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Productive infection in the murine central nervous system with avian influenza virus A (H5N1) after intranasal inoculation

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Abstract The H5N1 type of influenza A virus isolated from human patients in 1997 has a characteristic hemagglutinin and was considered to be directly transmitted from birds. Although neuropathogenicity of this virus was not demonstrated in human autopsy cases, some experimental studies using mice have disclosed that this virus infects the central nervous system (CNS) after intranasal inoculation. In this study we focused on the topographical localization of virus-infected cells in the murine CNS after intranasal inoculation. We immunohistochemically examined virus-infected cells in mouse tissues using a rabbit antiserum recognizing the nucleoprotein of influenza A virus. The virus-infected cells appeared initially in the respiratory tract. Thereafter, the virus antigen-positive cells appeared in the olfactory system and the cranial nerve nuclei innervating the facial region. This suggests that this virus is principally transmitted from the nasal cavity to CNS through the cranial nerves. Neurons were frequently infected and

glial and ependymal cells were also infected. Transneuronal transmission of the virus might play the important role of viral spread within the CNS.

Keywords Influenza virus · H5N1 · Nucleoprotein · Immunohistochemistry · Brain

Introduction

Influenza A virus is divided into subtypes by the antigenic nature of its surface glycoproteins of hemagglutinin (HA) and neuraminidase (NA) inserted in the envelope [12]. HA protein is known to be responsible for binding and penetration of virions to host cells. Among HA subtypes, three have been demonstrated to infect human beings and to have caused four pandemics in 1918 (H1N1, Spanish), 1957 (H2N2, Asian), 1968 (H3N2, Hong Kong) and 1977 (H1N1, Russian) [23]. As rare incidences, H7N7 virus was occasionally isolated from conjunctiva in 1980 and 1996 [11, 22]. H5N1 virus was first isolated from 18 human patients in Hong Kong in 1997 (6 of them died) [3, 19] and was considered to be directly transmitted from chicken. Biologically, H5N1 virus strains isolated during this outbreak were classified into two subgroups (Groups 1 and 2) by pathogenicity in mice [4] and by the antigenic character of HA protein [1]. In addition, H5N1 strains were again isolated from human patients in 2003–2004 in Thailand and Vietnam during the outbreak of avian influenza in Cambodia, China, Japan, Korea, Laos, Thailand and Vietnam.

Influenza A virus is transmitted by aerosol, and infects the mucosal epithelium [23]. Influenza virus infection within the central nervous system (CNS) has not been well demonstrated in human cases. Virological and pathological studies on experimental intranasal inoculation of H1N1 viruses revealed that the influenza virus infection was localized only in the columnar or alveolar epithelium of the respiratory

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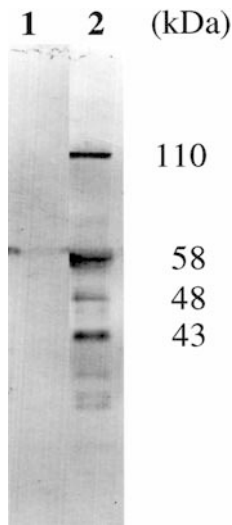


Fig. 1 Western blot analysis of the antibody recognizing the nucleoprotein of influenza A virus H5N1 used in the immunohistochemical study. *Lane 1*: MDCK cells without virus inoculation. *Lane 2*: MDCK cells inoculated with HK483 virus at 48 h p.i. (*p.i.* post inoculation)

tract [7, 9, 24, 25]. Only influenza viruses belonging to H5 and H7 subtypes have been demonstrated to infect CNS after intranasal inoculation in mice [4, 6, 13, 15, 16, 18]. In a previous report [15], we revealed that two Group 1 strains of H5N1 virus infected not only the respiratory tract, but also the CNS, adipose tissues, heart and liver after intranasal inoculation in mice. In this study, we further investigated the pathogenesis of CNS involvement in mice, using a Group 1 strain of H5N1 virus, by topographical analysis of virus-infected cells in the CNS.

Materials and methods

Mice and infection

All the experiments using a live H5N1 virus were performed in biosafety level 3 facilities under the recommended procedures. Animal care, breeding, virus inoculation, observation and sacrifice under deep

anesthesia were performed in accordance with the guidelines of the Institutional Committees. Specific pathogen-free (SPF) 4- to 7-week-old outbred female mice (ddY) were obtained from the Japan SLC (Hamamatsu, Japan). Influenza A/Hong Kong/483/97 (HK483; H5N1) virus [4], isolated in the Government Virus Unit, the Queen Mary Hospital, Hong Kong, was prepared in Mardin-Darby canine kidney (MDCK) cells without any special step for mouse adaptation. We also used influenza A/Puerto Rico/8/34 (PR8; H1N1) virus prepared as described previously [20] as a control of classical influenza A virus. Mice were lightly anesthetized with intraperitoneal injection of sodium amobarbital (0.25 mg), and were inoculated intranasally with 2 μ l or 20 μ l of virus suspension containing 2×10^5 plaque forming unit (PFU) HK483 / mouse into the left or both nostrils.

