



Human cytomegalovirus induces IL-6 and TNF α from macrophages and microglial cells: possible role in neurotoxicity

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Human cytomegalovirus (HCMV) can frequently infect the central nervous system (CNS) in the setting of immunosuppression such as transplantation and infection with the human immunodeficiency virus (HIV). Our laboratory previously reported that HCMV infection of human brain aggregates preferentially infected a microglial/macrophage (M/M) and caused a neuropathology that differed between strains and could occur in the absence of antigen expression. We extended these studies by infecting a human brain cell aggregate model with four low passage clinical isolates of HCMV. Two patterns of cytopathology emerged after infection; a lacy eosinophilic appearance or a glial nodular formation concomitant with a decreased aggregate size. None of the infections were positive for HCMV antigen; however, all were positive for HCMV DNA. We also infected primary macrophages and microglial cells with the same HCMV isolates. Microglial cells were more susceptible to HCMV infection resulting in a lytic infection. Production of potentially neurotoxic cytokines, IL-1, IL-6 and TNF α , from HCMV-infected macrophages and microglial cells were evaluated to explain brain aggregate cytopathology. Supernatants from HCMV-infected macrophages and microglial cells produced similar levels of TNF α (< 30 pg ml⁻¹) but showed strain and cell source variation in the production of IL-6; microglial cultures produced > 4 fold higher levels. None of the supernatants contained IL-1. Treatment of brain aggregates with either IL-6 or TNF α resulted in morphologic alterations and/or a decrease in size consistent with HCMV infection or supernatant treatment.

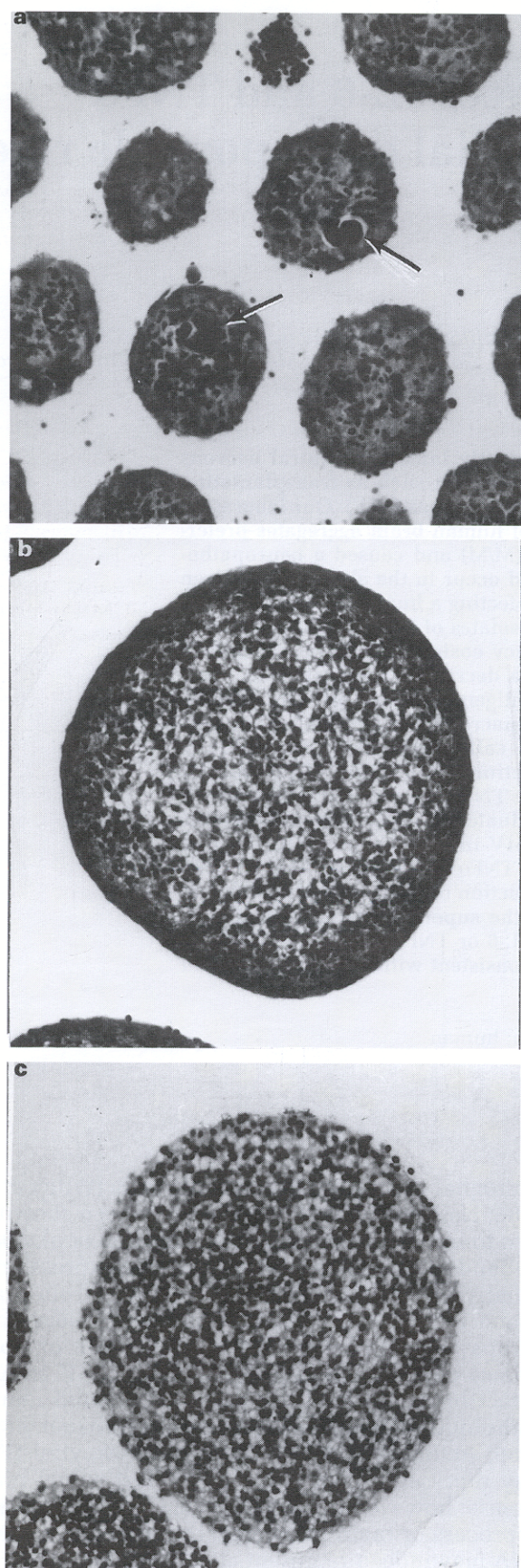
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Introduction

