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Robust expression of TNF-α, IL-1β, RANTES, and IP-10 by human microglial cells during nonproductive infection with herpes simplex virus

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Cytokine (TNF-α/β, IL-1β, IL-6, IL-18, IL-10, and IFN-α/β/γ) and chemokine (IL-8, IP-10, MCP-1, MIP-1α/β, and RANTES) production during herpes simplex virus (HSV) 1 infection of human brain cells was examined. Primary astrocytes as well as neurons were found to support HSV replication, but neither of these fully permissive cell types produced cytokines or chemokines in response to HSV. In contrast, microglia did not support extensive viral replication; however, ICP4 was detected by immunochemical staining, demonstrating these cells were infected. Late viral protein (nucleocapsid antigen) was detected in <10% of infected microglial cells. Microglia responded to nonpermissive viral infection by producing considerable amounts of TNF-α, IL-1β, IP-10, and RANTES, together with smaller amounts of IL-6, IL-8, and MIP-1α as detected by RPA and ELISA. Surprisingly, no interferons (α, β, or γ) were detected in response to viral infection. Pretreatment of fully permissive astrocytes with TNF-α prior to infection with HSV was found to dramatically inhibit replication, resulting in a 14-fold reduction of viral titer. In contrast, pretreatment of astrocytes with IL-1β had little effect on viral replication. When added to neuronal cultures, exogenous TNF-α or IL-1β did not suppress subsequent HSV replication. Exogenously added IP-10 inhibited HSV replication in neurons (with a 32-fold reduction in viral titer), however, similar IP-10 treatment did not affect viral replication in astrocytes. These results suggest that IP-10 possesses direct antiviral activity in neurons and support a role for microglia in both antiviral defense of the brain as well as amplification of immune responses during neuroinflammation. *Journal of NeuroVirology* (2001) **7,** 208–219.

Keywords: microglia; cytokines; encephalitis

Introduction

Infection of the central nervous system (CNS) with herpes simplex virus (HSV) 1 results in an acute focal, necrotizing encephalitis (HSE) along with severe neuroinflammation and swelling of the brain. Despite reductions in mortality following acyclovir or vidarabine therapy, most HSE patients who recover display significant long-term neuropathological manifestations (reviewed in Skoldenberg, 1996; McGrath *et al*, 1997). The mechanisms responsible for this neurological damage appear to involve both direct, virus-mediated as well as indirect, immune-mediated processes. Treatment with antiviral agents effectively controls viral replication but does not inhibit immune-mediated neuropathological sequelae. Studies have demonstrated that long-term neuroimmune activation and cytokine production persist following HSV infection in patients (Aurelius *et al*, 1994), as well as following experimental infections in mice (Cantin *et al*, 1995; Shimeld *et al*, 1995; Halford *et al*, 1996; Shimeld *et al*, 1997; Meyding-Lamade *et al*, 1998). The results of these studies suggest that HSE may develop into a chronic inflammatory disease in the CNS.

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Glial cells (microglia and astrocytes) form an intrinsic immune system within the CNS (Kreutzberg, 1996, Peterson *et al*, 1997a). Together with lymphocytes, monocytes, and neutrophils that migrate into the infected brain (Rowell and Griffin, 1999), glial cells have been proposed to defend the CNS against infectious agents (Carson and Sutcliffe, 1999). Activated microglia and astrocytes produce and respond to a number of immune mediators, including cytokines and chemokines (Benveniste, 1997). Through this complex network of immune mediators, glial cells appear to be active in regulating the local immune response in the brain during inflammation.

Cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-4, IL-10, and interferon-γ (IFN-γ), have been shown to possess antiviral properties in the CNS, and early local expression of cytokines in the CNS has been demonstrated to correlate with control of viral replication (Griffin, 1997). In addition, some cytokines have been proposed to have a direct antiviral effect on HSV (Feduchi *et al*, 1989; Chen *et al*, 1993). The release of cytokines by local neural cells, or infiltrating cells of the immune system, may directly inhibit viral spread in the CNS, or they may elicit antiviral activity through the induction of additional factors (e.g., nitric oxide). These host defense mechanisms in the CNS must balance control of the spread of invading microbes with undesirable toxic activity associated with inflammation, which may damage the brain.

Because of their chemotactic properties, chemokines likely play an important role in regulating brain inflammation during viral encephalitis. The migration of immune effector cells into the brain parenchyma is associated with the expression of chemokines (Weiss *et al*, 1998; Persidsky *et al*, 1999), which appear to be key signals for recruitment of cells of the immune system into and within the brain. Chronic inflammatory cell infiltrates, along with high level IFN-γ production, have been observed in ganglia during HSV infection (Cantin *et al*, 1995). $CD8^+$ T-lymphocytes have been shown to control HSV through nonlytic mechanisms most likely involving cytokines (Simmons and Tscharke, 1992). Microglia and astrocytes are the major source of T-cell attracting chemokines in the CNS (Peterson *et al*, 1997b; Oh *et al*, 1999).

Little is known about cytokine and chemokine production by brain cells in response to HSV infection. Thus, in this study we analyzed cytokine and chemokine production using homogeneous cultures of primary human brain cells infected with HSV. The purpose of this study was to characterize HSV infection of different human brain cell types and to determine cytokine and chemokine production patterns by these cells in response to viral infection. Gaining insight into the cytokine and chemokine networks that regulate persistent neuroinflammation may be an important first step towards new therapeutic approaches for management of the neuropathological sequelae subsequent to HSE.

Results

Replication of HSV in primary human brain cells

Before examining cytokine and chemokine production from human glial cells and neurons in response to HSV infection, we first examined viral growth characteristics in each of our primary brain cell culture systems. Productive HSV infection was observed in cultures of primary astrocytes, with a 3-log increase in viral titer over the 144-h time course of these experiments (Figure 1). Cultures of highly enriched human neurons, consisting of >90% neurons along with ~10% glial cells, were also found to be permissive for viral replication, with a 3-log increase, reaching peak titers by 48 h postinfection (p.i.) (Figure 1). In contrast to these findings with astrocytes and neurons, when purified microglial cells were infected with HSV, only limited replication was detected followed by a rapid decline in infectious virus (Figure 1).

