Herpes simplex virus 1 (HSV-1) DNA and immune complex (HSV-1–human IgG) elicit vigorous interleukin 6 release from infected corneal cells via Toll-like receptors

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INTRODUCTION

Corneal herpes infection continues to cause blindness, especially in developed countries, and the disease still constitutes a major cause of corneal blindness rated second to trauma in the United States (Kaye et al., 2000; Xu et al., 2002). Corneal herpes infection elicits a robust inflammatory response and eventually leads to a vision-threatening stromal keratitis as a sequela of frequent reactivation of latent virus from the trigeminal ganglion (Doymaz & Rouse, 1992; Streilein et al., 1997). Although there are effective antiviral drugs to treat acute virus replication in the corneal epithelium, the effective management of host inflammatory responses requires the use of an immunosuppressant, such as a steroid, to keep the affected cornea clear (Wilhelmus et al., 1994; Sudesh & Laibson, 1999). The aetiology of herpetic stromal keratitis (HSK) is thought to be an aberrant Th1 cytokine-mediated immunopathology (Niemialtowski & Rouse, 1992; Thomas & Rouse, 1997; Verjans et al., 1998). Stromal haze continues to increase and becomes a problem following acute virus replication. In this situation, the precise disease-triggering and/or driving mechanism(s) are still largely unknown.

Toll-like receptors (TLRs) are a family of evolutionarily conserved molecules that initially recognize pathogen-associated molecular patterns of invading microbes and trigger the initial host innate immune response. As a consequence, inflammatory cytokines are released (Takeda et al., 2003; Akira & Takeda, 2004; Iwasaki & Medzhitov, 2004; Netea et al., 2004). Among them, IL-6 is a critical cytokine component of corneal herpesvirus infection, because it vigorously attracts neutrophiles (Fenton et al., 2002) and also potently stimulates vascular endothelial growth factor (VEGF) production (Biswas et al., 2006).

Recent papers have reported that herpesvirus DNA is immunogenic (Lundberg et al., 2003) and that TLR-2, -3 and -9 are implicated as recognition molecules for some of the herpes simplex virus (HSV) moieties (Zheng et al., 2002; Lund et al., 2003; Kurt-Jones et al., 2004). TLR-3 is expressed on the cell surface of corneal epithelial cells and fibroblasts (Matsumoto et al., 2002; Ueta et al., 2005). It
recognizes double-stranded RNA, which is produced during the process of virus replication (Alexopoulou et al., 2001; Karikó et al., 2004; Tian et al., 2004; Sen & Sarkar, 2005). TLR-9 is expressed in the endosome and was originally identified as the receptor for unmethylated bacterial CpG DNA motifs, were shown to activate inflammatory cytokines and gamma interferon (IFN-γ) secretion via TLR-9 (Lundberg et al., 2003; Krug et al., 2004).

Data obtained from experimental studies implicated immune complexes (HSV-1-IgG) in the aetiology of HSK (Meyers & Pettit, 1973; Meyers & Chitjian, 1976; Meyers-Elliot et al., 1980). However, their roles have not been well described and are still largely unknown. We postulate that these viral DNA molecules and/or immune complexes deposited in corneal stroma may continue to trigger stromal inflammation by excessive cytokine release via TLR-3 or -9 long after the initial acute viral insult has subsided. In this study, we aimed to characterize the role of viral DNA and immune complexes in the release of IL-6 by corneal cells and to relate the phenomenon to TLR expression.

**METHODS**

**Cells.** Human corneal epithelial cells (HCEs; Araki-Sasaki et al., 1995) and primary human corneal fibroblasts (HCRFs), passage 2–5, were propagated to confluence on six-well plates with minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acid solution and 1× antibiotic/antimycotic solution (Invitrogen) containing 100 U penicillin ml⁻¹, 100 mg streptomycin ml⁻¹ and 250 ng amphotericin B ml⁻¹.

