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Cytomegalovirus induces cytokine and chemokine production differentially in microglia and astrocytes: Antiviral implications

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Glial cells function as sensors for infection within the brain and produce cytokines to limit viral replication and spread. We examined both cytokine (TNF-α, IL-1β, and IL-6) and chemokine (MCP-1, MIP-1α, RANTES, and IL-8) production by primary human glial cells in response to cytomegalovirus (CMV). Although CMV-infected astrocytes did not produce antiviral cytokines, they generated significant quantities of the chemokines MCP-1 and IL-8 in response to viral infection. On the other hand, supernatants from CMV-stimulated purified microglial cell cultures showed a marked increase in the production of TNF-α and IL-6, as well as chemokines. Supernatants from CMV-infected astrocyte cultures induced the migration of microglia towards chemotactic signals generated from infected astrocytes. Antibodies to MCP-1, but not to MIP-1α, RANTES, or IL-8, inhibited this migratory activity. These findings suggest that infected astrocytes may use MCP-1 to recruit antiviral cytokine-producing microglial cells to foci of infection. To test this hypothesis, cocultures of astrocytes and microglial cells were infected with CMV. Viral gene expression in these cocultures was 60% lower than in CMV infected purified astrocyte cultures lacking microglia. These results support the hypothesis that microglia play an important antiviral role in defense of the brain against CMV. The host defense function of microglial cells may be directed in part by chemokines, such as MCP-1, produced by infected astrocytes. *Journal of NeuroVirology* (2001) **7,** 135–147.

Keywords: chemokines; cytokines; cytomegalovirus; glia

Introduction

Human cytomegalovirus (CMV) encephalitis occurs primarily during fetal development and in the advanced stages of AIDS (Arribas *et al*, 1996). There is a wide range of clinical complications associated with CMV encephalitis, ranging from long-term cognitive defects, particularly in children, to death ensuing from unimpeded central nervous system (CNS) infection in AIDS patients (Cinque *et al*, 1997). Compared to AIDS patients, CMV encephalitis is relatively rare in other groups of immunocompromised patients (e.g., organ and bone marrow transplant recipients), although they are prone to development of other forms of CMV disease (Arribas *et al*, 1996). Hence, it seems likely that in addition to lymphocyte-mediated immunity, an intrinsic system within the brain parenchyma contributes to the defense of this organ system against CMV.

Due to strict viral host specificity, *in vivo* studies of human CMV have obvious limitations. The current state of knowledge of CMV pathogenesis and host defense against this virus has come largely from clinical observations, from studies using animal models, and from *in vitro* studies with cell lines or primary human cells (Sinzger and Jahn, 1996). *In vitro* studies from our laboratory (Lokensgard *et al*, 1999) and

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others (Ho *et al*, 1991; McCarthy *et al*, 1995; Poland *et al*, 1990), using primary human fetal brain cells have shown that CMV productively infects astrocytes, resulting in cell death. Little is known, however, about the mechanisms of host defense in the CNS against CMV.

In addition to neurons, the brain is constituted predominantly by glial cells that play a vital role in the preservation of CNS function. Astrocytes (which outnumber neurons about seven to one) and microglia (estimated to be found in equal numbers to neurons) form an intrinsic immune system within the brain (Kreutzberg, 1996; Xiao and Link, 1999). Together with immunocytes that migrate into the infected brain (Rowell and Griffin, 1999), these 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). Host defense mechanisms against pathogens in the CNS must not only control the spread of invading microbes but at the same time must minimize undesirable cytotoxic activity that may damage the brain. 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 without evidence of cytotoxicity (Griffin, 1997). Recently, we have found that treatment of astrocytes with proinflammatory cytokines (TNF-α, IL-1β, and IFN-γ), but not with anti-inflammatory cytokines (IL-4 and IL-10), markedly suppresses CMV replication (Cheeran *et al*, 2000). The expression of cytokines in the CNS appears to be restricted to foci of infection where they may facilitate local protection (Rowell and Griffin, 1999).

Leukocyte trafficking into infected tissues is the hallmark of an early immune response to viral infection. 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 immunocytes into and within the brain. CMV has been shown to induce chemokines in many cell types, including fibroblasts and endothelial cells (Grundy *et al*, 1998; Billstrom *et al*, 1999; Hirsch and Shenk, 1999). Microglia and astrocytes are major sources of chemokines in the CNS (Oh *et al*, 1999). Little is known, however, about cytokine and chemokine production by glial cells in response to CMV or about the role chemokines play in defense of the CNS. Thus, in this study we analyzed cytokine and chemokine production by primary astrocyte and microglial cells in response to CMV. We also used *in vitro* models to examine the biological function of immune mediators in defense of the brain against CMV.

Results

Cytokine and chemokine production by CMV-infected astrocytes

Recent experiments in our laboratory have demonstrated that proinflammatory cytokines suppress CMV replication in astrocytes (Cheeran *et al*, 2000). To evaluate whether these antiviral cytokines are produced by astrocytes in response to CMV infection, we screened astrocyte culture supernatants for the proinflammatory cytokines TNF-α, IL-1β, and IL-6. At all time points tested, TNF-α and IL-1β were undetectable (<10–20 pg/ml) in CMV-infected astrocyte culture supernatants (Figure 1A,B). To assess mRNA expression of these cytokines, total RNA was extracted from both uninfected and infected astrocytes at 3, 8, and 24 h p.i. and analyzed by RT-PCR. In both infected and uninfected astrocytes, no specific RNA message for IL-1β or TNF-α was detected (data not shown). When IL-6 was evaluated, small amounts of this cytokine were detected in supernatants from CMV-infected astrocytes (Figure 1C). Quantities of IL-6 measured at 24, 48, and 72 h p.i. were 0 pg/ml, 13 $\pm$ 1 pg/ml, and 30 $\pm$ 0 pg/ml, respectively, and mRNA for IL-6 was detected at 8 h and 24 h p.i. (Figure 1D).

Although CMV-infected astrocytes produced little or no proinflammatory cytokines, when culture supernatants were assayed for the presence of chemokines, substantial amounts of MCP-1 (Figure 2A), IL-8 (Figure 2B), and MIP-1α (Figure 2D) but not RANTES (Figure 2C) were found. MCP-1 and IL-8 protein levels in CMV-infected astrocyte culture supernatants peaked at 48 h p.i. reaching 41.36 $\pm$ 3.5 and 7.225 $\pm$ 0.015 ng/ml, respectively (Figure 2A,B). The mean levels of MCP-1 and IL-8, obtained from multiple experiments using cells from at least 3 different donors, were significantly higher in infected cultures at 24 and 48 h p.i. ($P = 0.0003$ and 0.014 for MCP-1 and IL-8, respectively) compared to uninfected astrocytes. MIP-1α levels in infected culture supernatants were relatively low, measuring 0.173 $\pm$ 0.006 ng/ml 48 h p.i. But, MIP-1α production was significantly greater ($P = 0.0003$, based on pooled data at 48 h p.i.) in infected cultures than from mock-infected or uninfected astrocyte culture supernatants (Figure 2D). RANTES levels were also low and not significantly different from control or mock-infected culture supernatants throughout the assay period (Figure 2C).

