Susceptibility of Murine CNS to OC43 Infection

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1. INTRODUCTION

Multiple sclerosis (MS), one of the most common neurological diseases of young adults, is accompanied by inflammatory demyelination and loss of oligodendrocytes in the central nervous system (CNS). Although its etiology remains unclear, a generally accepted hypothesis is that virus infections could initiate a CNS-directed immune process in a genetically predisposed host (Oldstone 1997). Amongst various animal models of virus-induced demyelination, studies on murine hepatitis virus (MHV) have revealed that this virus is capable of causing direct oligodendrocyte cytopathology, but may also elicit a variety of immunopathological responses (Lane and Buchmeier 1997). Given that MHV causes MS-like CNS demyelination, the related human coronaviruses (HCoV) represent a logical target of investigation as a potential microbial agent involved in MS pathogenesis.

HCoV are respiratory pathogens responsible for 10 to 35% of common colds (McIntosh 1990). They have occasionally been associated with other pathologies such as pneumonia or meningitis (Riski and Hovi 1980). We have reported their ability to replicate and persist in human brain cells (Bonavia *et al.* 1997; Arbour *et al.* 1999a, b) and even human brains (Arbour *et al.* 2000). Such neurotropic and neuroinvasive properties and analogies with MHV-induced MS-like disease in mice and rats have stimulated research on the possible implication of coronaviruses in MS.

The goal of the present study was to develop and characterize an experimental mouse model of HCoV-associated neuropathology. We first

The Nidoviruses (Coronaviruses and Arteriviruses).
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investigated *in vitro* the ability of primary cultures of murine cells to be infected by the two known viral serotypes (229E and OC43). We then studied the infection of mouse CNS by HCoV-OC43.

2. MATERIALS AND METHODS

2.1 Virus and cell culture

The 229E and OC43 strains of HCoV were originally obtained from ATCC, plaque-purified and grown on the human embryonic lung cell line L132 or the human rectal carcinoma cell line HRT-18, respectively. Virus stocks with a titer of about $5x10^6$ $TCID_{50}$/mL were kept at -80°C. Primary neural cell cultures were obtained by dissecting out the cortical brain of 1-day-old mice. A cell suspension was produced by passing the tissue several times through a syringe fitted with a 2mm gauge needle, then filtered on a 82 µm mesh. Cells were grown as monolayers at 37°C, in a humidified atmosphere containing 5% (v/v) CO_2, in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum.

After 10 days of growth, three series of four Petri dishes, containing approximately $1.5x10^6$ cells each, were infected by a 2-hour contact with a dilution of HCoV-229E or HCoV-OC43 virus stock to yield a MOI of 1. Infectious viral particle production was monitored for 8 weeks after infection. During this period, the entire volume of medium covering the cells was collected at weekly intervals and placed at -80°C until infectious virus titers could be determined. After each sampling, cell cultures were extensively washed in sterile PBS before adding new DMEM.

Infectious virus titers were determined by an indirect immunoperoxidase assay on susceptible cells (Arbour *et al.* 1999a). Simple and double immunofluorescence labeling identified cell types and which of them were infected by HCoV. A rabbit-anti-glial-fibrillary acidic protein antibody (GFAP, DAKO) was used to identify astrocytes and an ascites fluid from rat anti-MACII (ATCC) used to stain microglia/macrophages. For detection of viral antigens, ascites fluids containing MAbs directed to the N protein of HCoV-OC43 or -229E were used (Bonavia *et al.* 1997).

2.2 Animal model

Based on the results obtained in cell cultures, we only inoculated mice with HCoV-OC43. BALB/c mice were inoculated intracerebrally with 10 µl

of virus at 10^3 $TCID_{50}$/mL at the age of 8-days post-natal (P8). Infected sucklings were replaced with their mother until experimentation. Littermates of the same age were injected with 10 µl of PBS to serve as control animals.

2.2.1 Immunohistochemistry and electron microscopy

Mice were perfused by intraventricular injection of 4% paraformaldehyde under deep ketamine-xylazine anesthesia. Serial 50 µm-thick sections of brain or spinal cord tissue blocks were incubated overnight in anti-OC43 MAb (1/1000), then rinsed and processed in Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). Labeling was revealed with 0.03% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.01% H_2O_2. Samples for electron microscopy were postfixed for 2 hours with 2% osmium tetraoxide in 0.1M phosphate buffer, dehydrated in graded ethanol series, and embedded in Epon.

