Differential Expression of Tumor Necrosis Factor in Primary Glial Cell Cultures Infected with Demyelinating and Non-Demyelinating MHVs

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1. INTRODUCTION

TNF-α is a monocyte/macrophage derived cytokine with multiple biological activities that are antitumorigenic, cytotoxic, and pro-inflammatory in nature. TNF-α mediates cellular response through two distinct cell surface receptors, TNF-R1(p55) and TNF-R2(p75) (Barbara *et al* 1996). TNF-α appears to have a diverse range of functions in the CNS due to its effect on astrocytes and oligodendrocytes. These include: induction of MHC class I Ag on astrocytes (Lavi *et al* 1988; Mauerhoff et al 1988), induction of ICAM-1, up-regulation of MHC class II Ag induced by IFN-γ and/or virus on astrocytes, stimulation of IL-6 secretion by astrocytes, myelin damage (Selmaj and Raine 1988) and lysis of oligodendrocytes (Robbins *et al* 1987).

The multiple effects of this cytokine on various cell populations in the CNS, including autocrine stimulation of astrocytes, suggest that TNF-α may have a central role in augmenting intracerebral immune responses and inflammatory demyelination (Chung and Benveniste 1990; Chung *et al* 1991). The hypothesis of our study is that TNF-α may play a role in MHV-A59 induced demyelination. To test this hypothesis we infected astrocyte cultures and mice with viruses of different biologic phenotypes (demyelination-positive MHV-A59, and demyelination-negative MHV-2)

The Nidoviruses (Coronaviruses and Arteriviruses).
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and examined the levels of TNF-α mRNA and protein by quantitative RT-PCR and ELISA, respectively.

2. MATERIALS AND METHODS

2.1 Primary astrocytes cultures

Primary astrocytes cultures were prepared from neonatal C57BL/6 mouse brains following removal of the meninges (Mauerhoff *et al* 1988; Shahar *et al* 1989) and maintained in DMEM, 6 g/liter glucose, and 20% FBS. After the second passage the FBS concentration was reduced to 10%. The astrocytes were stained with monoclonal antibody to GFAP (1:4) for 30 min at room temperature, followed by 30 min incubation with Goat anti-mouse Ig-FITC (1:20). Astrocytes cultures were routinely > 95% positive for GFAP. The experiments were performed between 3-5 passages.

2.2 Mice, and viruses

Four- week-old C57BL/6 mice (Jackson Laboratories Bar Harbor, ME) were used in these experiments. The following viruses were used: MHV-A59 (Budzilowicz *et al* 1985; Lavi *et al* 1984), MHV-2 (Keck *et al* 1988).

2.3 TNF-a production by astrocytes

Primary mouse astrocytes were resuspended in DMEM containing 10% FBS, and plated at 1 X 10^6 cell/well into 6 well plates (Costa, cambridge, MA). The plates were incubated overnight to allow recovery of the cells from trypsinization and to assure adherence of the astrocytes. When the astrocytes reached confluence, the original media was aspirated off, and 2 ml 1 X DMEM containing 2% FBS was added to the wells. Astrocytes were infected with MHV-A59 (m.o.i.=1) or MHV-2 (m.o.i.=1) for one hour followed by washing with PBS. Alternatively, cultures were treated with LPS (1μg/ml), or a combination of the above (1 hour infection, followed by LPS treatment). Cultures were maintained for 12-18 hours following treatment, then supernatants were collected, centrifuged to remove contaminating cells, and stored at -70°C until use.

2.4 Infection of mice

Viruses were diluted in PBS containing 0.75% BSA. Mice were anesthetized with methoxyflurane (Methofane, Pittman-Moore, Mundelein, IL), then 25μl of diluted viruses were injected into the left cerebral hemisphere at the following doses: MHV-A59 3000pfu/ml, MHV-2 1000pfu/ml.

2.5 Quantitative PCR analysis

Livers, brains, and spinal cords were obtained from mice at 5 days (acute phase) or 30 days (chronic phase) post-infection. Total RNA was extracted from 1 x 10^7 Cells or 1g tissue using QIAGEN RNA easy Kit, then tested by qRT-PCR using a mimic method based on competitive PCR with non-homologous internal standards called PCR MIMICs (Clontech, Palo Alto, CA). Each PCR MIMIC consists of a heterologous DNA fragment with primer templates that are recognized by a pair of gene-specific primers. Thus, these templates " mimic " the target and are amplified during PCR.

Two composite primers are used. Each composite primer has the target gene primer sequence attached to a short, 20-nucleotide stretch of sequence designed to hybridize to opposite strands of a MIMIC DNA fragment. The desired primer sequences are thus incorporated during PCR reaction. A dilution of the first PCR reaction is then amplified again using only the gene specific primers. This ensures that all PCR MIMIC molecules have the gene specific primer sequences. Following the second PCR amplification the PCR MIMIC is purified by passage through CHROMA SPIN+TE-100 Columns. The yield of PCR MIMIC is calculated and diluted to 100 attomole/μl. Serial dilutions of PCR MIMICs are added to PCR amplification reactions containing constant amounts of experimental cDNA sample. Thus, PCR MIMIC and target templates, compete for the same primers in the same reactions. By knowing the amount of PCR MIMIC added to the reaction, we can determine the amount of target template, thus the initial mRNA levels.