Human cytomegalovirus (HCMV) is known to infect the central nervous system (CNS) and can cause devastating infections in the newborn and in patients with immune suppression. CMV encephalitis occurs in up to 30% of patients with acquired immunodeficiency syndrome (AIDS) and is also prevalent in adult recipients of organ transplantation. HCMV encephalitis has usually been a histologic diagnosis dependent on the presence of

microglial nodules or cytomegalic cells (Wiley *et al*, 1986; Nelson *et al*, 1988). HCMV has been shown *in vivo* to infect all cells of the CNS (Wiley *et al*, 1986; Holland *et al*, 1994); however, antigen expression is usually absent and recovery of HCMV from brain tissue is very rare. HCMV has been observed in autopsy studies by *in situ* hybridization or polymerase chain reaction (PCR) without CMV antigen detection and with little or no associated neuropathology (Wiley *et al*, 1986; Schmidbauer *et al*, 1989; Achim *et al*, 1994). In these cases, viral DNA may represent latency and not reflect actual brain disease; however, in the presence of an inflammatory process or brain pathology, the presence of CMV DNA may indeed represent an etiologic source.

My laboratory has previously shown, using a



human brain aggregate system, that the cell preferentially infected *in vitro* is the macrophage/microglial cell (Pulliam, 1991). HCMV produced two distinctly different patterns of cytopathology in human brain aggregates based on strain variation and multiplicity of infection. Microglial nodule formation was accompanied by HCMV antigen expression; however, fulminant cytopathology was not. Virions were observed intracellularly by electron microscopy in cells morphologically identified as macrophage/microglia. I speculated that the fulminant cytopathology was not due to direct virus replication, since the number of cells infected was very small, but rather a soluble factor produced by HCMV-infected macrophage/microglia.

In the present study, we extended the number of clinical isolates used to infect human brain aggregates to further evaluate the range of cytopathology. Because HCMV-infected macrophages are a potential source of HCMV in the brain as well as a vehicle for the spread of the infection and based on previous findings that infected macrophage/microglia elicited a neurotoxic pathology on human brain cultures, we evaluated the effects of HCMV-infected macrophage and microglial cell soluble factors on human brain cultures.

Results

Direct infection of human brain aggregates with HCMV

Four clinical HCMV isolates gave two distinct neuropathologies (Figure 1). Infection with isolates 1873 and 8303 resulted in cytomegalic cells, microglial nodule formation and a lacy eosinophilic matrix (Figure 1a) in one brain sample. All three aggregate preparations were smaller in size than control aggregates or aggregates infected with strains 1812 or 8802. Infection with HCMV strains 1812 and 8802 did not induce microglial nodule formation or cytomegalic cells but did produce a lacy eosinophilic matrix (Figure 1b) in all three brains infected. Brain aggregate size was not affected and was consistent with control aggregates (Figure 1c). HCMV antigen detection was not detected for any infected brain aggregates. This is in contrast to our previous study where antigen expression was occasionally observed in multinucleated giant cells and cytomegalic inclusions resulting from infection with laboratory strains

Figure 1 Human brain aggregates infected for 14 days with HCMV clinical isolates 1873 (a), 1812 (b) or mock uninfected control (c). Aggregates infected with strains 1873 (a) and 8303 were demonstrably smaller than control aggregates and showed microglial nodule formation (arrows). Aggregates infected with 1812 (b) or 8802 had a central lacy eosinophilic appearance. Magnification $\times 63$.

Table 1 Infectivity

Isolate	HCMV Source ^a	% infected ^b	
		Macrophages	Microglia
AD169	Laboratory	23.2 ± 2	19 ± 2
1812	AIDS	26.6 ± 6.5	11 ± 2
1873	AIDS	18.6 ± 3.8	9 ± 1
8303	Transplant	21.5 ± 4.8	8 ± 2
8802	Transplant	9.1 ± 1.8	9 ± 1

^a HCMV was isolated from the semen of patients with AIDS or organ transplants. AD169 is an isolate passaged many times in the laboratory

^b Immunohistochemistry staining for HCMV antigens on primary macrophage or microglial cultures using a monoclonal antibody to early antigens after 14 days of infection. Mean for three cultures ± s.d.

Towne or AD169 (Pulliam, 1991). HCMV DNA was detected by *in situ* hybridization in all infected cultures, but varied among strains. Between 1 and 10 foci per aggregate were detected either in microglial nodules or in the absence of these, distributed throughout the aggregate (data not shown). HCMV DNA was not detected in uninfected aggregate cultures.

