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Interleukin-6 induction in vitro in mouse brain endothelial cells and astrocytes by exposure to mouse hepatitis virus (MHV-4, JHM)

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Summary

Interleukin-6 (IL-6) induction, as detected by bioassay and Northern analysis, was examined in vitro in endothelial cells or astrocytes derived from BALB/c (susceptible) or SJL (resistant) mice following exposure to mouse hepatitis virus (MHV-4) or UV inactivated MHV-4 (UV-MHV-4). In BALB/c endothelial cells, up to 16-fold more IL-6 (> 640 U/ml) was induced, compared to SJL cells which showed a minimal response (40 U/ml), relative to basal levels (< 20 U/ml). In contrast, both BALB/c and SJL astrocytes showed a substantial IL-6 response to MHV-4 and UV-MHV-4 exposure, although a strain difference persisted. Despite strain and cell specific differences in released IL-6, equivalent levels of IL-6 mRNA were induced in all cell types following exposure to MHV-4 or UV-MHV-4.

Introduction

Mouse hepatitis virus (MHV-4, JHM) is a neurotropic coronavirus which causes a spectrum of disease ranging from fatal encephalomyelitis to demyelination in susceptible murine hosts (Bailey et al., 1949). Both direct infection of oligodendrocytes and immune mediated events have been reported to play a role in the pathologic events in the central nervous system (CNS) following MHV-4 infection (Knobler et al., 1981, 1982; Wang et al., 1990; Williamson et al., 1991). The identification of $CD8^+$ T-cells, NK cells, B-cells and PMN in the CNS of MHV-JHM infected mice suggests that immune mechanisms may be playing a role in the virus induced disease process (Knobler et al., 1982; Stohlman et al., 1983; Welsh et al., 1983; Natuck et al., 1987; Williamson et al., 1991; Zimprich et al., 1991).

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The role of locally released cytokines in the regulation of immune and inflammatory events in the CNS following mouse hepatitis virus infection has not yet been systematically studied. Cytokines play a critical role in the modulation of immune and inflammatory events, important in both anti-viral immunity, and the virus induced pathology observed in the CNS. Endothelial cells and astrocytes in the CNS are potential sources of cytokines and have been demonstrated to synthesize interleukin-6 (IL-6) in response to treatment with tumor necrosis factor (TNF), interleukin-1 (IL-1) and lipopolysaccharide (Frei et al., 1989; Jirik et al., 1989; Lieberman et al., 1989; Shalaby et al., 1989; Sironi et al., 1989; Beneveniste et al., 1990). Endothelial cells and astrocytes in the CNS are also readily infected by MHV-4 and the products released by these cells may be important in regulating immune mediated events in the CNS (Joseph et al., 1989, 1990, 1991).

IL-6 was chosen for this study because its release following infection has been demonstrated in other virus model systems (Frei et al., 1988, 1989; Houssiau

et al., 1988; Breen et al., 1990; Nishimoto et al., 1990). Additionally, IL-6 is a multifunctional cytokine with immunoregulatory effects on B-cells, T-cells and neutrophil functions (Cernetti et al., 1988; Takai et al., 1988; Borish et al., 1989; Le and Vilcek, 1989).

We demonstrate that IL-6 induction in MHV-4 exposed endothelial cells or astrocytes is strain dependent. BALB/c (MHV-4 susceptible) derived endothelial cells can produce up to 16-fold higher levels of IL-6, and release this earlier than SJL (MHV-4 resistant), as determined both by bioassay and Northern analysis. Active infection is not necessary since exposure to UV inactivated MHV-4 (UV-MHV-4) can also induce IL-6 in these cells.

Materials and Methods

Endothelial cell culture

Cerebral endothelial cells were isolated from the brains of BALB/c (MHV susceptible) and SJL (MHV resistant) mice as previously described (DeBault et al., 1981). Endothelial cell lines obtained were maintained in Medium 199 with 20% fetal bovine serum and additional supplements that included Basal Medium Eagle (BME) amino acids, BME vitamins, glutamine, Bacto-peptone and penicillin–streptomycin. Endothelial identity was established by studying the uptake of DiI-Ac-LDL (Biomedical Technologies, Stoughton, MA, USA) and specific binding of fluorescein labeled *Griffonia simplicifolia* agglutinin (Voyta et al., 1984; Sahagun et al., 1989). The purity of endothelial cell cultures was greater than 95%, as determined by these methods.