RT-PCR data obtained from infected astrocytes indicated that mRNA for IL-8, RANTES, and MIP-1α were induced in response to CMV infection (Figure 2B–D). Since MCP-1 was expressed constitutively in cultured primary astrocytes (Figure 2A), mRNA expression during CMV infection was examined by

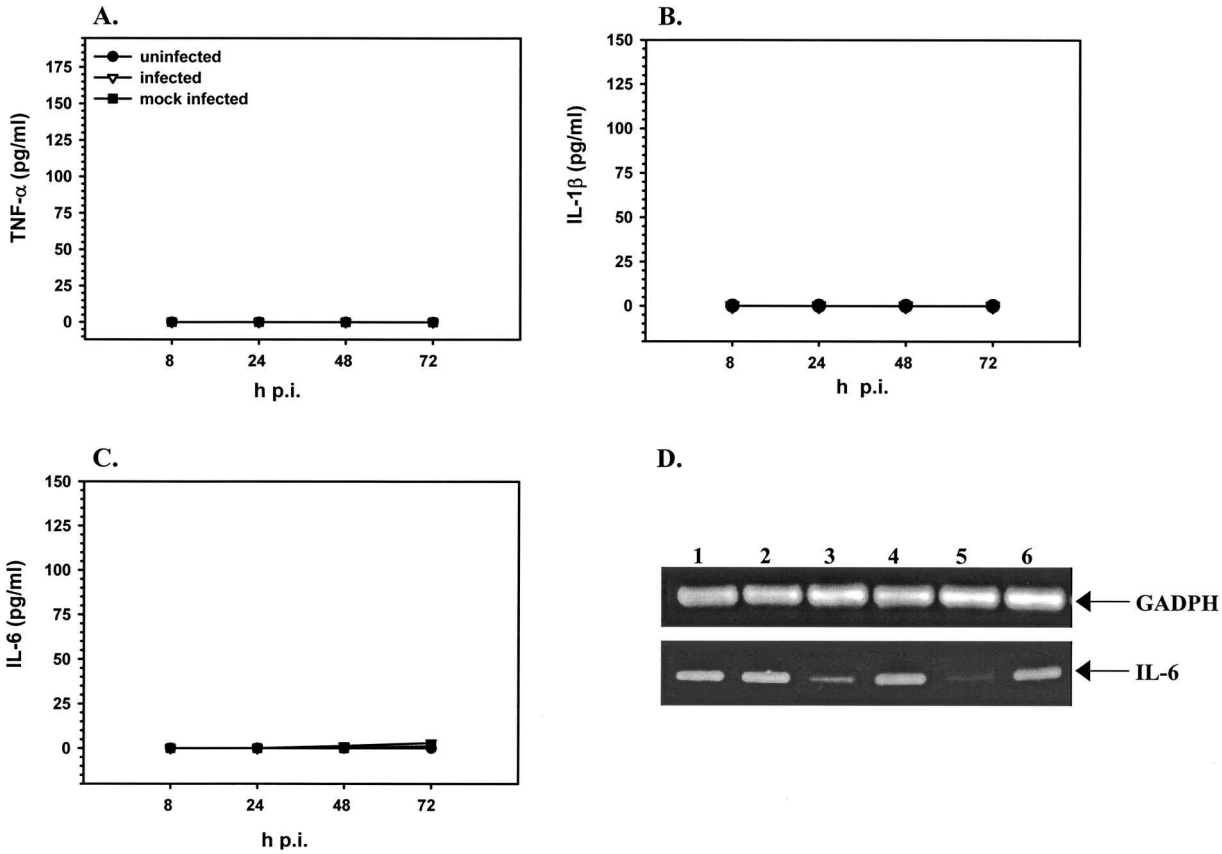

Figure 1 Antiviral cytokines are not induced during CMV infection of astrocytes. Astrocyte cultures (2×10^5 cells) were infected with CMV AD169 for 8, 24, 48, and 72 h. Cell-free supernatants were collected at these times and assayed for (**A**) TNF-α, (**B**) IL-1β, and (**C**) IL-6. Representative data from at least three independent experiments, using astrocytes from different brain specimens, are expressed as the mean ± SEM of triplicate samples for each time point tested. (**D**) RT-PCR was performed using total RNA (1.5 μg). IL-6 and GADPH specific amplification products obtained from infected and uninfected astrocyte cultures are shown; Lanes 2, 4, and 6 represent PCR products from infected astrocytes at 3 h, 8 h, and 24 h p.i., respectively; Lanes 1, 3, and 5 are from uninfected astrocytes at the same time points. TNF-α and IL-1β specific primers did not amplify any target cDNA at optimal conditions ($n = 3$, data not shown).

Northern blotting and analyzed by densitometry. MCP-1 mRNA was induced as early as 3 h p.i., demonstrating a 3–5-fold increase (normalized to GADPH) over uninfected controls by 24 h p.i. (Figure 2E).

Cytokine and chemokine production by CMV-infected microglial cells

Next, we tested the effect of CMV stimulation on production of cytokines by microglial cells. Microglial cell cultures were stimulated with CMV in the same manner as noted before with astrocyte cultures. Microglial cell culture supernatants were tested for TNF-α, IL-1β, and IL-6. In contrast to findings with astrocytes, CMV-stimulated microglial cells produced TNF-α (Figure 3A) and substantial amounts of IL-6 (Figure 3B). IL-1β, however, was not induced by CMV stimulation of microglia (data not shown). TNF-α levels peaked at 24 h between 160 ± 12.6 pg/ml and 174 ± 13.9 pg/ml in experiments using microglia from three brain specimens. The mean level of TNF-α detected in CMV-infected microglial cultures was significantly higher ($P = 0.00003$, based on pooled data at 24 h p.i.) compared to levels detected in mock-treated (30 pg/ml) or unstimulated microglia culture supernatants (10 pg/ml; Figure 3A). IL-6 levels peaked at 48 h p.i. and measured up to 760 ± 10 pg/ml in cultures derived from different brain specimens (Figure 3B). Mock-treated and uninfected cultures had significantly lower ($P = 0.01$, based on pooled data at 48 h p.i.) amounts of IL-6 (33 ± 3 and 37 ± 7.5 pg/ml, respectively).

Microglial cells also produced chemokines in response to CMV. Higher levels of MCP-1 and IL-8 were detected at 72 h p.i. (Figure 4A,B), while RANTES and MIP-1α levels peaked at 48 h p.i. (Figure 4C,D). Although constitutive synthesis of MCP-1, IL-8, and MIP-1α was detected in uninfected (7.4 ± 0.7, 64 ± 3.4, and 0.3 ± 0.09 ng/ml, respectively) and mock-treated (15.82 ± 0.3, 79.14 ± 10.4, and 0.53 ± 0.08 ng/ml) microglial culture supernatants, chemokine production in CMV-stimulated cultures was significantly higher (MCP-1: 34.28 ± 3.3 ng/ml; IL-8: 105.5 ± 3.1 ng/ml; and MIP-1α: 2.9 ±

0.29 ng/ml) at periods of peak production (Figure 4), with P values ranging between 0.004 to 0.04 for each chemokine based on data obtained using triplicate cultures derived from at least two different donors. Production of RANTES by CMV-stimulated microglial cells (1400 ± 100 pg/ml) was significantly greater ($P = 0.006$ based on pooled data at 48 h p.i.) than unstimulated (8 ± 0.5 pg/ml) or mock-treated (21 ± 6 pg/ml) cells (Figure 4C).

Viral replication is not required for induction of cytokines or chemokines

To investigate whether replication competent CMV was required to induce cytokines or chemokines in glial cells, we stimulated microglial cells and astrocytes with UV-inactivated virus and culture supernatants were assayed for TNF-α or MCP-1. Microglial cells responded to inactivated virus by producing similar quantities of TNF-α (190 ± 19 pg/ml) as those stimulated with replication competent CMV (160 ± 12 pg/ml) 24 h p.i. (Figure 5A). Similarly, astrocytes responded to UV-inactivated virus by producing MCP-1 (31.57 ± 0.44 ng/ml) at concentrations comparable to those infected with replication competent virus (32.4 ± 5.02 ng/ml) 48 h p.i. (Figure 5B).