**Viruses.** HSV-1 (KOS, McKrae and MP strains) and HSV-2 (MS strain) were propagated on Vero cell monolayers. Briefly, confluent monolayers of Vero cells were infected with HSV at an m.o.i. of 0.01. After 2 h adsorption, virus was aspirated and the monolayers were washed once with serum-free medium, then re-fed with fresh serum-free MEM and overlaid with serum-free virus suspension for 2 h, after which the monolayer was washed once with fresh serum-free MEM and overlaid with serum-free medium supplemented with 2% human IgG (human gamma globulins Cohn fraction II, III: anti-HSV neutralizing-antibody titre by 50% plaque reduction, 1:640; Sigma). The monolayers were incubated in a humidified 5% CO₂ incubator at 37°C for 48 h. Forty-eight hours after infection, viral plaques were counted and virus titre was determined by spectrophotometric reading of A₂₆₀.

HSV-1-anti-HSV IgG immune complex was made by mixing and incubating 1 ml purified UV-HSV-1 (McKrae strain: virus titre, 9 × 10⁹ p.f.u. ml⁻¹ before UV irradiation) and 30 ml 2% human gamma globulin (neutralization titre, 1:640) overnight at 4°C. The amount of the viral DNA in immune complexes was approximately 12 μg. After incubation, immune complex was centrifuged at 12 000 r.p.m. for 60 min and washed twice with PBS. The washed pellet, containing immune complex, was resuspended in 1 ml fresh PBS. Immune complex was not infectious when assayed on Vero cell monolayers.

**Quantification of TLR-3 and -9 expression by real-time PCR.** HCE and HCRF monolayers on a six-well plate were inoculated with live or UV-inactivated virus at an m.o.i. of 1:0 (for UV-inactivated virus, before the UV irradiation) and incubated for 2 h at 37°C in a humidified 5% CO₂ incubator. The inoculum was then aspirated and the monolayers were re-fed with 2 ml fresh serum-free medium and further incubated for 6 h for live virus-infected plates and 22 h for UV-inactivated virus-infected plates. Uninfected monolayers served as controls. At the end of the incubation, total monolayer cell RNA was extracted by using RNA STAT-60 extraction solution (Tel-Test Inc.) according to the manufacturer’s instructions.

Two micrograms of RNA was reverse-transcribed to make cDNAs by using TaqMan reverse-transcription reagents (Applied Biosystems). Production of cDNAs from individual RNAs was confirmed by detection of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in a 4% agarose gel by electrophoresis after amplification by 35 cycles of regular PCR. The synthesized cDNA was amplified for 40 cycles on an ABI Prism 7700 sequence detector to quantify expression of TLR-3 and -9 by real-time PCR using SYBR Green master mix (Applied Biosystems) and primers for TLR-3 and -9. For data analysis, the default settings of ABI Primer Express v. 1.6.3 software were used. The amplification of the housekeeping gene GAPDH was done for each sample as a control and to allow normalization between samples. Each assay comprised triplicate measurements. After the amplification, no primer-dimer formations were confirmed by agarose-gel electrophoresis. Specificity of the amplicons was confirmed by Southern blotting with the digoxigenin-labelled TLR-3, -9 and GAPDH probes. The following primers were used: TLR-3: forward 5'-GATCTGTCTATATGG-3'; reverse 5'-GACAGATTCGGATCCGTGTTG-3' (304 bp), probe 5'-CCACAGTGGTCCAGGGCTTA-3'; TLR-9: forward 5'- GTGCCCCACCTTCTCAGTTG-3'; reverse 5'-GGACAGCTACAGTGTTG-3' (259 bp), probe 5'-TTTGGGACCAGTGCCGGGCT-3'; GAPDH: forward 5'-AGCTGACGCGGAAAGGTCAGC-3', reverse 5'-GGATTGCGGTCGCTGTTGAGCT-3' (209 bp), probe 5'- GCCGGCCGCTCAAGGCGCAT-3'.