Astrocyte cultures

Astrocyte cultures were established from BALB/c and SJL mice using the previously described method (Knobler et al., 1987) adapted from McCarthy and deVellis (1980). Brain cortices derived from 14-day mouse embryos were mechanically dissociated through Nitex mesh bags (210 μm). Astrocytes were cultured in DMEM and Ham's F-12 nutrient medium, 1:1, containing 10% fetal bovine serum. Astrocytic identity of these cells was established by their positive reactivity to an antibody directed against glial fibrillary acidic protein. The purity of the astrocyte cultures was greater than 95%, as determined by this method.

Virus infection and collection of supernatants

Endothelial cells or astrocytes grown in T-25 flasks (1×10^6 cells/flask) were infected with MHV-4 or UV-MHV-4 for 1 h at 37°C using a multiplicity of infection (MOI) of 0.1. This MOI reflects the working titer of virus that elicits a cellular response without extensive cytolytic effects based on previous studies (Joseph et al., 1989, 1990, 1991). MHV-4 was grown to a stock containing 5×10^5 PFU/ml as previously described (Knobler et al., 1981). UV inactivation of virus (UV-MHV-4) was carried out for 30 min (1250 $\mu W/cm^2$) under a Spectroline UV-illuminator (Fisher Scientific, Springfield, NJ, USA). Virus infectivity was tested by plaque assays on L-2 cells (Knobler et al., 1982) to determine if the inactivation was complete following UV treatment.

After a 1-h incubation of the cells with MHV-4 or UV-MHV-4 the cultures were washed three times with phosphate buffered saline (PBS). After washes the cultures were fed with endothelial cell or astrocyte culture medium. Supernatants were collected on day 1–4 after virus exposure for testing in an IL-6 bioassay.

Bioassay for IL-6

The proliferation bioassay for IL-6 was performed using an IL-6 dependent B-cell hybridoma (T1165tc) (Jayaraman et al., 1990). The T1165tc cells were cultured at a density of 1×10^4 cells/well in the presence of endothelial or astrocyte supernatants collected on day 1–4 after MHV-4 or UV-MHV-4 treatment. The supernatants from MHV-4 infected cells were UV-inactivated prior to testing for IL-6 in order to prevent virus mediated killing of the IL-6 dependent cell line. On day 3 cells were pulsed with [^{3}H]thymidine for 6 h and then counted in triplicate in a beta counter as a measure of IL-6 induced proliferation. In order to test the specificity of the proliferative response to IL-6, a neutralizing rat anti-mouse IL-6 monoclonal antibody (Genzyme Corporation, Cambridge, MA, USA) was used. The anti-IL-6 antibody was used at a concentration of 5 μg/ml.

RNA isolation and Northern analysis

Four hours after exposure of endothelial cells and astrocytes to MHV-4, UV-MHV-4 or TNF (200 U/ml, Genzyme, Boston, MA, USA), total cellular RNA was isolated by immediate solubilization of cells in guanidine hydrochloride by standard procedures (Maniatis et al., 1982). The solution was sonicated and centrifuged to remove cellular debris. The RNA was ethanol precipitated, phenol extracted and then reprecipitated with ethanol. The RNA was quantified by absorbance at 260 nm and by visual estimation of ribosomal RNA content by ethidium bromide staining following electrophoresis in denaturing formaldehyde gels. RNA was transferred to nylon membranes (Hybond-N, Amersham, Arlington Heights, IL, USA) and fixed by UV irradiation. The RNA was prehybridized in 1 M NaCl, 0.1% SDS, 1.5 mg/ml salmon sperm DNA, and 10% dextran for 3 h at 45°C. The RNA was then hybridized overnight at 45°C with a radiolabeled murine IL-6 cDNA probe (obtained from Dr. Frank Lee, DNAX, Palo Alto, CA, USA). The probe was

radiolabeled (^{32}P) in low melting agarose by random hexamer priming. The blots were washed under high stringency conditions (0.1 × SSC, 0.1% SDS, 1 mM EDTA (pH 8), 10 mM sodium phosphate (pH 6.8) at 45°C) and analyzed by autoradiography with intensifying screens (Dupont, Hoffman Estates, IL, USA). Densitometry of the autoradiographs was carried out using a Macbeth densitometer (Model TD-932, Macbeth Process Measurements, Newburg, NY, USA).

