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Neurovirulence of H7N7 influenza A virus: Brain stem encephalitis accompanied with aspiration pneumonia in mice

Brief Report

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Summary. A mouse-adapted influenza A virus, A/equine/London/1416/73-MA (H7N7) caused viral pneumonia, ganglionitis and encephalitis after intranasal inoculation in mice. Virological and pathological data suggested that this virus spreads to the brain by both hematogenous and transneuronal routes, and produces encephalitic lesions similar to those seen in mice infected with H5 highly pathogenic avian influenza A viruses by intranasal infection. Some mice infected with this strain were affected by aspiration pneumonia, which may be caused by neurogenic dysfunction of the pharyngeal/laryngeal reflex due to brain stem encephalitis.

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Among the 15 subtypes of avian influenza viral hemagglutinin (HA), only two, H5 and H7, are found in highly pathogenic avian influenza (HPAI) viruses. HPAI viruses have been well characterized and are known to have a conserved amino acid at the cleavage site of HA, which is recognized by ubiquitous protease [5, 24], and this property allows the viruses to replicate in various organs, including the brain, of the infected host. Some highly pathogenic influenza A virus strains have shown significant neurovirulence in animal models [8, 11, 20, 25]. Detailed pathological studies have revealed that highly pathogenic H5 strains accomplish brain invasion by viremic or transneural spread in infected animal [8, 11, 15, 20]. However, despite recent outbreaks of highly pathogenic avian

H7N7 influenza virus [3], the pathogenicity of H7 influenza viruses in animals is ill-defined.

To date, H7 viruses have been isolated from avian species, seal, horse and a recent human victim of an H7N7 HPAI outbreak [3, 16]. One of these H7 viruses, A/equine/London/1416/73 (Equine/London), has highly cleavable HA, which exhibited virulence in chickens when it was recombined with the internal genes of an avian-derived strain. Although the equine strain itself showed no pathogenicity in chickens [1], it had significant virulence in mice [6]. To further the pathogenic characterization of this equine-derived H7 virus, we virologically and pathologically studied infection with a mouse lung-adapted Equine/London (Equine/London-MA) [6] in mice.

In order to investigate the growth properties of this H7N7 virus, we inoculated 5-week-old female BALB/c mice with 50 μl of virus fluid [$10^{5.0}$ of 50% egg infectious dose (EID_{50})/ml] by the intranasal or intravenous route (9 mice per group). Three mice from each infected group were sacrificed on days 1, 3 and 6 postinfection (p.i.) for virus titration. We harvested and homogenized the nasal turbinate, lungs, spleen, liver, kidneys, brain, pancreas, colon, and blood, performing titrations on 10-day-old embryonated chicken eggs by calculating mean EID_{50} value. The virus recovery data showed that the viral distribution in mice after inoculation was similar, irrespective of inoculation route, and that temporal systemic spread of virus occurs within 24 h p.i. by viremia (Table 1). There was significant pneumo- and neuro-tropism with this strain.

We then studied the growth properties of the virus in passive immunized 5-week-old female ddY mice by intraperitoneal injection of chicken anti-H7 polyclonal antibody [800 hemagglutinin inhibition unit (HIU)/mouse] 12 h before

Table 1. Distribution of Equine/London-MA[a] virus in mice

Virus inoculation route	Days after infection	Virus titer (log_{10} EID_{50}/g) in[b]:								
		Lungs	Nasal turbinates	Spleen	Liver	Kidneys	Brain	Pancreas	Colon	Blood
IN	1	7.8	8.0	1.7	3.2	1.6	5.8	–	–	–
	3	8.0	7.7	–[c]	–	–	4.7	–	–	–
	6	7.6	5.0	–	–	–	1.6	–	–	–
IV	1	7.0	2.8	2.8	4.4	2.4	2.3	–	2.4	–
	3	7.5	6.5	–	–	–	2.5	–	–	–
	6	–	7.2	–	–	–	3.2	–	–	–

[a]Mouse-adapted A/equine/London/1416/73 virus after passaging nine times in mouse lungs

[b]5-week-old, female, BALB/c mice, anesthetized with diethylether, were infected intranasally or intravenously with 50 μl of virus fluid ($10^{5.0}EID_{50}$/ml). Three mice per group were sacrificed on postinfection days 1, 3 and 6 for virus titration

[c]Virus was not isolated

intranasal inoculation with 50 µl of virus fluid ($10^{5.0}$ EID_{50}/ml). The differences of the dose required to kill 50% of mice (MLD_{50}) value between BALB/c and ddY mice was less than 1 log, $10^{1.9}$ and $10^{1.7}$ EID_{50}/mouse respectively, however, the temporal systemic viral spread previously seen in BALB/c mice within 24 h of viral exposure was not detectable by routine recovery test, due to the antiserum immunization that was performed preceding inoculation. The virus was recovered from the respiratory organs at days 1, 5, and 8 p.i. and from the brain at the days 5 and 8 p.i., thus reconfirming the pneumo- and neuro-tropic nature of this virus (Table 2). The transneuronal spreading of the virus strain to the central nervous system (CNS) was suggested, as temporal systemic spread of the virus might have been prevented by passive immunization. The recovery data also

