JOURNAL OF VIROLOGY, July 1991, p. 3891–3894
0022-538X/91/073891-04$02.00/0

Vol. 65, No. 7

Equine H7N7 Influenza A Viruses Are Highly Pathogenic in Mice without Adaptation: Potential Use as an Animal Model

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Received 17 December 1990/Accepted 27 March 1991

Equine H7N7 influenza A viruses, representing a broad range of isolates, were lethal in mice without adaptation. After repeated passages, A/Equine/London/1416/73 acquired neurotropism upon intranasal infection. Thus, mice infected with equine influenza A viruses provide a model system for the study of highly virulent mammalian influenza viruses.

Influenza A viruses naturally infect a variety of animal species, including humans, pigs, horses, and avian species. Unlike the pandemic strain that killed 20 million people worldwide in 1918 and 1919, currently circulating mammalian influenza viruses are not lethal unless complications develop in the host. However, highly virulent influenza viruses exist in avian species, serving as a reminder that highly virulent strains could reappear in humans.

Mouse adaptation of human influenza A viruses has been studied extensively (for review, see reference 8). In general, human influenza viruses are not lethal in mice and do not cause lung lesions without adaptation (8). After repeated mouse-to-mouse passages, the viruses that replicate to high titers in the lungs are selected and acquire the ability to kill mice. In rare cases, human influenza viruses are lethal in mice on the first passage, but this property stems from the high titer of the inoculum and not from any inherent virulence of the viruses, as the lungs of the dead mice contain a limited amount of infectious virus (22). Use of reassortant viruses (avirulent versus mouse-adapted strains) to study the molecular basis of the acquisition of virulence by these mouse-adapted viruses has disclosed the involvement of the HA, M, and NS genes (3).

Cleavability of the hemagglutinin (HA) molecule is associated with the virulence of avian influenza A viruses (2). In tissue culture, the HAs of the virulent viruses are cleaved in the absence of trypsin (i.e., high cleavability), whereas those of the avirulent viruses require trypsin for cleavage. A series of basic amino acids at the cleavage site between HA1 and HA2 is required for high cleavability of the HA molecule, making it a key determinant of the virulence of avian influenza A viruses (2, 12). Although equine H7N7 viruses cause only mild respiratory symptoms in horses, Gibson et al. (5) recently found that the HAs of these viruses contain a series of basic amino acids between HA1 and HA2. This structural feature is also required for the neurovirulence of influenza A viruses in mice (1). Therefore, the pathogenicity of equine H7N7 influenza A viruses in mice was assessed, with the thought of establishing a model system for the study of highly virulent mammalian influenza viruses.

Although all of the H7N7 equine viruses examined by Gibson et al. (5) contain multiple basic amino acids at the cleavage site of the HA, only one (A/Equine/Prague/1/56) has been shown to be cleaved in tissue culture in the absence of trypsin (4). To determine whether high cleavability of the HA is a common property of H7N7 equine influenza viruses, the HA cleavage of seven H7N7 equine influenza viruses isolated from different geographic regions during different years was examined. Chicken embryo fibroblasts infected with these viruses were radiolabeled with [^{3}H]mannose and immunoprecipitated with monoclonal antibodies to the HA. Polyacrylamide gel analysis (Fig. 1) showed that all the HAs of these viruses were cleaved, although not completely, in the absence of trypsin, demonstrating high cleavability of the equine influenza virus HA.

Cleavage of the HA is required for influenza virus infectivity (14, 16). In tissue culture, virulent avian influenza A viruses with highly cleavable HAs undergo multiple cycles of replication in the absence of trypsin, whereas other viruses, including human influenza viruses, require trypsin. Therefore, the equine influenza viruses were examined to determine whether high cleavability of the HA permits them to undergo multiple replication cycles in tissue culture in the absence of trypsin. In chicken embryo fibroblasts, all of the equine H7N7 viruses tested underwent multiple cycles of replication, although A/Equine/Prague/1/56 and A/Equine/Kentucky/58 were less efficient than other strains (data not shown). Thus, the ability of the viruses to undergo multiple cycles of replication in the absence of trypsin in these cells is related to the cleavability of their HAs.