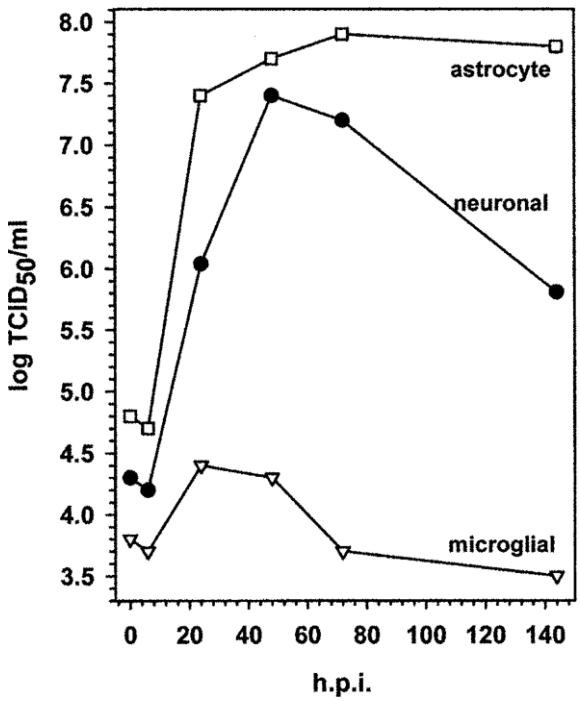

Figure 1 HSV replication in primary human brain cells. Astrocyte (>99% $GFAP^+$, 2×10^5 cells/well), neuronal (>90% NSE^+, 5×10^5 cells/well) and microglial (>99% $CD68^+$, 2×10^5 cells/well) cell cultures were infected with HSV (strain 17 syn^+, MOI = 1) for 0, 6, 24, 48, 72, and 144 h. Infected brain cells were harvested at the indicated time points and viral titers in the cryolysates were determined by 50% tissue culture infectious dose ($TCID_{50}$) assay on rabbit skin indicator cells. The viral titer at time 0 represents the amount of HSV detected following the 2-h adsorption and subsequent washings. Data presented are representative of three independent experiments using cells isolated from different brain specimens.

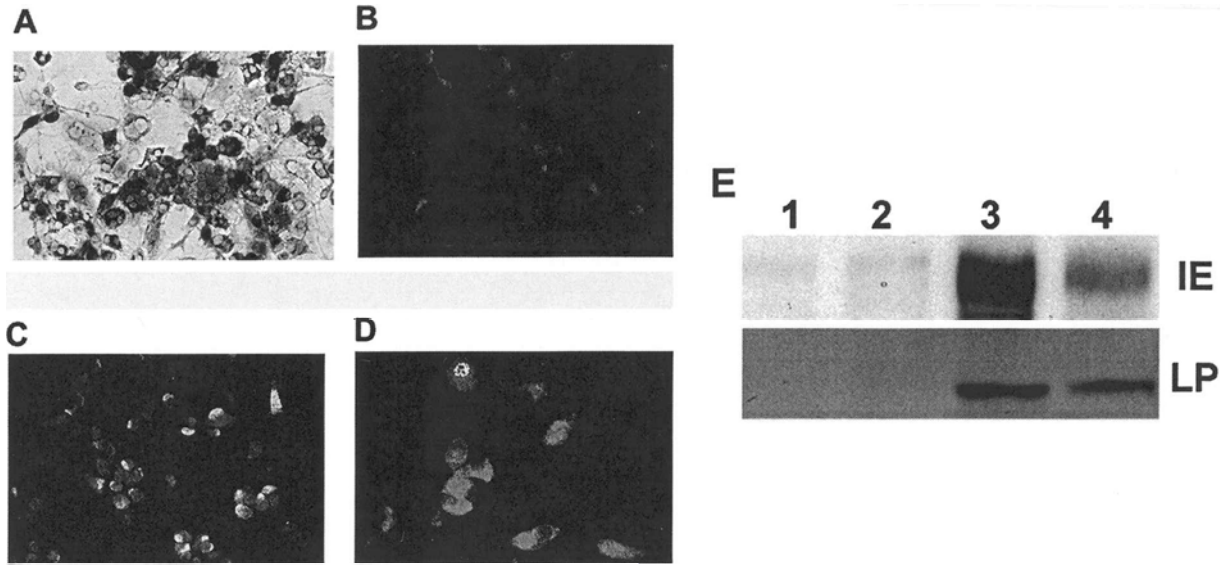

Figure 2 HSV infection and antigen expression in microglial cells. (**A**) Microglia were infected overnight with the Lac Z-containing recombinant HSV 17' LAT-MT1. β-galactosidase activity indicative of LAT-MT1 promoter activity was detected (blue staining) with X-gal (1mg/ml) at 24 h p.i. (**B**) Uninfected and (**C**) infected microglial cell cultures were probed using antibodies against the immediate early (IE) protein ICP4 and (**D**) the 155-kD viral nucleocapsid protein (Biodesign International, Saco, ME) by immunofluorescence at 3, 8, 24, and 48 h p.i. Data shown were obtained at 8 and 24 h for ICP4 and nucelocapsid protein, respectively. Late protein production was detected by immunoflourescence in <10% of the infected microglial cells. (**E**) Western blot analysis of HSV protein production. Total protein was extracted from HSV-infected primary human microglial cells (2×10^6 cells). Then, 20 μg of protein was blotted and probed with antibodies to HSV ICP4 (IE), Advanced Biotechnologies, or nucleocapsid protein (LP). Lane 1, protein extract from uninfected microglial cells; lanes 2–4, extracts from microglial cells at 6 h, 20 h, and 45 h following infection with HSV, respectively.

HSV infection of human microglial cells

Given the lack of robust viral growth in microglial cell cultures, we set out to determine if microglia were nonproductively infected with HSV. In these experiments, we employed a Lac-Z expressing recombinant virus (17' LAT-MT1), kindly provided by LT Feldman (UCLA). 17' LAT-MT1 contains a binary fusion between the HSV LAT promoter and the murine metallothionein promoter driving expression of Lac-Z, which is active in both productively and latently infected cells (Lokensgard *et al*, 1994). When 17' LAT-MT1-exposed microglial cells were stained with X-gal (1 mg/ml), an intense blue staining was observed (Figure 2A). This result demonstrates that although there is limited viral growth, microglial cells are infected with HSV.