Results

IL-6 induction in cerebral endothelial cells

Tables 1 and 2 demonstrate that IL-6 is induced in both BALB/c and SJL derived endothelial cells following exposure to MHV-4 as determined by bioassays, albeit at very different levels. In the MHV-4 susceptible BALB/c cells, the peak of IL-6 induction occurred on day 2 of infection, and the levels of IL-6 were 16-fold higher (> 640 U/ml) than those obtained from the MHV-4 resistant SJL cells (Table 2). The level of IL-6 release from SJL endothelial cells (40 U/ml), following exposure to MHV-4, was not substantially different from the basal levels (< 20 U/ml) detected in control cultures. Active infection of the cells was not apparently necessary since UV inactivated virus also induced IL-6 in a strain dependent fashion. In Table 1 the drop of IL-6 levels on day 3 and the subsequent increase on day 4 following UV-MHV-4 treatment of BALB/c endothelial cells was an isolated observation in the representative experiment presented. The general trend was toward a gradual decline in IL-6 levels from day 3 onward.

IL-6 induction in astrocytes

Tables 3 and 4 demonstrate that IL-6 was induced in both BALB/c and SJL astrocytes by MHV-4, as determined by bioassay of the culture supernatant.

TABLE 1

Quantitation of IL-6 levels induced following MHV-4 treatment of BALB/c (MHV susceptible) endothelial cells

	MHV-4 (U/ml) A	MHV-4 (U/ml) B	UV-MHV-4 (U/ml) C	UV-MHV-4 (U/ml) D
Day 1	288	0	115	0
Day 2	≫ 640	0	> 640	0
Day 3	> 640	≪ 10	254	0
Day 4	490	≪ 10	416	0

IL-6 units were determined by the dilution of supernatants yielding half maximal proliferation. The data presented in rows B and D show the effect of rat anti-mouse IL-6 monoclonal antibody (5 μg/ml) in neutralizing the IL-6 activity in the supernatants. IL-6 levels in cell culture supernatants on day 0 were ≪ 20 U/ml.

TABLE 2

Quantitation of IL-6 levels induced following MHV-4 treatment of SJL (MHV resistant) endothelial cells

	MHV-4 (U/ml) A	MHV-4 (U/ml) B	UV-MHV-4 (U/ml) C	UV-MHV-4 (U/ml) D
Day 1	40	0	≪ 20	0
Day 2	40	0	≪ 20	0
Day 3	40	0	≪ 20	0
Day 4	ND	ND	40	0

IL-6 units were determined by the dilution of supernatants yielding half maximal proliferation. The data presented in rows B and D show the effect of rat anti-mouse IL-6 monoclonal antibody (5 μg/ml) in neutralizing the IL-6 activity in the supernatants. IL-6 levels in cell culture supernatants on day 0 were ≪ 20 U/ml.

Levels of IL-6 were maximal on day 1 (> 640 U/ml) after MHV-4 infection of BALB/c astrocytes. The levels of IL-6 continued to remain high even 4 days post infection. In contrast, IL-6 activity in SJL astrocytes peaked later, on day 2 (402 U/ml), but were not sustained, declining from day 3 onward. As seen with the endothelial cell cultures, UV inactivated virus was also able to induce IL-6 (Table 2).

Testing for specificity of IL-6 activity

To confirm that IL-6 in the supernatants being tested was the inductive signal for proliferation of the IL-6 dependent cell line a neutralizing monoclonal anti-mouse IL-6 antibody was used. As shown in Tables 1–4, the proliferative response in all cases was blocked by the antibody to IL-6. This confirms that IL-6 activity was present in supernatants collected after exposure of endothelial cells or astrocytes to MHV-4 and UV-MHV-4.

Testing for the presence of TNF

The MHV-4 preparation used in these studies was obtained by infection and sonication of L-cells. It is

TABLE 3

Quantitation of IL-6 levels induced following MHV-4 treatment of BALB/c (MHV susceptible) astrocytes

	MHV-4 (U/ml) A	MHV-4 (U/ml) B	UV-MHV-4 (U/ml) C	UV-MHV-4 (U/ml) D
Day 1	> 640	≪ 10	561	0
Day 2	> 640	< 10	518	0
Day 3	> 640	10	> 640	≪ 10
Day 4	> 640	< 10	ND	ND

IL-6 units were determined by the dilution of supernatants yielding half maximal proliferation. The data presented in rows B and D show the effect of rat anti-mouse IL-6 monoclonal antibody (5 μg/ml) in neutralizing the IL-6 activity in the supernatants. IL-6 levels in cell culture supernatants on day 0 were ≪ 20 U/ml.