Table 2. Distribution of Equine/London-MA[a] virus in passive-immunized mice

Days after infection	Virus titer (log_{10} EID_{50}/g) in[b]:								
	Lungs	Nasal turbinates	Spleen	Liver	Kidneys	Brain	Pancreas	Colon	Blood
1	4.0	3.5	–[c]	–	–	–	–	–	–
5	8.5	5.0	–	–	–	3.5	–	–	–
8	6.5	3.0	–	–	–	5.3	–	–	NT[d]
30	–	–	–	–	–	–	–	–	–

[a]Mouse-adapted A/equine/London/1416/73 virus after passaging nine times in mouse lungs

[b]5-week-old, female, ddY mice were anesthetized with diethylether, were infected intranasally with 50 µl of virus fluid ($10^{5.0}$$EID_{50}$/ml) 12 h after intraperitoneal injection with 800HI of chicken anti-H7 polyclonal antiserum. The mice were sacrificed on postinfection days 1, 5, 8, and 30 for virus titration

[c]Virus was not isolated

[d]Not tested

Table 3. Lesions in passive immunized mice infected with Equine/London-MA[a] virus

Days after infection	Inflammatory lesion/viral antigen in[b]:						
	Lungs	Ganglions					Brain
		Vagal	Trigeminal	Superior cervical	Inferior cervical	Sympathetic	
1	+/−[c]	−/−	−/−	−/−	−/−	−/−	−/−
2	+/+	+/−	−/−	−/−	−/−	−/−	−/−
3	+/−	++/+	++/+	++/−	+/−	−/−	−/−
4	+/+	++/+	++/−	+++/−	−/−	−/−	+/−
5	++/+	++/+	++/−	++/−	+/−	−/−	++/+

[a]Mouse-adapted A/equine/London/1416/73 virus after passaging nine times in mouse lungs

[b]5-week-old, female, ddY mice were anesthetized with diethylether, were infected intranasally with 50 µl of virus fluid ($10^{5.0}$$EID_{50}$/ml) 12 h after intraperitoneal injection with 800HI of chicken anti-H7 polyclonal antiserum. The mice were sacrificed on postinfection days 1 to 5 for pathological examination

[c]Degree of inflammation in detected lesions and viral antigens are indicated by: +++; many, ++; moderate, +; few, −; not detected

indicated that replication in the respiratory organs may be essential for neuronal invasion.

In a further effort to understand the pathogenic characteristics of this virus, we performed pathological examination of the lungs, ganglions and brain of passive-immunized mice under the same conditions as for virologic examination. On days 1 through 5, the lungs, ganglions and brain were sampled and embedded in paraffin for histologic examination. Additional samples of the lungs and brain were collected on days 7–9, days 11–13, and day 30 p.i. for histologic study. With time after infection, inflammatory lesions with/without expression of viral antigen were detected in the lungs, extending to the cranial ganglions, and finally distributed in the brain with 1- to 3-day time lags (Table 3). In the lungs, early lesions