All of the equine H7N7 viruses were lethal (i.e., the majority of deaths occurred by 9 days) to BALB/c mice which were infected intranasally with approximately 10^6 50% egg-infectious dose (EID_{50}) of virus (Fig. 2). Although A/Equine/Santiago/77 was less lethal (three of six mice survived) than other H7N7 equine viruses, the pulmonary virus titers of mice infected with this virus ($10^{6.83}$ EID_{50}) were similar to those of mice infected with A/Equine/Kentucky/77 ($10^{6.5}$ EID_{50}) or with A/Equine/London/1416/73 ($10^{7.5}$ EID_{50}). The virus dose lethal to 50% of the mice was calculated to be $10^{5.5}$ EID_{50} for A/Equine/London/1416/73.

None of the mice infected with the other subtypes of influenza virus from horse (A/Equine/Tennessee/5/86 [H3N8] and A/Equine/California/103/82 [H3N8]), human (A/Aichi/2/68 [H3N2] and A/USSR/1/77 [H1N1]), avian (A/Duck/Hokkaido/8/80 [H3N8]), or swine (A/Swine/Iowa/15/30 [H1N1]) strains showed any disease symptoms, nor did any die during the 2-week observation period. Virus titers in the lungs of mice infected intranasally with approximately 10^6 EID_{50} of virus were low ($10^{3.5}$ EID_{50}/g) for A/Equine/Tennessee/5/86 but relatively high ($10^{6.83}$ EID_{50}/g) for A/Aichi/2/68 (H3N2) and A/Equine/London/1416/73 ($10^{7.5}$ EID_{50}/g). The virus doses required to infect 50% of the mice were similar among these viruses: $10^{3.1}$, $10^{2.0}$, and $10^{2.3}$ EID_{50} for

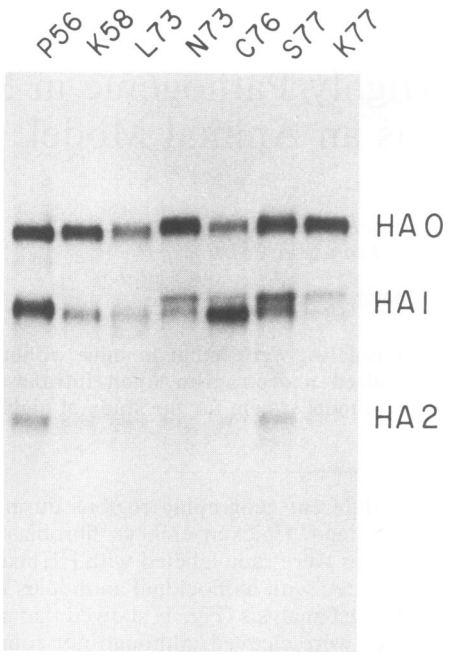

FIG. 1. Comparison of HA cleavage among H7N7 equine influenza A viruses. Cleavage of the HA molecule in chicken embryo fibroblasts was examined by infecting cell monolayers with A/Equine/Prague/1/56 (P56), A/Equine/Kentucky/58 (K58), A/Equine/London/1416/73 (L73), A/Equine/New York/73 (N73), A/Equine/Cordova/76 (C76), A/Equine/Santiago/77 (S77), and A/Equine/Kentucky/77 (K77); 3 h later, the HAs were labeled with [^{3}H]mannose for 5 h at 37°C. Cell extracts were immunoprecipitated with monoclonal antibodies to the HA and analyzed on a 12.5% polyacrylamide gel as described before (15).

