Parainfluenza virus type 1 infects olfactory neurons and establishes long-term persistence in the nerve tissue

Isamu Mori,1,2 Takayuki Komatsu,1 Kenji Takeuchi,1 Kazuya Nakakuki,3 Masakatsu Sudo2 and Yoshinobu Kimura1*

Departments of 1 Microbiology, 2 Pediatrics and 3 Pathology, Fukui Medical School, Fukui 910-11, Japan

A mouse model of Sendai virus infection was adopted to examine the in vivo neurovirulence of parainfluenza viruses. A nested polymerase chain reaction detected the Sendai virus nucleoprotein gene in the olfactory bulbs of intranasally infected mice for at least 168 days post-infection (p.i.) and virus-specific messenger RNAs for 28 days p.i. Viral proteins were histochemically detected in some olfactory neurons for 7 days p.i. They were also found in glomeruli of the olfactory bulbs but not in the mitral cells and the tufted cells. No virus was detected in the whole brain not including the olfactory bulbs. When mice were inoculated with UV-inactivated virus, the viral RNA was present in the olfactory bulbs for a short period of 14 days, with no demonstrable viraemia. These results demonstrate that the parainfluenza virus directly accesses the central nervous system via olfactory neurons and establishes long-term persistence in the nerve tissue.

Human parainfluenza viruses are a major cause of morbidity in infancy and almost exclusively cause respiratory disease. Central nervous system (CNS) diseases associated with parainfluenza virus have been documented, including Reye's syndrome (Powell et al., 1973), Guillain-Barré syndrome (Román et al., 1978), aseptic meningitis (Vreede et al., 1992), and multiple sclerosis (Goswami et al., 1987). However, the aetiological relation of the virus to CNS diseases has not been fully established.

The Sendai strain of parainfluenza virus usually causes a pneumonia in mice. Since the olfactory pathway is one of the most important routes of infection by some neurotropic viruses (Tomlinson & Esiri, 1983; Lundh et al., 1987; Lafay et al., 1991; Barnett & Perlman, 1993), we investigated the olfactory nerve tissues of intranasally infected mice for the presence of the virus by using the highly sensitive polymerase chain reaction (PCR).

The Nagoya strain of Sendai virus was propagated routinely by allantoic inoculation of 10-day-old embryonated eggs with 10−4 diluted seed virus. Virus infectivity was assayed by plaque titration on LLCMK2 cell monolayers as described previously (Sugita et al., 1974). For UV-inactivation, stock virus suspension was exposed to a 15 W UV lamp at a distance of 30 cm at 4 °C with continuous and gentle stirring. After irradiation, infectivity was reduced to < 10−7 of the original, while haemagglutination activity was well preserved.

Six- to seven-week-old male C3H/HeJ mice (Clea Japan) were mildly anaesthetized with diethyl ether and inoculated in the right nostril with 105 p.f.u. of Sendai virus in 25 μl of PBS. In some experiments, mice were inoculated with 25 μl of UV-inactivated virus at the same dilution. At intervals, mice were anaesthetized and perfused with 4% paraformaldehyde. The nose, brain and lung were post-fixed with the same fixative at 4 °C for 24 h, and the nose was then decalcified in 4% EDTA for 5 days. These tissues were dehydrated and embedded in paraffin wax. Coronal sections 2 μm thick were cut on a microtome and stained for Sendai virus antigens by the streptavidin–biotin–peroxidase method using a Histofine kit (Nichirei). The deparaffinized slides were successively reacted with 3% hydrogen peroxide in methanol, normal goat serum, rabbit anti-Sendai virus serum diluted 1:2000, biotinylated goat anti-rabbit immunoglobulin, streptavidin-conjugated peroxidase and finally with 3,3'-diaminobenzidine tetrachloride. The slides were counterstained with haematoxylin. A group of three to five mice was used for each time point. Tissue sections from infected mice treated with preimmune serum were used as a negative control. Tissues from uninfected mice were also used as an additional control.