Titration of virus

Viral titration of the brain and the lung tissues of infected mice was performed by a plaque assay method using MDCK cells. Tissue homogenate (10% w/v) was prepared in PBS (pH 7.2). The supernatant of homogenates after centrifugation at 3,000 rpm for 20 min was inoculated into the cells in the presence of 10 μ g/ml acetylated trypsin (Sigma, St. Louis, MO).

Antibody and its characterization

For detection of HK483 virus, we used a polyclonal anti-influenza A nucleoprotein rabbit serum prepared by immunizing rabbits with the purified nucleoprotein of PR8 [20]. The specificity of this antibody to recognize the nucleoprotein of HK483 virus was confirmed by Western blot analysis. HK483 virus-infected and uninfected MDCK cells were lysed in Laemmli sample buffer with 2-mercaptoethanol, boiled for 10 min, and then subjected to 12% SDS-polyacrylamide gel electrophoresis. After electrophoretic transfer of proteins from gels to PVDF filters (Immobilon, Millipore, Bedford, MA), the filters were incubated in

Fig. 2 Survival and virus titers of ddY mice after intranasal inoculation of HK483 virus. **A** Survival of mice after intranasal inoculation of 2 μ l HK483 virus. **B** Mean \pm SD of virus titers in the lungs (*open circles*), brain (*closed circles*) and blood (*open square*) of three mice on days 2, 3, 4, 5, and 6 p.i. Virus titers in the blood were examined on days 4 and 6

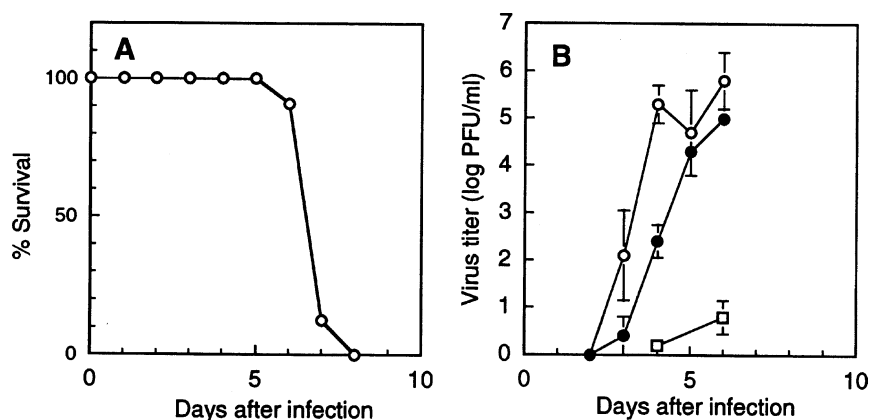


Table 1 Immunohistochemical detection of the virus-infected cells in mice infected by influenza A virus (H5N1, HK/483/1997). Values indicate the number of mice (*p.i.* post inoculation, *CNS* central nervous system, *NE* not examined)

Day <i>p.i.</i>	2- μ l inoculum (<i>n</i> = 5)			20- μ l inoculum (<i>n</i> = 5)		
	Nose	Lungs	CNS	Nose	Lungs	CNS
2	5/5	0/5	0/3	5/5	5/5	0/3
3	5/5	0/5	3/3	5/5	5/5	3/3
4	5/5	3/5	3/3	5/5	5/5	3/3
5	5/5	4/5	3/3	5/5	5/5	3/3
7	1/1 ^a	1/1 ^a	1/1 ^a	NE	NE	NE

^aOnly surviving mice were examined

2% non-fat dry milk in PBS and reacted with the antiserum. After washing in PBS-Tween 20, the filters were incubated with biotinylated goat anti-rabbit IgG, followed by streptavidin-alkaline phosphatase. The filters were developed in NBT/BCIP.

Histology and immunohistochemistry

The tissue obtained at autopsy was fixed in 4% formaldehyde prepared in PBS, and subsequently embedded in paraffin. For bony tissue, decalcification was achieved in EDTA solution before embedding. The maxillary region containing the nasal cavity was embedded in four portions after step-wise coronal sections. The lungs were transversely cut three times. The brain was cut four times at the frontal plane. Paraffin sections were analyzed by histological and immunohistochemical methods. Immunohistochemistry for detection of the viral antigen was performed by the avidin-streptavidin peroxidase method using diaminobenzidine as chromogen, as described previously [9, 15]. Topographical analysis was performed on 10- μ m-step frontal or sagittal sections of whole mount-embedded brain tissue.