TABLE 4

Quantitation of IL-6 levels induced following MHV-4 treatment of SJL (MHV resistant) astrocytes

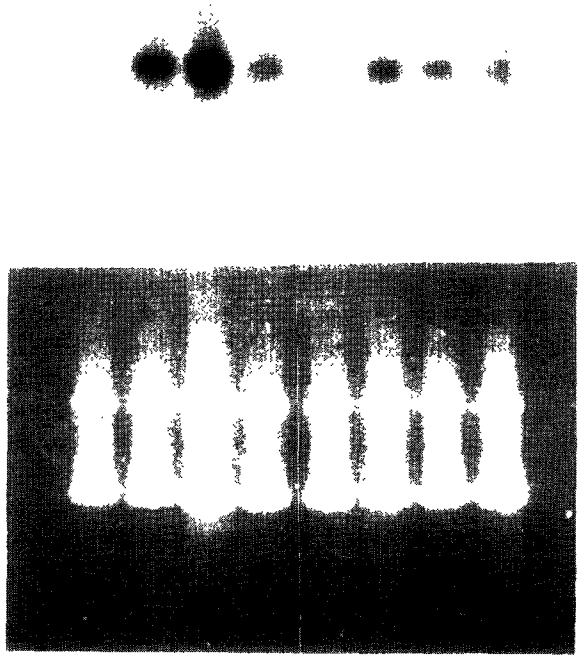
	MHV-4 (U/ml) A	MHV-4 (U/ml) B	UV-MHV-4 (U/ml) C	UV-MHV-4 (U/ml) D
Day 1	73	0	106	0
Day 2	402	< 10	128	0
Day 3	176	< 10	70	0
Day 4	126	< 10	47	0

IL-6 units were determined by the dilution of supernatants yielding half maximal proliferation. The data presented in rows B and D show the effect of rat anti-mouse IL-6 monoclonal antibody (5 μg/ml) in neutralizing the IL-6 activity in the supernatants. IL-6 levels in cell culture supernatants on day 0 were < 20 U/ml.

possible that TNF was produced by the L-cells during MHV-4 production. TNF can induce IL-6 in various cell types, including endothelial cells and astrocytes (Shalaby et al., 1989; Beneveniste et al., 1990). Therefore a bioassay was employed to rule out the presence of TNF in the virus preparation used in our studies. This bioassay is based on the ability of TNF to inhibit proliferation of a mouse fibrosarcoma cell line, WEHI 164.8.5. UV-MHV-4 L-cell lysates (virus stock) did not inhibit the proliferation of the WEHI 164.8.5 cells. This documented the absence of detectable TNF activity in the L-cell derived virus preparation used for the IL-6 induction studies. UV-MHV-4 was used in these studies to avoid the cytolytic effects of MHV-4 on the WEHI 164.8.5 cells. A positive control using recombinant murine TNF (Genzyme, Cambridge, MA, USA) at doses as low as 6 U/ml caused a 100-fold decrease in proliferation of the WEHI 164.8.5 cells.

Induction of IL-6 mRNA in astrocytes

Northern analysis was performed to look for differences in the induction of IL-6 mRNA between BALB/c and SJL derived astrocytes (Fig. 1). The IL-6 cDNA probe (moIL-6 cDNA, DNAX, Palo Alto, CA, USA), used in our studies, hybridizes to a 1.3-kb species (Frei et al., 1989). The RNA from untreated cells (lanes A and E) showed no evidence for induction of IL-6 mRNA. In contrast, IL-6 mRNA induction was noted in lanes B–D and F–G. IL-6 mRNA was induced to a comparable degree in both BALB/c (susceptible) and SJL (resistant) derived astrocytes following exposure to either MHV-4 (lanes B and F), UV-MHV-4 (lanes C and G) or TNF at 200 U/ml (lanes D and H) for 4 h. TNF exposure was included as a positive control for IL-6 induction. Densitometric analysis did not indicate major differences in IL-6 mRNA induction in BALB/c astrocytes relative to SJL cells (Table 5). A similar pattern of MHV-4 induction was obtained when evaluating IL-6 mRNA of endothelial cells (data not shown).



