CYTOKINE INDUCTION *IN VITRO* IN MOUSE BRAIN ENDOTHELIAL CELLS AND ASTROCYTES BY EXPOSURE TO MOUSE HEPATITIS VIRUS (MHV-4, JHM)

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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 (1). 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 (2-5). 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 (3-9).

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 IL-6 in response to treatment with tumor necrosis factor (TNF), interleukin-1 (IL-1) and lipopolysaccharide (10-16). 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 (17-19).

The cytokine IL-6 was chosen for this study because its release following infection has been demonstrated in other virus model systems (11, 20-22). Additionally, IL-6 is a multifunctional cytokine with immunoregulatory effects on B-cells, T-cells and neutrophil functions (23-26).

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

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to sixteen 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

Virus Infection and Collection of Supernatants

Cerebral endothelial cell and astrocyte cultures were established from the brains of BALB/c (MHV-susceptible) and SJL (MHV-resistant) strains of mice as previously described (27-29). Endothelial cells or astrocytes grown in T-25 flasks (1 x 10^6 cells/flask) were infected with MHV-4 or UV-MHV-4 for 1 hour at 37^{0}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 (17-19).

After a one hour 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 proliferation bioassay described below.

Bioassay for IL-6

The proliferation bioassay for IL-6 was performed using an IL-6 dependent B cell hybridoma (T1165tc) (30). 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) was used.

RNA Isolation and Northern Analysis

Four hours after exposure of endothelial cells and astrocytes to MHV-4, UV-MHV-4 or tumor necrosis factor (200U/ml, Genzyme, Boston, MA), total cellular RNA was isolated by immediate solubilization of cells in guanidine hydrochloride by standard procedures (Maniatis et al., 1982). The RNA was then hybridized overnight at 45^{0}C with a radiolabeled murine IL-6 cDNA probe (obtained from Dr. Frank Lee, DNAX, Palo Alto, CA), washed under high stringency conditions 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).

RESULTS

Interleukin-6 Induction in Cerebral Endothelial Cells

Table 1 demonstrates 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 sixteen fold higher ($>$640 U/ml) than those obtained from the MHV-4 resistant SJL cells. The level of IL-6 release from SJL endothelial cells (40 U/ml), following exposure to MHV-4, was not substantially different from the basal levels ($<$20U/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.

Interleukin-6 Induction in Astrocytes

Table 2 demonstrates that IL-6 was induced in both BALB/c and SJL astrocytes by MHV-4, as determined by bioassay of the culture supernatant. Levels of IL-6 were maximal on day 1 (>640U/ml) after MHV-4 infection of BALB/c astrocytes. The levels of IL-6 continued to remain high even four days post-infection. In contrast, IL-6 activity in SJL astrocytes peaked later, on day 2 (402U/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). In order to confirm the specificity of the proliferative signal a neutralizing rat anti-mouse monoclonal IL-6 antibody was used (Tables 1 and 2).

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 1.3kb species (11). 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 200U/ml (lanes D and H) for 4 hours. TNF exposure was included as a positive control for IL-6 induction. A similar pattern of MHV-4 induction was obtained when evaluating IL-6 mRNA of endothelial cells (data not shown). 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.

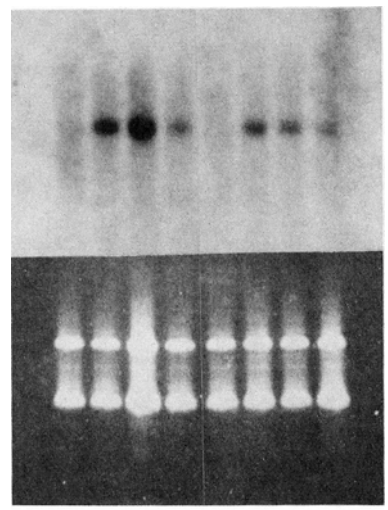

Fig. 1. Northern blot analysis of IL-6 mRNA induction. Lanes **A-D** shows RNA derived from Balb/c (MHV-susceptible) astrocytes. Lanes **E-H** shows RNA derived from SJL (MHV-resistant) astrocytes. Cells were treated for 4 hours as follows, Lane **A**. Untreated, **B**. MHV-4, **C**. UV-inactivated MHV-4, **D**. Tumor Necrosis factor (Genzyme, 200U/ml), **E**. Untreated, **F**. MHV-4, **G**. UV-inactivated MHV-4, **H**. Tumor Necrosis Factor (Genzyme, 200U/ml). The top panel shows the autoradiograph and the lower panel is the ethidium bromide profile of the gel (5μgms of RNA loaded per lane)

Table 1. Quantitation of IL-6 levels induced following MHV-4 treatment of endothelial cells

		MHV-4		U.V. MHV-4	
Day p.i.		A	B	C	D
1	Balb/c	288*	0	115	0
	SJL	40	0	20	0
2	Balb/c	640	0	640	0
	SJL	40	0	20	0
3	Balb/c	640	10	254	0
	SJL	40	0	20	0
4	Balb/c	490	10	416	0
	SJL	ND	ND	40	0

Table 2. Quantitation of IL-6 levels induced following MHV-4 treatment of astrocytes

		MHV-4		U.V. MHV-4	
Day p.i.		A	B	C	D
1	Balb/c	640*	10	561	0
	SJL	73	0	106	0
2	Balb/c	640	10	518	0
	SJL	402	10	128	0
3	Balb/c	640	10	640	10
	SJL	176	10	70	0
4	Balb/c	640	10	ND	ND
	SJL	126	10	47	0

*units/ml

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

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 (10, 11, 20-26).

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 ($>$640U/ml), and at earlier time points, than resistant SJL derived cells. The SJL endothelial cells yield barely more (40U/ml) than basal levels ($<$20U/ml) of IL-6 in response to MHV-4. In contrast, SJL astrocytes are induced to release a significant quantity of IL-6 (402 U/ml), although not as great as in the BALB/c astrocytes ($>$640U/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 is 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 (32). 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 suggests 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 interleukin-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 (19).

The greater degree of differences 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 (3), 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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