Results

Antibody characterization

We characterized the antigens of HK483 virus recognized by the anti-influenza A nucleoprotein antiserum prepared by immunizing rabbits with a purified nucleoprotein of influenza virus A H1N1 (PR8) [20]. In a Western blot analysis, this rabbit serum was shown to react with a 58-kDa band, consistent with the expected size of nucleoprotein in MDCK cells at 48 h after inoculation with HK483 virus. In addition, three minor bands of 110 (dimeric form), 48 and 43 kDa were recognized. A faintly stained band at 60 kDa was seen in uninfected cells (Fig. 1). Thus, this antibody was shown to principally recognize the HK483 virus.

Survival of mice after intranasal inoculation

We infected eight 4-week-old SPF ddY mice with 2 μ l inoculum containing 1×10^5 PFU of HK483 virus into both nostrils. All of inoculated mice died within 8 days post inoculation (*p.i.*) (Fig. 2A). In contrast, the five control and five mice inoculated with 2 μ l phosphate buffer or PR8 showed no fatal outcome.

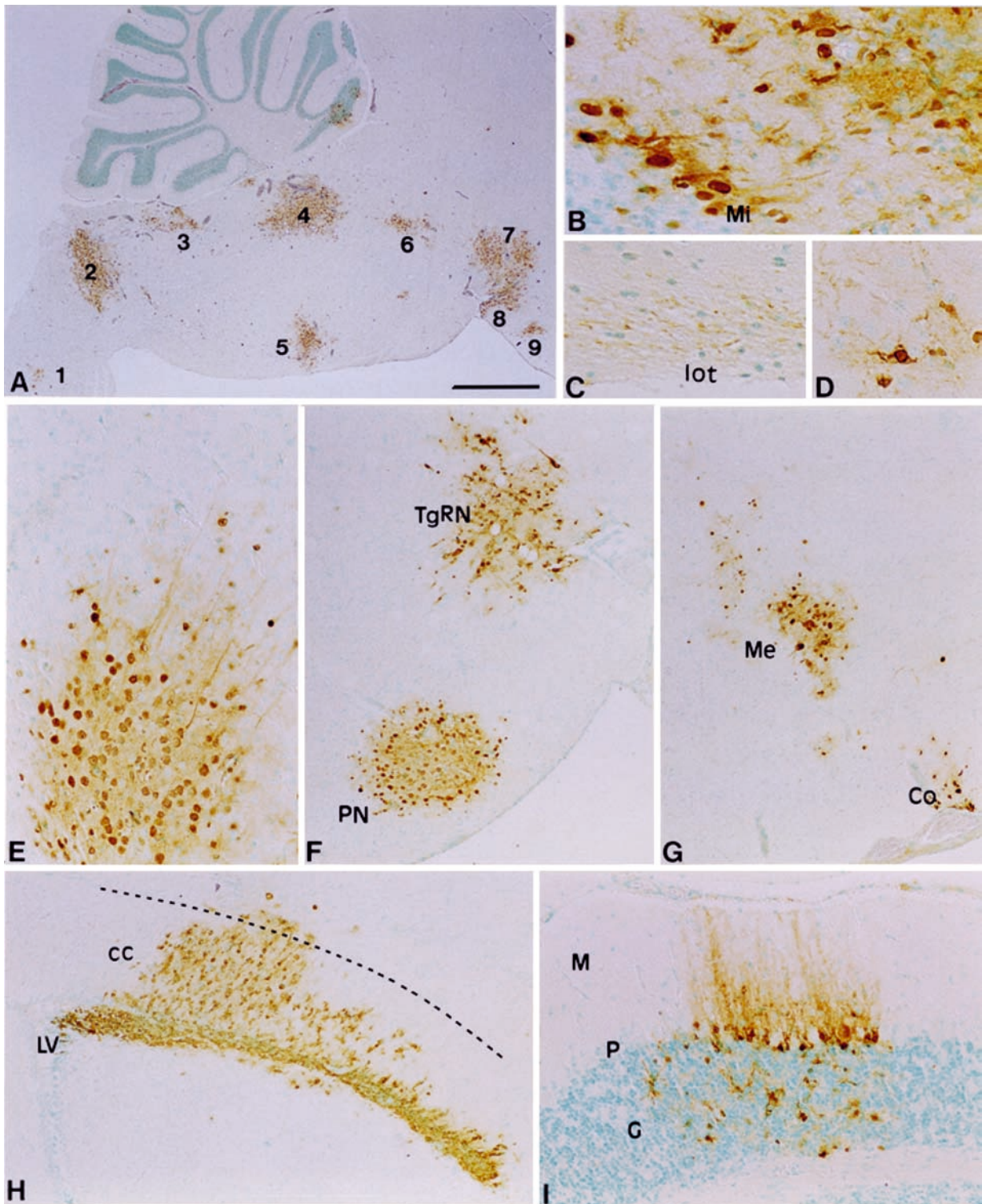
Virus titers in the brain, lung and blood after intranasal inoculation