Chemotaxis of microglial cells towards supernatants from CMV-infected astrocytes

Because CMV-infected astrocytes produced several chemokines that hypothetically could serve as signals for the migration of microglia towards infected foci within the brain, we used a chemotaxis chamber to assess the chemotactic activity of microglial cells towards supernatants from

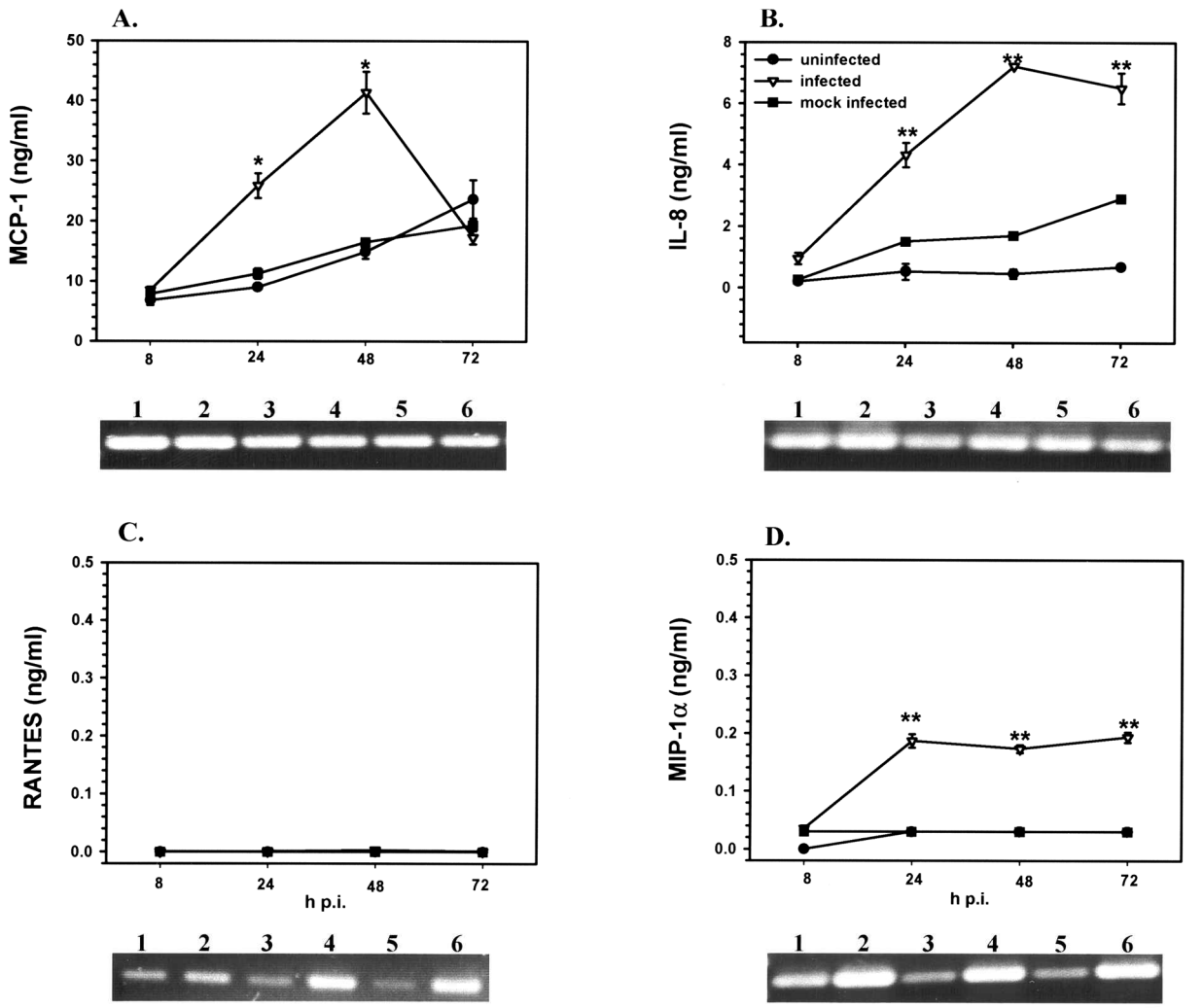

Figure 2 CMV infection induces chemokine production by astrocytes. Astrocyte cultures (2×10^5cells) were infected with CMV AD169 for 8, 24, 48, and 72 h. Cell free supernatants were collected at these time points and assayed for (**A**) MCP-1, (**B**) IL-8, (**C**) RANTES, and (**D**) MIP-1α. Representative data from three independent experiments for each chemokine are presented. Chemokine concentrations are expressed as a mean ± SEM of triplicate samples for each time point tested. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus uninfected astrocyte cultures. Insets: RT-PCR was performed using 1.5 μg of total RNA isolated from infected or control astrocytes. Chemokine specific amplification products are shown; Lanes 2, 4, and 6 represent PCR products from infected astrocytes at 3 h, 8 h, and 24 h p.i. respectively; Lanes 1, 3, and 5 represent uninfected astrocytes at the same time points. *(Continued)*

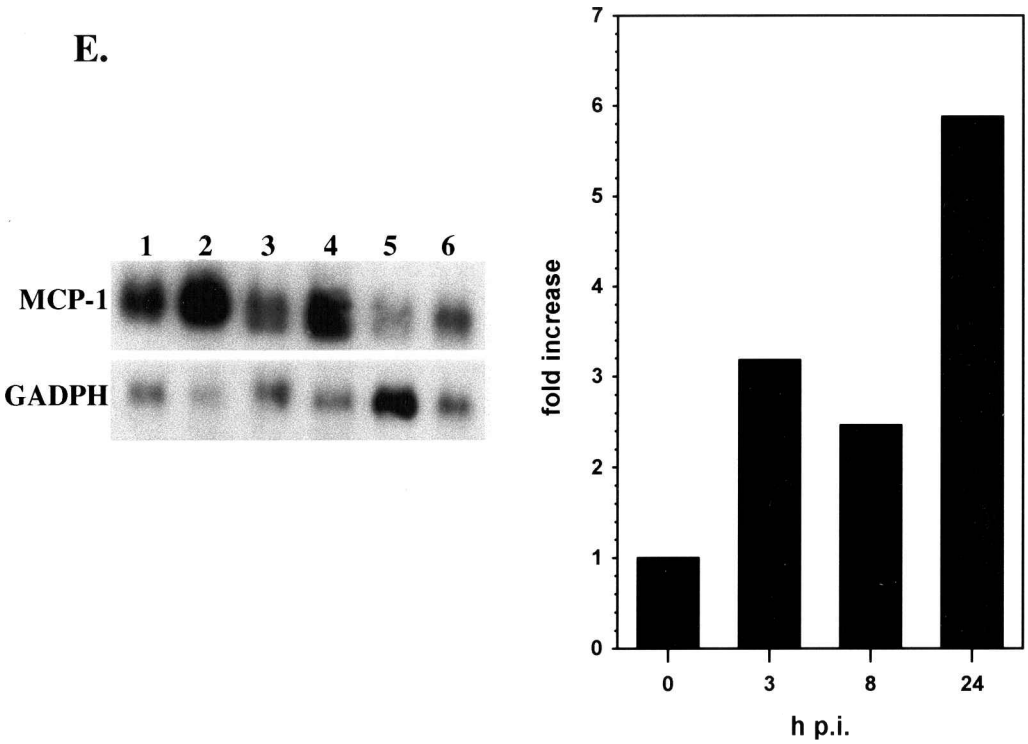

Figure 2 (Continued) (**E**) Northern blot analysis of MCP-1 expression. Lanes 2, 4, and 6 represent MCP-1 and GADPH specific mRNA from CMV-infected astrocytes at 3, 8, and 24 h p.i., respectively. Lanes 1, 3, and 5 correspond to uninfected astrocytes at the same time points. Densitometry and ImageQuant analysis of a blot representative of two separate experiments is shown.