Infection of macrophage and microglial cultures with HCMV

Three separate preparations of each cell type were infected with five different HCMV strains (Table 1). Both cell types were productively infected although the course of infection was different. Supernatants harvested as early as 5 days after infection and before ultracentrifugation were able to infect human

foreskin fibroblasts (HFF); no infectious virus was detectable after ultracentrifugation. Infected macrophages became morphologically refractile 5 days after infection; however, the cells remained viable throughout the infection until harvest at day 14. In contrast, microglial cells at the same MOI of 0.1 pfu per cell became infected, rounded up and detached from the monolayer in approximately 5 days. At a reduced MOI (0.01 pfu per cell), fewer cells were infected and the infection proceeded in a less lytic manner leaving intact cells after 14 days.

Treatment of brain aggregates with supernatants from HCMV-treated macrophage and microglial cultures

Untreated control brain cultures and brain cultures treated with uninfected macrophage or microglial culture supernatants contained less than 1% dead cells as determined by trypan blue exclusion. Histology was unremarkable with brain aggregates appearing within a compact extracellular matrix and normal nuclei. In contrast, aggregates treated with 20% supernatants from either HCMV-infected macrophages or microglial cells were smaller than uninfected supernatant control aggregates, with a lacy eosinophilic matrix appearing around the periphery (Figure 2). The smaller aggregate size was similar to that observed with direct HCMV-infected

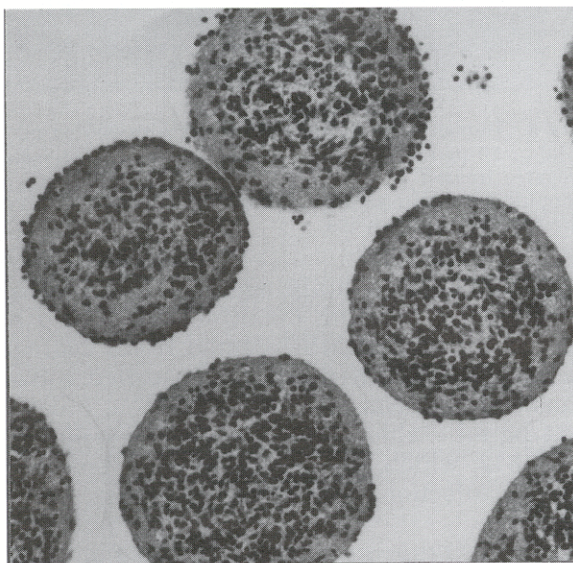


Figure 2 Human brain aggregates treated for 7 days with supernatant from HCMV (8303)-infected microglial cells. Aggregates are smaller than control aggregates (1c) and are beginning to have an eosinophilic periphery. Magnification ×63.

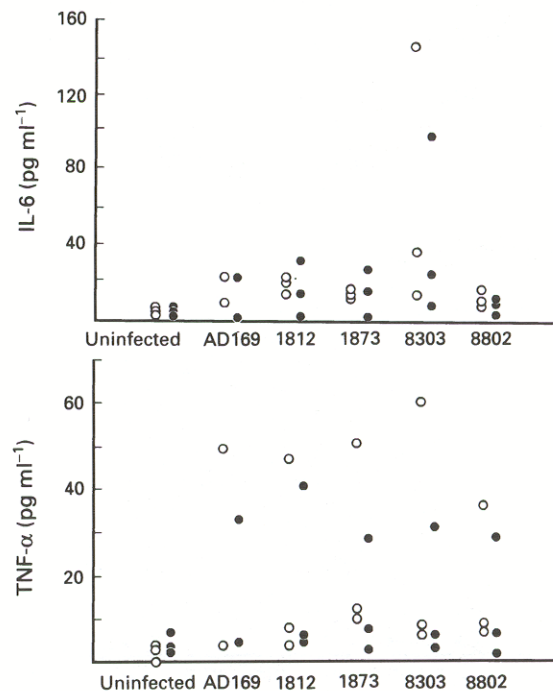


Figure 3 Cytokine production from supernatants of HCMV-infected macrophage cultures compared to supernatants from uninfected macrophage cultures 5 (○) and 10 days (●) after infection. All infected supernatants were significantly higher than uninfected controls ($P < 0.02$).

brain aggregates (Figure 1a).

Cytokine production

Supernatants from HCMV-infected macrophage or microglial cell cultures were collected at 5 and 10 days after infection and assayed for the presence of IL-1B, IL-6 or TNF α . There was no detectable IL-1B in any of the supernatants, HCMV-infected or uninfected. IL-6 production from supernatants of HCMV-infected macrophages was low (Figure 3); however, all infected supernatant levels were significantly higher than control uninfected macrophage supernatants ($P = < 0.02$). Uninfected macrophage and microglial cell supernatants contained low and in some cases undetectable levels of IL-6. Levels of IL-6 from supernatants of HCMV-infected microglial cultures varied between strains (Figure 4) although all were significantly different from uninfected controls ($P = < 0.02$) except supernatant from AD169 after 10 days.