To investigate the viral dissemination in the mice inoculated with 2 μ l virus suspension containing 2×10^5 PFU of HK483 virus, the virus amount in the lung and brain of three infected mice was determined on days 2, 3, 4, 5 and 6 *p.i.* In addition, viral titers in the blood were also examined on days 4 and 6 *p.i.* Virus was isolated from the brain and lungs from day 2 *p.i.* onwards, but first became measurable on day 3 *p.i.* in both tissues (Fig. 2B). Although the viral titers were higher in the lungs than in the brain, the virus replication in the lungs and in the brain rose in a similar pattern. Virus was also isolated from the blood but titers were less than one fifth of those in lungs and brain.

Distribution of virus-infected cells in the nasal cavity, lungs and brain

We analyzed the localization of the virus antigen-positive cells in the paraffin sections of tissue from three to five mice by an immunohistochemical method using anti-influenza virus A nucleoprotein antiserum. Initially, we confirmed that there was no cross-reaction of this antiserum with normal mouse tissues (without viral infection, data not shown). Mice were inoculated with 2 μ l or 20 μ l of virus solutions containing the same titer of HK483 virus and autopsied from days 2 to 7 *p.i.* On day 2 *p.i.* virus-infected cells were detected in the nasal cavity in mice infected with both 2 μ l and 20 μ l, but only in the lungs in mice inoculated with the 20- μ l volume (Table 1). On day 3 *p.i.* infected cells were observed in the brains of mice inoculated with the different volumes; however, no antigen-positive cells were detected in the three transverse sections of the lungs of mice infected with a 2- μ l inoculum. For these mice, virus-infected cells first appeared in the lungs from day 4 *p.i.* It therefore became apparent that CNS involvement was not correlated with viral infection in the lungs.

In the CNS of mice inoculated with a 2- μ l volume, virus-infected cells were initially detected in the trigeminal nuclei and ganglia, and thereafter increased in number and foci. To clarify which cells were susceptible to this virus and the pathway of viral spread within the CNS, we examined the topographical localization of virus-infected cells in CNS by serial sectioning of the whole brain of mice inoculated with a 20- μ l volume.



In the CNS, HK483 virus mainly infected neurons (Figs. 3A–I, 4A–C). Around these infected neurons, some glial cells were also positive for the viral antigens (Figs. 3B, D, E; 5A, B). In the center of lesion, both the nuclei and cytoplasm of neurons were frequently positive, whereas in their periphery only the nuclei were positive (Fig. 3E). Some ependymal cells were also in-

fectured (Fig. 3H). Viral infection was recognized in the cervical spinal cord, the caudal brain and rostral cerebellar folium on day 4 p.i., and was widespread on day 7 p.i. (Table 2). In the brainstem, the nuclei of nerves innervating the facial region (trigeminal and vestibular nuclei), pontine reticular formation, and substantia nigra were infected (Fig. 3A, F). In addition, the olfactory



Fig. 3 Parasagittal section (rostral to the *right*) of a mouse intranasally inoculated with 20 μ l of H5N1 virus on day 4 p.i. **A** Infected foci in the cervical spinal cord (1) and the caudal brain; the caudal spinal trigeminal nucleus (2), the nucleus of solitary tract (3), the medial vestibular nucleus (4), perifacial pontine reticular region (5), the rostral pontine reticular formation (6), the ventral tegmental area (7) and the substantia nigra (pars compacta, 8; and pars reticulata, 9). Rostral cerebellar folium is also infected. **B–G** Positive cells in the olfactory bulb (**B**) (*Mi* mitral cells), positive fibers in the lateral olfactory tract (**C**), positive cells in the olfactory tubercle (**D**), the visual cortex (**E**), the pontine tegmental reticular nucleus (*TgRN*), and the pontine nuclei proper (*PN*) (**F**), and the medial (*Me*) and cortical (*Co*) nuclei of the amygdaloid complex (**G**). **H** Positive ependymal cells of the lateral ventricle (*LV*), glial cells in the corpus callosum (*cc*), and deep cortical neurons. **I** Positive cells in the cerebellar cortex (*G* granular layer, *P* Purkinje cell layer, *M* molecular layer). Immunohistochemistry (peroxidase/diaminobenzidine) for the nucleoprotein of influenza A virus counterstained with methylgreen. *Bar* **A** 1 μ m; **B–D** 50 μ m; **E–I** 200 μ m