The heparinized blood was fractionated by Ficoll-Paque (Pharmacia) density gradient centrifugation into peripheral blood mononuclear cells (PBMC), red blood cells (RBC) and plasma. Total RNA was extracted from the olfactory bulbs, blood fractions or lung using TRIzol reagent (Life Technologies). A cDNA copy was synthesized using Moloney murine leukaemia virus reverse
transcriptase (Life Technologies) with 10 pmol sense primer (5' ACCAAACAAGAG 3'). For detection of virus-specific mRNAs, 50 pmol of oligo(dT) was used as a primer. The cDNA samples were amplified in a single tube by nested or semi-nested PCR using a DNA Thermal Cycler (Perkin-Elmer Cetus). Primers for the Sendai virus nucleoprotein (NP) gene had the following sequences: external primers, 5' CGGGATCCTGAAGTTATACAGGAT 3' (sense) and 5' CCAGCACAATC-CAGACTTGGAC 3' (antisense); internal primers, 5' CGGGATCCAGACCCCTTTGCTTTGC 3' (sense) and 5' ATTTGACATCGGCGTTACTCG 3' (antisense). The final nested product was 340 bp in length. The first PCR was carried out in a 20 μl solution containing 2 μl 10 × PCR buffer (15 mM-MgCl₂, pH 9.0), 0.4 μl dNTPs (each 10 mM), 0.3 μl each of the external primers (5 pmol/μl), 0.2 U Tub DNA polymerase (Amersham), and 4 μl of the cDNA sample. After two drops of liquid paraffin were added, the tube was briefly centrifuged. The thermal cycling program was: 94 °C for 3 min, 25 cycles of 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 1 min, and 72 °C for 5 min. For the second amplification, the first PCR product was mixed in the same tube with a 30 μl solution containing 3 μl 10 × PCR buffer, 1 μl dNTPs (each 10 mM), 1 μl each of the internal primers (50 pmol/μl), and 0.5 U Tub DNA polymerase. The tube was then briefly centrifuged. The thermal cycling program consisted of: 94 °C for 3 min, 35 cycles of 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 1 min, followed by 72 °C for 5 min. The final PCR product was analysed by electrophoresis on a 2% agarose gel, visualized by ethidium bromide staining and confirmed by Southern hybridization with a digoxigenin (DIG; Boehringer Mannheim)-labelled PCR probe, which was successively detected with a DIG Luminescent Detection Kit (Boehringer Mannheim). The PCR probe was synthesized using a DNA thermal cycler (Perkin-Elmer Cetus) in a 50 μl solution containing 5 μl 10 × PCR buffer, 4 μl DIG DNA labelling mixture (Boehringer Mannheim), 1 μl each of the internal primers (50 pmol/μl), 0.5 U Tub DNA polymerase and 1 μl of the PCR product synthesized with the Sendai virus cDNA. The thermal cycling program was: 94 °C for 3 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, followed by 72 °C for 5 min. For detection of the NP-specific mRNA, the sense primer adopted in the first PCR was substituted by that of the second PCR, because the former had the extracistronic sequence. The primers used in this research were designed with reference to the published sequences of Sendai virus gene (Shioda et al., 1983).

As reported previously (Kimura et al., 1979; Iwata et al., 1990), virus growth peaked in nasal turbinates, tracheas, and lungs at 5 days post-infection (p.i.). Thereafter, the immune response against Sendai virus excluded progeny virus from the respiratory tracts within the subsequent 5 days. It should be noted that no infectious progeny virus could be isolated from the brain or blood by plaque assay during the course of infection (data not shown).
**Table 1. Detection of Sendai virus genomic RNA in the olfactory bulbs, lung, and blood fractions of mice intranasally inoculated with live or UV-inactivated virus**

<table>
<thead>
<tr>
<th>Time post-inoculation (days)</th>
<th>Live virus</th>
<th>Inactivated virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>OB†</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>OB†</td>
<td>- - -</td>
<td>+++</td>
</tr>
<tr>
<td>Live virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OB†</td>
<td>- - -</td>
<td>+++</td>
</tr>
<tr>
<td>Lung</td>
<td>- + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>OB†</td>
<td>- - -</td>
<td>+++</td>
</tr>
<tr>
<td>Inactivated virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OB†</td>
<td>- - -</td>
<td>+ + +</td>
</tr>
<tr>
<td>Lung</td>
<td>- + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>PBMC</td>
<td>- - -</td>
<td>+++</td>
</tr>
<tr>
<td>RBC</td>
<td>- - -</td>
<td>+ + +</td>
</tr>
<tr>
<td>Plasma</td>
<td>- - -</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

* Three mice were used at each time point, hence three symbols for each tissue and time point.
† OB, Olfactory bulbs.
ND, Not done.