Expression of viral antigens in microglial cells

Having determined that microglia can be infected by HSV, we set out to examine the extent of viral gene expression. By immunochemical staining of HSV-infected microglia (at 3, 8, 24, and 48 h p.i.), high levels of the immediate early (IE) ICP4 antigen were detected in most of the infected cells at 8 h p.i. (Figure 2B,C). In contrast, when the infected cells were stained using antibodies to the 155-kD nucleocapsid protein (a late viral antigen) <10% of the infected cells displayed late viral antigen expression at 24 h p.i. (Figure 2D), with few cells remaining positive by 48 h. Western blot analysis confirmed that even in the absence of viral replication, IE proteins are produced in HSV-infected microglia, along with some expression of late viral antigens (Figure 2E).

Cytokine and chemokine production by microglial cells in response to nonproductive HSV infection

To evaluate whether selected cytokines and chemokines are produced by human brain cells in response to HSV infection, we screened supernatants from each of our three infected brain cell cultures for the cytokines TNF-α and β, IL-1β, IL-6, IL-18, IL-10, and IFN-α, β, and γ; and the chemokines IL-8, IFN-γ inducible protein 10 kD (IP-10), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and β, and regulated upon activation, normal T cell expressed and secreted (RANTES). Although primary astrocytes and neurons were found to support HSV replication, neither of these fully permissive cell types produced any cytokines or chemokines in response to HSV infection (Table 1). In contrast, microglial cells did not support extensive viral replication, however, infection with HSV stimulated large amounts of the proinflammatory cytokines TNF-α and IL-1β and the chemokines RANTES and IP-10. Lesser amounts of HSV-induced IL-6, MIP-1α and β, MCP-1, and IL-8 were also detected (Table 1). Surprisingly, levels of INF-α, β, or γ were undetectable (<10 pg/ml) following HSV infection of microglial cells.

Table 1 Microglial cells produce proinflammatory cytokines and chemokines in response to nonproductive HSV infection[a]

Cytokine	*Microglial cells*	*Astrocytes*	*Neurons*
TNF-α	++	–	–
IL-1β	+++	–	–
IL-6	+	–	–
IL-18	–	–	–
TNF-β	–	–	–
IL-10	–	–	–
IFN-α	–	–	–
IFN-β	–	–	–
IFN-γ	–	–	–
RANTES	+++	–	–
MIP-1α	+	–	–
MIP-1β	+	–	–
MCP-1	+	–	–
IL-8	+	–	–
IP-10	++++	–	–

[a]Cell-free supernatants were collected at 24 h and 48 h postinfection.

Kinetics of cytokine and chemokine production

Production of TNF-α was rapidly induced following HSV infection reaching levels of 342 pg/ml by 24 h p.i. with highest levels (405 $\pm$ 18.5 pg/ml) at 72 h p.i. (Figure 3A). Expression of IL-1β was delayed relative to TNF-α reaching levels of 92 $\pm$ 11 pg/ml and 726 $\pm$ 16 pg/ml at 24 and 48 h p.i., respectively (Figure 3B). Mock-treated and unstimulated microglial culture supernatants contained little or no detectable TNF-α or IL-1β. The time-course of chemokine production by HSV-infected microglial cells was similar for RANTES and IP-10 with levels rising throughout the duration of these studies (Figure 3C,D). The chemokine and cytokine production results obtained by ELISA were confirmed using ribonuclease protection assay (RPA). mRNA for TNF-α and IL-1β was induced early (3 h) following infection and declined after 24 h (Figure 4). Constitutive levels of IL-1β mRNA can be observed. Expression of RANTES and IP-10 mRNA was slightly delayed compared to IL-1β; they were first detectable at 8 h p.i., and persisted over the 24-h time point (Figure 4).

Production of TNF-α and IL-1β requires replication competent virus

To investigate whether replication competent HSV was required to induce cytokine or chemokine production in microglial cells, we stimulated microglia with ultraviolet (UV) light- (256 nm, 30 min) or heat- (56°C, 45 min) inactivated virus and culture supernatants were assayed for TNF-α, IL-1β, and RANTES. We found that UV inactivation of the virus completely abolished microglial cell production of TNF-α, and these cells also produced markedly less IL-1β (18.5 $\pm$ 1.5 pg/ml) than cells infected with replication competent virus (237 $\pm$ 26 pg/ml and 630 $\pm$ 30 pg/ml for TNF-α and IL-1β, respectively) at 24 h p.i. (Figure 5). Interestingly, similar levels of RANTES were produced by microglial cells in response to replication competent (170 $\pm$ 16 pg/ml) or UV-inactivated virus, 179 $\pm$ 4.5 pg/ml (Figure 5). Heat-inactivated HSV did not stimulate TNF-α, IL-1β, or RANTES production by microglial cells.

Involvement of signal transduction pathways

We next examined the involvement of two MAP kinase signal transduction cascades which could be activated by stimulation of microglial cells with HSV. In these studies, HSV-induced cytokine (TNF-α and IL-1β) production by human microglia was found to be susceptible to inhibition by both the p38 mitogen-activated protein (MAP) kinase inhibitor SB202190 (1.0 to 10 μM) and the MAP kinase kinase (MEK) 1/2 inhibitor UO126 (Figure 6A,B). In contrast, HSV-induced chemokine production (RANTES and IP-10) was suppressed by SB202190, but the MEK 1/2 inhibitor UO126 had no inhibitory effect (Figure 6C,D). The corresponding negative controls SB202474 and UO124 had no inhibitory effect at any concentration tested (data not shown). These data suggest that the p38 MAP kinase pathway, but not the MEK1/2 pathway, plays a role in HSV-induced chemokine production in human microglia.

Cytokine and chemokine-mediated inhibition of HSV replication in permissive human brain cells

Recent experiments performed in our laboratory have demonstrated that proinflammatory cytokine treatment of permissive astrocytes suppresses the subsequent replication of human cytomegalovirus (Cheeran *et al*, 2000). In the present study, pretreatment of highly purified, fully permissive astrocytes with recombinant human TNF-α (20 ng/ml) for 72 h prior to infection with HSV was found to dramatically inhibit viral replication resulting in a 14-fold reduction in titers as determined by plaque assay (Figure 7A). In contrast, pretreatment of astrocyte cultures with exogenous IL-1β (10 ng/ml) had little effect on viral replication. The addition of TNF-α to neuronal cultures had no effect on subsequent HSV replication, nor did exogenously added IL-1β suppress HSV replication (Figure 7A). When the chemokine IP-10 (100 ng/ml) was added to primary human neurons prior to viral infection, it was found to potently inhibit HSV replication with a 32-fold reduction in viral titer (Figure 7B). Similar IP-10 treatment did not affect viral replication in astrocytes. Additionally, treatment of either neurons or astrocytes with exogenously added RANTES did not result in significant decreases in viral titers (Figure 7B).

