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Induction of Interleukin-8 Gene Expression Is Associated with Herpes Simplex Virus Infection of Human Corneal Keratocytes but Not Human Corneal Epithelial Cells

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Interleukin-8 (IL-8) is a proinflammatory cytokine released at sites of tissue damage by various cell types. One important function of IL-8 is to recruit neutrophils into sites of inflammation and to activate their biological activity. Stromal keratitis induced by herpes simplex virus type 1 (HSV-1) is characterized by an initial infiltration of neutrophils. This study was carried out to determine whether cells resident in the cornea synthesize IL-8 after virus infection. Pure cultures of epithelial cells and keratocytes established from human corneas were infected with HSV-1, and the medium overlying the cells was subsequently assayed for IL-8 by an enzyme-linked immunosorbent assay. Cytokine mRNA levels in cell lysates were monitored by Northern (RNA) blot analysis. It was found that virus infection of keratocyte cultures led to the synthesis of IL-8-specific mRNA with more than 30 ng of IL-8 made per 10^6 cells. Neither UV-inactivated virus nor virus-free filtrates collected from HSV-1-infected keratocytes could induce IL-8 protein or mRNA, suggesting that viral gene expression was needed for induction of IL-8 gene expression. Unlike keratocytes, HSV-1-infected epithelial cells failed to synthesize IL-8 protein or mRNA. However, these cells readily produced both molecules following tumor necrosis factor alpha stimulation. HSV-1 had similar titers in both cell types. Thus, the failure to induce IL-8 synthesis was not due to an inability of the virus to replicate in epithelial cells. The capacity of HSV-1-infected corneal keratocytes to synthesize IL-8 suggests that these cells can contribute to the induction of the acute inflammatory response seen in herpes stromal keratitis.

The outer surface of the cornea consists of several layers of epithelial cells resting on a layer of connective tissue called the stroma. The stroma consists of fibroblast-like cells called corneal keratocytes embedded in a transparent extracellular matrix composed of collagens and proteoglycans (6). Both epithelial and stromal layers of the cornea are highly susceptible to herpes simplex virus (HSV) infection (35, 37–39). However, HSV infection of the epithelial layer is usually self-limiting and does not lead to vision loss. In contrast, virus infection of the stroma initiates a severe inflammatory response in the eye that is initially characterized by an infiltration of neutrophils (36, 37, 39), which may be followed several days later by an infiltration of lymphocytes (54). The infiltration of neutrophils and lymphocytes into the stroma can result in damage to the corneal surface and permanent vision loss (7, 34, 54).

It has recently been found that a number of cell types can synthesize and release significant amounts of the proinflammatory cytokine interleukin-8 (IL-8) (3, 9, 27, 42, 58, 61, 64). IL-8 is a potent chemoattractant for both neutrophils and lymphocytes (2, 23, 26, 32). In addition to its chemoattractant properties, IL-8 can also activate neutrophils after they have arrived at sites of infection (1, 32, 67). Since IL-8 maintains its physiological activity over a wide range of pHs and is resistant to mild proteolytic degradation (1, 32, 49, 67), the production of IL-8 by corneal cells during HSV infection could result in the recruitment of neutrophils and lymphocytes to infected tissues for prolonged periods.

HSV infection has been shown to induce expression of various cellular genes including those encoding the cytokines IL-6 and interferon (14–18, 44, 50, 57, 62). However, it is not known whether HSV type 1 (HSV-1) activates IL-8 gene expression following ocular infection of its natural host. This is an important question, since induction of IL-8 synthesis in infected corneal epithelial cells and stromal keratocytes could play an important role in the immunopathogenesis of HSV stromal keratitis. The goal of this study, therefore, was to determine whether HSV infection of human corneal epithelial cells and human corneal keratocytes induces expression of the gene encoding IL-8. It was found that HSV-1 infection significantly enhanced the expression of the IL-8 gene in cultures of corneal keratocytes but not in cultures of corneal epithelial cells.

MATERIALS AND METHODS

Establishment of human corneal epithelial and keratocyte cell cultures from human corneas. Corneas were obtained from the National Disease Research Interchange (Philadelphia, Pa.) or the Alabama Eye and Tissue Bank (Mobile, Ala.) and placed into culture within 4 days of enucleation. Human corneal epithelial and keratocyte cell cultures were prepared by a modification of published techniques (8, 19, 20). Briefly, corneoscleral rims were first trimmed from donor corneas. After off the endothelial cell layers were peeled from the bottom of the corneas, the corneas were washed repeatedly in RPMI 1640 medium. The epithelial layer was then removed by placing the cornea concave side down onto a drop of Dispase (grade II; Boehringer Mannheim, Indianapolis, Ind.) containing 25 caseinolytic units per ml of Dispase and 20 μg of gentamicin per ml in Hanks balanced salt solution. The corneas were then incubated with Dispase at 4°C for 24 h in a humidified environment.