CMV-infected astrocyte cultures. The number of microglia migrating towards supernatants from CMV-infected astrocytes (92.1 ± 4.9 cells/5HPF; $n = 8$) increased almost threefold over migration towards uninfected culture supernatants (33.2 ± 4.8 cells/5HPF; $n = 5$) and by greater than fourfold over random migration (18.4 ± 3.2 cells/5HPF; $n = 5$) (Figure 6A). Directed migration of microglia towards supernatants from infected astrocytes was similar to that observed with recombinant chemokines (MCP-1, 10 ng/ml, 98 ± 12.05 cells/5HPF; MIP-1α, 30 ng/ml, 84.8 ± 0.75 cells/5HPF; and RANTES, 30 ng/ml, 101 ± 8 cells/5HPF).

To investigate which chemokines released from CMV-infected astrocytes were responsible for microglial cell chemotaxis, supernatants from infected astrocytes were treated with antibodies (10 μg/ml) to MCP-1, MIP-1α, RANTES, or IL-8. Antibodies to MCP-1 decreased the number of microglial cells migrating towards CMV-infected culture supernatants. The number of microglial cells migrating towards anti-MCP-1 antibody-treated infected astrocyte supernatants (35.1 ± 2.3 cells/5HPF; $n = 7$) was comparable to the number migrating towards uninfected astrocyte culture supernatants (33.2 ± 4.8 cells/5HPF). While anti-MCP-1 antibodies reduced CMV-induced migration of microglial cells by 96.7%, the addition of antibodies to MIP-1α, RANTES, and IL-8 to astrocyte culture supernatants had no detectable effect (Figure 6B). These results suggest that, among those tested, MCP-1 is the main chemokine in CMV-infected astrocyte supernatants responsible for microglial cell chemotaxis.

Decreased viral gene expression in cocultures of microglial cells and astrocytes

Since microglia produce antiviral cytokines and migrate towards MCP-1 produced by CMV-infected astrocytes, we investigated whether microglia could suppress CMV expression in astrocytes. For this purpose, a coculture system of astrocytes and microglia was used. Twenty-four hours after being constituted, the cocultures were infected with CMV (RC256). At a coculture ratio of 2 astrocytes to 1 microglial cell, CMV gene expression was lower than that in cultures containing only astrocytes (Figure 7). At a MOI of 1, viral gene expression was suppressed by $60 \pm 10.1\%$ and at a MOI of 0.2 viral gene expression was reduced by $61 \pm 2.5\%$. Similar suppression was also seen when microglia were added to the astrocyte cultures 3 h p.i. ($54.3 \pm 8.9\%$; data not shown).

To determine if TNF-α, the antiviral cytokine which was present in CMV-stimulated microglial cell culture supernatants, was produced in astrocyte/microglial cell cocultures, supernatants from CMV infected cocultures were analyzed at 8, 24, 48, and 72 h p.i. TNF-α levels peaked at 24 h, measuring 36.67 ± 1.7 pg/ml in CMV-infected coculture supernatants. Mock-treated and unstimulated coculture supernatants contained little or

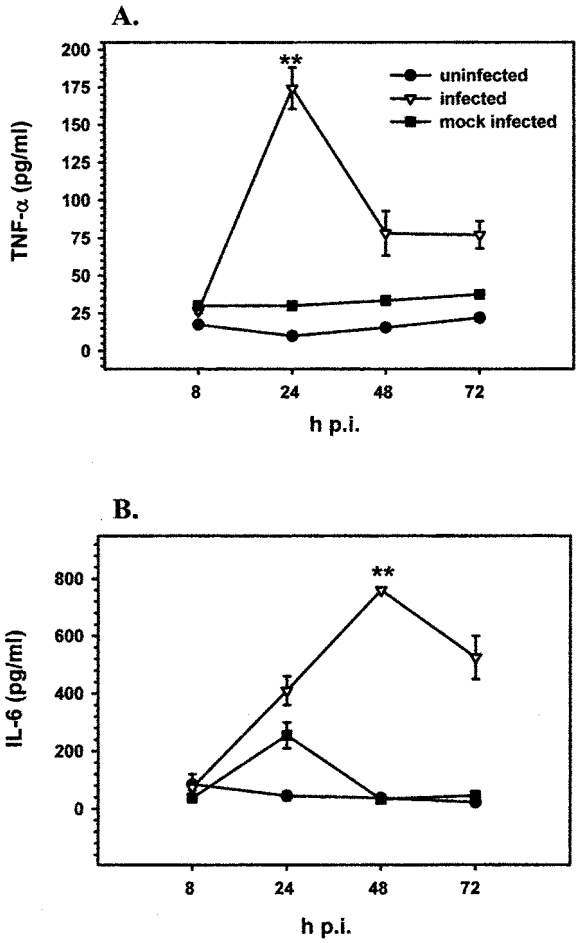

Figure 3 TNF-α and IL-6 are induced in CMV-stimulated microglia. Purified microglial cell cultures (2×10^5 cells) were infected with CMV AD169 for 8, 24, 48, and 72 hours. Cell-free supernatants were assayed for (**A**) TNF-α and (**B**) IL-6. Representative data from at least three independent experiments, using microglial cells from different brain specimens, are expressed as the mean ± SEM of triplicate samples for each time point tested. $^{**}P < 0.01$ versus uninfected microglial cell cultures.

no detectable TNF-α. In addition to TNFα, IL-6 (0.6 ± 0.3 ng/ml), MCP-1 (46.7 ± 2.5 ng/ml), MIP-1α ($0.196.6 \pm 0.008$ ng/ml), and IL-8 (104.8 ± 9.3 ng/ml) were detected in CMV-infected cocultures and their production peaked at 48 h p.i., while RANTES (0.315 ± 0.015 ng/ml) production peaked at 24 h p.i.

Discussion

The current state of knowledge regarding the pathogenesis of human CMV encephalitis is based largely upon clinical features and postmortem studies, which reflect the final stages of disease progression. Little is known about the role of glial cells in preventing intracerebral virus spread. Activated glial cells are known to produce a wide variety of cytokines and chemokines (Benveniste, 1997) and these soluble immune mediators have been proposed to play a role in the clearance of viral infection from the CNS (Schneider-Schaulies *et al*, 1997). Recent experiments performed in our laboratory have demonstrated that CMV productively infects human astrocytes (Lokensgard *et al*, 1999) and that selected proinflammatory cytokines (i.e. TNF-α, IL-1β, and IFN-γ) profoundly suppress CMV replication in this population of glial cells (Cheeran *et al*, 2000). In the present study, we found that astrocytes do not produce these antiviral cytokines in response to CMV infection. However, CMV-infected astrocytes do release substantial amounts of the chemokines MCP-1 and IL-8. Based on these results it seems reasonable to hypothesize that astrocytes, the major brain cell type supporting productive CMV replication (Lokensgard *et al*, 1999), may recruit other immune cells to the site of infection. In contrast to astrocytes, CMV-stimulated microglia were found to produce the antiviral cytokine TNF-α, in addition to IL-6, MCP-1, IL-8, RANTES, and MIP-1α. Taken together, these observations suggest that although astrocytes may not be capable of defending themselves against CMV infection, they have the capacity to produce chemokines that recruit antiviral immunocytes, such as microglia, to sites of infection.