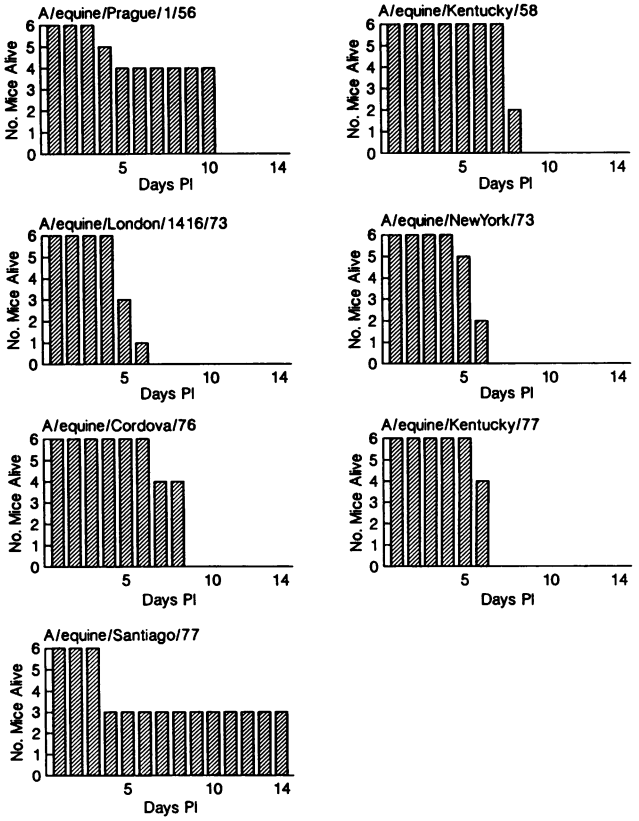

FIG. 2. Virulence of equine H7N7 influenza viruses in mice. The virulence of each virus was examined by inoculating groups of six mice with approximately 10^6 EID_{50} of each virus. Mice were observed for 2 weeks. PI, postinfection.

A/Equine/Tennessee/5/86, A/Aichi/2/68, and A/Equine/London/1416/73, respectively. These findings indicate that the ability to replicate to a high titer in the lungs and relative infectivity in mice do not necessarily correlate with the lethality of influenza A viruses in mice.

High cleavability of the HA is associated with the neurovirulence of influenza viruses in both chickens (2) and mice (1). Therefore, the tissue tropism of A/Equine/London/1416/73 in mice was examined by determining the virus titer in organs (lung, kidney, spleen, liver, brain, and lower intestine) 3 days after infection with 10^6 EID_{50} of virus. Virus was recovered only from the lungs ($10^{5.5}$ EID_{50}/g); except on rare occasions, a limited amount of virus ($10^{2.2}$ EID_{50}/g) was recovered from the brain.

Because human influenza A viruses are lethal to mice after repeated mouse-to-mouse passaging, it was asked whether this procedure would increase the virulence of A/Equine/London/1416/73. The virus was passaged by inoculating mice intranasally with a pooled 10% lung homogenate (50 μl) from two infected mice. Although the virus did not kill mice more rapidly after 11 passages, it did acquire the ability to spread to organs other than the lungs. After eight passages, the virus was recovered from a variety of tissues, including brain, indicating that it had acquired the ability to spread systemically (Table 1). By contrast, the replication of a mouse-adapted human influenza A virus, A/Aichi/2/68 (H3N2), was limited to respiratory organs and the liver; it did not spread to the brain. Because isolation of virus in the livers of mice infected with the mouse-adapted human virus was unexpected, the experiments were repeated. Replication of mouse-adapted human viruses was observed repeatedly in the livers of most of the infected mice (data not shown).