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The epithelial cell layer from each cornea was then lifted from the stroma with a pair of jeweler's forceps, and single-cell suspensions were prepared by trypsin digestion. The cells were then cultured in Falcon Primaria positively charged 25-cm^2 tissue culture flasks (Becton Dickinson, Lincoln Park, N.J.) in Keratinocyte Serum Free Medium (KSFM) (GIBCO) containing 5 μg of gentamicin per ml. KSFM is a medium selective for epithelial cells and inhibits the growth of fibroblasts and macrophages. Keratocytes were freed from corneal stromas by collagenase type I digestion, as previously described (20). The cells were then cultured in 75-cm^2 flasks containing Dulbecco's modified Eagle's medium containing 20% fetal calf serum and antibiotics.

Immunofluorescence cell staining. Antikeratin and fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G antibodies were obtained from Boehringer Mannheim. Antibodies were diluted in phosphate-buffered saline containing 2% bovine serum albumin and 0.1% sodium azide. Human corneal keratocytes and corneal epithelial cells were grown in Lab-Tek eight-well chamber slides (Miles Laboratories, Inc., Naperville, Ill.) until approximately 75% confluent. The cells were then fixed as described by Sun and Green (63). Fixed-cell cultures were subsequently incubated with antikeratin AE1-AE3 or with anti-HSV glycoprotein D (29) at 37°C for 1.5 h. The monolayers were then washed and reacted with the secondary fluorescein-labeled antibody for an additional 1.5 h prior to a final wash and examination with a Zeiss Axiovert 35 fluorescence microscope.

Virus preparations. HSV-1(RE) was obtained from Ysolina Centifanto-Fitzgerald (Tulane University, New Orleans, La.). Human corneal keratocyte monolayers were grown to confluency in minimal essential medium plus 5% fetal calf serum. The cells were washed with serum-free Opti-MEM (GIBCO) and cultured for an additional 3 days in Opti-MEM with daily changes of medium. The cells were then infected at a multiplicity of infection (MOI) of 3 and maintained in Opti-MEM at 37°C until the cells exhibited a cytopathic effect score of 4+ 22 h postinfection. Infected cells were scraped from flasks and sonicated for 10 to 20 s with a Sonic 300 Dismembrator (Arteck Systems Incorporated, Farmingdale, N.Y.) to release infectious virus. Cellular debris was removed by centrifugation, and the titers of virus-containing supernatants on kerotocyte monolayers were determined. Mock-infected inocula were prepared in an identical fashion, except that keratocyte monolayers were not infected with HSV-1. All virus preparations and cell lysates were found to be negative for endotoxins by the *Limulus* amebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, Mass.).

Filtered virus inocula were prepared by filtering a 3-ml virus stock containing 7.5×10^8 PFU/ml through a Microsep microconcentrator with a cutoff of 300,000 molecular weight (Filtron Technology Corp., Northborough, Mass.) at 3,000 × *g* for 12 h at 4°C. After centrifugation, 0.5 ml of virus suspension trapped on the membrane was removed from the top of the filter apparatus and resuspended in 0.5 ml of Opti-MEM. The number of PFUs was then determined by plaquing on keratocyte cell monolayers. The filtrate collected from the bottom of the filter apparatus was added to keratocyte cell monolayers in IL-8 induction experiments.

UV inactivation was done by the protocol described by Lausch et al. (28). Briefly, 1 ml of HSV-1 at a titer of 1.2×10^9 was placed in a 30-mm-diameter petri dish on ice and then exposed to a 365-nm wavelength (long-wave) UV source for 10 min. Samples of irradiated virus were then used to infect human corneal keratocytes. A portion of the UV light-irradiated virus was assayed to determine the infectious titer.

In vitro growth curves. Monolayers of corneal epithelial cells and keratocytes in six-well tissue culture plates (Costar, Atlanta, Ga.) were infected at a MOI of 3.0. The virus inoculum was allowed to adsorb for 1 h at room temperature. The unabsorbed virus was then removed by washing the cells three times with RPMI 1640 medium. The cells were then incubated in appropriate medium at 37°C. Infected cells were scraped from wells at various times postinfection, washed three times, and then disrupted by sonication for 20 s to release intracellular virus. The amount of intracellular virus per sample was determined by assaying the samples on Vero cell monolayers.

Exposure of epithelial and keratocyte cultures to TNF-α and HSV-1. Epithelial cultures were infected at a MOI of 3.0. After a 30-min adsorption period at room temperature, the cultures were replenished with KSFM. In tumor necrosis factor alpha (TNF-α) treatment cultures, the medium was aspirated and replaced with 2 ml of KSFM containing 500 U of human recombinant TNF-α (Genzyme, Cambridge, Mass.). At selected times posttreatment, the medium was removed and frozen at −20°C for subsequent cytokine analysis. In experiments with keratocytes, cultures were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum until approximately 90% confluent. Dulbecco's modified Eagle's medium was then replaced with Opti-MEM, which was changed daily for 3 days before induction experiments. Cultures were then infected at a MOI of 3.0. After a 30-min absorption period, the cells were overlaid with Opti-MEM. For TNF-α induction, the medium was aspirated from cultures and replaced with Opti-MEM containing 500 U of human TNF-α per ml. Supernatants were then removed from flasks of treated cells at various time intervals and frozen at −20°C for subsequent cytokine analysis.