**ELISA for IL-6.** HCEs and HCRFs were grown to confluence on 24-well plates. They were inoculated with live or UV-inactivated virus (m.o.i. of 1:0) and/or 200 μl immune complex for 2 h or were transfected with 1 μg HSV DNA by using Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions. After 2 h incubation, the inocula were removed and the monolayers were washed once with serum-free medium. They were re-fed with 1 ml fresh serum-free medium and incubated at 37°C in a humidified, 5% CO₂ incubator. Supernatants were harvested at 8 h p.i. for live virus-infected monolayers or at 24 h p.i. for UV-inactivated virus-infected, immune complex-inoculated and/or viral DNA-transfected monolayers. Supernatants obtained at 8 and 24 h after incubation from uninfected monolayers served as controls. IL-6 concentration was determined by ELISA according to the
manufacturer’s instructions (Biosource). Each experiment was repeated at least three times. The data were analysed by using Student’s t-test and considered statistically significant if the P values were <0.05.

Inhibition of IL-6 release. HCRFs grown on a 24-well plate were treated with 200 μl immune complex or transfected with 1 μg viral DNA with Effectene transfection reagent (QIAGEN). After 2 h incubation, the transfection complex and/or immune complex was removed and the cells were washed with fresh medium and re-fed with serum-free medium supplemented with one of the following reagents: the TLR-9-inhibitory oligomer ODN TTAGGG (iODN; InvivoGen), the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (InvivoGen) and/or anti-TLR-3 goat IgG antibody (Santa Cruz Biotechnology Inc.). Two hours prior to and throughout the transfection/treatment period, HCRF monolayers were incubated with either iODN (10–100 μM) or LY294002 (50–100 μM). To selected monolayers, 5 μg anti-TLR-3 antibody was added to the overlay medium, starting at the beginning of the infection or transfection and continuing throughout the entire incubation period. After 24 h incubation at 37°C in a humidified 5% CO2/air incubator, supernatants were removed and assayed for IL-6. Supernatants obtained from HCRF monolayers treated with iODN or LY294002 only served as controls.

RESULTS

Induction of TLR-3 and -9

Expression of TLR-3 and -9 in live virus-infected (8 h.p.i.), UV-inactivated virus-infected (24 h.p.i.) and uninfected control cells were quantified by real-time PCR. In HCEs, TLR-3 gene expression was upregulated when they were infected with either live or UV-inactivated HSV-1 strains [KOS (6.2x), McKrae (8.3x), MP (5.8x), UV-KOS (6.5x), UV-McKrae (2.7x), UV-MP (8.7x)] or UV-HSV-2 strain MS (3.7x) (Fig. 1a). TLR-9 gene expression was also slightly enhanced in HCEs when they were infected with live McKrae strain (1.57x). Greater augmentation of TLR-9 gene expression was obtained in HCEs when UV-McKrae (8.2x) or UV-MP (14.6x) strains of HSV-1 were used (Fig. 1a).

As the major affected site in chronic herpetic keratitis is the corneal stroma, we next studied TLR-3 and -9 gene expression in HCRFs infected with HSV. All live HSV-1 strains [KOS (50x), McKrae (20x) and MP (24x)] and all UV-HSV-1 strains [UV-KOS (22x), UV-McKrae (26x) and UV-MP (7.3x)] and UV-HSV-2 strains [UV-MS (3.1x)] augmented TLR-3 gene expression in HCRFs. Enhanced gene expression of TLR-9 was observed in live HSV [KOS (1.8x), McKrae (4.5x), MP (5.6x), MS (1.7x)] and UV-HSV [UV-KOS (1.8x), UV-McKrae (6.0x), UV-MP (3.5x), UV-MS (6.2x)] strain-infected HCRFs (Fig. 1b). Expression of both the TLR-3 and -9 genes was increased in infected HCEs and HCRFs; however, TLR-3 gene expression was more prominent than

![Fig. 1. Induction of TLR-3 and -9 with live and UV-inactivated HSV.](http://vir.sgmjournals.org)
TLR-9 in most of the infected cells, with the exception of UV-MP-infected HCEs and UV-MS-infected HCRFs. In HCRFs, increased ratios of TLR-3 were greater than those observed in HCEs. Variations of the increased TLR-3 and -9 gene expression were noted with some strains of live and UV-inactivated HSV. Currently, we have not identified factors contributing to these variations.

