface receptors

Productive viral replication is not required for chlamydial persistence induction (Deka *et al.*, 2007), suggesting that viral glycoprotein–host receptor interactions alone might induce persistence. Earlier studies indicate that molecules

present on the surface of fixed cells can interact with molecules on the surface of viable responder cells and activate signal transduction pathways (Savage *et al.*, 1991). Mock- or HSV-2-infected HeLa cells were paraformaldehyde-fixed (Savage *et al.*, 1991) and immunostained, and it was confirmed that gB, gC, gD and gH were surface-exposed on HSV-infected (HI) inducer cells but not mock-infected (MI) cells (Fig. 2a). No specific staining was

observed with secondary antibody alone or with the irrelevant control antibody α -Sal (data not shown). Replicate viable HeLa monolayers were then mock- (not shown) or *C. trachomatis*-infected for 24 h, overlaid with fixed MI or HI cells and incubated for various times (hours post co-incubation; h.p.c.i.). Co-incubation of HI cells with viable *C. trachomatis*-infected responder cells for 20 h (day 1) reduced chlamydial infectivity similarly to that observed in HSV-2 co-infected controls (Fig. 2b). TEM studies (Fig. 2c) indicated that when *C. trachomatis*-infected responder cells were co-incubated with HI cells for 20 h, the RBs within were swollen and misshapen (Fig. 2c, grey arrow), typical of persistent chlamydiae and similar to those observed previously (Deka *et al.*, 2006, 2007). Conversely, co-incubation with MI cells altered neither RB morphology (Fig. 2c, white arrow) nor EB development (Fig. 2c, black arrow). While EB production in HI co-incubated samples remained significantly reduced at 44 h.p.c.i. (day 2), it was unaffected at 68 h.p.c.i. (day 3) or 140 h.p.c.i. (day 6) (Fig. 2b). Viral plaque assays and PCR studies indicated that (i) no viable HSV was present and (ii) no HSV replication occurred (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.

HSV gD-specific antibody prevents induction of co-infection-induced chlamydial persistence

gD- and gH-specific NuAbs inhibit viral entry and replication. In contrast, anti-gB mAbs are only partially inhibitory and anti-gC mAbs do not inhibit these processes to any observable degree (Fuller & Spear, 1987; Fuller *et al.*, 1989; Parry *et al.*, 2005). Thus, we used HSV glycoprotein-specific mAbs and NuAbs to determine whether the viral gD and gH glycoprotein(s) are required for altering chlamydial development during co-infection. A strain of HSV-1 (HSV-1 tk12) expressing the β -gal gene was used in these experiments so that viral entry could be monitored. HSV-1 tk12 was pre-incubated with equal quantities of α -gB-, α -gC-, α -gD- or α -gH-specific NuAbs/mAbs. α -Sal, α -human papilloma virus 18 (α -HPV), concentrated medium + 10% serum (cMEM) and medium + 10% serum + α -HPV (cMEM/ α -HPV), none of which affect viral entry or replication, were used as negative controls (data not shown and Fuller & Spear, 1987). HeLa cell monolayers were co-infected with *C. trachomatis* and antibody-pre-incubated HSV-1 tk12 and incubated for an additional 20 h post-co-infection. As expected, β -gal activity was evident in HSV-1 tk12-infected monolayers, as well as those infected with HSV-1 tk12 + cMEM, cMEM/ α -HPV, α -Sal, α -gB or α -gC (Fig. 3a). Only α -gD and α -gH inhibited HSV-1 tk12 entry and, hence, β -gal expression and activity (Fig. 3a) (Fuller *et al.*, 1989; Montgomery *et al.*, 1996; Nicola *et al.*, 1998). EB production was significantly reduced in chlamydia-infected cells that were co-infected with either HSV-1 tk12 alone or virus + α -Sal

cMEM or cMEM/ α -HPV. Of the HSV-specific antibodies tested, only α -gD prevented the HSV-induced reduction in chlamydial titre (Fig. 3b, c). Control plaque assays confirmed that only α -gD or α -gH pre-incubation decreased viral replication and release (data not shown). These data indicate that HSV gD–host co-receptor interaction is required for co-infection-induced chlamydial persistence.

HSV-2 gD–host cell co-receptor interaction is sufficient to stimulate chlamydial persistence

Antibody neutralization data suggest that HSV gD is required to induce *C. trachomatis* persistence in this model system. If HSV gD is sufficient for this effect, purified gD should induce persistence in the absence of other virion proteins.