Cytokine assays. IL-8 was quantified with enzyme-linked immunosorbent assay (ELISA) kits obtained from R&D Systems (Minneapolis, Minn.). The lower limit of detection for IL-8 was 5 pg/ml. Colorimetric results were read at 450 nm with a EL308 microplate reader (Bio-tek Instruments, Winooski, Vt.). For each donor, separate flasks of epithelial cells and keratocytes were used at each dose and time point. Significant differences between cytokine levels were evaluated by small-sample paired *t* statistics.

RNA isolation and Northern (RNA) blot analysis. Total cellular RNA was isolated from HSV-1-infected cultures, cultures treated with a specific cytokine, or untreated cultures by the rapid total RNA isolation method described by Sambrook et al. (55). RNA (10 μg) was fractionated on a 1.0% agarose gel containing 2.2 M formaldehyde (55) and then stained with 1 μg of ethidium bromide per ml to confirm that all lanes contained equal amounts of RNA. Gels were then blotted onto MagnaGraph nylon filters by capillary transfer overnight (Micron Separations Inc., Westborough, Mass.) and then baked for 1 h at 80°C under vacuum. Membranes were hybridized overnight at 42°C with ^{32}P-labeled cDNA probe for IL-8 following by washing at high stringency as described by the membrane manufacturer's instructions. Probes were then stripped from filters as described by the manufacturer and rehybridized with additional probes as needed. Membranes were autoradiographed with an image-intensifying screen at −70°C.

Preparation of radiolabeled probes. The IL-8 cDNA clone was a gift from J. Oppenheim (Laboratory of Molecular

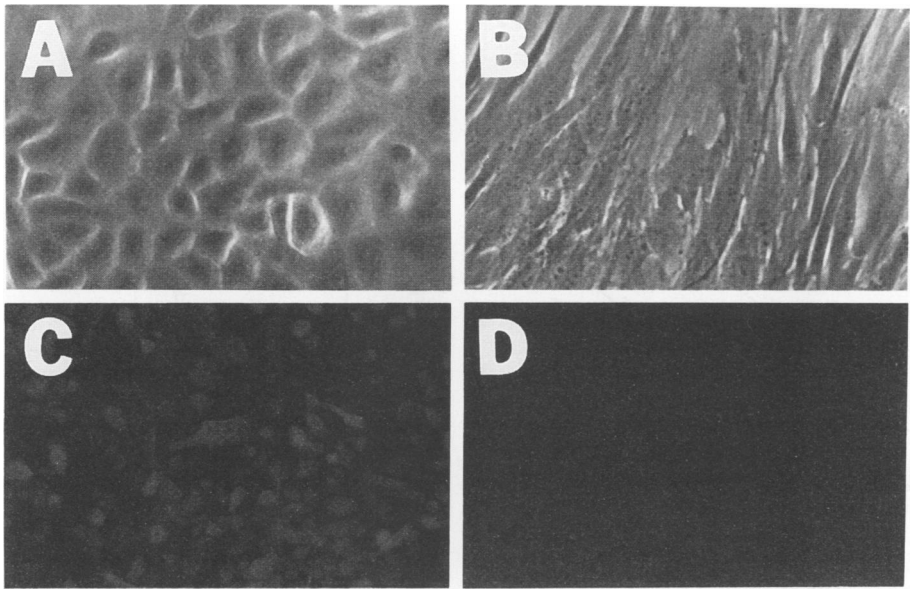

FIG. 1. Indirect immunofluorescence staining of human corneal epithelial cell and keratocyte cell cultures following staining with anticytokeratin antibodies. Human corneal epithelial cells and keratocytes were established as cultures as described in Materials and Methods. The cultures were then reacted with anticytokeratin antibody. Phase-contrast photomicrograph of unstained confluent cultures of epithelial cells (A) and keratocytes (B) at ×490 magnification. Subconfluent cultures of epithelial cells (C) and keratocytes (D) stained with anticytokeratin antibody at ×290 magnification. Neither cell type reacted with a monoclonal antibody made against HSV-1 glycoprotein D (data not shown).

Immunoregulation, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Md.). The cDNA clone for 28S rRNA was kindly provided by Warren Zimmer (University of South Alabama). The probe for glyceraldehyde-3-phosphate dehydrogenase (GAPD) was purchased from the American Type Culture Collection (Rockville, Md.). Probes were labeled by random-primer extension with the DECAprime DNA labeling kit (Ambion, Inc., Austin, Tex.) and [α-^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.) to a specific activity of $\geq 4 \times 10^8$ cpm/μg.