We next studied the effect of HSV DNA on TLR gene expression in corneal cells, because HSV DNA is detected in patient corneas even in the chronic or silent stages of infection. In this experiment, we used McKrae and MP DNA, because both strains are pathogenic in the cornea (Wander et al., 1980; Centifanto-Fitzgerald et al., 1982) and KOS DNA does not persist long in HSK corneas (Maggs et al., 1998). When HCEs were transfected with McKrae or MP DNA, gene expression of TLR-3 was enhanced by 3-7 and 22×, respectively, compared with untransfected cells (Fig. 2a). TLR-9 gene expression was increased slightly in McKrae DNA-transfected cells (1-67×). When HCRFs were transfected with McKrae or MP DNA, TLR-3 gene expression was enhanced in HCRFs by 2 and 3-5× more than uninfected-control HCRFs, respectively (Fig. 2b). TLR-9 was augmented slightly (2-4×) in HCRFs with McKrae DNA transfection. Although we obtained varied augmentations of TLR-3 and -9 gene expression in HCEs and HCRFs with McKrae DNA or MP DNA transfection, this may be related to strain differences rather than virulent factors. However, at this time, we are not addressing the nature of virulent factors associated with different HSV strains. By indirect immunofluorescence, transfected cells showed positive fluorescence in the nucleus and perinuclear cytoplasm with an anti-HSV ICP0 mAb at 24 h post-transfection. Positive fluorescence was also seen in the cytoplasm when stained with an anti-HSV gD mAb (data not shown).

**Increased release of cytokine (IL-6) from infected cells**

Culture supernatants obtained from live HSV-infected (at 8 h p.i.), UV-HSV-infected (at 24 h p.i.), HSV DNA-transfected (at 24 h post-transfection) and uninfected-control cells were assayed for IL-6 by ELISA (Fig. 3a, b).

When IL-6 produced by live and UV-HSV-1-infected HCEs was quantified, significantly enhanced levels were detected (Fig. 3a); however, they were far lower than those synthesized by HCRFs (Fig. 3). McKrae DNA- or MP DNA-transfected HCEs also exhibited increased levels of IL-6 release (Fig. 3); however, these values were also far below than those obtained from HCRFs (Fig. 3b). HCRFs infected with live McKrae strain or UV-HSV-1 viruses released more IL-6 than uninfected cells (3-8× in HSV-1-infected and 5-16× times in UV-HSV-1-infected HCRFs) (Fig. 3b). UV-MP, the strain known for causing stromal opacity in experimental animals (Centifanto-Fitzgerald et al., 1982), greatly enhanced (16×) IL-6 release when compared with uninfected controls. However, maximal IL-6 release was obtained when HCRFs were transfected with HSV-1 DNA purified from McKrae and MP strains (27-6 and 30-3×, respectively) (Fig. 3b). Clinically, HSV-2 infection in the cornea is rare and, interestingly, live or UV-HSV-2 did not enhance IL-6 release from HCRFs. However, mechanisms behind this phenomenon await further investigation.

**Ability of HSV immune complexes to augment TLR gene expression and induce IL-6 release**

HSV recurrent infections are frequently associated with the presence of high levels of neutralizing antibody. We next evaluated the ability of HSV–anti-HSV IgG immune complexes to interact with TLRs and trigger IL-6 production. As is seen in Fig. 2(a), HSV immune complex-treated HCEs enhanced both TLR-3 and -9 gene expression (2-5 and...
and a heterodimer of gH–gL with cell-surface herpesvirus-entry mediator (HVeM), a member of the tumour necrosis factor (TNF) receptor family (Montgomery et al., 1996; Marsters et al., 1997). This process of the virus entry itself may trigger nuclear factor kappa B (NF-κB) activation. Therefore, to study whether IL-6 release obtained after HSV infection is mediated directly via the TLR-3 or -9 pathways and subsequent activation of NF-κB, we tried to bypass the virus-entry processes by transfecting the purified viral DNA directly into the cells. We then looked at the release of IL-6 from the HSV DNA-transfected or immune complex-treated HCRFs in the presence of a TLR-9 inhibitor, iODN. iODN is known to block the colocalization of CpG DNA with TLR-9 within endosomal vesicles (Gursel et al., 2003). Treatment of HCRFs transfected with McKrae strain DNA with varying doses of iODN demonstrated dose-dependent inhibition of IL-6 release. Approximately 70–75 % of IL-6 release was inhibited by 50–100 μM iODN (Fig. 4). However, inhibition was not complete with iODN. Therefore, we next tried to inhibit IL-6 release by adding anti-TLR-3 antibody (final dilution in overlay medium, 40×) in addition to iODN.