TNF α levels from supernatants of HCMV-infected macrophages or microglial cells (Figures 3 and 4) were significantly higher than uninfected controls ($P = < 0.02$) although there was variability between brain preparations.

Supernatants from HCMV-infected brain aggregates were also tested for IL-6 and TNF α by ELISA.

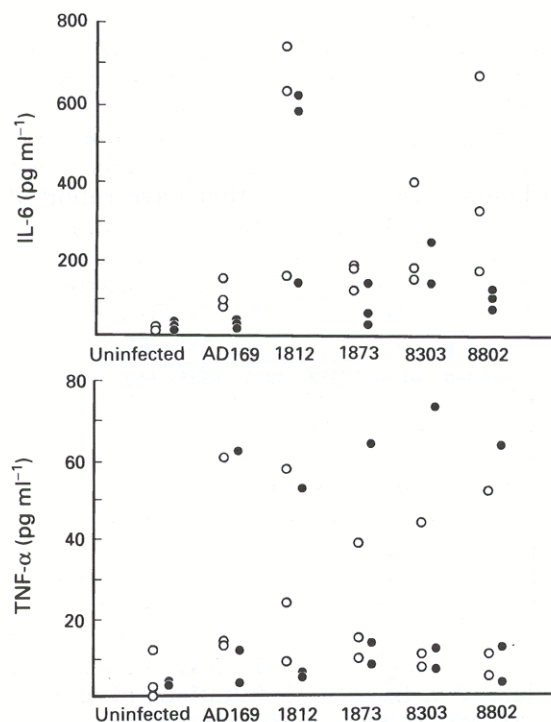


Figure 4 Cytokine production from supernatants of HCMV-infected microglial cultures compared to supernatants from uninfected microglial cultures 5 (○) and 10 days (●) after infection. All infected supernatants were significantly higher than uninfected controls ($P = < 0.02$) except IL-6 levels in supernatant from AD169-infected cells after 10 days.

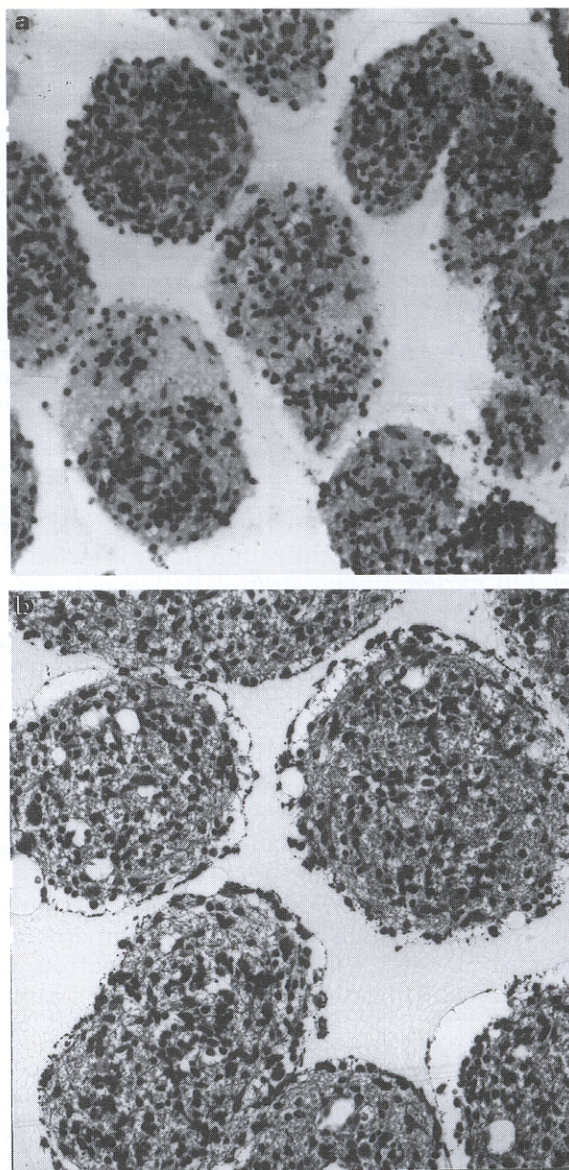


Figure 5 Human brain aggregates treated with 500 pg ml⁻¹ of IL-6 (a) or 50 pg ml⁻¹ of TNF α (b) for 7 days. Morphologically, aggregates treated with either IL-6 or TNF α were lacy and eosinophilic and appeared smaller than control aggregates (see Figure 1c). Magnification $\times 63$.