Microglial cells are killed by nonproductive HSV infection

Even with limited viral replication, cytopathic effects were evident in the HSV-infected microglia, with some cells detaching from the plate and floating into the culture supernatants. Microglial cell viability, as assessed by MTT dye uptake, revealed ~5%, 35%, and 95% of microglial cells were dead at 48,

72, and 96 h p.i., respectively. To characterize the phenomenon of cell death, we then examined the infected microglial cells for apoptotic changes using a sandwich ELISA method that detects nucleosomal oligomers. DNA fragmentation indicative of apoptosis was rapidly induced by HSV infection of microglial cells. Twenty-four h following viral infection apoptotic DNA fragmentation was 241% greater than in uninfected control cells and increased to 486% by 48 h p.i., followed by a decline at later time points (Figure 8). The oligonucleosomal DNA fragmentation that was observed following viral infection

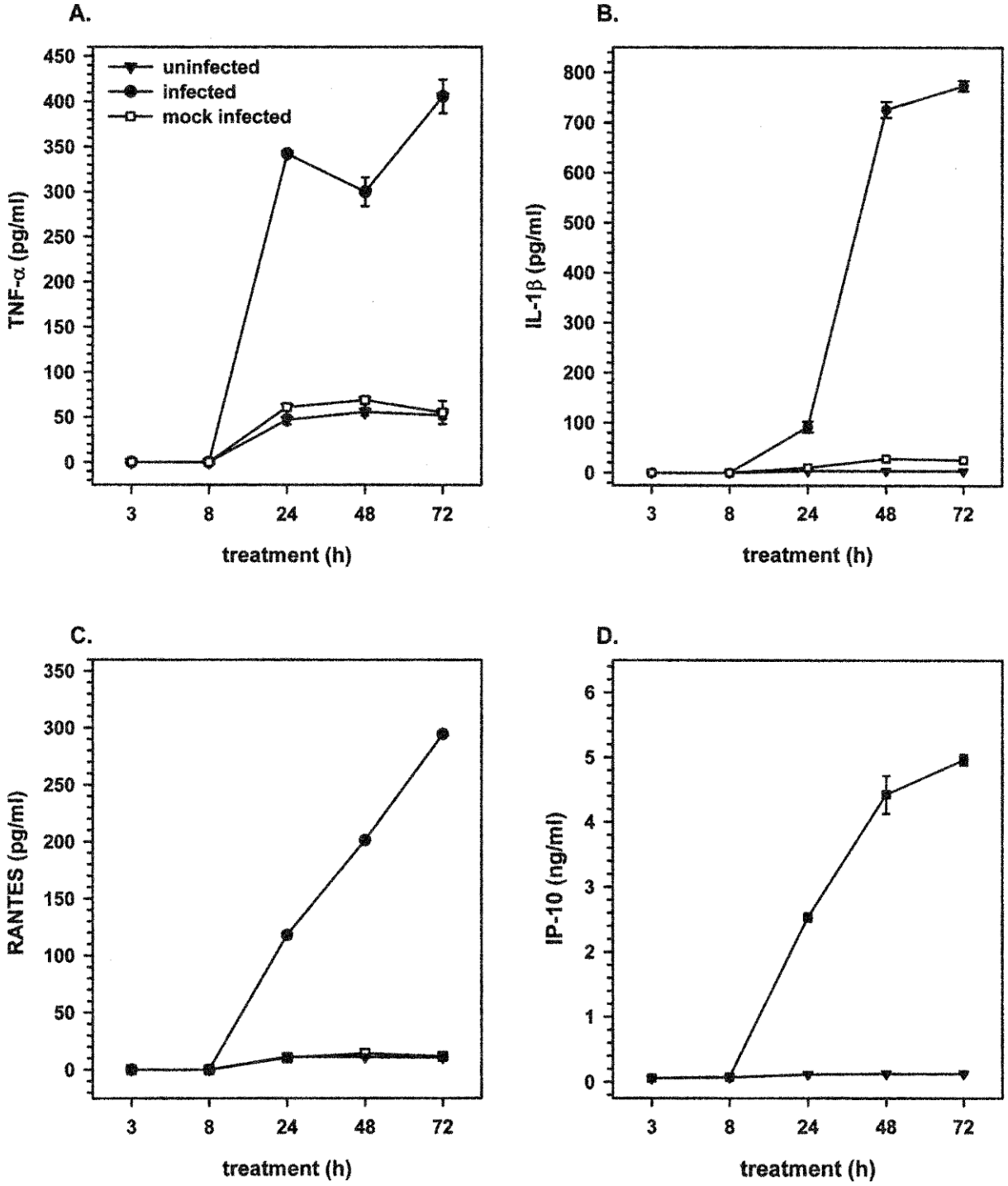

Figure 3 Kinetics of cytokine and chemokine production by human microglial cells in response to HSV infection. Cell-free supernatants were collected at 3, 8, 24, 48, and 72 h p.i. and assayed for (**A**) TNF-α, (**B**) IL-1β, (**C**) RANTES, and (**D**) IP-10. Representative data from at least four independent experiments, using microglial cells from different brain specimens, are expressed as the mean ± SEM of triplicate samples for each time point tested.