RESULTS

Growth of HSV-1 in human corneal epithelial cells and human corneal keratocytes. Before studying the effects of HSV-1 infection on the synthesis of IL-8 in human corneal epithelial cells and keratocytes, it was necessary to establish that pure cultures of the two cell types were obtained in tissue culture and that the two cell types supported virus growth.

Cultures of corneal epithelial cells were found to be composed of polygonal-shaped cells that were characteristic of stratified squamous epithelium, and in immunofluorescence tests, these cells reacted with anticytokeratin antibody (Fig. 1A and C). In contrast, corneal keratocytes were found to be fibroblastic in appearance and did not react with anticytokeratin antibody (Fig. 1B and D). The uniform presence of cytokeratin, a cytoskeletal protein found in corneal epithelial cells but not keratocytes (24, 56, 68), indicated that the epithelial cell cultures did not contain keratocytes. The absence of cytokeratin in keratocyte cultures demonstrated that they were not contaminated with epithelial cells.

The capacities of pure epithelial and keratocyte cultures to support HSV-1 replication were then investigated. It was found that the kinetics of virus growth was similar in both cell types and that cultures of epithelial cells and keratocytes produced indistinguishable numbers of progeny 22 h postinfection (Fig. 2). Similar results were obtained in a second experiment using cells derived from a different donor (data

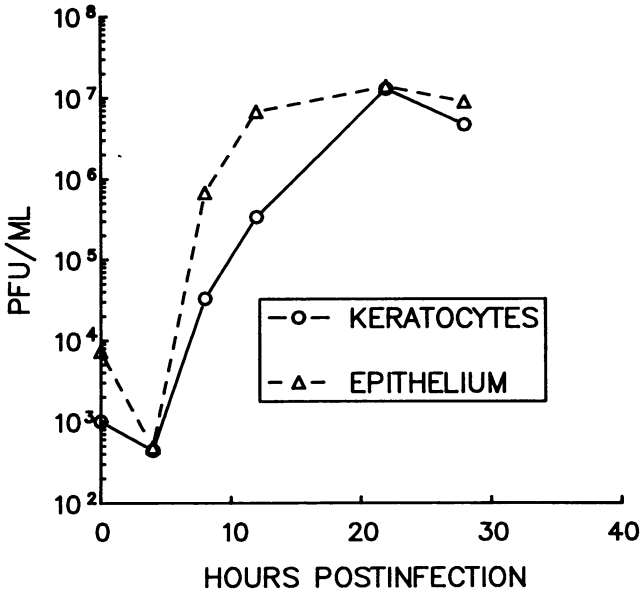

FIG. 2. One-step growth curve of HSV-1 in human corneal keratocytes and epithelial cells. Human corneal keratocyte and epithelial cell cultures were infected at a MOI of 3. After a 1-h adsorption period, the monolayers were washed three times and replenished with Opti-MEM (keratocytes) or KSFM (epithelial cells). At the indicated times, cultures were assayed for intracellular virus.

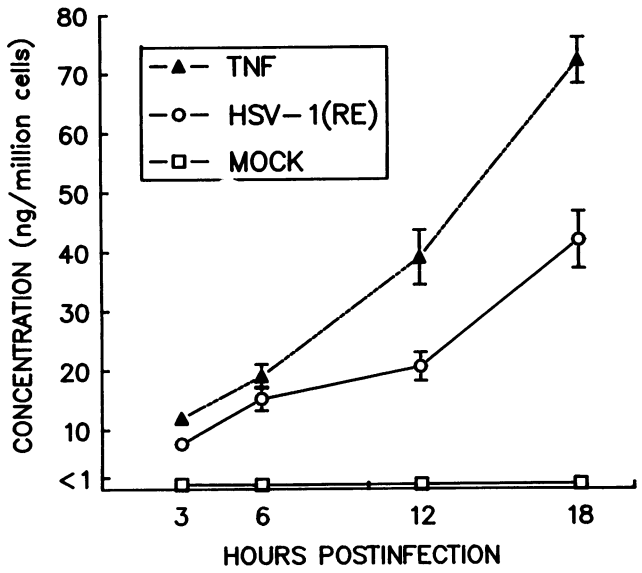

FIG. 3. IL-8 synthesis in HSV-1-infected human corneal keratocytes. Keratocyte cultures were exposed to HSV-1 or TNF-α or were mock infected. At the indicated times, culture supernatants were harvested, and the IL-8 content was determined by ELISA. The points represent the means ± standard errors of the means from experiments performed on identically prepared cultures established from four corneal donors. Statistically significant differences were found between HSV-1-exposed cultures and mock-infected cultures ($P < 0.01$).