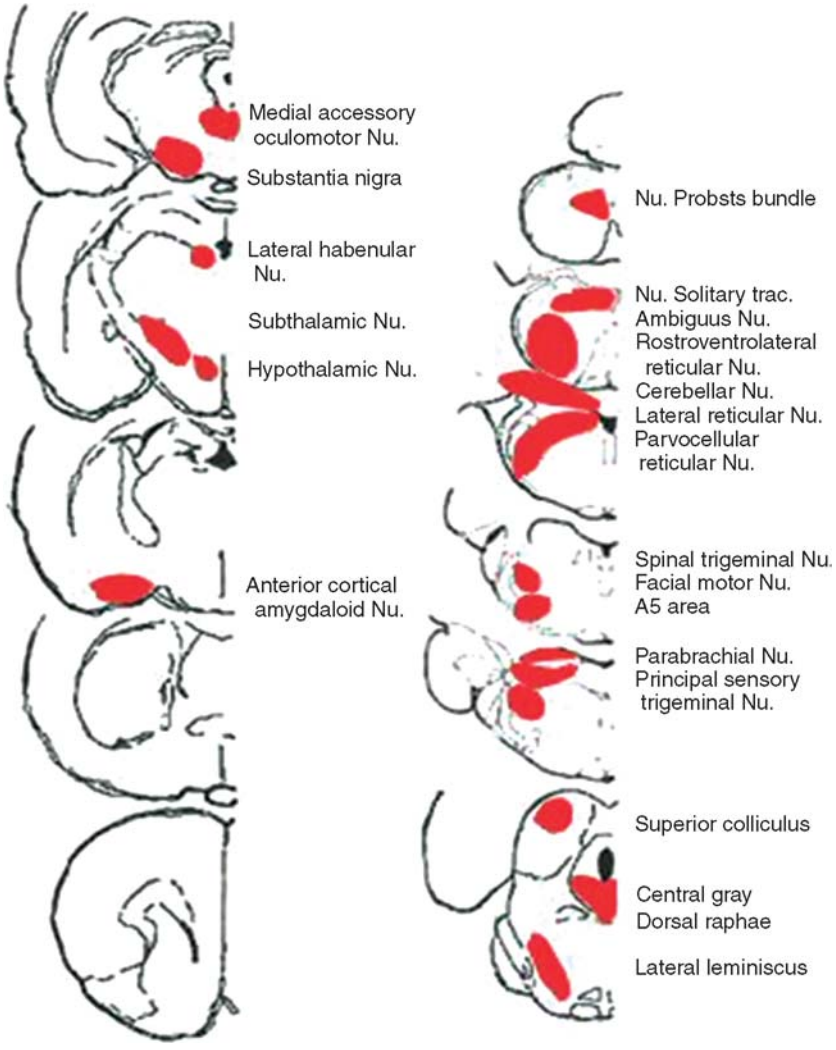

Fig. 1. Distribution of inflammatory lesions in mice infected with the Equine/London-MA virus after passive immunization. (Red area). Lesions are converged on the brain stem and basal ganglia, including substantia nigra

were detected as bronchitis and bronchiolitis, developing to bronchoalveolitis and finally resulting in non-suppurative pneumonia with prominent congestive edema. Ten percent of infected mice had lesions of aspiration pneumonia with foreign body reactions against food particles. Perivascular cuffing of mononuclear cells, gliosis, and degeneration of neurons were significant and viral antigens were detected in the neurons and the astrocytes of the nervous tissues. Sequential changes on the distribution of viral antigen/inflammatory response in such organs were similar to those of mice infected with highly pathogenic avian H5 viruses [11, 20]. Moreover, the encephalitic lesions were centered on the brain stem and basal ganglia, including the substantia nigra (Figs. 1, 2A), and this distribution pattern was the same as that observed in CNS lesions of mice infected with highly pathogenic H5 viruses [11, 20].

In addition to respiratory tropism, the neurotropic nature of this equine strain was reconfirmed by virological and pathological examination. It is noteworthy that this equine-derived H7 virus enhanced its pathogenicity after several passages in mouse lungs [6], and produced similar distributional lesions in mouse CNS as H5 HPAI viruses. In the case of H5N1 strains isolated from human patients during the 1997 Hong Kong outbreak, even low pathogenic strains in mice can undergo multi-cycle replication in mouse CNS by direct intracerebral inoculation, and a single amino acid substitution in the viral internal protein (PB2) allowed the virus to distribute systemically, including the brain, after intranasal infection [19]. These facts suggest that many HPAI have neurovirulent potential that is restricted by the function of internal proteins. Roles of HA receptor-binding affinity and cleavability in neurovirulence have been indicated [10]. Our data demonstrated that the equine-derived virus with highly cleavable HA also showed significant neurovirulence in mice after intranasal infection and produced encephalitic lesions that were similar to those seen in mice with CNS infection by H5 HPAI viruses.

The aspiration pneumonia lesions detected in this study were recognized as novel in influenza infection models. Food particles were scattered in the lungs and exhibited conspicuous foreign body reactions (Fig. 2B). Because this type of pneumonia is unusual during the course of lethal infection with non-neurovirulent influenza A virus in mice, neurogenic dysfunction of the pharyngeal/laryngeal reflex was presumed. These reflexes are triggered by sensory input from the pharyngeal and laryngeal mucosa to the nuclei in the medulla oblongata [7], and laryngopharyngeal muscles are dominated by the motor neurons at the ambiguus nucleus of the medulla oblongata. Although one neurotransmitter, substance P, is believed to play a major role in the sensory pathways of these reflex reactions [17], involvement of the 9^{th} to 11^{th} cranial nerve nuclei in the brain stem encephalitis by Equine/London-MA infection (Table 3, Fig. 1) may be the direct cause of neurogenic dysfunction of the pharyngeal/laryngeal reflex and aspiration pneumonia in mice. Viral encephalitis is unusual during respiratory viral infection, except during opportunistic CNS infection in immune-compromised infant or elderly patients [2, 4, 12–14, 18, 21–23]. Aspiration pneumonia resulting from brain stem lesions can become serious in these patients. The present results indicate a mechanism for aspiration pneumonia in these patients.

