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Membranes of Herpes Simplex Virus Type-1-Infected Human Corneal Epithelial Cells Are Not Permeabilized to Macromolecules and Therefore Do Not Release IL-1α

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Nanogram amounts of the proinflammatory cytokine interleukin-1α (IL-1α) were detected in uninfected cultures of human corneal epithelial cells (HCEC). Although HSV-1 replicated $>10^4$-fold in these cells and caused extensive cytopathic effects, virus infection was not accompanied by significant extracellular release of IL-1α. Additional studies showed that release of radiolabeled cytosolic proteins from virus-infected HCEC was no greater than that released by mock-infected cells. These findings indicate that HSV-1 infection of HCEC does not result in IL-1α release because newly formed virus progeny can escape infected cells without disrupting cell membranes.

INTRODUCTION

Interleukin 1α (IL-1α) is a cytokine produced in response to infection and is involved in regulation of acute inflammation and specific immune responses as well as in the activation of secondary cytokines involved in host defenses (Dinarello, 1994a,b). Epithelial cells found at many epidermal surfaces can initiate inflammatory reactions in response to disease or injury through their capacity to synthesize this proinflammatory mediator constitutively (Gahring *et al.,* 1985; Hauser *et al.,* 1986; Kameda and Sato, 1994; Kupper *et al.,* 1986; Sauder *et al.,* 1982). Even though epithelial cells synthesize IL-1α, they do not secrete the cytokine by the classical secretory pathway but store the molecule in the cytoplasm in a biologically active form until it is passively released when cellular membranes are disrupted by disease or injury (Bakouche *et al.,* 1987; Matushima *et al.,* 1986; Stevenson *et al.,* 1992). Once IL-1α escapes the cell, it initiates local inflammatory responses by upregulating the synthesis of chemokines, which then induce extravasation of inflammatory cells from blood vessels and chemoattract them into sites of tissue injury (Dustin *et al.,* 1986; Furie and Randolph, 1995; Groves *et al.,* 1992; Strieter *et al.,* 1996; Waltz *et al.,* 1990).

The corneal surface is composed of a layer of transparent epithelial cells resting upon a stratum of connective tissue called the stroma. Ocular herpes is a disease initiated by infection of the epithelial and stromal layers of the eye surface with HSV-1, resulting in the infiltration of lymphocytes, neutrophils, and monocytes into the stromal layer of the cornea. Although these white blood cells are important in providing protection against the pathogen, their biological activities can also damage corneal tissues, leading to scar formation and vision loss (Hendricks and Tumpey, 1990; Metcalf and Kaufman, 1976; Meyers and Pettit, 1974; Meyers-Elliot and Chitjian, 1981; Pepose, 1991; Thomas *et al.,* 1997). The precise mechanisms whereby inflammation is initiated within HSV-1-infected corneas is not known. Many pathogens initiate inflammation by inducing release of preformed cytoplasmic IL-1α from epithelial cells (Agace *et al.,* 1993; Eckmann *et al.,* 1995; Rasmussen *et al.,* 1997; Walters *et al.,* 1995). Since HSV-1 replication kills infected cells (Roizman and Sears, 1996), it is reasonable to propose that inflammatory processes which develop during ocular herpes are also initiated in part by release of IL-1α from damaged corneal epithelial cells. This study was initiated to test the hypothesis that HSV-1 infection results in IL-1α release from human corneal epithelial cells. To our surprise, we found that the virus growth cycle goes to completion without inducing significant release of preformed intracellular IL-1α.

RESULTS

The effects of HSV-1 infection upon human corneal epithelial cells

Epithelial cell monolayers infected with HSV-1 demonstrated extensive cytopathic effects by 24 h postinfection. By 36 h postinfection, >90% of the cells had detached from the flask to become suspended in the culture medium. At this time, it was found that the number of both extracellular and intracellular plaque forming units had increased by greater than four logs (Fig. 1). The number