TLR-3 is a receptor for double-stranded RNA, which is generated during the symmetrical transcription of HSV DNA. In fact, TLR-3 gene expression in HCRFs infected with live or UV-inactivated McKrae strain was upregulated. When anti-TLR-3 antibody was added to the overlay medium, IL-6 release from HCRFs was inhibited by about 40 %. iODN inhibited 70 % of IL-6 release from HCRFs and inhibitory activity was further enhanced up to 80 % when anti-TLR-3 antibody and iODN were combined (Fig. 5). On the contrary, IL-6 release from immune complex-treated HCRFs was not inhibited significantly by iODN (Fig. 4).

Phosphatidylinositol 3-kinase (PI3K) plays a role in active TLR-3 signalling (Sarkar et al., 2004) and in shuttling CpG DNA to TLR-9 (Ishii et al., 2002). Therefore, use of the specific inhibitor of PI3K LY294002 (Vlahos et al., 1994) is crucial for deciphering the roles of these TLRs in cellular-signalling processes. LY294002 (50–100 μM) inhibited IL-6 release from both immune complex-treated and McKrae DNA-transfected HCRFs by 80 to over 90 %, respectively (Fig. 6). Control monolayers treated with only iODN or LY294002 released <520 pg IL-6 ml⁻¹. Results obtained from these inhibition experiments, therefore, indicate that IL-6 release from HSV-infected HCRFs is at least in part mediated via both the TLR-3 and TLR-9 pathways.

**DISCUSSION**

These studies demonstrated that IL-6 was released from both corneal epithelial cells and fibroblasts in response to HSV infection. Interestingly, HCRFs released significantly more IL-6 than HCEs when infected with live or UV-inactivated HSV. Maximum release, however, was obtained when HCRFs were transfected with viral DNA or treated with anti-HSV–IgG immune complex. Sight-threatening

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**Fig. 3.** IL-6 secretion with live or UV-inactivated HSV infection, viral DNA transfection or treatment with immune complex. (a) HCEs and (b) HCRFs were infected with live or UV-inactivated HSV-1 (KOS, McKrae and MP strains) or HSV-2 (MS strain) at an m.o.i. of 1·0 (for UV-inactivated virus, before UV), treated with 200 μl immune complex (IC) or transected with 1 μg purified HSV DNA (McKrae or MP strain). After 8 h (live virus) or 24 h (UV-inactivated virus, immune-complex treatment or transfection), supernatants were removed and assayed for IFN-γ and IL-6 by ELISA. Supernatant obtained from uninfected/untreated cells served as the control. Each column represents the mean ± SD value of released IL-6 (pg ml⁻¹) obtained from three wells. Fig. 3 is representative of three separate experiments with similar results. *P<0·05 compared with control.

3·1×, respectively). Likewise, HSV immune-complex treatment of HCRFs enhanced TLR-3 (6·9×) and TLR-9 (8·6×) gene expression significantly in HCRFs (Fig. 2b). Human gamma globulin alone (200 μl ml⁻¹) did not enhance TLR-3 or -9 gene expression (data not shown). Moreover, when HCEs or HCRFs were treated with HSV immune complexes, a significant increase in IL-6 release was observed. These data indicate that the neutralized HSV immune complexes were as potent as HSV DNA in their ability to augment TLR gene expression and to induce the release of IL-6.