Chemokines are pivotal in the inflammatory processes of the CNS. Inflammatory infiltrates within the CNS observed during viral encephalitis are presumably a consequence of chemokine expression by infected cells (Glabinski and Ransohoff, 1999). CMV-induced production of chemokines has been studied in various cell culture systems. Induction of MCP-1 (Hirsch and Shenk, 1999) and RANTES (Michelson *et al*, 1997) in fibroblasts, and IL-8 in endothelial cells (Almeida-Porada *et al*, 1997) and monocytes (Murayama *et al*, 1997) in response to CMV have been described. Cerebrospinal fluid of AIDS patients with CMV encephalitis shows elevated MCP-1 levels (Bernasconi *et al*, 1996), although the role of MCP-1 *in vivo* is speculative. From previous studies in our laboratory, it was found that chemokines have no effect on CMV replication in primary human astrocytes (Cheeran *et al*, 2000). We found in this study, however, that microglial cells migrate towards CMV-infected astrocyte culture supernatants. We also showed that microglial cell migration towards supernatants from infected astrocyte cultures is mediated by MCP-1. Such responses to chemotactic signals from CMV-infected cells are not restricted to microglia. CMV-infected human umbilical vein endothelial cells enhance movement of selective subsets of T lymphocytes across a porous membrane (Borthwick *et al*, 1997). Interestingly, the CMV genome encodes chemokine-receptor homologues that are potentially involved in immune evasion mechanisms (Bodaghi *et al*, 1998; Billstrom *et al*, 1999). Although the relevance of findings from *in vitro* studies has not been demonstrated *in vivo*, it has been postulated that CMV evolved these mechanisms to evade recruitment of immune

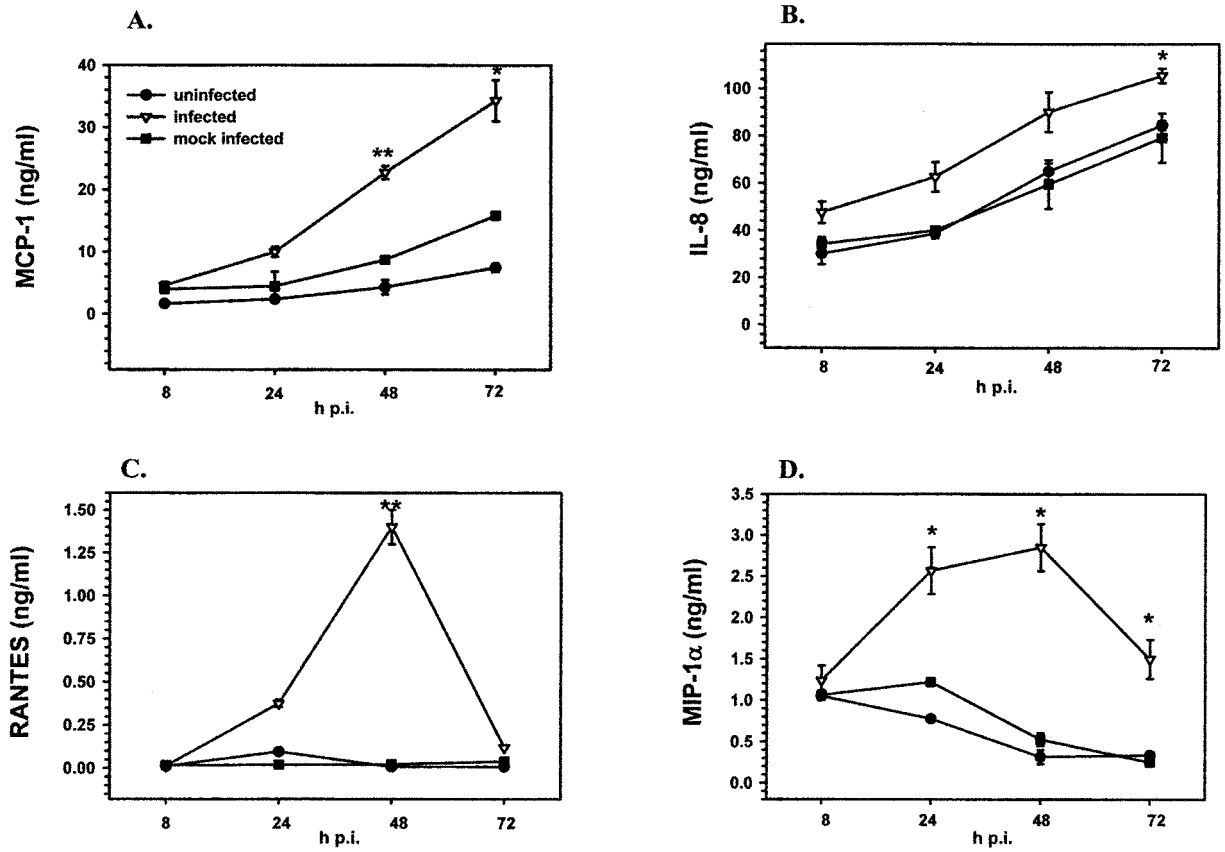

Figure 4 Microglial cells produce chemokines in response to CMV stimulation. Microglial cell cultures were infected with CMV AD169. Cell-free supernatants collected at 8, 24, 48, and 72 hours p.i. were assayed for (**A**) MCP-1, (**B**) IL-8, (**C**) RANTES, and (**D**) MIP-1α. Representative data from at least three independent experiments, using microglial cells from different brain specimens, are expressed as the mean ± SEM of triplicate samples for each time point tested. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus uninfected microglial cell cultures.

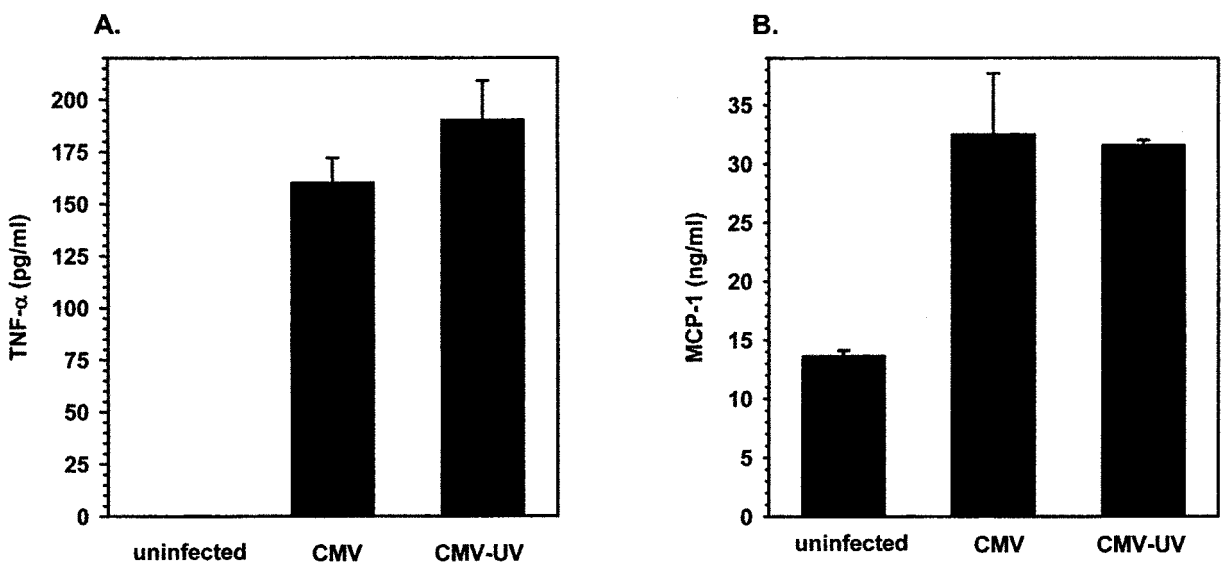

Figure 5 Effect of UV-inactivated CMV on cytokine induction in glial cells. (**A**) Microglial cells cultures were stimulated with UV-inactivated CMV AD169 (MOI equivalent to 2.5) in triplicate. Cell-free supernatants collected at 24 h p.i. were tested for TNF-α production. (**B**) Cell-free supernatants from astrocytes cultures stimulated with UV-inactivated CMV AD169 were assayed 48 h p.i. for MCP-1. Data are representative of two independent experiments.