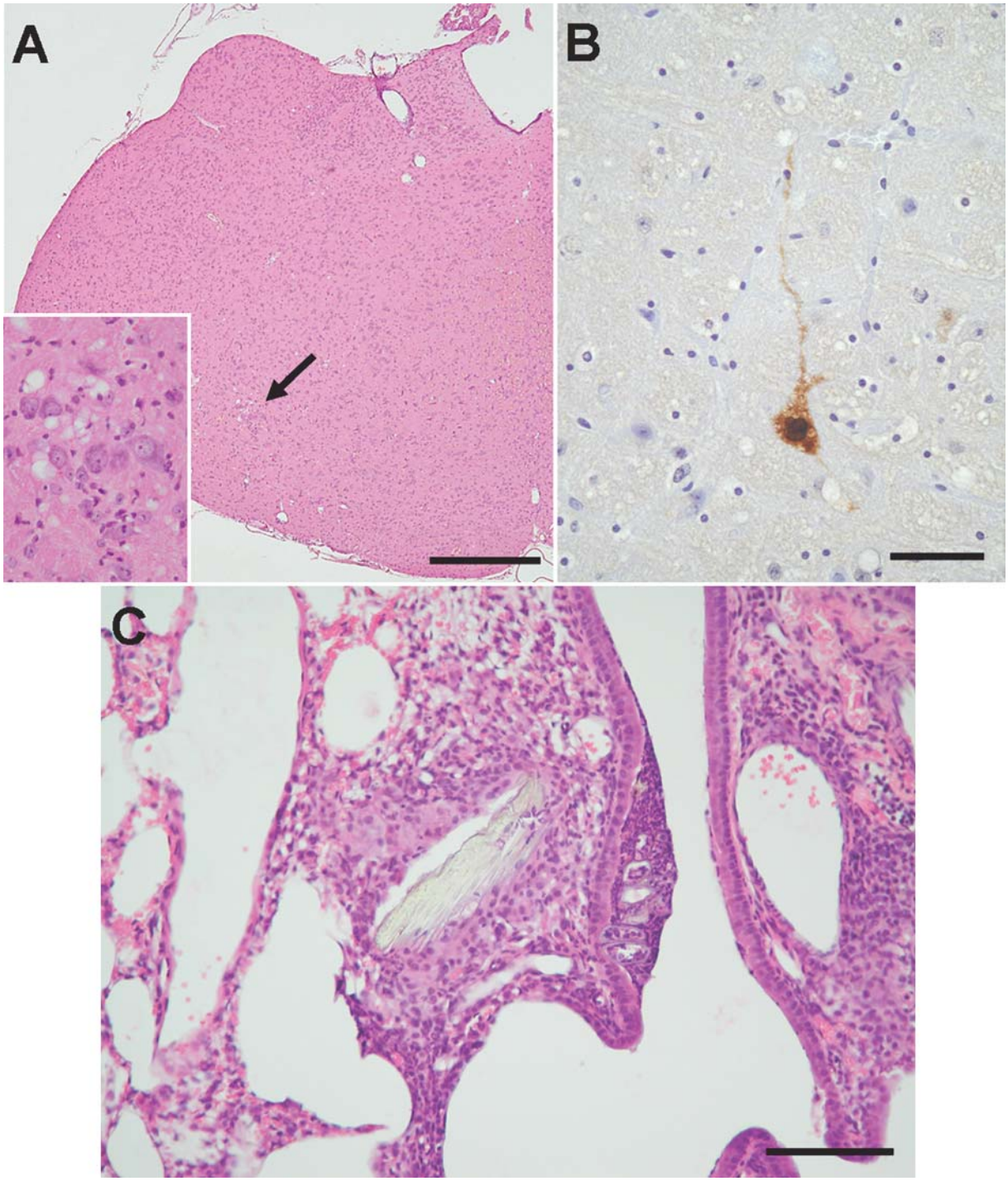

Fig. 2. Pathological findings detected in mice infected with the Equine/London-MA virus after passive immunization. **A**: Non-suppurative inflammation in the brain (arrows). Higher magnification (insert) of the lesion (arrow) illustrates microgliosis and spongiosis of the neuropile. Bar = 500 μm. **B**: Viral antigen in the brain. Bar = 50 μm. **C**: Foreign body reaction to a food particle in the lung. Bar = 100 μm

In conclusion, we characterized the pathological properties of the mouse-adapted equine-derived H7N7 virus in mice. This virus caused viral pneumonia, ganglionitis and encephalitis after intranasal inoculation. Virological and pathological data suggested that this virus can spread to the brain by both hematogenous and transneuronal routes, and can produce encephalitic lesions similar to those seen in mice with CNS infection by H5 HPAI viruses. Aspiration pneumonia detected in some mice infected with this virus was suggested to be the result of neurogenic dysfunction of the pharyngeal/laryngeal reflex due to brain stem lesions.

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