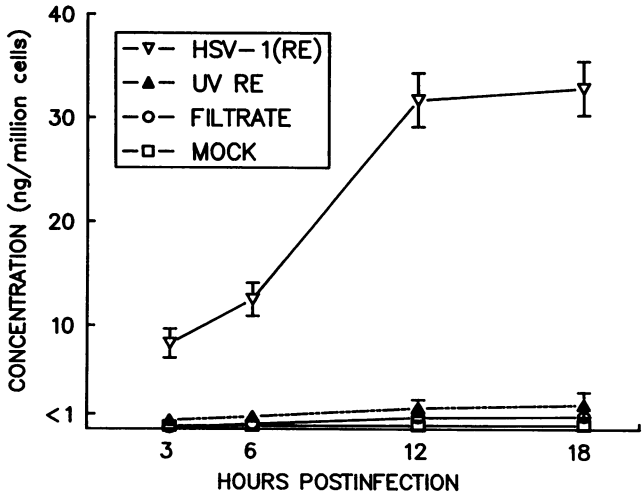

FIG. 4. IL-8 synthesis in human corneal keratocytes exposed to UV-inactivated HSV-1 or to filtered virus inocula. Virus inocula prepared in keratocyte cultures were filtered through a Filtron filter with a cutoff of 300,000 molecular weight. An aliquot of the virus preparation collected from the top of the filter was UV irradiated, while a second aliquot was left untreated. Keratocyte cultures were then exposed to UV-inactivated HSV-1, infectious HSV-1, or virus-free filtrate collected from the bottom of the filter, or were mock infected. At the indicated times, culture supernatants were harvested, and the IL-8 content was determined by ELISA. The points represent the means ± standard errors of the means from four separate experiments using identically prepared cultures established from four corneal donors. Statistically significant differences were found between IL-8 levels in cultures exposed to HSV-1 and cultures exposed to UV-inactivated virus or to virus-free filtrates ($P < 0.01$).

not shown). Thus, it can be concluded from these experiments that the two cell types did not differ significantly in their capacity to support HSV-1 replication.

HSV-1 infection enhances IL-8 synthesis in human corneal keratocytes. To test whether HSV-1 infection could stimulate IL-8 synthesis, confluent cultures of human corneal keratocytes were infected at a MOI of 3 PFU per cell. At various intervals postinfection, culture supernatants were harvested and assayed for IL-8 by ELISA. As a positive control, additional cultures were treated with 500 U of TNF-α per ml. TNF-α is a potent stimulator of IL-8 synthesis in many cell lines (27, 31, 60, 61), and this dose of TNF-α (500 U/ml) stimulates optimal levels of IL-8 production in human corneal keratocytes and human corneal epithelial cells (unpublished observations). The results in Fig. 3 are representative of four separate experiments involving four different corneal donors. It was found that HSV-1-infected cultures synthesized more than 30 ng/10^6 cells of IL-8, while TNF-α-induced cultures synthesized nearly 70 ng/10^6 cells. Mock-infected cultures synthesized less than 500 pg/10^6 cells of IL-8 during 18 h of incubation. Thus, keratocyte cultures exposed to HSV-1 or TNF-α synthesized greater than 50-fold more IL-8 than did mock-infected keratocyte cultures.

UV irradiation and ultrafiltration abrogate the capacity of HSV inocula to enhance IL-8 synthesis. The above results suggested that HSV-1 infection of human corneal keratocytes enhanced IL-8 synthesis. Additional experiments were performed to establish that the infecting virus, not some extraneous factor within the virus inoculum, was responsible for IL-8 induction. In these experiments, virus inocula were filtered to remove infectious virus particles. An aliquot of the virus suspension trapped on the membrane and an aliquot of the virus-free filtrate were then used to treat cultures of keratocytes. An equivalent amount of each virus suspension removed from the filter was also UV irradiated prior to being added to keratocyte monolayers to reduce infectivity by 99%. In four independent experiments, it was found that keratocyte cultures exposed to virus-free filtrates or to UV-inactivated virus did not produce significantly more IL-8 than that produced by mock-infected controls (Fig. 4). In contrast, infectious virus recovered from the filter surface induced the synthesis of nanogram amounts of IL-8. Thus, elimination of infectious particles from virus inocula by either ultrafiltration or UV irradiation abrogated their capacity to induce IL-8 synthesis.

HSV-1 infection of keratocytes enhances steady-state levels of IL-8 mRNA. The kinetics of IL-8 mRNA synthesis in HSV-1-infected keratocyte cultures and TNF-α-treated cell cultures were compared. Total RNA was extracted from keratocyte cultures at selected times posttreatment and then analyzed for IL-8 mRNA levels by Northern hybridization. Figure 5 shows that in TNF-α-treated cultures, maximum IL-8 mRNA levels were reached at 3 h postexposure (lane 4). Levels began to decline at 6 h and were close to unstimulated levels at 18 h (lanes 9, 14, and 19). In HSV-1-infected cells, maximal levels of IL-8 mRNA were also seen at 3 h postinfection (lane 5). However, IL-8-specific mRNA levels in HSV-1-infected cells rapidly declined at 6 h postinfection (lane 10). IL-8 mRNA levels then rebounded at 12 h postinfection, reaching levels greater than that seen in TNF-α-stimulated cultures (lane 15). Subsequently, IL-8 mRNA levels declined to levels seen in TNF-α-stimulated cultures at 18 h postinfection (lane 20). The same pattern of a decline in IL-8 mRNA levels 6 h postinfection followed by a rise in