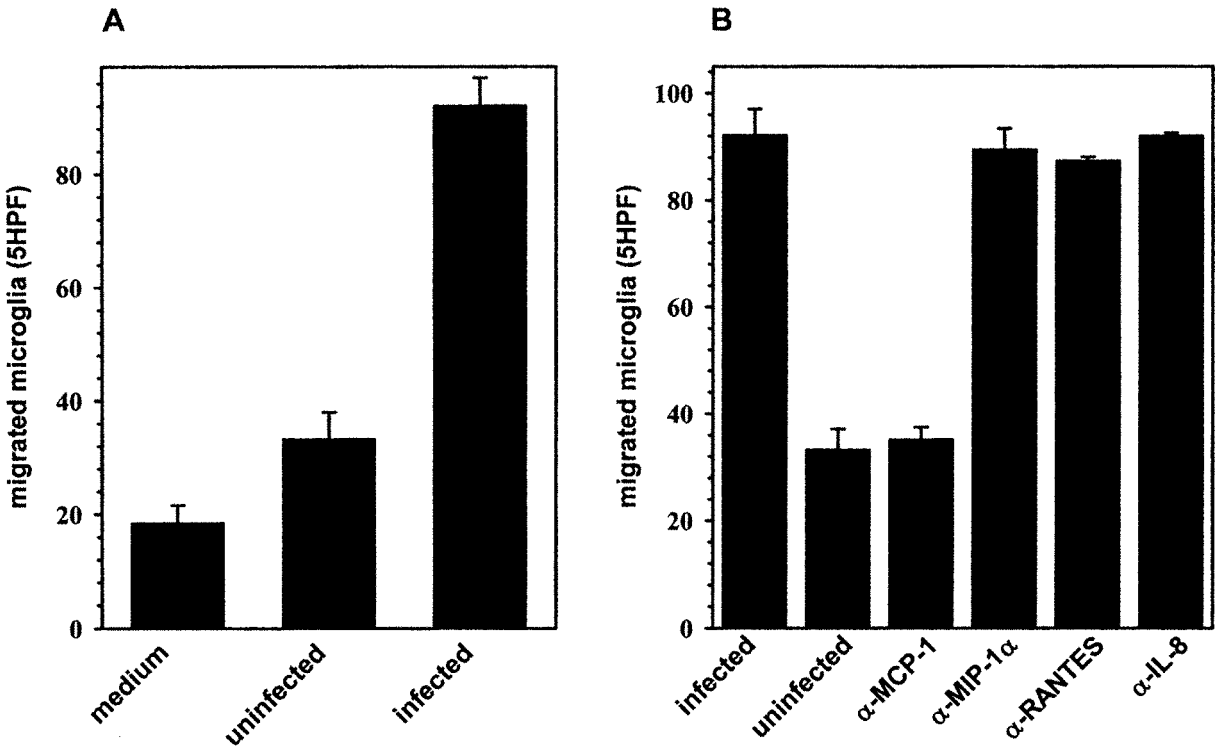

Figure 6 Migration of microglial cells towards supernatants from CMV-infected astrocytes is mediated by MCP-1. Supernatants from CMV-infected astrocyte (2×10^5 cells) cultures were applied to the lower wells of a Boynton chamber. (**A**) Migration of microglial cells (3×10^4 cells) through a 5-μm membrane towards infected culture supernatants was assayed by counting number of cells migrating through the filter towards medium (random migration), or supernatants from uninfected or infected astrocyte cultures. Number of cells is determined from means of triplicate wells for each sample per 5 high power fields. Data presented are obtained from 12 experiments performed using microglial cells from at least 3 different brain specimens. (**B**) Migration toward supernatant from CMV-infected cultures is abrogated when astrocyte supernatants were pre-treated with antibody (10 μg/ml) to MCP-1 but not by antibodies to MIP-1α, RANTES, and IL-8.

effectors to sites of infection. The recruitment of antiviral cytokine producing microglial cells (or other immune effectors, such as lymphocytes) towards chemical signals from infected astrocytes may be a host defense mechanism used to thwart acute CMV brain infection.

Cytokine production in response to CMV has been described in other cell types, such as TNF-α production by T cells (Davignon *et al*, 1996), monocytes (Yurochko and Huang, 1999), and macrophages/microglia (Pulliam *et al*, 1995). TNF-α inhibits CMV replication in astroglial cell lines (Davignon *et al*, 1996) and primary human astrocytes (Cheeran *et al*, 2000). In mice, TNF-α is associated with the clearance of CMV infection (Pavic *et al*, 1993). We demonstrated in this study that the presence of microglia was associated with suppressed CMV gene expression in astrocytes, suggesting microglial cells may perform an antiviral function. TNF-α production, along with other cytokines and chemokines, was also measured in cocultures of astrocytes and microglia in response to CMV. Both replication competent and UV-inactivated CMV stimulated microglia to produce TNF-α. This observation suggests that viral binding to specific receptors on microglia may be sufficient to induce production of this cytokine. CMV binding to the appropriate cell surface receptors in monocytes and fibroblasts also induces translocation of NF-κB into the nucleus resulting in the expression of immunoregulatory genes (Yurochko and Huang, 1999). Previous experiments performed in our laboratory have demonstrated that binding of CMV to microglial cells induced activation of NF-κB (unpublished observation), which may be responsible for the expression of TNF-α in these cells. These results suggest that the production and local release of antiviral cytokines by CMV-activated microglia may protect uninfected astrocytes from CMV infection.

Microglial nodule formation is a pathological feature of CMV encephalitis (Arribas *et al*, 1996). These nodules consist of aggregates of macrophages/microglia surrounding inclusion-bearing cells (Cinque *et al*, 1997). Although these nodules appear to be the centers of CMV replication, we have found that microglia are not productively infected with CMV (Lokensgard *et al*, 1999). When microglial cells were cocultured with astrocytes, nodule formation was seen in CMV-infected but not in uninfected cultures 48 h p.i. Thus, it seems reasonable to hypothesize that microglial nodules develop as a result of microglial cell migration toward the chemokine