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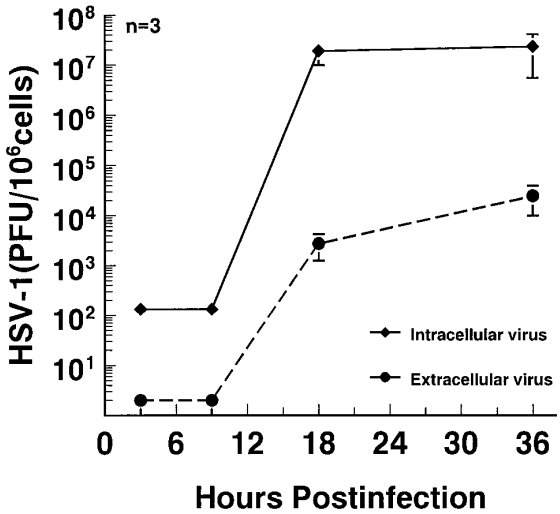

FIG. 1. Growth of HSV-1 in cultured human corneal epithelial cells. Confluent cultures of human corneal epithelial cells were infected with HSV-1 at a m.o.i. of 3. At selected times postinfection, supernatants were removed and the cells were scraped from the flasks and sonicated to release infectious virus. Supernatants and cellular lysates were assayed for infectious virus particles on cultured human corneal fibroblast cells. The values shown represent means ± SEM of identical experiments performed in cultures established from three corneal donors.

of cells killed during the virus growth cycle was determined by trypan blue exclusion (Strober *et al.,* 1997). It was found that by 36 h postinfection, >70% of the cells in the infected cell monolayers were not viable (Fig. 2). These results demonstrated that HSV-1 replicates in

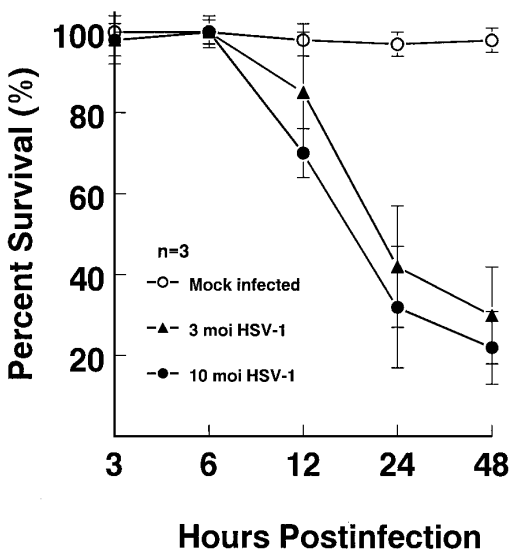

FIG. 2. Survival of HSV-1 (RE)-infected human corneal epithelial cells. Monolayers of epithelial cells were either mock-infected or infected with HSV-1 (RE). At selected times postinfection, the viability of the cells was estimated by the trypan blue exclusion assay. The values shown represent means ± SEM of identical experiments in cultures established from three corneal donors.

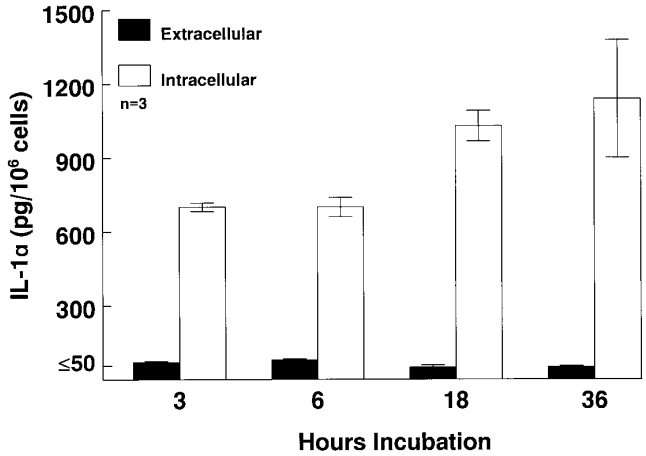

FIG. 3. Constitutive IL-1α production in cultures of human corneal epithelial cells. Confluent cultures of human corneal epithelial cells were grown in 25-cm^2 flasks. Following the addition of fresh medium, extracellular IL-1α was collected by removing the medium from individual flasks at regular intervals. The cells were then harvested and intracellular IL-1α was released by sonication. Supernatants and cell lysates were assayed for IL-1α by ELISA. The values shown represent means ± SEM of identical experiments performed in cultures established from three corneal donors.

HCEC and that the production of viral progeny results in the death of infected cells.