system (olfactory epithelium, olfactory bulb, lateral olfactory tract and olfactory tubercle) (Figs. 3B–G), visual (Fig. 3E) and cerebellar cortices (Fig. 3I), and ventricular ependymal cells (Fig. 3H) were involved. On 7 day p.i., viral infection became extensive and the virus-infected cells were disseminated in most of caudal brainstem and cerebellar nuclei, globus pallidus, and hypothalamic area (Fig. 4A–C).

The principal histological changes of the CNS were necrotic changes of neurons and glial and ependymal cells with granulocytic infiltration (Fig. 6). Some neurons showed karyorrhexis or pyknosis. Subarachnoid infiltration was focally observed but perivascular cuffing and glial nodules were not conspicuous. In the nasal cavity, virus infected the columnar and olfactory epithelial cells, and the subepithelial cells beneath the olfactory epithelium (Fig. 7) on day 3 p.i. Mice infected with the PR8 virus showed a localized infection of the respiratory tract without viral dissemination to the brain, spinal cord, heart or liver; only the epithelial cells were infected by PR8.

Discussion

Although no histological analysis of the brains of fatal human cases has been reported, animal models of H5N1 virus infection in mice and birds disclosed that it causes a generalized infection with marked neural involvement [15]. In this study, we have shown that nasal infection without marked pulmonary involvement is sufficient to cause CNS infection. Usual influenza A viruses, such as H1N1, H2N2, and H3N1, become infectious after cleavage of HA protein to HA₁ and HA₂ subunits by extracellular protease present in the respiratory lumen [10]. However, H5N1 virus is made infectious by intracellular protease [8], and may therefore spread in tissue without any extracellular proteases or with inhibitors to these proteases. This difference in HA proteins is important in viral invasiveness. H5N1 virus can infect

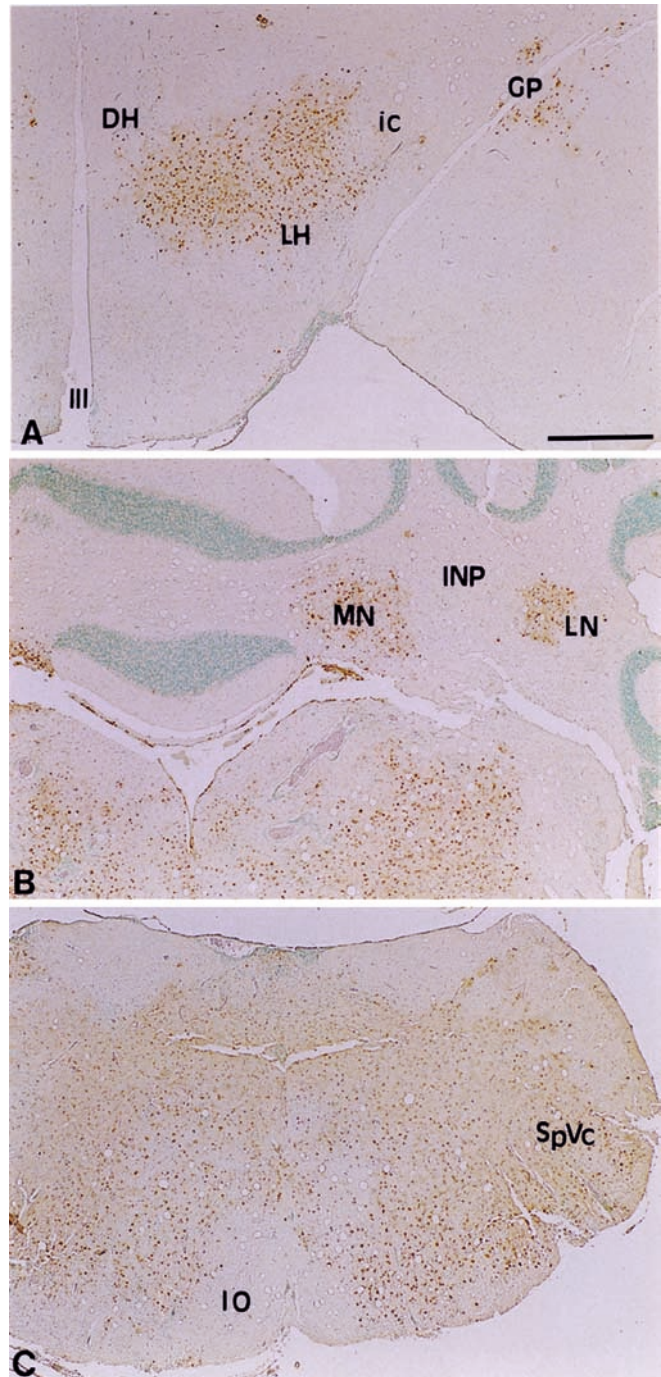
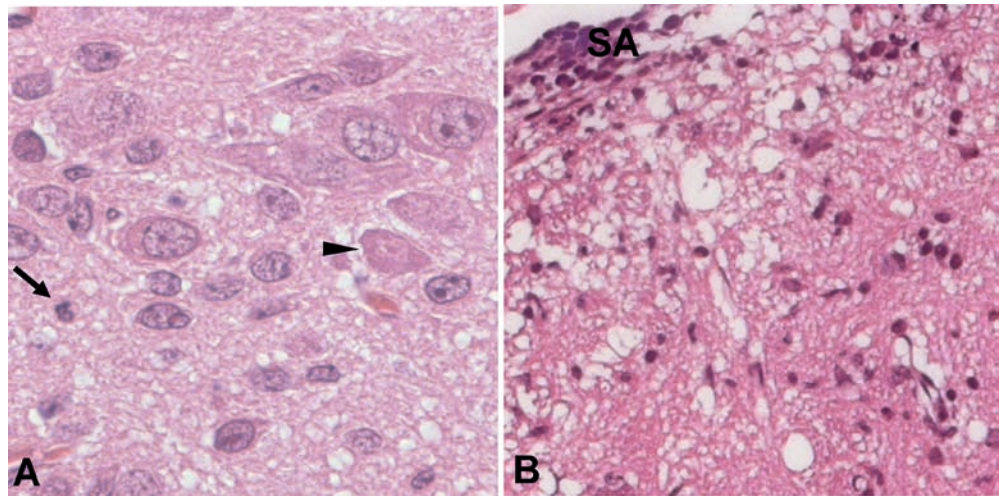