To test this hypothesis, HSV-2 gD/rabbit IgG Fc (gD:Fc) and the rabbit IgG Fc control proteins (Blk:Fc) were expressed and purified (Yoon *et al.*, 2003). Immunofluorescent staining confirmed that gD:Fc binds to the HeLa cell surface (Fig. 4a). To determine whether gD:Fc–host cell interaction alters *C. trachomatis* development, *C. trachomatis* singly infected cultures were exposed to various fusion proteins for 20 h. Because the HSV co-receptor nectin-1 must dimerize before initiating cellular signalling cascades (Ogita & Takai, 2006), gD:Fc was clustered by addition of anti-rabbit IgG in some samples (Fig. 4b). As a positive control, parallel HeLa cell monolayers were co-infected with *C. trachomatis* and HSV-2 (Fig. 4c, Ct/HSV). No significant difference in EB production was observed between *C. trachomatis* singly infected samples and those exposed to anti-rabbit IgG alone (α IgG), gD:Fc, Blk:Fc or the anti-rabbit IgG + Blk:Fc (α IgG/Blk:Fc) negative control (Fig. 4c), although the chlamydial titre in gD:Fc-exposed cultures was consistently depressed by 10–20%. In contrast, anti-rabbit IgG + gD:Fc (α IgG/gD:Fc) exposure significantly reduced EB titre. To ensure that the observed effect was gD-specific, α IgG/gD:Fc protein complexes were pre-incubated with α -gD NuAb (α IgG/gD:Fc + α gD). α -gD completely reversed the α IgG/gD:Fc-mediated decrease in EB production (Fig. 4d). Overall, these data indicate that (i) HSV-2 gD interaction with the host cell surface is sufficient to induce chlamydial persistence, and (ii) ligand clustering is likely required for maximal inhibitory effect.

DISCUSSION

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 on the inducer cell surface and their associated host cell receptors is sufficient to stimulate chlamydial persistence. Viral attachment to and entry into the host cell is a dynamic process that can stimulate cellular signalling cascades. For example,

Fig. 3. Co-infection of *C. trachomatis* with antibody-pre-incubated HSV-1 tk12. (a) Cultures of HeLa cells were mock-, singly or co-infected with *C. trachomatis* and HSV-1 tk12 or HSV-1 tk12 + α -Sal, α -gB, α -gC, α -gD, α -gH, cMEM or cMEM/ α -HPV and assayed for β -gal activity. (b, c) Replicate HeLa monolayers were similarly infected and processed for chlamydial titration. EB titres are expressed as IFU (ml sample)⁻¹; error bars, SEM, $n=3$. Asterisks indicate titres that are significantly different (by t test) from those of *C. trachomatis* singly infected cells ($P<0.05$).

both HSV-1 gD protein and UV-inactivated virions induce host NF- κ B activity at 1–3 h post-infection (Amici *et al.*, 2006; Sciortino *et al.*, 2007). HSV gD can also block apoptosis in herpes viral entry mediator (HVEM)-expressing U937 cells through an NF- κ B-dependent pathway (Sciortino *et al.*, 2008). Increased intracellular calcium uptake and activation of Rac1 and Cdc42 are also observed early during HSV-1 infection (Cheshenko *et al.*, 2003; Hoppe *et al.*, 2006). Thus, it is feasible that HSV attachment to host co-receptors transmits cellular signals that have downstream effects on both the host cell and upon developing chlamydiae.

The observations that (i) gD-specific antibody blocks HSV co-infection- and gD fusion protein-induced persistence, and (ii) clustered, soluble gD:Fc fusion protein decreases *C. trachomatis* infectivity indicate that HSV gD interaction with the host cell surface is sufficient to stimulate persistence and that gB/C–host receptor interaction is insufficient to induce this effect. These data do not, however, eliminate the possibility that HSV envelope glycoproteins other than gD modulate the efficiency with which chlamydial persistence is induced. Notably, the primary role of gD during viral attachment is to bind one of several co-receptors on the host cell surface: HVEM, nectin-1, nectin-2 or 3-*O*-sulfated heparan sulfate (Spear, 2004). HVEM, a member of the tumour necrosis factor

receptor family, activates NF- κ B when complexed to its natural ligand, LIGHT (Mauri *et al.*, 1998). HVEM is expressed by epithelial cells and is used with equal efficiency by both HSV-1 and HSV-2 for host cell entry (Hsu *et al.*, 1997; Kwon *et al.*, 2006; Marsters *et al.*, 1997; Montgomery *et al.*, 1996; Spear, 2004). Nectin-1 and nectin-2 are members of the immunoglobulin superfamily that are involved in the formation of cell junctional complexes (Cocchi *et al.*, 1998). When stimulated, nectins activate the Cdc42 and Rac small G proteins via c-Src, leading to modulation of host cell gene expression via the c-Jun N-terminal kinase (JNK) pathway (Nakanishi & Takai, 2004; Shimizu & Takai, 2003). While nectin-1 and nectin-2 are both expressed on epithelial cells, nectin-2 serves primarily as an HSV-2 co-receptor. Nectin-1, however, is used with equal efficiency by both HSV-1 and HSV-2 (Spear, 2004). 3-*O*-Sulfated heparan sulfate is expressed on numerous cell types, but is only used as a co-receptor by HSV-1 (Shukla *et al.*, 1999).