The effects of HSV-1 infection upon intracellular IL-1α stores

We next determined the quantities of IL-1α stored in HCEC and whether IL-1α is released during the virus growth cycle. It was found that HCEC incubated in growth medium synthesize >1 ng/10^6 cells of intracellular IL-1α (Fig. 3). However, during 36 h of incubation <4.0% of the IL-1α was released into the culture medium (Fig. 3). In order to determine if significant quantities of IL-1α are released following damage to cytoplasmic membranes, cells were rinsed three times with phosphate-buffered saline (PBS) and then exposed to 80 μg/ml digitonin for 10 min at room temperature. Digitonin is a non-ionic detergent which permeabilizes plasma membranes, releasing cytoplasmic proteins (Adam *et al.,* 1990; Cserpán and Udvardy, 1995). When supernatants collected from digitonin-treated cells were assayed for IL-1α, they were found to contain >1 ng of the cytokine/10^6 cells (Fig. 4). Thus, permeabilized HCEC release nearly all of their intracellular IL-1α. In contrast to digitonin treatment, however, infection of epithelial cells with HSV-1 did not significantly increase the amounts of IL-1α released even when they were infected at a m.o.i. of 10 ($P < 0.01$) (Fig. 4). Intracellular stores of IL-1α were not destroyed during the virus growth cycle because infected cells possessed >1 ng of IL-1α/10^6 cells at 48 and 72 h postinfection (Fig. 5). This is close to the amount of intracellular IL-1α found to be associated with noninfected cells in earlier experiments (Fig. 3).

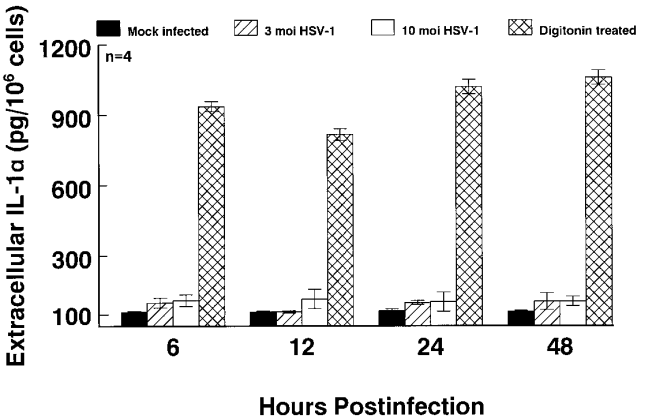

FIG. 4. Effect of HSV-1 infection on IL-1α release from human corneal epithelial cells. Confluent cultures of epithelial cells were either mock-infected or infected with HSV-1 At selected times postinfection, extracellular IL-1α was collected by removing the medium from individual flasks. Cytoplasmic IL-1α was released from uninfected cells by permeabilization of cell membranes with 80 μg/ml digitonin for 10 min at room temperature. Extracellular medium and cell lysates were assayed for IL-1α by ELISA. The values shown represent means ± SEM of identical experiments performed in cultures established from four corneal donors.

The effects of HSV-1 infection upon cell permeability

The failure of HSV-1 infection to cause significant release of IL-1α may be due to the fact that infected corneal epithelial cell membranes are not sufficiently damaged to allow IL-1α to escape. To test this hypothesis, we determined whether the amount of soluble proteins released from HCEC is enhanced by virus infection. Uninfected HCEC were found to release approximately 30% of their proteins into the growth medium during 48 h

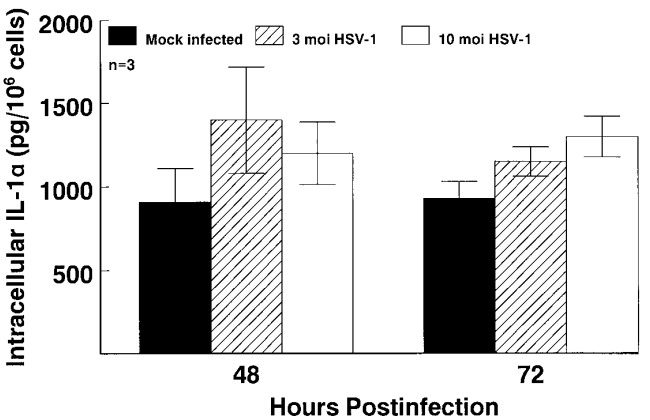

FIG. 5. Levels of intracellular IL-1α in HSV-1-infected human corneal epithelial cells. Confluent cultures of human corneal epithelial cells were infected with HSV-1 At selected times postinfection, intracellular IL-1α was collected by trypsinizing and sonicating the cells to release their intracellular contents. Sonicated samples were assayed for IL-1α by ELISA. The values shown represent means ± SEM of identical experiments performed in cultures established from three corneal donors.