Fig. 4 Extensive viral spread in the central nervous system of a mouse inoculated with 20 μ l inoculum on day 7 p.i. **A** The lateral hypothalamic area (*LH*), globus pallidus (*GP*), dorsomedial hypothalamus (*DH*), third ventricle (*III*). **B** Pons and the cerebellum: medial (*MN*) and lateral (*LN*) deep cerebellar nuclei (*INP* interposed nuclei). **C** The caudal brainstem: the caudolateral regions of inferior olive (*IO*) is intact (*SpVc* caudal spinal trigeminal nucleus). Immunohistochemistry (peroxidase/diaminobenzidine) for the nucleoprotein of influenza A virus counterstained with methylgreen. *Bar* **A–C** 500 μ m

the subepithelial cells directly and spread in a cell to cell fashion, as observed in this study, but usual influenza A virus only infects the surface epithelium and never

Fig. 5 Antigen-positive lesions in the vestibular nucleus of a mouse inoculated with HK483 on day 3 p.i. (A) and the pterygopalatine ganglion on day 5 p.i. (B). The antigen is detected not only in the neurons but also in glial and satellite cells. Late-infected cells become positive in the nucleus and cytoplasm (arrowheads) and early-infected cells only for nucleus (arrows). Immunohistochemistry (peroxidase/diaminobenzidine) for the nucleoprotein of influenza A virus counterstained with methylgreen



spreads within lumen containing extracellular proteases [9]. The distributions of the antigen-positive cells in mice inoculated with H5N1 (HK483) and H1N1 (PR8) viruses correlate well with this concept.

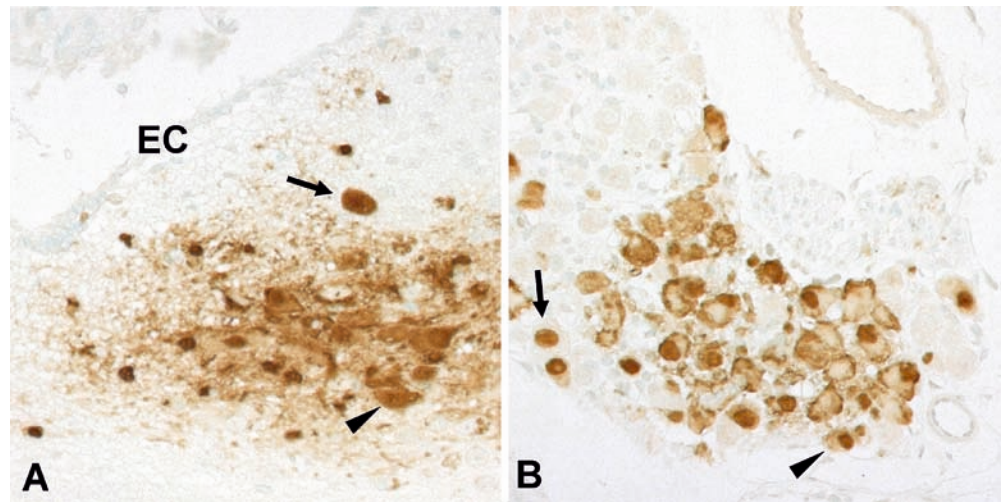
Virus titers in the brain and lungs differed markedly in mice inoculated with either a 2- μ l (2×10^5 PFU; present study) or 20- μ l (10^6 PFU, [15]) volume. In mice inoculated with 20 μ l, a high level replication of H5N1 virus was recognized only in the lungs on day 1 p.i. Viral