Uninfected control brain aggregate supernatants were negative for both cytokines. No TNF α was detected for any HCMV-infected brain aggregate supernatant tested. IL-6 production varied between strains from a mean of 51–78 pg ml⁻¹ at 5 days to 54–74 pg ml⁻¹ after 10 days with the exception of strain 1812, which produced 236 pg ml⁻¹ of IL-6 10 days after infection.

Treatment of brain aggregates with IL-6 and TNF α

Aggregates were treated for 7 days with 500 pg ml⁻¹ IL-6 or 50 pg ml⁻¹ of TNF α to determine whether the changes observed with the HCMV direct infections and supernatant treatments were consistent

with these cytokines. Both treatments resulted in small eosinophilic lacy aggregates with a decrease in aggregate integrity (Figure 5). The decrease in aggregate size is consistent with HCMV infection by several strains and treatment with HCMV-infected macrophage and microglial cell supernatants; however, the degree of aggregate disruption was greater in the IL-6 and TNF α treatments compared to aggregates treated with supernatants from HCMV-infected macrophage and microglial cells.

Discussion

This study extends the findings previously reported (Pulliam, 1991). In the previous study, the laboratory isolates AD169 and Towne plus the clinical isolate W were used to infect brain aggregates. Antigen expression was only present within microglial nodules or multinucleated cells and this cytopathology was only demonstrated by the laboratory strains. In the direct HCMV infection studies presented here, only low passage clinical isolates were tested. Human CMV infection of brain aggregates demonstrated strain to strain variation in neuropathology. Direct infection by all strains resulted in a loose matrix giving a lacy appearance. Cytomegalic cells and microglial nodules were only observed with strains 1873 and 8303 in one of the brains infected but not the other two. This culture variability may reflect differences in brain regions or age of gestation. Other investigators have shown CMV localization in selected regions of the brain (Wiley *et al*, 1986; Nelson *et al*, 1988). Previous studies using *in situ* hybridization or polymerase chain reaction (PCR) documented HCMV DNA in the brain without antigen expression or neuropathology (Wiley *et al*, 1986; Achim *et al*, 1994). How this finding relates to brain disease caused by HCMV infection is unknown. Prior autopsy studies confirmed that HCMV antigen is most often observed in microglial nodules and cytomegalic cells (Wiley *et al*, 1986; Nelson *et al*, 1988; Pulliam, 1991; Holland *et al*, 1994). Our previous findings are consistent with those seen *in vivo*; that is, HCMV antigen expression can coincide with the presence of microglial nodules. The lack of antigen expression may not reflect the absence of antigen but rather the antisera used may not recognize the appropriate early or late proteins. The present study underlies the importance that in the absence of HCMV antigen and in the presence of HCMV DNA with accompanying neuropathology, damage due to this virus should not be overlooked.

Human HCMV DNA has been detected in neurons and astrocytes as well as microglial cells (Wiley *et al*, 1986; Nelson *et al*, 1988). Using the human brain aggregate system, HCMV DNA or the presence of HCMV virions by ultrastructure examination (Pulliam, 1991) was only detected in microglial cells. The detection of HCMV DNA in

neurons and astrocytes in autopsy studies without accompanying antigen inflammation and/or neuropathology may suggest latency and the significance of this to brain dysfunction is unknown.

Human HCMV has been shown recently to productively infect differentiated macrophages (Ibanez *et al*, 1991; Minton *et al*, 1994). The degree of differentiation appears to be related to the permissiveness to HCMV. Likewise, the degree of activation may affect infectivity. Infection in these studies was performed on adherent differentiated macrophage and microglial cells. The difference in susceptibility may be related to the fetal nature of the microglial cells or the degree of activation. Macrophages were not killed by HCMV infection in contrast to the microglial cells which were lytically infected. The less lytic infection of HCMV in brain aggregates may reflect a protective environment provided by the aggregate matrix which contains other neural cells compared to the primarily unicellular monolayer culture system. The aggregate cultures may also reflect a less activated state for the microglial cells. Productive replication of murine CMV in microglial cells isolated from newborn mice was recently reported (Schut *et al*, 1994). We concluded from these previous results and those presented here that microglial cells derived from fetal tissue are highly susceptible to HCMV infection. HCMV susceptibility of adult microglial cells *in vitro* has yet to be determined.