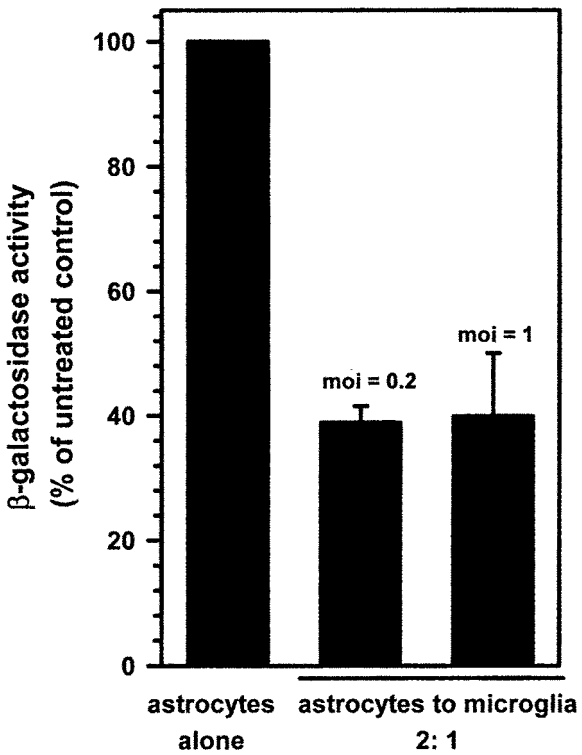

Figure 7 CMV gene expression in infected astrocytes is decreased by the presence of microglial cells. Astrocytes (2×10^5) and microglial cells (1×10^5) were cocultured in 24-well tissue culture plates. Cocultures were infected for 72 h with RC256 at an MOI of 0.2 or 1. Infected cells were collected and lysed by three freeze–thaw cycles in PBS. Cell lysates were assayed for β-galactosidase activity using CPRG. Levels of viral expression in cocultures are presented as % of the value obtained using purified astrocytes in the absence of microglia (mean ± SEM of triplicate samples). Data are representative of four independent experiments performed with cells from different donors.

signals produced by CMV-infected astrocytes. Microglial nodules are seen in the brains of AIDS patients and also transplant patients (Arribas *et al*, 1996), which may reflect the formation of a focal immune response intended to prevent viral replication and spread. Because acute CMV encephalitis occurs commonly during advanced AIDS, it is possible that HIV-1 infection of microglia could potentially subvert brain defenses against CMV through dysregulation of the defense function of microglial cells. It can be envisioned that along with the profound lack of $CD4^+$ T cells, a local deficiency of microglial cell mediated defense mechanisms in advanced neuroAIDS patients may render them susceptible to CMV brain disease due to the unrestricted spread of the virus.

Based on these results and reports of other investigators a model of host defense against CMV brain infection is proposed (Figure 8), which involves (1) production of chemokines (MCP-1) from infected astrocytes, (2) recruitment and activation of resting microglia, and (3) production of antiviral cytokines by CMV-stimulated microglia conferring an antiviral state in bystander cells. This response by microglia may then initiate a cascade of signals required to recruit other immunocytes, such as lymphocytes and monocytes, to the site of infection.

Materials and methods

Brain cell culture

Purified human fetal astrocyte and microglial cell cultures were prepared as described previously (Chao *et al*, 1996). Briefly, 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. Trypsin digestion was stopped using 10% fetal bovine serum (FBS). The cells were washed 3× with Hank's buffer and resuspended by gentle pipetting in fresh Dulbecco's Modified Eagle's medium (DMEM) with 10% heat inactivated FBS, 100 u/ml of penicillin, and 100 μg/ml of streptomycin. The cell suspension was seeded at 75–100 × 10^6 cells in 75 cm^2 tissue culture flasks and was incubated in a humidified incubator at 37°C with 10% CO_2. 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. The harvested cells were seeded into 24-well tissue culture plates (2×10^5 cells/well) and washed after 60 min incubation at 37°C. Microglial cells used in these experiments were ≥99% pure, as determined by CD68 antibody staining. Less that 1% of the cells stained with antibodies to glial fibrillary acidic protein (GFAP), an astrocyte marker.

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.

Viruses

Sucrose purified human CMV AD169 and RC256, a Lac-Z containing recombinant CMV strain (American Type Culture Collection, Rockville, MD, USA) (Spaete and Mocarski, 1987), were used in this study. Human foreskin fibroblasts (HFF) were used to propagate viral stocks. Infected HFF cultures were harvested at 80–100% cytopathic effect and subjected

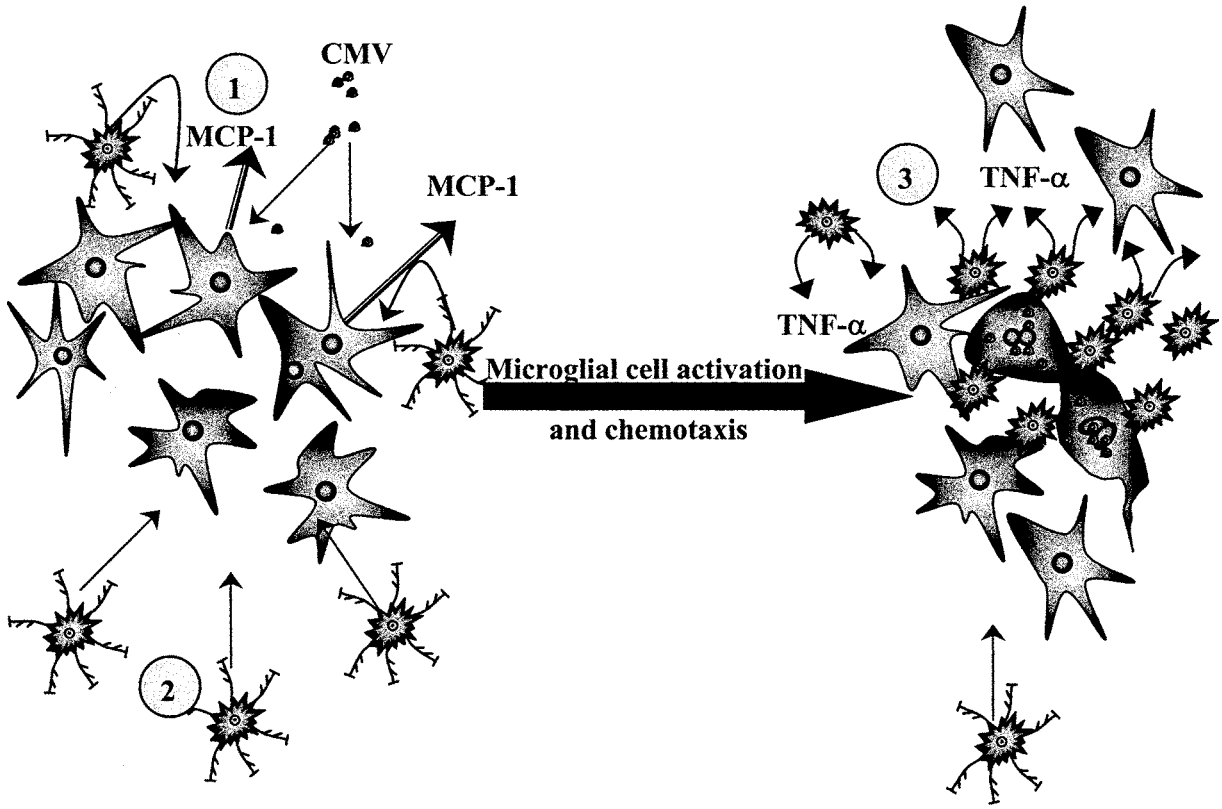

Figure 8 Model for microglial cell-mediated protection of astrocytes from cytopathic CMV infection. In response to viral infection, astrocytes may produce chemoattractants including MCP-1 (**1**). Microglial cells, responding to these chemotactic factors, move towards foci of infection (**2**). Activated microglia produce antiviral, proinflammatory cytokines, including TNF-α (**3**), thereby preventing spread of infection to neighboring cells (bystander effect).