2.6 TNF-α protein assay

Samples were assayed by ELISA according to the modified protocol from Pharmingen. The 96 well plates were read on a Titertek Multiscan using a wavelength of 405nm.

2.7 Measurement of viral titers

Samples of media from infected cultures were collected at 48 hours post infection and stored at -70^0C until tested. Viral titers were measured by a 6-well-plate plaque assay of duplicate 10-fold dilutions of the samples.

3. RESULTS

3.1 Detection of TNF-α mRNA in MHV-A59 and MHV-2 infected astrocytes by competitive RT-PCR

RNA was isolated from uninfected MHV-A59-infected or MHV-2-infected astrocytes. We synthesized the corresponding cDNA by reverse transcription, and then used PCR to amplify a specific sequence of the TNF-α cDNA and TNF-α MIMIC cDNA. The amplified TNF-α cDNA was detected in astrocytes infected with MHV-A59 and MHV-2 but not in uninfected astrocyte cultures. Competitive PCR analysis showed that the level of TNF-α mRNA in MHV-A59 infected astrocytes was 80-fold higher than in astrocytes infected with MHV-2. To rule out that the differences were due to differences in viral replication in the cultures viral titers were measured and found to be similar (approximately 10^5PFU/ml in both infections).

3.2 Detection of TNF-α mRNA in tissues of mice infected with MHV-A59 and MHV-2

Mice were injected intracerebrally with MHV-A59 or MHV-2 and sacrificed at 5 or 30 days post infection. RNA was isolated from the livers, brains and spinal cords. Competitive RT-PCR was used to compare the level of TNF-α mRNA in tissues infected with the different viruses.

As shown in table 1, the amplified TNF-α mRNA was detected in livers, brains and spinal cords in both MHV-A59 and MHV-2 infected mice at 5 days post infection, but not in uninfected mice. TNF-α mRNA in the MHV-A59 infected brain and spinal cord was 2.5 and 10 fold higher than the brain and spinal cord of MHV-2 infected mice respectively. Furthermore, at 30 days post infection, TNF-α mRNA was only detected in the MHV-A59 infected brains and spinal cords but not in MHV-2 infected mice.

3.3 The effect of MHV-A59 and MHV-2 infection on TNF-α production in mouse astrocytes cultures

Astrocytes were incubated with either MHV-A59 or MHV-2 (m.o.i.=1) for 1 hour, then viruses were washed out and cultures were maintained for the interval of 18 hours. In this experiment, the LPS (1μg/ml) was used as a positive control and also combined with MHV-A59 or MHV-2 separately. After 18 hours, the supernatants were harvested and assayed for the concentration of soluble TNF-α. Unstimulated astrocytes did not produce detectable levels of TNF-α. MHV-2 alone did not induce astrocytes to secrete TNF-α, but when combined with LPS the induction of TNF-α was significantly increased. Astrocytes could also be stimulated by a combination of LPS and MHV-A59 to secrete TNF-α. The TNF-α secretion stimulated by MHV-A59 was significantly higher than LPS alone or MHV-2 combined with LPS.

Table 1. TNF-α mRNA Detected by Competitive PCR in Mice and astrocyte cultures.

		Control	A59	MHV-2
Astrocytes		–	80X	1X
5D	Liver	–	10X	10X
	Brain	–	5X	2X
	SC	–	2X	0.2X
30D	Liver	–	1X	0.1X
	Brain	–	+	–
	SC	–	+	–

Astrocyte cultures were prepared from newborn C57BL/6 mouse brains, infected at m.o.i=1 of each virus and tested for TNF-α mRNA at 48 hours post infection. Tissues were sampled at 5 days post infection (peak of acute phase) and at 30 days post infection (during the chronic inflammatory stage). SC: Spinal cord. The units are all arbitrary relative units.

4. DISCUSSION

4.1 Demyelination positive virus MHV-A59 induced TNF-α mRNA at a significantly higher level than demyelination negative virus MHV-2 in both astrocytes culture and in mouse tissues. The difference was most significant in the CNS.

4.2 ELISA assay results suggest that the secretion of the TNF-α protein into the medium of infected cultures is also more significantly up-regulated in MHV-A59 infected astrocytes than in MHV-2 infected astrocytes. The level of viral replication in both cases is similar, suggesting that there is an intrinsic difference between these two viruses in their interaction with astrocytes.

4.3 These findings suggest that TNF-α may play a role in neurotropism and demyelination, which may explain the differential biologic phenotypes of these two closely related strains of viruses.

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