2.2.2 Infectious Tests

At 3, 4, 6, 8 and 10 days post-infection, four animals were sacrificed. Brain and spinal cords were dissected out homogenized in sterile PBS and tissue passed through a 0.22 µm filter-fitted syringe. The filtered extracts were processed for the presence of infectious virus.

2.2.3 Western Blot Analyses

Tissues were homogenized in SUB buffer, as previously described (Jacomy *et al.* 1999). Samples (5 µg total proteins) were fractionated on a 7.5% polyacrylamide gel and either visualized by Coomassie Blue staining or transferred to nitrocellulose for Western blot analysis. Membranes were incubated for 2 hours with the OC43-specific antibody. After several rinses with TS buffer containing 0.05% (v/v) Tween 20, membranes were incubated for one hour with peroxidase-conjugated anti-mouse IgG (1/1000, DAKO). Bands were visualized using a Western blot chemoluminescent kit (Super Signal, PIERCE, Rockford, MD, USA).

2.2.4 Preparation of RNA and RT-PCR

CNS tissues were dissected out at different time points post-infection. Total RNA was extracted by homogenization in Trizol (GibcoBRL, Burlington, CA, USA). For RT-PCR, one pair of HCoV-OC43 primers was designed to amplify a region containing 920 nucleotides (primers O1 and O9) of the gene coding for the N protein (Arbour *et al.* 1999b).

Approximately 5 μg of RNA were reverse transcribed and the product was added to a PCR mix followed by 30 amplification cycles of 2 min at 72°C, 1 min at 95°C and 2 min at 60°C. Ten μl of reaction product was loaded onto a 1.2% (w/v) agarose gel containing 5 μl (v/v) ethidium bromide.

3. RESULTS AND DISCUSSION

3.1 Cell Cultures

Using double immunofluorescence detection, we showed numerous GFAP-positive astrocytes also staining for HCoV-OC43 antigens. Some of the MAC-II-stained microglia/macrophages also contained viral antigens. These results demonstrate that both murine microglia/macrophages and astrocytes were susceptible to an infection by HCoV-OC43. Moreover, measurements of infectious virus titers in cell culture supernatants demonstrated that glial cell cultures produced infectious viral particles and were therefore productively infected. At 4 days post-infection, titers reached about 10^7 $TCID_{50}$/ml (Table 1). Relatively stable amounts of infectious virions were detected at all time points. Titers of about 10^5 $TCID_{50}$/mL observed after 8 weeks are consistent with a persistent infection of primary murine CNS glial cell cultures by HCoV-OC43.

Table 1: Yield of infectious virions from persistent HCoV-OC43 infections of primary glial cell cultures. The highest level of infectious virus titer was obtained at 4 days post-infection. Glial cells produced infectious virions at least until 8 weeks.

Weeks post-infection	Yield of infectious virions (log_{10} $TCID_{50}$/mL)
0.5 (~ 4 days)	7.02 (± 0.90)
2	5.49 (±0.86)
4	5.02 (±0.03)
6	4.25 (±0.00)
8	5.13 (±0.63)

Pearson and Mims (1985) reported that only neuronal cell types were able to produce infectious HCoV-OC43 virions, and that astrocytic cells could produce viral antigens but not infectious virus. Our culture conditions avoided the possibility of neurons playing a role in the production of infectious viral particles. Indeed, no neurons were able to live in the culture conditioned medium used and no neurons could survive after 2 months of culture. Thus, this is the first report of a productive and persistent HCoV-OC43 infection of murine astrocytes and microglial/macrophages.

No infection could be detected with HCoV-229E, probably due to the absence on murine cell membranes of the aminopeptidase-N (CD13) molecule, which is necessary for infection (Lachance *et al.*, 1998).