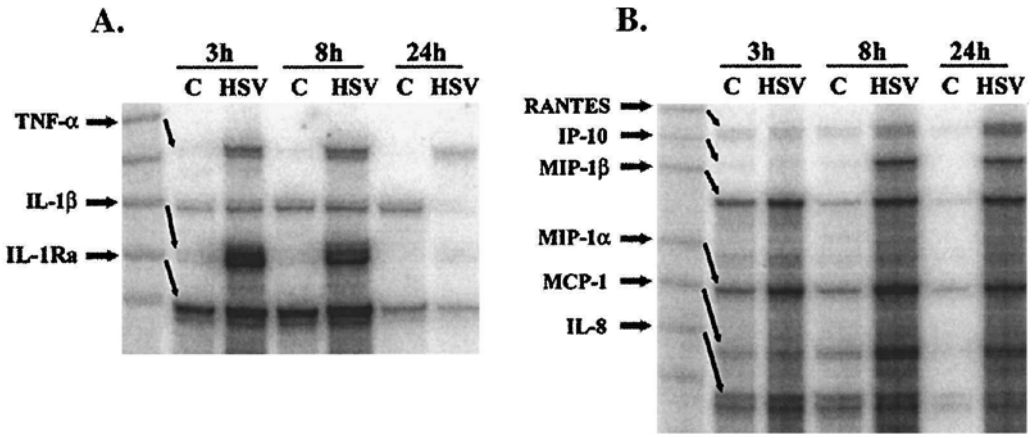

Figure 4 (**A**) Cytokine and (**B**) chemokine mRNA expression in HSV-infected human microglial cell cultures. Total RNA was extracted from control (**C**) and infected (HSV) cultures at 3, 8, and 24 h postinfection. Then, 4 μg of total RNA was used in the multiprobe RNAse Protection Assay (RPA) according to the manufacturer's instruction (Pharmingen, San Diego, CA).

of neurons appeared to be delayed as compared with microglial cells (Figure 8). Interestingly, apoptosis was not induced following HSV infection of primary astrocytes (Figure 8).

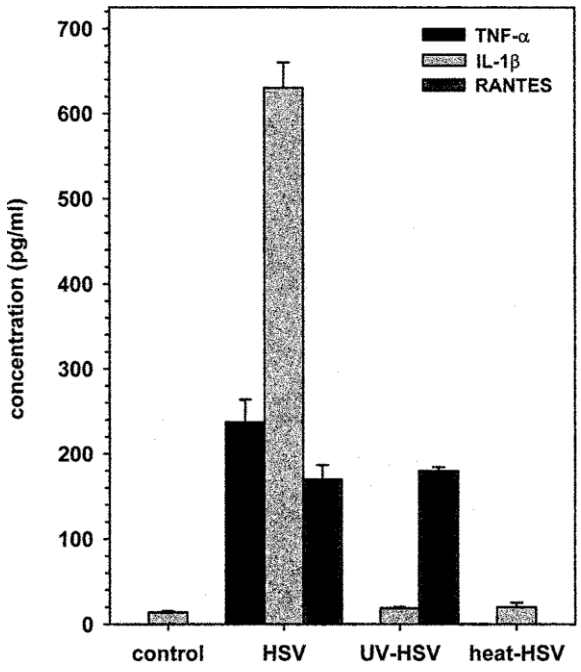

Figure 5 Effect of HSV inactivation on cytokine and chemokine production. HSV was UV-inactivated (254 nm, 30 min) or heat-inactivated (56°C, 45 min). Microglial cell cultures were infected with inactivated HSV at an amount equivalent to MOI = 1. Cell-free supernatants were collected at 24 h p.i. and tested for TNF-α. Supernatants collected at 48 h p.i. were tested for IL-1β and RANTES production. Data are expressed as the mean ± SEM of triplicate samples and are representative of at least three independent experiments.

Discussion

The results of this study clearly demonstrate that human microglial cells respond to HSV infection by producing select proinflammatory cytokines and chemokines. A vigorous host immune response is critical for inhibiting primary viral replication and viral-mediated neuronal damage, but overly aggressive brain defense mechanisms may have deleterious, long-term consequences. The role of immune-mediated pathology in brain damage during and after HSE still needs to be determined. During both HSE in humans and in experimental models, viral load in the cerebrospinal fluid or brain tissue does not correlate absolutely with the severity of structural damage or clinical findings (Wildemann *et al*, 1997). The absence of a clear correlation between viral load and morbidity suggests a role for indirect mechanisms of brain injury during HSV infection, perhaps mediated by microglial cell-produced cytokines and chemokines.

Within HSV-infected microglial cells, high levels of both ICP4 and reporter gene expression were observed. A subset of microglial cells (<10%) displayed late viral antigen (nucleocapsid) expression. Similar findings have been reported using blood-derived mononuclear phagocytes. In these cells, permissiveness to HSV replication was found to be dependent on the stage of blood monocyte differentiation (Bruun *et al*, 1998). It is likely that comparable differentiation stage-dependent viral replication occurs in microglial cells.

Results generated during these studies indicated that TNF-α and IL-1β were the predominant cytokines found to be produced by human microglial cells in response to nonpermissive infection by HSV. There are several published studies that investigated cytokine production in the trigeminal ganglia (Walev *et al*, 1995; Halford *et al*, 1996; Shimeld *et al*, 1997;