Since HVEM, nectin-1 and nectin-2 are linked to cellular signal transduction pathways, gD interaction with any of the three could potentially activate an anti-chlamydial host response. However, several lines of evidence suggest that nectin-1 is the most likely candidate. First, both HSV-1 and HSV-2 induce chlamydial persistence (Deka *et al.*, 2007),

Fig. 4. Interaction of HSV-2 gD:Fc fusion proteins with *C. trachomatis*-infected cultures decreases chlamydial infectivity. (a) HeLa cell cultures were immunostained with 1 $\times$ PBS (Mock), Blk:Fc or gD:Fc with or without Texas red-conjugated anti-Rabbit IgG Fc antibodies (α IgG-TR). Cells were photographed at $\times 320$ magnification; bar, 120 μ m. (b) Model for activation of signalling from HSV co-receptors by antibody–gD cross-linking. The gD fusion protein is cross-linked by addition of α -IgG FcAb, which may increase activation of receptor-associated signal transduction pathways by promoting receptor aggregation in the plasma membrane. (c) Replicate *C. trachomatis* singly infected samples were incubated with α -IgG, gD:Fc, Blk:Fc or mixtures of α -IgG + gD:Fc (α IgG/gD:Fc) or α -IgG + Blk:Fc (α IgG/Blk:Fc) and harvested for EB titration. Replicate HeLa cultures were co-infected with both *C. trachomatis* and HSV-2 (Ct/HSV). (d) *C. trachomatis* singly infected samples were incubated with α -IgG + gD:Fc (α IgG/gD:Fc) or α -IgG + gD:Fc + α -gD NuAb (α IgG/gD:Fc + α gD) and harvested for EB titration. Asterisks indicate titres that are significantly different (by *t* test) from those of parallel *C. trachomatis* singly infected cultures ($P < 0.05$).

suggesting that either HVEM or nectin-1 might initiate this response. However, anti-HVEM serum only marginally reduces HSV-1 entry into HeLa cells, suggesting that HVEM is not the principal HSV-1 co-receptor in HeLa cells (Montgomery *et al.*, 1996). Secondly, we demonstrated that IgG pre-clustering of gD:Fc was required for maximal effect on chlamydial infectivity. Similarly, ligand clustering and subsequent nectin-1 aggregation are required for initiation of downstream signalling cascades (Ogita & Takai, 2006). Collectively, these observations suggest that HSV gD–nectin-1 interaction initiates co-infection-induced persistence.

However, caution must be exercised when eliminating potential cellular mediators of 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 sufficient signalling to alter chlamydial development. Although nectin-2 does not efficiently mediate HSV-1 entry, the low affinity of HSV-1 gD for nectin-2 could also be sufficient to stimulate anti-chlamydial signalling pathways. HSV-2 also enters Chinese hamster ovary cells using an unknown co-receptor (Spear, 2004). Finally, interaction between gB and the paired immunoglobulin-like type 2 receptor (PILR) α has recently been shown to mediate HSV entry (Sato *et al.*, 2008). Our observation that cellular interaction with recombinant gD alone reduces chlamydial infectivity suggests that PILR is not involved; however, we cannot completely eliminate the possibility that an alternative co-receptor, a currently uncharacterized HSV receptor or multiple co-receptors could mediate HSV-induced chlamydial persistence.

Investigations of mechanisms that drive developing chlamydiae to enter persistence have illuminated multiple

aspects of the biology and pathogenesis of this fascinating organism (Fehlner-Gardiner *et al.*, 2002; Nelson *et al.*, 2005). HVEM, nectin-1 and nectin-2 have endogenous ligands (Granger & Rickert, 2003; Hsu *et al.*, 1997; Nakanishi & Takai, 2004), raising the possibility that stimulation of these receptors by host ligands could restrict chlamydial development in the absence of viral co-infection. If so, this novel host pathway may limit the spread of chlamydiae *in vivo*, much like the well-characterized IFN- γ -induced anti-chlamydial response. Therefore, investigation of pathways activated by HSV co-receptors and how these cascades interact with developing chlamydiae could potentially reveal new and captivating aspects of the relationship that *C. trachomatis* fosters with its host cell.

ACKNOWLEDGEMENTS

The authors want to thank Dr Priscilla B. Wyrick (East Tennessee State University; ETSU), Dr Udayasankar Kumaraguru (ETSU), Dr Patricia Spear (Northwestern University) and Dr Helena Browne (University of Cambridge) for providing reagents and for many helpful discussions. We want to acknowledge the Electron Microscopy Core Facility, Department of Pathology, Quillen College of Medicine. This work was supported by NIH grant number 5R21AI59563 to R.V.S., ETSU RDC grant number 04-024M to R.V.S., and an ETSU Graduate Council research grant awarded to J.V.

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Edited by: K. Fields