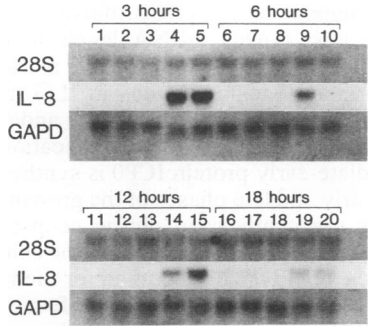

FIG. 5. Comparison of IL-8 mRNA levels in human keratocyte cultures exposed to TNF-α with IL-8 mRNA levels in cultures exposed to HSV-1 or filtered virus inocula. Keratocytes from three corneal donors were independently established in tissue culture. Infectious virus, UV-inactivated virus, and filtered virus inocula were prepared as described in the legend to Fig. 4. Human corneal keratocyte cultures were then mock infected (lanes 1, 6, 11, and 16) or exposed to filtered virus inocula (lanes 2, 7, 12, and 17), UV-inactivated HSV-1 (lanes 3, 8, 13, and 18), TNF-α (lanes 4, 9, 14, and 19) or infectious HSV-1 (lanes 5, 10, 15, and 20). At the indicated times, total cellular RNA was prepared and 10 μg was added to each lane of a 1.0% agarose gel containing 2.2 M formaldehyde. The RNA was electrophoretically separated, blotted onto a nylon membrane, and successively hybridized with different ^{32}P-labeled cDNA probes. A representative experiment performed on keratocyte cultures established from one corneal donor is shown.

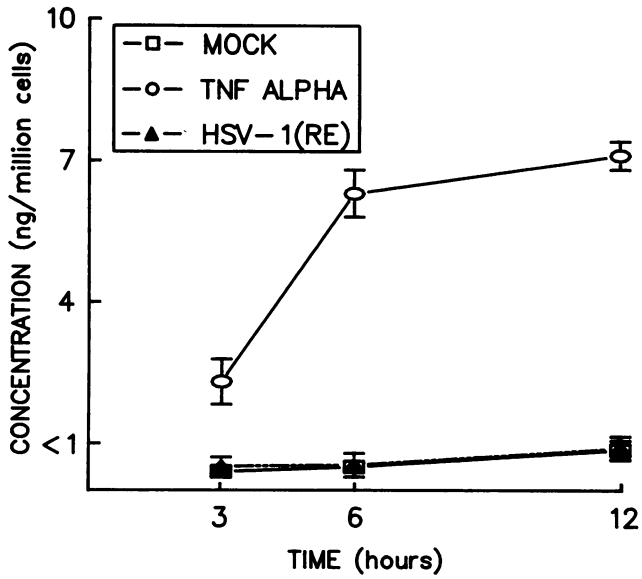

FIG. 6. IL-8 synthesis in HSV-1-infected human corneal epithelial cell cultures. Epithelial cell cultures were exposed to HSV-1 or TNF-α or were mock infected. At the indicated times, culture supernatants were harvested, and the IL-8 content was determined. The points represent the means ± standard errors of the means for experiments performed on identically prepared cultures established from five corneal donors. Statistically significant differences were not found between mock-infected cultures and cultures exposed to HSV-1 ($P > 0.5$).

IL-8 mRNA levels at 12 h postinfection was observed in two additional experiments (data not shown). In contrast to HSV-1-infected and TNF-α-treated keratocyte cultures, IL-8 mRNA could not be detected in mock-infected cultures, in cultures infected with UV-inactivated virus, or in cultures exposed to filtered virus inocula. The results also show that the levels of mRNA encoding the housekeeping gene GAPD were not affected by HSV-1 infection until 18 h postinfection (lanes 5, 10, 15, and 20).

HSV-1 infection of human corneal epithelial cells does not enhance IL-8 gene expression. We next investigated whether IL-8 gene expression could be stimulated in corneal epithelial cells following HSV-1 infection. Human corneal epithelial cell cultures were infected with HSV-1, and the culture supernatants were analyzed for IL-8 by ELISA. Surprisingly, virus infection did not stimulate IL-8 synthesis above the IL-8 levels in mock-infected cultures (Fig. 6). The failure of infected cells to produce IL-8 was not due to an inability of epithelial cells to synthesize this cytokine, since cultures treated with TNF-α produced more than 7 ng/10^6 cells of IL-8. We also isolated IL-8 mRNA from HSV-1-infected epithelial cells to determine the effects of HSV-1 infection upon IL-8 mRNA synthesis. It was found that HSV-1-infected cells failed to synthesize detectable amounts of IL-8-specific mRNA (Fig. 7, lanes 4, 8, and 12). In contrast, TNF-α-treated epithelial cells expressed readily detectable levels of IL-8 mRNA at 3, 6, and 12 h posttreatment (lanes 3, 7, and 11). GAPD mRNA levels were nearly indistinguishable in mock-infected cells, HSV-1-infected cells, and TNF-α treated cultures at 3 and 6 h postinfection (lanes 1 to 8). However, at 12 h postinfection, GAPD mRNA levels in HSV-1-infected cells were much less than that seen in mock-infected or TNF-α-treated cells (lane 12).