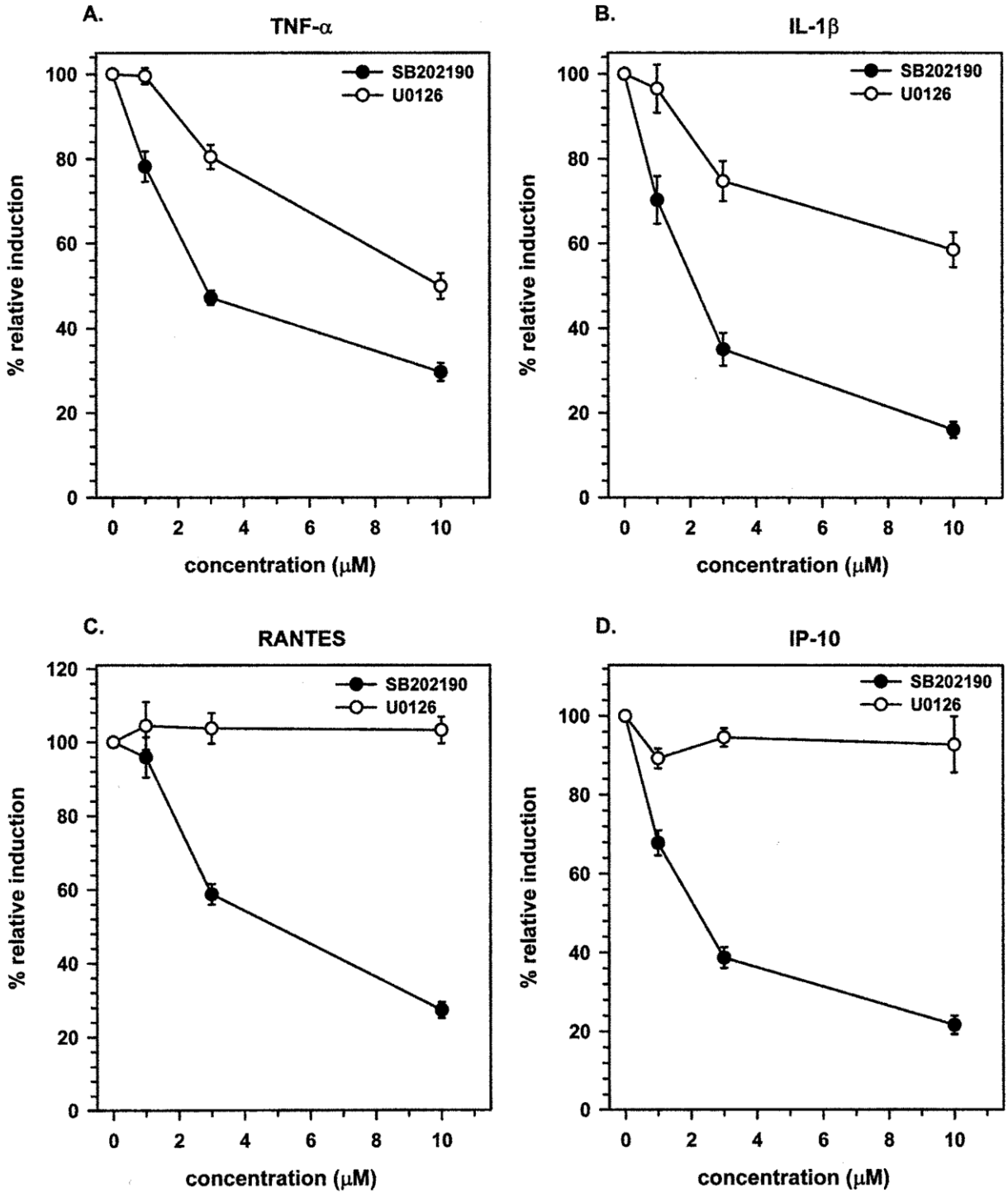

Figure 6 Effect of signaling pathway inhibitor treatment on HSV-induced cytokine and chemokine production by human microglial cells. Microglia were pretreated with the p38 MAPK inhibitor SB202190 or the MEK1/2 inhibitor UO126 (Calbiochem, San Diego, CA) for 30 min at the indicated concentrations (1.0 to 10 μM) prior to stimulation of the cells with HSV (MOI = 1). Cell-free supernatants were collected at 24 (TNF-α) or 48 h p.i. and assayed for (**A**) TNF-α, (**B**) IL-1β, (**C**) RANTES, and (**D**) IP-10 production by ELISA. Data are presented as relative induction of each cytokine from treated microglial cells in comparison to the cytokine levels observed in HSV-stimulated cells without signaling pathway inhibitors. Bars represent the mean ± SEM of triplicate samples, which are representative of three independent experiments.

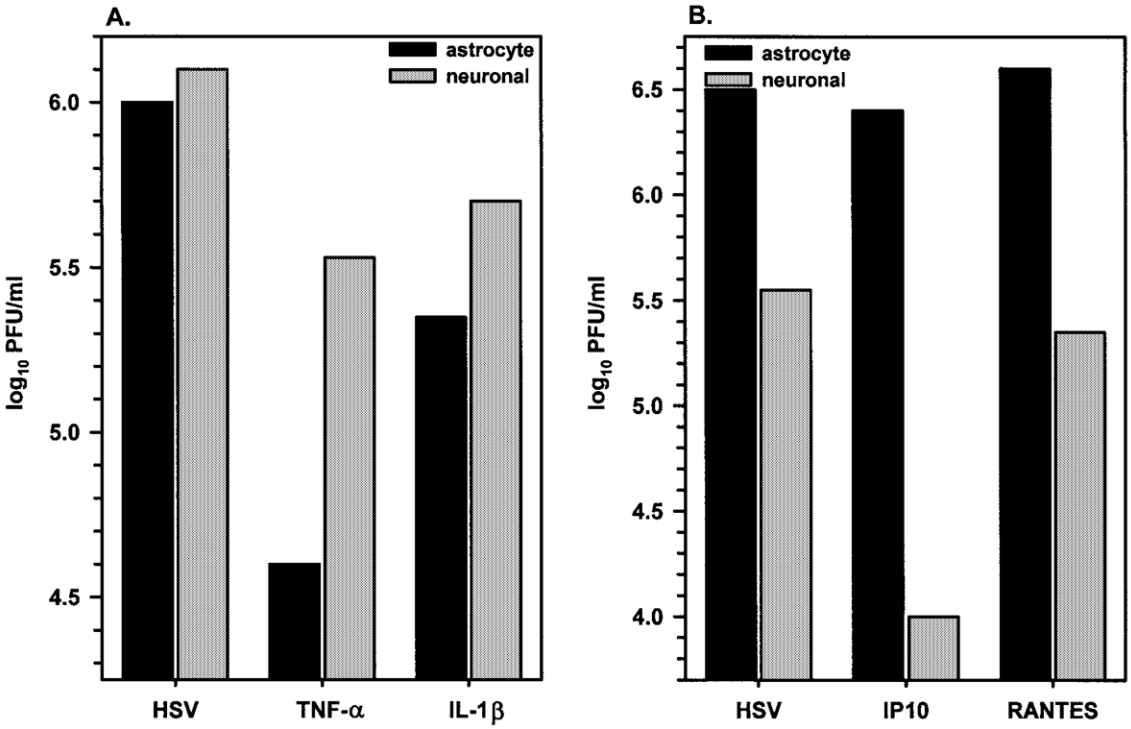

Figure 7 Effect of exogenous (**A**) cytokine (TNF-α, IL-1β) or (**B**) chemokine (RANTES, IP-10) treatment on HSV replication in permissive human brain cells. Highly enriched (>99% GFAP$^+$) primary astrocytes (2×10^5 cells/well) and neurons (>90% NSE$^+$, 5×10^5 cells/well) were treated with TNF-α (20 ng/ml), IL-1β (10 ng/ml), RANTES (100 ng/ml), or IP-10 (100 ng/ml) for 72 h prior to infection with HSV (17 syn$^+$). Then, 72 h post infection, the brain cell cultures were subjected to three cycles of freeze–thaw lysis, and lysate dilutions were plated onto rabbit skin fibroblasts for determination of viral titer by plaque assay. Data are expressed as mean titers of duplicate samples and are representative of three independent experiments with each cytokine or chemokine using brain cells isolated from different brain specimens.