3.2 Mouse model

Empiric experimentation was used to obtain a sub-lethal infection by HCoV-OC43 in the hope of establishment of life-long persistence. Given a previous report on the age-dependent resistance of mice to HCoV-OC43 infection (Pearson and Mims, 1983), intracerebral inoculations were performed on mice at various post-natal ages, using 10 µl of various dilutions of the virus stock, to determine the 50% lethal dose (LD_{50}). BALB/c mice were selected in view of their relative susceptibility to both respiratory and enteric strains of MHV. Intracerebral inoculations were used to favor a CNS infection. Finally, we determined the optimal experimental conditions to be as follows: 10 µl of a diluted virus solution at about 100 $TCID_{50}$ inoculated intracerebrally at 8 days post-natal. In these conditions, about 50% of the pups died within the first 8 days post-infection. All inoculated mice developed signs of acute disease characterized by apathy, hunched posture, ruffled fur and tremor, comparable to pathological signs described after MHV infection (Kristensson *et al.* 1986). Mice which survived after 8 days showed clinical remission and have remained well for 10 months.

Viral infections initiated by intracerebral inoculations were quickly disseminated. Indeed, cells positive for viral antigens were detected throughout the CNS. At the electron microscopic level, virions were observed in the spinal cord 8 days after injection in the brain. These particles were mostly localized in the cell cytoplasm, closely associated with the Golgi apparatus, or in extracellular spaces, between dendrites and the axonal endings. This reflects a replication of the virus in the cytoplasm of the cells and probably cell-to-cell transport of the virions, as was described after MHV infection (Lavi *et al.*, 1988; Sun and Perlman, 1995).

Infectious virus could be isolated from the CNS between 2 and 8 days post-infection. The highest level of infectious virions was obtained at 6 days post-infection; this titer could reach about 10^7 $TCID_{50}$/g for the brain and about 10^5 $TCID_{50}$/g for the spinal cord extracts. These high CNS virus titers were indicative of a generalized infection during the first week post-inoculation. No infectious virions could be detected starting at 10 days post-infection, as was also reported with MHV (Woyciechowska *et al.*, 1984).

Viral proteins were detected in the brain and the spinal cord between 4 to 8 days post-infection. Coomassie blue-stained gels of protein extracts revealed the presence of new protein bands in infected compared to control

animals, indicative of viral protein synthesis (Fig. 1A). Indeed, proteins transferred onto nitrocellulose membranes and stained with antibodies against the N protein of HCoV-OC43 recognized these new bands in all the isolates of virus-infected animals, without staining any proteins in control animals (Fig. 1B). Moreover, Western blot analysis revealed a second N protein band in infected mice, compared to the single N protein band of the viral stock solution. This second band probably represents a phosphorylated form of the N protein which may account for high viral synthesis in infected mice. Viral proteins were found in 90% of mice at 4 days after infection, but only in 22% of the animals at 8 days. Viral proteins were not detectable after 10 days.

Having demonstrated persistent infection in murine CNS cell cultures and a generalized infection of CNS during the first 8 days post-infection, we wanted to detect a possible persistent infection in the CNS of infected mice. By RT-PCR analyses, viral RNA could be found in some animals at least until 5 months post-infection (Fig. 1C).

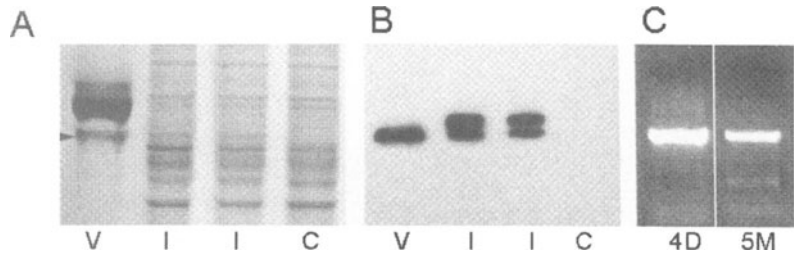

Figure 1: HCoV-OC43 nucleocapsid protein and RNA in mouse brain. (A) Coomassie blue stained gel; I: infected mice and C: control animal. (B) Western blot confirmation of the presence of N protein in infected mouse brain. (C) RT-PCR: viral RNA was detected at 4 days (4D) but also 5 months (5M) post-infection. V: virion controls.

4. CONCLUSIONS

These results demonstrate that the CNS of BALB/c mice is susceptible to an acute and persistent HCoV-OC43 infection, with viral spread and replication, including a productive infection of astrocytes and macrophages/microglial. Viral persistence observed in the CNS may play a role in the development of chronic pathologies, as it was suggested for MHV infection. It will be interesting to study demyelination and immunopathology in this new animal model.

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