A B C D E F G H

Fig. 1. Northern blot analysis of IL-6 mRNA induction. Lanes A–D show RNA derived from BALB/c (MHV susceptible) astrocytes. The IL-6 cDNA probe (moIL-6 cDNA, DNAX, Palo Alto, CA, USA), used in our studies, hybridizes to a 1.3-kb species (Frei et al., 1989). Lanes E–H show RNA derived from SJL (MHV resistant) astrocytes. Cells were treated for 4 h as follows. Lane A, untreated; B, MHV-4; C, UV inactivated MHV-4; D, TNF (Genzyme, 200 U/ml); E, untreated; F, MHV-4; G, UV inactivated MHV-4; H, TNF (Genzyme, 200 U/ml). The top panel shows the autoradiograph and the lower panel is the ethidium bromide profile of the gel (5 μg of RNA loaded per lane).

These results show that IL-6 mRNA is induced at comparable levels in the different strains and cell types, although there are dramatic differences in the quantity of IL-6 released. This suggests that these differences may be accounted for by altered translation or post-translational processing.

TABLE 5

Densitometric analysis using an autoradiograph of Northern blot detecting IL-6 mRNA from MHV susceptible and resistant strain derived astrocytes

	BALB/c	SJL
MHV-4	0.89	0.74 [a]
UV-MHV-4	1.48	0.66
TNF	0.70	0.53

[a] Arbitrary optical density units determined using a Macbeth densitometer (Model TD-932, Macbeth Process Measurements, Newburg, NY, USA).

Discussion

The study of the immune and inflammatory events in the CNS following infection with MHV-4 is important in understanding the pathologic events and mechanisms of virus induced demyelination. Immunoregulatory cytokines can profoundly affect the cascades of both humoral and/or cell mediated immune events in the CNS. However, there has not yet been a systematic analysis of cytokine induction following MHV infection of CNS derived cells.

The cytokine IL-6 was studied because of (1) demonstration of its release following viral infections with LCMV (lymphocytic choriomeningitis virus), VSV (vesicular stomatitis virus), HIV (human immunodeficiency virus) and HTLV-1 (human T-cell leukemia virus), and (2) its multifunctional immunoregulatory role in modulating T, B and neutrophil functions (Cernetti et al., 1988; Frei et al., 1988, 1989; Houssiau et al., 1988; Takai et al., 1988; Borish et al., 1989; Le and Vilcek, 1989; Breen et al., 1990; Nishimoto et al., 1990).

We report on the induction of IL-6 in cultures of cerebral endothelial cells or astrocytes following infection with MHV-4. The MHV susceptible BALB/c derived endothelial cells and astrocytes produce substantially higher levels of IL-6 (> 640 U/ml), and at earlier time points, than resistant SJL derived cells. The SJL endothelial cells yield barely more (40 U/ml) than basal levels (< 20 U/ml) of IL-6 in response to MHV-4. In contrast, SJL astrocytes are induced to release a significant quantity of IL-6 (402 U/ml), although not as great as in the BALB/c astrocytes (> 640 U/ml). Therefore, the strain differences in IL-6 induction in response to MHV-4 are more striking between the endothelial cells than with the astrocytes.

The mechanisms regulating this strain dependent induction of IL-6 are under further study. One possible explanation may reflect the differences in MHV receptor expression on endothelial cells and astrocytes. MHV binding receptors have been demonstrated on BALB/c but not SJL derived intestinal brush border and liver cells (Williams et al., 1990). The binding of the virus to its receptor on the cell surface may activate a signal transduction pathway for IL-6 production.

The results obtained with UV-MHV-4 suggest that infection is not required and that the binding of the viral particles to its receptor on the cell surface may be sufficient to trigger the release of IL-6. This ability of UV-MHV-4 to exert a biological effect is not unique to the induction of IL-6. Previous studies in our laboratory have demonstrated that UV-MHV-4 can block γ-interferon induced MHC class II antigen expression on endothelial cells to the same degree as infectious virus (Joseph et al., 1991).

The greater degree of difference in IL-6 induction noted in cerebral endothelial cells compared to astrocytes is intriguing in the face of the potential role of this cytokine in triggering a variety of immune mediated inflammatory mechanisms. Since the MHV-4 replication in BALB/c mouse brain is two to three logs greater than in SJL mice (Knobler et al., 1982), and there is a correspondingly greater inflammatory response to MHV-4 infection in BALB/c than SJL mice, this observation deserves further investigation. Current studies are directed at elucidating these mechanisms.

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