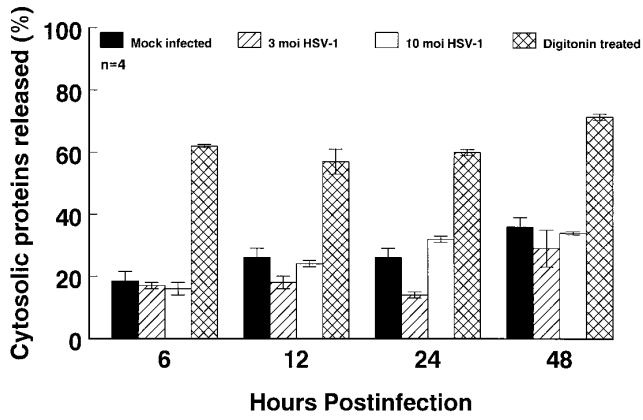

FIG. 6. The effect of HSV-1 infection upon cell permeability. Confluent cultures of human corneal epithelial cells in 24-well plates were labeled for 3 h before infection with translabel (25 μCi/ml sp act). The cells were washed 2× with PBS and then either infected with HSV-1 or mock-infected. At selected times postinfection, medium was collected from flasks for analysis of extracellular radiolabeled proteins. Any detached cells were removed by centrifugation prior to analyzing released proteins. Uninfected cell monolayers were washed and then permeabilized with 80 μg/ml digitonin for 10 min at room temperature to release soluble cytosolic proteins. Sodium dodecyl sulfate (0.6%) was then added to both infected and permeabilized cells to solubilize cellular components. The amounts of radioactive proteins released by the treatments were quantitated by liquid scintillation counting and are reported as the percentage released compared to the total radioactivity present (supernatants plus cells). The values shown represent means ± SEM of identical experiments performed in cultures established from four corneal donors.

of incubation (Fig. 6). In order to determine the quantities of soluble cytosolic protein retained in epithelial cells, we treated cells at selected times postlabeling with digitonin. Exposure of epithelial cells to this detergent enhanced the amount of protein released from epithelial cells by >40% (Fig. 6). Extension of the incubation time or use of higher digitonin concentrations did not significantly increase the amount of ^{35}S-labeled protein released (data not shown). When monolayers of epithelial cells were infected with HSV-1, the amount of intracellular proteins released was not significantly enhanced over uninfected cells even after infection at a m.o.i. of 10 (Fig. 6). These results suggest that HSV-1-infected human corneal epithelial cells (HCEC) are not lysed during the virus growth cycle.

DISCUSSION

Our results provide evidence that HSV-1 can complete its growth cycle in human corneal epithelial cells without inducing significant release of preformed IL-1α stored within the cell. Since infected cultures produce $>10^4$ PFU/ml of infectious extracellular virus particles 36 h after infection, it appears that significant numbers of newly formed progeny can exit cells without damaging the permeability barrier of cell membranes. HSV-1 replication is generally thought to be lytic for many types of

cells (Roizman and Sears, 1996). Consequently, how is it possible for infected HCEC to secrete newly formed virus progeny without releasing IL-1α? Mature enveloped virus particles escape infected cells by being carried in transport vesicles through the cells secretory pathway to the plasma membrane, where they are actively secreted (Banfield and Tufaro, 1990; Johnson and Spear, 1982). Since IL-1α lacks a signal sequence and cannot interact with intracellular secretory pathways (Bakouche *et al.,* 1987; Matushima *et al.,* 1986; Stevenson *et al.,* 1992), it cannot be incorporated into vesicles carrying virions to the cell surface. Therefore, it is likely that HSV-1-infected HCEC release viral progeny without releasing IL-1α because HSV-1 escapes infected epithelial cells by exclusive use of the exocytosis pathway.