DISCUSSION

Initiation of inflammation at body surfaces involves the synthesis of a number of proinflammatory cytokines (22). However, it is not known whether HSV-1-infected cells directly participate in the amplification of inflammation by producing one or more of these cytokines. In this study, we investigated whether human corneal keratocytes and/or corneal epithelial cells, two cell types which support virus replication during ocular HSV infection, synthesize IL-8. This cytokine was investigated, because the immunopathology of ocular herpes involves infiltration of neutrophils and IL-8 is a potent neutrophil chemotaxin (1, 32, 40, 41, 67).

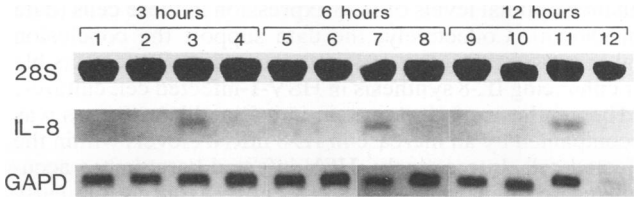

FIG. 7. Comparison of IL-8 mRNA levels in human epithelial cells exposed to TNF-α with cultures infected with HSV-1. Epithelial cells from two corneal donors were independently established in tissue culture. The cultures were then placed in KSFM (lanes 1, 5, and 9), mock infected (lanes 2, 6, and 10), TNF-α treated (lanes 3, 7, and 11), or HSV-1 infected (lanes 4, 8, and 12). At the indicated times, total cellular RNA was prepared and 10 μg was added to each lane of a 1.0% agarose gel containing 2.2 M formaldehyde. The RNA was electrophoretically separated, blotted onto a nylon membrane, and successively hybridized with ^{32}P-labeled cDNA probes. A representative experiment performed on epithelial cell cultures established from one corneal donor is shown.

It was found that human corneal keratocytes synthesized more than 30 ng/10^6 cells of IL-8 following HSV-1 infection. This amount was within the same order of magnitude as the amounts of IL-8 synthesized by cells stimulated with the potent IL-8 inducer TNF-α. It is possible that HSV-1 infection activated IL-8 gene expression by binding to virus receptors on the surface of individual cells. Such a mechanism has been reported to be responsible for induction of IL-6 synthesis in peripheral blood mononuclear cells (14). HSV-1 also carries a *trans*-activating protein called VP16 within its virion which can enhance gene expression by activating host transcriptional factors (52). Thus, it is possible that VP16 could have been involved in inducing IL-8 gene expression following penetration of virus from the cell surface into the cytoplasm of susceptible cells. However, our results suggest that neither virus adsorption nor VP16 was involved, because UV irradiation, which does not inhibit virus adsorption and uptake or the activity of VP16, abrogated the capacity of the virus to induce IL-8 gene expression (4). These results suggest, therefore, that one or more virus-specified proteins synthesized during virus replication are needed for induction of IL-8 synthesis within infected keratocytes.

We also considered the possibility that IL-8 was produced by HSV-1-infected keratocyte cultures, because virus-cell interactions induced synthesis of a primary proinflammatory cytokine such as IL-1 or TNF-α which then bound to infected cells and enhanced IL-8 gene expression. However, several observations are evidence against this hypothesis. First, IL-8 synthesis in HSV-1-infected keratocytes was enhanced within 3 h postinfection. Therefore, it is unlikely that HSV-1 could have stimulated synthesis of another cytokine in quantities sufficient to turn on IL-8 synthesis to the same magnitude as that seen in cells stimulated with optimal concentrations of TNF-α in such a short time. Second, filtrates of virus inocula prepared in keratocytes did not induce IL-8 synthesis. The molecular weight cutoff of the filters used in these experiments was 300,000, nearly 10 times more than the molecular weight of any known cytokine (22). Thus, if HSV-1-infected keratocytes had the capacity to produce cytokines capable of turning on IL-8 gene expression, these molecules would have passed through the filter and enhanced IL-8 gene expression in treated cultures. Finally, we have been unable to find either IL-1 or TNF-α in medium harvested from HSV-1-infected keratocyte by ELISAs sensitive enough to detect 15 pg of these cytokines per ml. This is less than the amount of IL-1 or TNF-α needed to enhance optimal levels of IL-8 expression in these cells (data not shown). Collectively, the data support the conclusion that virus-specific gene products were directly responsible for enhancing IL-8 synthesis in HSV-1-infected cell cultures.