replication in the brain started at a low level on day 2 p.i. and increased on day 3 p.i. In contrast, virological analysis of the mice inoculated with a 2- μ l volume disclosed an almost simultaneous replication of H5N1 virus in CNS and in the lungs, although virus antigen-positive cells were detected 1 day earlier in the brain. This discrepancy in histological detection of virus-infected cells and virus titration can be explained by the sensitivity of these methods. From these lines of evidence, we con-

Table 2 Viral involvement in the murine central nervous system. Two mice were inoculated with 20- μ l inoculum of H5N1 virus (2×10^5 PFU/mice) and examined on days 4 and 7 p.i., respectively

Area	Day p.i.		Area	Day p.i.	
	4	7		4	7
Olfactory bulb	2+	+/-	Pretectum	2+	2+
Olfactory ventricle	2+	-	Superior colliculus	2+	+
Lateral olfactory tract	2+	+/-	Inferior colliculus	-	2+
Anterior olfactory nuclei	+	+/-	Nuclei of lateral lemniscus	+	2+
Olfactory tubercle	+	+/-	Oculomotor nucleus	+	+
Nucleus of diagonal band	2+	+/-	Trochlear nucleus	+	+
Septum	+	+/-	Pontine nuclei	2+	+
Preoptic area	+	2+	Pontine tegmental reticular nucleus	2+	2+
Hypothalamic nuclei	+	2+	Pontine reticular formation	2+	2+
Amygdaloid nuclei	2+	+/-	Perifacial region	3+	3+
Piriform cortex	+	+/-	Perilobular region	2+	3+
Entorhinal cortex	2+	+/-	Other region	+	2+
Hippocampus	2+	+/-	Pedunculopontine nucleus	+	2+
Lateral ventricle	2+	+/-	Superior olivary complex	+	3+
Corpus callosum	2+	-	Nuclei of trigeminal nerve		
Cerebral neocortex	2+	+	Motor	+	2+
Thalamic nuclei			Principal sensory	2+	3+
Mediodorsal nucleus	+	-	Spinal trigeminal	3+	3+
Ventral posteromedial nucleus	+	+	Facial nucleus	+/-	2+
Lateral geniculate body	+	-	Ventral cochlear nuclei	+	+
Reticular nucleus	2+	+/-	Vestibular nuclei	2+	3+
Caudate putamen	2+	+/-	Nucleus of solitary tract	3+	3+
Globus pallidus	+	2+	Dorsal motor nucleus of vagus nerve	+	2+
Zona incerta	+	+	Perihypoglossal nucleus	+	2+
Subthalamic nucleus	2+	+	Hypoglossal nucleus	+/-	2+
Entopeduncular nucleus	2+	+	Cuneate/External cuneate nucleus	2+	+
Substantia nigra	+	2+	Gracile nucleus	-	+
Ventral tegmental area	2+	2+	Inferior olive	+	+
Red nucleus, magnocellular	-	+	Medullary reticular formation	2+	3+
Periruber reticular formation	+	2+	Cervical spinal cord	+	3+
Cerebellar cortex	2+	+			
Cerebellar nuclei	+	2+			

Fig. 6 Histology of the pons of an infected mouse inoculated with HK483 virus on day 3 p.i. (A) and day 5 p.i. (B). **A** A neuron (arrowhead) becomes necrotic in the vestibular nucleus. Granulocytic infiltration (arrow) is observed. **B** Inflammatory cell infiltration is observed within subarachnoid space (SA) and white matter with spongiosis



sidered that the virus infection starts independently in the CNS and lungs in mice inoculated with a 2- μ l volume.