At 3 and 7 days p.i., Sendai virus proteins were detected in the cytoplasm of some olfactory neurons (Fig. 1). At 14 days p.i. and later, they were not found in these cells. Supporting cells in the olfactory epithelium were not infected. The histochemistry also disclosed the presence of virus in glomeruli of the olfactory bulbs, recognizable as specific brown grains, at 7 days p.i., but not at 3 or 14 days p.i. In glomeruli, olfactory neurons synapse with dendrites of the mitral cells and the tufted cells (Halász & Shepherd, 1983). It is of note that these secondary neurons remained negative for viral proteins during the course of infection (Fig. 1). Viral antigens did not appear in other portions of the brain. The histochemistry detected viral antigens in the respiratory epithelium at 3 days p.i., but not at 7 days p.i. This method could not detect viral antigens in the olfactory and respiratory epithelium of mice inoculated with the UV-inactivated virus.

PCR run in nested configuration detected the NP gene in starting material that contained as little as 5 p.f.u. of Sendai virus. Because nose tissue consists of a variety of cell populations other than olfactory neurons (such as respiratory epithelial cells and cells of muscle, bone and lymphoid tissue origin) we investigated the presence of viral genetic material in the olfactory bulbs, which contain the nerve terminals of olfactory neurons. The PCR detected the NP gene in the olfactory bulbs at least up to 168 days p.i. (Table 1), but it did not show the invasion of other portions of the brain (Fig. 2). The PCR also detected the NP-specific mRNA in the olfactory bulbs for 28 days p.i. (data not shown). This finding was further confirmed by detecting mRNA coding for the matrix and fusion proteins. The lung was positive for the NP gene up to 28 days p.i. (Table 1). In blood fractions, the NP gene appeared during a short period from 2 to 5 days p.i., suggesting the induction of a transient viraemia. When mice were inoculated with UV-inactivated virus, the NP gene was detected in the olfactory bulbs and the lung for 14 days p.i., but it was not found in the blood samples even at 3 and 5 days p.i. (Table 1). Sendai virus-infected mice showed mild respiratory symptoms but displayed no appreciable neurological symptoms.

These experiments demonstrated that Sendai virus infects olfactory neurons of immunocompetent mice. There have been reports that indicate the neurovirulence of Sendai virus by intranasal or intracerebral infection of newborn mice (Shimokata et al., 1976; Kristensson et al., 1984; Ruttkay-Nedecký et al., 1987).

The virus appears to access the CNS not via the haematogenous route but directly through the olfactory pathway because of (i) evidence of virus infection of olfactory neurons, (ii) no evidence of virus invasion of the brain other than the olfactory bulbs and (iii) the ability of UV-inactivated virus to invade the CNS, when no demonstrable viraemia occurred. The inactivated virus may be transported via the axonal transport within olfactory neurons. Virus entry via the olfactory pathway has been documented for some viruses such as rabies virus (Lafay et al., 1991), vesicular stomatitis virus (Lundh et al., 1987), mouse hepatitis virus (Barnett & Perlman, 1993), and herpes simplex virus (Tomlinson & Esiri, 1983). These viruses without exception spread to other areas of the brain. However, under the present experimental conditions, Sendai virus remained in olfactory neurons.
It should be noted that the virus genome persisted in the nerve tissue for a long period, whereas it was cleared from the lung. Intact functions of the virus genome appear to be essential for the establishment of long-term persistence of virus genome in olfactory neurons, since the UV-inactivated virus could not persist in nerve tissue. Major ingredients for in vivo establishment of persistent infection are the ability of virus to assume a non-cytolytic phenotype and to escape from the host immune response (Oldstone, 1993). Virus infection of neurons often meets these qualities. It is tempting to speculate that human parainfluenza viruses frequently invade the human CNS through the olfactory pathway, in rare instances provoking certain neurological diseases.

We thank Sayuri Kubo for technical assistance and Nobuo Takimoto for providing us with excellent histologic preparations. This research was supported in part by a grant-in-aid from the Ministry of Education, Science and Culture, Tokyo.

References


(Received 28 November 1994; Accepted 1 February 1995)