The synthesis of IL-8 protein in infected keratocytes was accompanied by an increase in IL-8 mRNA levels within the infected cell. Interestingly, HSV-infected keratocytes accumulated the highest levels of IL-8 mRNA during the early part of the virus growth cycle. A number of virus-encoded *trans*-acting regulatory proteins expressed immediately after HSV-1 infection can interact with host proteins in the activation of select cellular promoters (10, 11, 16, 17, 47, 48, 50). Since immediate-early proteins are synthesized in the largest quantities soon after infection, the results suggest that one or more of these proteins may be involved in enhancing IL-8 gene expression. Curiously, IL-8 mRNA levels decreased in HSV-1-infected keratocytes at 6 h postinfection. The decline in IL-8 mRNA levels was not caused by a general degradation of host mRNAs by the virion host shutoff (*vhs*) polypeptide carried by infecting virions (12, 25, 46, 51), since host GAPD mRNA levels in infected and mock-infected cells were indistinguishable at this time point. Thus, it is possible that fluctuations in IL-8 mRNA levels were caused by changes in the abundance and/or activity of immediate-early proteins during virus replication. For example, the immediate-early protein ICP0 is synthesized at high levels during early and late phases of the growth cycle, while small amounts are synthesized during eclipse (69). Thus, enhanced synthesis of ICP0 at the end of the eclipse phase of the growth cycle could have accounted for the resurgence in IL-8 mRNA levels noted at 12 h postinfection. It is also known that HSV-1 can activate the cellular transcriptional activator NF-κB within the cytoplasm of infected cells (53). Since the IL-8 gene promoter possesses NF-κB-binding sites (43), activation of NF-κB within the cytoplasm of infected keratocytes could have resulted in its binding to the IL-8 gene promoter activating gene expression. Thus, it is possible that fluctuations in intracellular levels of this factor during the virus growth cycle contributed to the reduced IL-8 mRNA levels noted at 6 h postinfection. Finally, the return of IL-8 mRNA at 12 h may reflect the fact that a late gene product, such as a virion component, is involved in enhancing IL-8 gene expression.

Clearly, HSV-1 infection was not able to induce detectable levels of IL-8 protein or IL-8 mRNA in epithelial cells, even though HSV-1 replicated equally well in corneal epithelial cells and corneal keratocytes. This result suggests that not all of the cell types which support virus replication in the host will synthesize IL-8 in response to HSV-1 infection. The failure of HSV to induce IL-8 gene expression in epithelial cells was also not caused by a general destabilization of host mRNAs by the *vhs* polypeptide, because GAPD mRNA levels were not affected by HSV-1 infection during the first 6 h of the virus growth cycle. One possible explanation for differences in the capacity of HSV-1 to enhance IL-8 gene expression in the two cell types could be related to the fact that dissimilar cell types often express distinct transcriptional regulatory proteins (30, 33). Thus, *trans*-acting regulatory proteins expressed by keratocytes may have interacted more efficiently with HSV-1-specific immediate-early proteins within the IL-8 promoter than did transcriptional factors expressed by epithelial cells. This would result in increased rates of gene transcription in keratocytes and enhanced IL-8 synthesis. Alternatively, the results could reflect a general instability of IL-8-specific mRNA within infected epithelial cells. It is known that IL-8 mRNA contains sequences within its 3′ end which hasten its own degradation (5, 31) and that the *vhs* polypeptide shuts off host macromolecular synthesis by degrading host mRNAs (12, 13, 25, 46, 59). Since *trans*-acting cellular proteins may participate in *vhs* functions (21), epithelial cell proteins may have interacted more efficiently with *vhs* polypeptides than did analogous keratocyte proteins, resulting in more rapid degradation of IL-8 mRNA during the virus growth cycle. It can also be noted that epithelial cells were less responsive to TNF-α than were keratocytes. Thus, it is possible that HSV-1 failed to induce IL-8 synthesis in these cells, because IL-8 gene expression is less sensitive to stimulation than is IL-8 gene expression in keratocytes.

In humans as well as in experimental animals, HSV-1 infection of corneal epithelium elicits very little infiltration of inflammatory cells, whereas HSV-1 infection of keratocytes within the stromal layer is characterized by an intense infiltration of neutrophils and lymphocytes (40, 41, 65). It is tempting to speculate therefore that the differences in the

capacities of the two cell types to produce IL-8 following HSV-1 infection contribute to the differences in the numbers of inflammatory cells seen in the two layers of infected corneas. However, IL-8 is just one member of a superfamily of neutrophil-activating peptides (45, 66). It will be of importance to determine whether other members of this gene family can be synthesized by human corneal epithelial cells and/or keratocytes following ocular HSV-1 infection.

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