The small number of HCMV-infected cells in the brain aggregates, relative to the high degree of pathology, argues for the production of a soluble neurotoxic factor. Both macrophages and microglia are capable of producing a variety of cytokines and other inflammatory mediators that have reported neurotoxic effects (Chao *et al*, 1992; Morganti-Kossmann *et al*, 1992). Several cytokines have emerged as initiators of a potentially destructive cytokine cascade; they are IL-1, TNF α and IL-6. TNF α mRNA was previously shown to be localized to CMV-infected macrophages in colonic mucosa from AIDS patients (Smith *et al*, 1992). Supernatants from HCMV-infected brain aggregates had elevated levels of IL-6 with no detectable TNF α . This may suggest that TNF α was more locally produced within brain parenchyma as has been shown in HIV-infected brain tissue (Wesselingh *et al*, 1993). In contrast, IL-6 levels were elevated in the supernatant of infected aggregates. This is reminiscent of findings from brain tissue and CSF of AIDS patients at autopsy in which CSF levels did not correlate with tissue concentrations of various compounds and in particular IL-6 (Achim *et al*, 1993). In that study, IL-6 was substantially increased in the CSF and low in brain parenchyma. Microglial/macrophages are thought to be the most immunologically active cells in the central nervous system; however, astrocytes also produce cytokines, in particular IL-1 and TNF α , in response to a vari-

ety of stimuli, (Fontana *et al*, 1982; Sawada *et al*, 1989; Chung and Benveniste, 1990; Benveniste, 1992). The cultures used in these studies were highly enriched for microglial cells although astrocyte contamination cannot be ruled out. Cytokines produced from microglial/macrophages may also induce further production of cytokines from astrocytes in the brain aggregates, including TGF- β or IFN γ . For these reasons, the morphologic alterations observed from direct treatment of brain aggregates with IL-6 and TNF α may not entirely be a result of the cytokine itself but a combination of interacting processes, especially since brain aggregates contain approximately 40% astrocytes compared to only 10% microglial cells. The absence of IL-1 in HCMV-infected macrophage or microglial cell supernatants is surprising. IL-1 is known to be produced by microglia and astrocytes by a variety of stimuli (Fontana *et al*, 1982; Malipiero *et al*, 1990). Astrocytes are also known to produce IL-6 in response to IL-1 and TNF α (Frei *et al*, 1989), IL-6 and TNF α are known to induce each other and TNF α has been shown *in vitro* to cause oligodendrocyte death by apoptosis (Selmaj *et al*, 1991). The disruptive cytopathology from these treatments needs to be further studied to determine what cell is damaged and how.

HCMV infection of the central nervous system in adults is often seen in the setting of cell-mediated immunosuppression such as organ transplantation and HIV infection. In a recent report, HCMV encephalitis in the setting of HIV infection was characterized both clinically and pathologically to differentiate it from AIDS dementia (Holland *et al*, 1994). Pathologically, HCMV encephalitis was defined by the presence of intranuclear HCMV inclusions associated with tissue disruption and necrosis. The findings presented here emphasize the alternative; neuropathology associated with HCMV infection may be present in the absence of microglial nodules or antigen; therefore we suggest that this criteria for a HCMV encephalitis diagnosis may be too restrictive (Holland *et al*, 1994).

Previous work by others has shown HIV did not induce IL-1, IL-6 or TNF α in mononuclear cells infected *in vitro* (Molina *et al*, 1990). Our laboratory also showed that *in vitro* HIV infection of primary mononuclear cells did not induce IFN γ or TNF α (Pulliam *et al*, 1991) and we concluded that the morphologic alterations observed in human brain aggregates treated with macrophage supernatants were not due to these cytokines. Brain aggregates treated with HIV-infected mononuclear cell supernatants showed severe morphologic damage that was not consistent with neurotoxicity observed in aggregates treated with HCMV-infected macrophages. The results presented here suggest that mononuclear cells infected with HCMV respond differently than infection with HIV. In the setting of HIV infection, this point may be irrelevant

since HCMV is a common co-infection and may stimulate HIV-infected macrophage/microglial cells to produce cytokines.

In summary, HCMV infection of human brain aggregates produced strain specific neuropathology. HCMV infection of primary macrophages and microglial cells induced soluble factors that produced neurotoxicity. These soluble factors included elevated levels of IL-6 and TNF α . When brain aggregates were treated with exogenous IL-6 and TNF α , a similar neuropathology was observed.

Materials and methods

Virus isolates

Four strains of low passage clinical HCMV were used in these studies along with the laboratory strain AD169. All clinical strains were isolated from semen. Strain 1812 was isolated from an AIDS patient with CMV retinitis; strain 1873 was isolated from an AIDS patient with generalized CMV infection. Strains 8303 and 8802 were isolated from organ transplant recipients.