Although HSV-1 infection of corneal epithelial cells does not result in the detectable release of IL-1α *in vitro,* one could envision that IL-1α could be released by these cells following infection *in vivo,* for example, by the eventual disintegration of infected cells or by the course of engulfment by phagocytic cells. However, it is known that injured corneal epithelial cells are shed into the tear film, where they are removed by the lavaging action of tears (Davson, 1990). Thus, IL-1α leakage into corneal tissue *in vivo* may also be minimal. This view is consistent with the clinical observation that little or no inflammation develops in corneas of individuals when HSV-1 infections are limited to epithelial layers of the cornea (Pepose *et al.,* 1996).

Following corneal infection, HSV-1 spreads into sensory neurons which innervate the ocular surface (Fraser *et al.,* 1991). The release of intracellular stores of IL-1α is believed to be an early warning signal by which epithelial cells damaged by disease or injury alert host defenses of microbial invasion (Dinarello, 1994a,b). The failure of HSV-1 to induce significant IL-1α release from infected HCEC may contribute to the pathogenesis of HSV-1 by delaying induction of antiviral host responses, thereby enhancing prospects for the virus to penetrate sensory nerve endings and establish a latent infection in the trigeminal ganglion. In addition to corneal epithelial cells, HSV-1 also infects epithelial cells at other body surfaces. It is not known if IL-1α is also retained in these cells following HSV-1 infection.

MATERIALS AND METHODS

Cell culture and preparation of virus stocks

Human corneas were obtained from the National Research Interchange (Philadelphia, PA). Epithelial cells were isolated from the corneas and established in tissue culture as previously described (Cubitt *et al.,* 1993). Virus stocks were prepared and titered on monolayers of human corneal fibroblasts by established procedures (Oakes *et al.,* 1993).

Kinetics of virus growth

Confluent cultures were infected with HSV-1 (RE) at a m.o.i. of 3. At selected times postinfection, supernatants were removed from the cultures and cells were then dislodged from the plates with trypsin and sonicated to release intracellular virus. The number of plaque-forming units (PFU) in supernatants and cell lysates released was determined by standard plaque assays on human corneal fibroblasts (Oakes *et al.,* 1993).

Assay of intracellular and extracellular IL-1α

Extracellular levels of IL-1α secreted from cell monolayers were determined by harvesting supernatants from flasks at selected intervals postinfection. Intracellular IL-1α levels were then determined by trypsinizing the cell monolayers in 0.05% trypsin with EDTA (Gibco BRL, Grand Island, NY). The cell membranes were then disrupted by centrifuging cells at 14,000 *g* for 5 min at 4°C and then sonicating the pelleted cells for 20 s in a Sonic 300 Dismembrator (Antek Systems Corp., Farmingdale, NY). After resuspending the sonicated cells in 1 ml of Keratinocyte Serum Free Medium (KSFM) (Gibco BRL), both sonicated samples and supernatants were assayed for IL-1α by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN). The sensitivity of the assay was 0.5 pg/ml. The significance of differences between cytokine levels were evaluated by using small sample paired *t* statistics.

Measurement of cell permeability in HSV-1-infected cells

Epithelial cells were grown in KSFM containing half the normal concentration of methionine and cysteine for 12 h. The medium was aspirated and the cells labeled with methionine-free and cysteine-free DMEM containing labeled [^{35}S]methionine and [^{35}S]cysteine (Translabel, ICN Pharmaceutical, Irvine, CA) for 3 h. The labeling medium was removed from the flasks and the monolayers were washed three times with PBS. The washed monolayers were then either mock-infected or infected with HSV-1. After virus absorption, the flasks were replenished with KSFM and the monolayers incubated at 37°C. For digitonin permeabilization, cells were rinsed three times with PBS and then exposed to 80 μg/ml digitonin for 10 min at room temperature. After removal of intact cells by centrifugation, soluble proteins released into the supernatant from HSV-1-infected cells, mock-infected cells, or digitonin-permeabilized cells were quantitated by scintillation counting in a Beckman LX500CE scintillation counter (Beckman Inc., Fullerton, CA). The remaining cell-associated proteins were then collected by washing cell monolayers with 0.2 ml PBS followed by treatment with 0.6% sodium duodecyl sulfate (SDS) in 10 mM EDTA for 2 min. Cell lysates were collected and

centrifuged for 14,000 *g* for 5 min at 4°C. Aliquots were counted in a liquid scintillation cocktail.

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