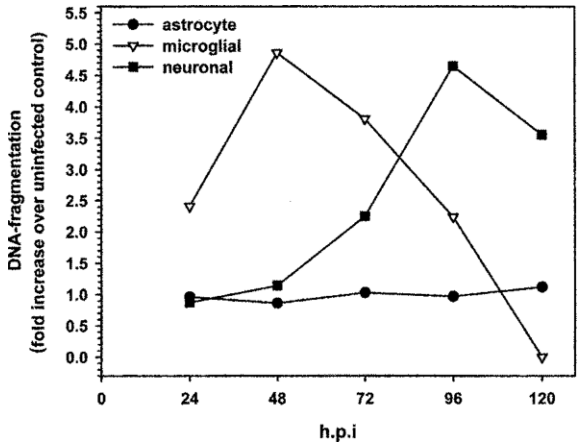

Figure 8 HSV infection induces apoptotic cell death in microglia. Primary human brain cells were examined for apoptotic DNA fragmentation using an oligonucleosomal ELISA (Boehringer Mannheim) at 24, 48, 72, 96, and 120 h post-HSV infection. Data are presented as fold increase in DNA fragmentation in infected cells over uninfected control cells at the same time points and are representative of four experiments done on tissues from two different donors.

Carr *et al*, 1998; Kodukula *et al*,1999) or in cultured keratinocytes (Mikloska *et al*, 1998) following infection with HSV. However, the immune response in the brain may differ from that of the peripheral nervous system, or other peripheral tissues, because of the blood–brain barrier and the presence of different types of glial cells in the CNS. In the CNS, astrocytes at the dorsal root entry zone have been reported to produce TNF-α and IL-6 during clearance of HSV and up to day 30 p.i., the latest time point tested (Shimeld *et al*, 1997). This result suggests that glial cell-produced TNF-α plays a role throughout the acute and latent infection.

There is no consensus on which cytokines are of primary importance during clearance of the virus. The antiviral activity of TNF-α has previously been reported using a number of systems (Mestan *et al*, 1986; Wong and Goeddel, 1986; Schijns *et al*, 1991; Pavic *et al*, 1993) including HSV (Feduchi *et al*, 1989; Chen *et al*, 1993). However, the antiviral effects of particular cytokines depend on receptors present on specific infected cell types, hence the importance of using primary cells. Recent experiments performed in our laboratory have demonstrated that

TNF-α and IL-1β profoundly suppress the replication of human cytomegalovirus in human astrocytes (Cheeran *et al*, 2000). In the present study, we demonstrated that TNF-α suppresses HSV replication in astrocytes but not in neurons. In contrast to our findings using cytomegalovirus, IL-1β did not suppress HSV replication in astrocytes.

The role of chemokines in CNS diseases is not completely understood; however, increasing evidence suggests that chemokines, like cytokines, possess a number of properties that extend well beyond leukocyte recruitment (Asensio and Campbell, 1999). Inflammatory infiltrates within the CNS observed during viral encephalitis are presumably a consequence of chemokine expression by infected cells (Glabinski and Ransohoff, 1999). Control of HSV in the nervous system by both $CD4^+$ and $CD8^+$ lymphocytes has been demonstrated (Simmons and Tscharke, 1992; Manickan and Rouse, 1995). Lymphocyte infiltration into the cornea following HSV infection is facilitated by chemokines (Su *et al*, 1996). The presence of IP-10 and RANTES, chemokines that attract T-cells (Farber, 1997), in microglial cell supernatants following infection with HSV suggests that they may have a role in the recruitment of T-lymphocytes into the CNS. It has recently been demonstrated that IP-10 exerts direct antiviral activity *in vivo* (Mahalingam *et al*, 1999). Data obtained during the present experiments clearly demonstrate that exogenously added IP-10 potently inhibits HSV replication in primary neurons (with a 32-fold reduction in viral titer), but similar IP-10 treatment does not affect viral replication in astrocytes. Treatment of either neurons or astrocytes with exogenously added RANTES does not result in significant decreases in viral titers. Thus, the results of these new experiments clearly show that IP-10, but not RANTES, possesses direct antiviral activity in human neurons.

Results generated from this study show that production of TNF-α and IL-1β requires replication competent virus. This finding suggests that binding of the virus to the microglial cell membrane is an inadequate stimulus for the production of these cytokines. In contrast, UV-inactivated virus induces microglial cell production of RANTES. Binding of the virus to microglial cells, without *de novo* protein synthesis, appears to be sufficient for the induction of chemokines. The p38 MAP kinase pathway plays an important role in the regulation of expression of immune mediators in microglia stimulated by inflammatory factors (Lee *et al*, 2000). Results of the present study suggest that the p38 MAP kinase pathway, but not the MEK1/2 pathway, plays a role in HSV-induced chemokine production in human microglia. A similar signal transduction pathway leading to induced IP-10 mRNA production by astrocytes stimulated by HIV-1 Tat has recently been reported (Kutsch *et al*, 2000).

These *in vitro* findings suggest that IP-10 possesses direct antiviral activity in neurons and support a role for human microglial cells in both antiviral defense of the brain as well as amplification of immune responses during neuroinflammation. Implications regarding cytokine production by microglia, chemokine-mediated recruitment and retention of cells of the immune system, and brain injury secondary to viral clearance should be tested in animal models. Understanding the defense mechanisms associated with clearance of HSV from the brain may bring about adjunctive treatments designed to alleviate immune-mediated neuropathological sequelae using immunomodulatory agents.

Materials and methods

Brain cell culture

Purified human fetal microglial, astrocyte, and neuronal cell cultures were prepared as described previously with minor modifications (Chao *et al*, 1994; Chao and Hu, 1994; Chao *et al*, 1996). Fetal brain tissues were obtained from human abortuses at 16–22 weeks of gestation, under a protocol approved by our Institutional Human Research Subjects Committee. The brain tissue was cleared of meninges and dissociated by passing repeatedly through a pipette under sterile conditions. The triturated tissue was incubated with 0.125% trypsin for 45 min at 37°C to make a single cell suspension. The cell suspension was seeded at 75–100 $\times$ 10^6 cells in 75-cm^2 tissue culture flasks and incubated in a humidified incubator at 37°C. Cultures were grown for 2 weeks with weekly changes of medium. After 2 weeks in culture, microglial cells floating in the medium and those loosely attached to the monolayer were harvested by gentle shaking. Microglial cell cultures used in these experiments were >99% pure, as determined by CD68 antibody staining.