**Release of IL-6 is partially inhibited by anti-TLR-3 antibody and TLR-9-inhibitory oligomer**

HSV adsors to cell-surface heparan sulfate, then enters the cellular cytoplasm by fusing envelope glycoproteins gD, gB...
corneal haze in the corneal stroma caused by an excessive cytokine release without active virus replication and IL-6 have been well documented as some of the contributing factors for massive neutrophil attraction to the lesion and induction of VEGF in experimental corneal herpes infection (Niemialtowski & Rouse, 1992; Tang et al., 1997; Fenton et al., 2004; Biswas et al., 2006).

It is well known that the virulence of each HSV strain is different, which may affect in part their ability to induce IL-6 production. We demonstrated that HCRFs released larger amounts of IL-6 with UV-McKrae and UV-MP strain than with the UV-inactivated avirulent KOS strain, although live KOS induced TLR-3 and TLR-9.

McKrae and MP strains are both highly pathogenic to the cornea (Wander et al., 1980; Centifanto-Fitzgerald et al., 1982). Transfection of HCRFs with viral DNA also showed that virulent-strain DNAs were more potent inducers of IL-6 release and may well contribute to aberrant immune responses in HSK, where live virus is not present.

We have recently reported that, during corneal-transplantation surgery when the recipient cornea had a history of herpetic keratitis, HSV-1 DNA was detected in the majority of cases. In fact, corneas were positive in seven out of eight cases, with up to 1.6 x 10^6 DNA copies (mg tissue)^−1. Even without a history of herpetic keratitis, 10-8% (4/37) of the recipients’ corneas were positive for the HSV genome, with a mean of 8.7 DNA copies (mg tissue)^−1. From these samples, active replicating virus was not isolated (Y. Shimomura, T. Deai, M. Fukuda, S. Higaki, L. Hooper & K. Hayashi, unpublished results). These studies indicated that viral DNA resided long after the subsidence of acute viral growth in the patient’s cornea. Mitchell et al. (1994) reported that HSV-1 DNA presenting in the cornea was associated closely with inflammatory lesions long after infectious HSV-1 had cleared.

The host response to HSV infection is characterized by the production of high levels of anti-HSV IgG and subsequent generation of virus–antibody complexes. The role of the anti-HSV antibody in stromal keratitis has been documented in experimental animals (Meyers & Pettit, 1973; Meyers & Chitjian, 1976; Meyers-Elliot et al., 1980). HSV antigens
the cell (Matsumoto et al. express TLR-3 receptors both on the cell surface and inside with anti-TLR-3 antibody. Expression of TLR-3 is quite cell inhibitory activity was obtained when iODN was combined 2003). Inhibition was not complete, however, and better has been reported previously (Krieg et al., 2002). Suppressive activity of the same iODN conclusion, when iODN was used with McKrae DNA-generated in response to HSV-1 infection is mediated at 200 mDNA; McKrae-anti-HSV IgG.

Previously, interaction of HSV with TLR-9 has been reported with plasmacytoid dendritic cells (Kurt-Jones et al., 2004). Additionally, peritoneal macrophages and human embryonic kidney cells express TLR-2 upon infection with HSV-1, eliciting robust cytokine responses (Zheng et al., 2002). Although we did not study TLR-2 expression, this is the first report demonstrating that TLR-3 and -9 are expressed in corneal fibroblasts upon HSV DNA transfection and/or immune-complex treatment, which leads to subsequent release of IL-6 via these signal-transduction pathways. A novel and intriguing observation made in this report is the ability of HSV DNA and HSV–antibody complexes to generate IL-6 through TLRs in the corneal cells. Other parameters relative to the TLR-3- and TLR-9-induced IL-6 secretion in HSV-1-infected corneal cells are still under study. Actually, this may be a more generalized phenomenon. Recently, studies from Luster’s laboratory demonstrated that DNA–anti-DNA antibody complexes found in systemic lupus erythematosus patients were capable of inducing IFN-α through TLR-9 (Means et al., 2005). In this report, we demonstrated that TLR-3 and TLR-9 mediate the activation of corneal cells by HSV, HSV DNA and HSV–antibody complexes. These findings suggest that cytokine release via TLR-3 and TLR-9 may be an appropriate target for interventional strategies in stromal keratitis.

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