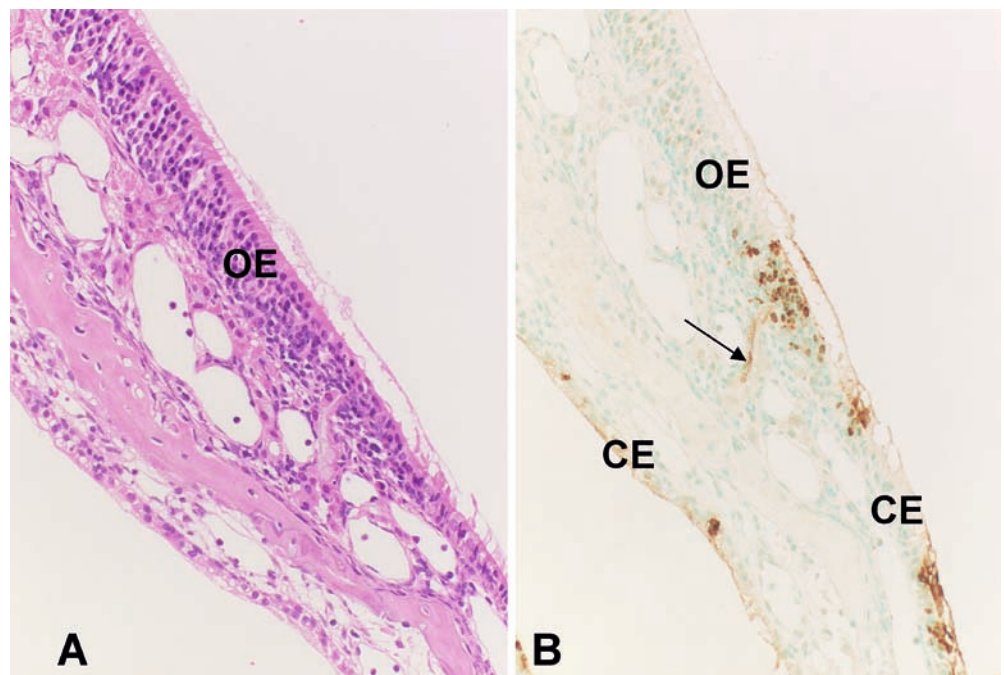
The histological characteristics of H5N1 virus-infected CNS are neuronal degeneration and necrosis with granulocytic infiltration. These changes are consistent with viral encephalitis, although typical characteristics of viral encephalitis, such as vascular cuffing and glial nodules, were not conspicuous. The early neuronal changes are rather difficult to identify and the immunohistochemical detection of the nucleocapsid proteins is helpful in making a correct diagnosis.

The distribution of infected cells in the CNS supports the new concept of H5N1 virus penetration into the CNS through direct neural spread from the cranial nerve endings innervating the respiratory tract, as proposed by

Park et al. [16]. A hematogenous route might play some roles in viral dissemination in the inoculated mice since virus was isolated from the blood. However, the early appearance of infected cells in the CNS, other than in the respiratory tract, suggests that the neural spread from the nasal cavity was more predominant than the hematogenous spread. Neural spread is observed in infection of herpes simplex virus [22], rabies virus [14], cercopithecine virus-1 (B virus) [2] and Venezuelan equine encephalitis virus [21].

The neuroarchitectural relationship of the infected cells observed in mice inoculated with a 20- μ l volume at 4 and 7 days p.i. suggests the possibility of viral spread through the neural tissue and cerebrospinal fluid (CSF) in the CNS (Table 2). The afferent nerve endings may initially play an important role in viral spread because of

Fig. 7 Histology (A, hematoxylin-eosin) and immunohistochemistry for the viral antigen (B) of the nasal cavity of the mouse inoculated with 2 μ l of HK483 virus suspension on day 3 p.i. Positive cells are observed in and beneath (arrow) the olfactory epithelium (OE) and in some columnar epithelium (CE). Immunohistochemistry (peroxidase/diaminobenzidine) for the nucleoprotein of influenza A virus counterstained with methylgreen



the early appearance of virus-infected cells in the sensory nuclei, which suggests a retrograde transport of H5N1 virus. Thereafter, virus is transmitted through trans-neuronal pathways. The nucleoprotein of influenza virus appears initially in the nucleus and subsequently in both the nucleus and cytoplasm [9]. The frequency of nucleus-positive and cytoplasm-negative glial cells, in relation to nucleus-positive and cytoplasm-positive neurons, suggests that these glial cells get the virus from infected neurons.

Ependymal cell infection of H5N1 virus, recognized in this study, can cause virus dissemination through the CSF. The clinical significance of ependymal cell involvement remains to be clarified, and further studies, especially by examination of cytological changes of and viral detection in CSF obtained from patients infected with this virus, are needed to resolve this point.

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