To prepare purified astrocyte cultures, the flasks were shaken after 21 days in culture at 180–200 rpm for 16–18 h. The cultures were then washed with Hank's buffer to remove any floating, nonastroglial cells. The adherent monolayer was then trypsinized with 0.125% trypsin for 20 min at 37°C, washed, and seeded into fresh flasks. Medium was changed 24 h after plating. The procedure was repeated three to four times at weekly intervals. The final cultures, which contained >99% astrocytes (GFAP positive cells), were plated at a density of 2×10^5 cells per well in a 24-well plate. To prepare neuronal cultures, dispersed cortical cells were plated for 24 h, extensively washed, and cultured in DMEM containing 10% FBS. On day 5, the culture medium was replaced with DMEM containing 10% fetal bovine serum, uridine (33.6 mg/ml, Sigma Chemical Co, St. Louis, MO) and fluorodeoxyuridine (13.6 mg/ml, Sigma Chemical Co) to prevent overgrowth of astroglial

cells. On day 12, the neuronal cell cultures contained differentiated neurons. These highly enriched neuronal cell cultures consisted of approximately 90% neurons, 5% astrocytes, and <5% microglia.

Viruses
HSV strains 17 syn$^+$ and 17$^+$ LAT-MT1, a Lac-Z containing recombinant HSV strain (Lokensgard *et al*, 1994) were kindly provided by LT Feldman (UCLA). Rabbit skin fibroblasts (RS) were used to propagate viral stocks. Infected RS cultures were harvested at 80–100% cytopathic effect and subjected to three freeze–thaw cycles. Cellular debris was removed by centrifugation (1000 $\times$ g) at 4°C and the virus was pelleted through a 35% sucrose cushion (in Tris-buffered saline; 50 mM Tris-HCl and 150 mM NaCl; pH 7.4) at 23000 $\times$ g for 2 h at 4°C. Viral stocks were titered by plaque assay on RS cells. Mock-infected RS cultures were processed in exactly the same manner as virus stocks. Glial cell cultures treated with mock-infected culture preparations were used to evaluate nonspecific cell stimulation. UV-inactivated virus was prepared by placing 3 ml of purified virus in a 50-mm tissue culture dish at a distance of 8 cm from a 256 nm UV light source, on ice, for 30 min. HSV was heat-inactivated at 56°C for 45 min. The titers of the UV- and heat-inactivated virus were 5 $\log_{10}$ PFU lower than the replication competent virus.

Western blot analysis
Preparation of cell lysates, electrophoresis, and protein transfer was performed using standard procedures. Briefly, 0.5 $\times$ 10^6 HSV-infected microglial cells were rinsed in PBS and extracted with protein lysis buffer (Pierce, Rockford, IL). After protein concentration was determined (BioRad, Hercules, CA), 15 μg of protein was boiled in sample buffer and loaded on a 5% SDS-Page gel system (BioRad, Hercules, CA). The gels were transferred on to nitrocellulose membrane (Micron Separations Inc, Westborough, MA) and probed using the appropriate antibodies. For analysis of IE protein expression in infected microglia, monoclonal antibodies to ICP4 (Advanced Biotechnologies, Columbia, MD) and the nucleocapsid protein (Biodesign International, Saco, ME) were used. The corresponding proteins were detected using a chemiluminescence detection kit (ECL) according to the manufacturer's instructions (Amersham Pharmacia Biotech, NJ).

Cytokine and chemokine ELISAs
A sandwich ELISA-based system previously described (Peterson *et al*, 1997b) was used to quantify cytokine and chemokine levels from glial cell culture supernatants. ELISA plates (96-well) were coated with a mouse–anti-human cytokine or chemokine capture antibody (R&D systems, Minneapolis, MN) at 1–2 μg/ml overnight at 4°C. The plates were washed (0.05% Tween-20 in phosphate-buffered saline, PBS) and blocked with 1% BSA in PBS for 1 h at 37°C. Serial dilutions of known concentrations of the respective cytokine or chemokine were used in each assay to generate a standard concentration curve. HSV-infected, uninfected or mock-treated culture supernatants were incubated in capture antibody-coated wells for 2 h at 37°C. Detection antibodies (goat–anti-human cytokine or chemokine antibodies, 1–2 μg/ml; R&D systems, Minneapolis, MN) were added for 90 min at 37°C followed by donkey–anti-goat lgG horseradish–peroxidase conjugate (1:10 000; Jackson Immunoresearch, West Grove, PA) for 45 min. A chromogenic substrate (K-blue; Neogen Corporation, Lexington, KY) was then added for 10–20 min at room temperature. Color development was stopped with 1M H_2SO_4. Absorbance values at 450 nm were used to quantify the levels of cytokines and chemokines in the culture supernatants from the standard concentration curve.

RNase protection assay
For cytokine and chemokine mRNA expression analysis, 4 μg of total RNA was used in the multiprobe RNAse Protection Assay (RPA) according to the manufacturer's instructions (Pharmingen, San Diego, CA). Total RNA was extracted from control and infected (MOI = 1) cultures at 3, 8, 24 h postinfection.

Oligonucleosomal ELISA
DNA fragmentation subunits generated during apoptosis in response to HSV infection were detected using a sandwich-enzyme-immunoassay system (Boehringer Mannheim, Indianapolis, IN). The assay was performed at different time points postinfection (p.i.) according to the directions from the manufacturer. In short, cell lysates from infected and mock-infected brain cultures at each time point were placed in a streptavidin-coated microtiter plate along with a mixture of anti-histone biotinylated antibody and anti-DNA peroxidase antibody. This mixture was incubated for 2 h, during which the anti-histone antibody binds the histone component of the oligonucleosomal unit and fixes it to the plate, by the streptavidin–biotin bond. The anti-DNA antibody binds the DNA strands associated with the oligomer and were detected by a chromogenic enzyme-substrate reaction.

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