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 23 000 $\times$ g for 2 h at 4°C. The pellet was resuspended in equal volumes of DMEM and 35% sorbitol (in Tris-buffered saline; 50 mM Tris-HCl and 150 mM NaCl; pH 7.4). Viral stocks were titered on HFF cells as 50% tissue culture infectious dose ($TCID_{50}$) per ml. A multiplicity of infection (MOI) of 2.5 $TCID_{50}$ units was used in all experiments.

Mock-infected HFF cultures were processed in exactly the same manner as virus stocks. Glial cell cultures treated with mock-infected culture preparations were used to evaluate non-specific 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. The titer of the UV-inactivated virus was 4 $\log_{10}$ $TCID_{50}$ lower than the replication competent virus.

RT-PCR

RNA extraction, reverse transcription, and PCR were performed using standard procedures (Chomczynski and Sacchi, 1987). Briefly, total cellular RNA was extracted from 1×10^6 astrocytes or microglia at 3, 8, 24, and 48 h postinfection (p.i.) with CMV. RNA (1.5 μg) was reverse transcribed using Superscript II RNaseH-reverse transcriptase, as directed by the manufacturer (Life Technologies, Gaithersburg, MD). The cDNA obtained was used in PCR reactions with the primer sets described. The reaction mixture contained 10$\times$ PCR buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0), and 1% triton X-100], 25 mM MgCl2, 10 mM dNTP mixture, 0.22 μg/μl Taq-start antibody (Clontech, Palo Alto, CA), 25 μM primer set (sense and antisense), cDNA, and 5U Taq polymerase (Promega, Madison, WI). Annealing temperatures and cycle numbers were optimized for each primer set: GADPH, 5′-CCACCCATGGCAAATTCCATGGCA-3′ (sense), 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (antisense), 65°C for 22-cycles (600 bp); TNF-α, 5′-CAGA GGGAAGAGTTCCCCAG-3′ (sense), 5′-CCTTGGTC TGGTAGGAGACG-3′ (antisense), 62°C for 33 cycles (325 bp); IL-1β, 5′-AAACAGATGAAGTG CTCCTTCAGG-3′ (sense), 5′-TGGAGAACACCA CTTGTTGCTCCA-3′ (antisense), 65°C for 33 cycles, (391 bp); IL-6, 5′-ATGAACTCCTTCTCCAC AAGCGC-3′ (sense), 5′-GAAGAGCCCTCAGGCTG GACTG-3′ (antisense), 65°C for 33 cycles (628 bp);

MCP-1, 5′-CCCCAGTCACCTGCTGTTATAACTTCA C-3′ (sense), 5′-GAGTGAGTGTTCAAGTCTTCGGAG TTTG-3′ (antisense), 65°C for 18 cycles (222 bp); MIP-1α, 5′-ACCAGTTCTCTGCATCACTTGCTG-3′ (sense), 5′-AACAACCAGTCCATAGAAGAGGTAGC TG-3′ antisense, 65°C for 35 cycles, (343 bp); RANTES, 5′-ACCACACCCTGCTGCTTTGCCTACAT TGCC-3′ (sense), 5′-CTCCCGAACCCATTTCTTCTCT GGGTTGGC-3′ (antisense), 68°C for 35 cycles (162 bp); and IL-8, 5′-ATGAACTCCTTCTCCACAAG CGC-3′ (sense), 5′-TCTCAGCCCTCTTCAAAAACTT CTC-3′ antisense, 65°C for 22 cycles (289 bp). Control reactions using GADPH primers were included for each sample.

Northern blot analysis

Total RNA, extracted from CMV-infected and uninfected astrocytes, was analyzed by a Northern blot technique using standard procedures. Briefly, 2×10^6 astrocytes were infected with sucrose purified CMV (AD169). RNA was extracted from the infected astrocytes 3, 8, and 24 h p.i. Then, 5 μg of total RNA was separated on 1.5% denaturing formaldehyde agarose gels and transferred to nylon membranes (Magna Graph, Micron Separation Inc, Westborough, MA, USA). The blots were probed using PCR amplification products of MCP-1 specific primers, described previously. The amplified product was purified by electroelution and labeled with ^{32}P-dATP by primer extension using random primers (DECAprimeII^{TM}, Ambion Inc, Austin, TX). Blots were hybridized overnight in 50% formamide at 42°C, then washed under high stringency conditions (0.1× SSPE at 65°C), developed on a phosphoimager (Molecular Dynamics, Sunnyvale, CA), and analyzed using ImageQuant software (Molecular Dynamics). Expression of GADPH was used to normalize MCP-1 band intensity. Ratios of MCP-1 to GADPH band density were calculated for infected and uninfected samples at each time point, to determine the increase in CMV-induced MCP-1 mRNA expression.

ELISA

A sandwich-ELISA based system previously described (Peterson *et al*, 1997) was used to quantify cytokine (TNF-α, IL-1β, and IL-6) and chemokine (RANTES, MIP-1α, MCP-1, and IL-8) 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. CMV-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 IgG 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 1 M 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. ELISA assay sensitivity for each cytokine and chemokine tested was: TNF-α, 20 pg/ml; IL-1β, 10 pg/ml; IL-6, 10 pg/ml; MCP-1, 15.6 pg/ml; MIP-1α, 15.6 pg/ml; RANTES, 20 pg/ml; and IL-8, 10 pg/ml (Lokensgard *et al*, 1997; Peterson *et al*, 1997; Ehrlich *et al*, 1998).

Chemotaxis assay

Microglial cell migration towards culture supernatants from CMV-infected astrocytes, assay medium (DMEM) containing 1% FBS (random migration), or recombinant chemokine (positive control) was assayed using a 48-well microchemotaxis Boynton chamber (Neuro Probe, Cabin John, MD), as previously described (Peterson *et al*, 1997) with minor modifications. A 5-μm polyvinylpyrrolidone-free filter was used to separate the upper and lower compartments of the chamber. Microglial cells (2×10^4 cells/well) were added to the upper chamber while the supernatants or recombinant chemokines were added to the lower chamber. After 3 h incubation at 37°C, nonmigrating cells were gently scraped off the upper surface of the filter, and the cells in the lower surface were fixed in methanol and stained (Diff-Quik, Baxter, McGraw Park, IL). The number of cells migrating through the filter to the lower surface was then counted microscopically under an oil immersion objective (400×). Five high power fields (HPF)/well of triplicate samples were counted for each treatment and the mean cell numbers were determined.

To identify the chemokines in astrocyte culture supernatants responsible for microglial cell migration, the supernatants were treated with antibodies (10 μg/ml) to various chemokines for 30 min (37°C) before adding them into the wells of the lower chamber.

β-Galactosidase assay

The recombinant CMV strain, RC256 (Spaete and Mocarski, 1987) expresses a single copy Lac-Z reporter gene under the control of the major early promoter and has replication properties similar to wild-type CMV. Expression of β-galactosidase in CMV-infected astrocytes has been shown to correlate with viral replication (Cheeran *et al*, 2000). Infected cell lysates collected at 48 h p.i. were used to measure

β-galactosidase activity in a colorimetric assay using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (CPRG, 1 mg/ml; Boehringer Mannheim, Indianapolis, IN) as substrate. Optical density at 593 nm (OD_{593}) was used to quantify color change in the reaction, indicative of viral gene expression.

Statistical analysis

Data were expressed as mean ± SEM of triplicate samples. Student's *t*-test was applied to both pooled data from multiple experiments, using cultures derived from at least two different donors, and on data obtained from triplicate cultures derived from the same donor. Differences in mean cytokine production values, from infected versus uninfected glial cells, with alpha levels of 95% ($P \leq 0.05$) considered statistically significant.

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