Virus stocks were prepared by growing the isolates in human foreskin fibroblasts (HFF). HCMV-infected HFF cells were sonicated, centrifuged and the supernatants mixed with sterile sucrose phosphate freezing buffer (20% sucrose, 0.1 M Na₂HPO₄, pH 7.2) and frozen at -70°C . Virus stocks were titrated in a plaque assay using HFF cells. Media and fetal calf serum (FCS) were certified endotoxin free; mycoplasma was not detected in HCMV stock cultures (ELISA, Boehringer-Mannheim, Indianapolis).

Macrophage cultures

Peripheral blood mononuclear cells (PBMC) were isolated from normal healthy donors and separated through a Ficoll-Hypaque gradient (Sigma Chemical Co, St Louis, Mo.), washed three times with sterile saline and suspended in endotoxin free RPMI-1640 medium supplemented with 10% heat-inactivated human serum, 2 mM L-glutamine, 1.0 mM sodium pyruvate and 50 $\mu\text{g ml}^{-1}$ gentamicin at a density of 1.0×10^6 cells ml^{-1} . One ml per well of suspension was seeded into a 12 well tissue culture plate. The macrophages were allowed to adhere in a humidified, 5% CO₂ incubator at 37°C . After 2–3 days, non-adherent cells were aspirated and the adherent macrophages given fresh medium. Thereafter, medium was changed every 3–4 days until approximately 10–14 days after seeding, when one well of a 12 well plate contained approximately 5×10^4 cells.

Infection of macrophage cultures

Macrophage cultures were infected at a multiplicity of infection (MOI) of 0.1 plaque forming units (pfu) cell $^{-1}$. Virus was allowed to adhere for 60 min at which time the cells were washed and the medium

replaced. Media from both infected and uninfected cultures were collected 5 and 10 days after infection and stored at -70°C .

Microglial cultures

Human fetal brain cells were dissociated through nylon screens as described below for brain aggregate cultures. Neural cells within endotoxin free DMEM medium containing 0.6% glucose, $50\text{ }\mu\text{g ml}^{-1}$ gentamicin and 10% heat-inactivated FCS were diluted to a final concentration of 1×10^6 cells per 25 cm^2 tissue culture flask. After 5 days in culture, the flasks were sealed tightly and rotated for 2 h at approximately 125 rpm. The non-adherent cells were resuspended in medium with the additional supplement of 50 U ml^{-1} recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF, Genzyme Inc, Cambridge MA) and plated in 12 well tissue culture plates. Cultures were subconfluent after approximately 10–14 days. Microglia were identified by immunohistochemical staining with the biotinylated lectin *Ricinus communis* agglutinin-1 (RCA-1, Vector Laboratories, Burlingame CA) for 45 min at a dilution of 1:200. Normal rabbit serum (1:80) served as a negative control. Binding of RCA-1 was resolved by incubation with streptavidin-conjugated alkaline phosphatase (Vector Laboratories) followed by the chromagen substrate, NBT/BCIP. Cultures were greater than 95% positive for RCA-1.

Infection of microglial cultures

Subconfluent microglial cultures were infected at an initial MOI of 0.1 pfu ml^{-1} . Virus was allowed to adsorb for 60 min, washed and medium containing GM-CSF replaced. Medium was half exchanged after 5 days. Experiments were terminated after 10 days of infection and the cells were washed and fixed in 2% paraformaldehyde and stored in PBS at 4°C . Microglial cultures infected at an MOI of 0.1 were lytically infected in less than 5 days and the cells were destroyed. For this reason, the MOI was dropped to 0.01 pfu cell^{-1} for these studies. Microglial cultures were immunohistochemically stained for both RCA-1 and the presence of HCMV antigens. HCMV antigens were identified by incubation with a monoclonal antibody cocktail against HCMV immediate early and early antigens (Dako, Carpinteria, CA) diluted 1:40 for 60 min at room temperature. Binding of the primary antibody was resolved using an avidin-biotin peroxidase kit (Vector Laboratories) and the substrate diaminobenzidine (DAB).

Cytokine production from HCMV-infected macrophages/microglia

After 5 days of HCMV infection, one half of the medium was exchanged and saved; after 10 days, medium was collected and the experiment terminated. Supernatants were ultracentrifuged over a

20% sucrose cushion to remove all virus particles and stored at -70°C until further study. An aliquot was assayed on HFF to confirm that the supernatants were free of virus. Supernatants were tested for mycoplasma using an ELISA kit (Boehringer-Mannheim) and for the cytokines $\text{TNF}\alpha$, IL-6 and IL-1B using the Quantikine ELISA kits (R & D Systems Inc, Minneapolis, MN).

Brain aggregate cultures

Human fetal brain tissue between 16 and 20 weeks gestation was obtained from elective abortuses in accordance with guidelines established by the University of California, San Francisco Committee on Human Experimentation. This model system has been characterized previously (Pulliam et al, 1988). Briefly, brain tissue was gently dissociated through nylon screens to obtain single cells and viability was determined by trypan blue exclusion. Cells were counted on a hemocytometer and 4×10^7 cells in 4 ml Dulbecco minimum essential media (DMEM) supplemented with 0.6% dextrose, $50\text{ }\mu\text{g ml}^{-1}$ gentamicin and 10% FCS were distributed into 25 ml DeLong flasks. Aggregate cultures were incubated at 37°C in an atmosphere of 10% CO_2 with constant rotation. After 2 to 3 days, the brain cultures were transferred to 50 ml DeLong flasks and 5 ml DMEM supplemented with 15% FCS (exchange media) was added to each flask. Media were exchanged every other day throughout experimentation. After 10 days in culture and before each experiment, aggregates were tested for viability by trypan blue exclusion and embedded for histology. Brain aggregates were thought to be indicative of mature neural cells since they stained immunohistochemically with neuronal and astrocytic markers (neuron specific enolase and glial fibrillary acidic protein). Brain aggregates consisted of the predominant cells of the central nervous system including approximately 40% neurons, 40% astrocytes, 10% oligodendrocytes and 10% microglia.

HCMV infection of brain aggregates, virus detection and IL-6 and $\text{TNF}\alpha$ production

After 10–12 days in culture, brain aggregates were infected with HCMV at an MOI of 1 pfu per aggregate. Virus was allowed to adsorb for 60 min with constant rotation. Aggregates were washed and resuspended in exchange media. Five ml of media was exchanged every 2–3 days. Mock-infected brain cultures were prepared and sampled in parallel with the HCMV-infected cultures. After 5 and 10 days of infection, samples of supernatant were taken for cytokine production. Supernatants were ultracentrifuged and assayed on HFF to confirm that they were free of virus. Supernatants were tested for the cytokines $\text{TNF}\alpha$ and IL-6 by ELISA.

After 14 days of infection, aggregates were washed with PBS, fixed in 2% paraformaldehyde and embedded for histology, immunohistochem-

istry and *in situ* hybridization. HCMV antigens were identified by incubation with a monoclonal antibody cocktail against HCMV immediate early and early antigens (Dako) diluted 1:40 for 60 min at room temperature. Binding of the primary antibody was resolved using an avidin-biotin peroxidase kit (Vector Laboratories) and the substrate DAB. *In situ* hybridization for CMV DNA was performed on deparaffinized brain aggregate sections using a kit from ENZO diagnostics (Farmingdale, New York), according to the manufacturer's directions.

Treatment of brain aggregates with HCMV-infected macrophage and microglial cell supernatants

Supernatants from HCMV-infected and uninfected macrophages and microglial cells were processed as described above. Aggregates were treated with 20% HCMV-infected or uninfected macrophage or microglial supernatants within exchange media for 7 days. Half of the media plus treatment was exchanged after the third day. After treatment, the aggregates were sampled for trypan blue exclusion and fixed in 2% paraformaldehyde for histology.

Treatment of brain aggregates with IL-6 and TNF α

Brain aggregates were treated with 500 pg ml⁻¹ of human recombinant IL-6 (Genzyme Inc, Cambridge, MA) and 50 pg ml⁻¹ of human recombinant TNF α (Sigma Chemical Co, St Louis, MO) for 7 days. After treatment, aggregates were fixed in 2% paraformaldehyde for histology.

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Statistical analysis

Levels of IL-6 and TNF α from HCMV-infected macrophage and microglial cell cultures were compared to uninfected cultures using a modified *t*-statistic (Brownie *et al*, 1990). This modified *t*-statistic allows the standard deviations to be larger in the infected than in the uninfected; the standard *t* test assumes that the standard deviation for the infected results are equal to the standard deviation for the uninfected. The modified *t*-statistic is given by

$$\text{mod-}t = \frac{X_2 - X_1}{s_1 \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \quad (1)$$

where X_1 and X_2 are the means of the uninfected and infected, s_1 is the standard deviation of the uninfected and n_1 and n_2 are the sample sizes for the uninfected and infected, respectively. Under the null hypothesis that the uninfected and infected have the same distribution, this statistic has a *t* distribution with $n_1 - 1$ degrees of freedom.

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