The current study demonstrates the existence of mammalian influenza A viruses with highly cleavable HAs that could be lethal in mammals. It should be emphasized that all other

TABLE 1. Comparison of tissue tropism in mice between A/equine/London/1416/73 (H7N7) and A/Aichi/2/68 (H3N2) after repeated passages in mice[a]

Virus	Days after infection	Virus titer (log_{10} EID_{50}/g)				
		Lung	Colon	Liver	Kidney	Brain
A/Equine/London/1416/73 (H7N7)	3	5.5	2.2	3.8	3.2	3.5
	6	5.5	NR[b]	2.2	NR	3.2
A/Aichi/2/68 (H3N2)	3	5.5	NR	5.2	NR	NR
	6	5.5	NR	2.9	NR	NR

[a] Mice (BALB/c) were inoculated intranasally with approximately 10^6 EID_{50} of virus. Samples from two mice were collected 3 or 6 days after infection and pooled. Samples on day 6 were obtained from dead mice. To avoid contamination with virus from respiratory organs, the samples were taken in the following order: brain, liver, kidney, colon, and lung. Samples of virus grown from each organ were identified in hemagglutination inhibition tests with specific antisera. A/Equine/London/1416/73 and A/Aichi/2/68 were passaged in mice 8 and 13 times, respectively.

[b] NR, virus not recovered.

influenza viruses that are virulent in mice have been adapted in the laboratory. Moreover, the H7N7 equine virus causes systemic infection after intranasal inoculation and spread to the brain after repeated passages in mice, in contrast to other mouse-adapted viruses, whose replication in tissues other than the respiratory organs is rare. Although some mouse-adapted neurovirulent viruses do exist, they require intracerebral inoculation (i.e., WSN strain). By contrast, the equine H7N7 virus in this study causes systemic infection after intranasal inoculation, with spread to the brain.

The molecular basis for the enhanced pathogenicity of equine H7N7 influenza viruses was not examined in this study. Recent evolutionary studies indicate that the internal genes of A/Equine/London/1416/73 (H7N7) were not derived from the prototype A/Equine/Prague/1/56-like (H7N7) virus, but were derived instead from equine H3N8 influenza viruses by genetic reassortment (6, 11, 13, 19). Since equine H3N8 viruses are not pathogenic without adaptation, one could speculate that the genes encoding the surface proteins but not the internal proteins are important for the virulence of the H7N7 viruses. Previous reassortment experiments between avirulent and mouse-adapted virulent viruses also demonstrated that the HA gene plays a role in the virulence of human viruses in mice, although only a single arginine is present at the HA cleavage site in such viruses (3).

The current study shows that a mouse-adapted human virus (A/Aichi/2/68) can replicate not only in the lungs but also in the liver, suggesting that mouse adaptation of the virus expands its tissue tropism. This was also the case for A/Equine/London/1416/73 (H7N7). However, the equine virus acquired the ability to reach the brain, a characteristic not shared by A/Aichi/2/68 (H3N2), although only single-passage lines of each strain have been examined thus far. It is unclear whether recovery of a mouse-adapted A/Equine/London/1416/73 strain from the brains of infected mice reflects actual virus replication in the brain or viremia. Direct examination of virus replication in different organs is needed to answer this question. Whether the expanded tissue tropism of the mouse-adapted A/Equine/London/1416/73 (H7N7) reflects the presence of a series of basic amino acids at the HA cleavage site remains to be determined.

Why equine viruses are highly pathogenic in mice but not in horses is unclear. Genetic studies indicate that the equine viruses have been maintained for a long time in this species (10), suggesting that they have evolved to a point where they are able to replicate in horses without causing severe disease, much like avian influenza viruses in wild ducks (7). The possibility exists that the HAs of these viruses, which are highly cleavable in tissue culture cells, may not be highly cleavable in the cells of horses.

Whether the HA cleavability of the 1918 human pandemic strain was similar to that of virulent avian viruses is not known. However, results of pathology studies of human tissue from the 1918 influenza pandemic suggest that the causative virus was neurotropic (9, 18), a characteristic associated with high cleavability of the HA (1, 17, 21). The importance of high cleavability of the HA for the neurotropism of influenza viruses in mice has been well established through the use of reassortment viruses between virulent avian and human strains (1, 20), although other components of human viruses, such as the M, PB1, and PA genes, are also important in allowing efficient virus replication in mice (1, 20).

There are a few alternative models for studying highly pathogenic influenza viruses in animals. Mouse-adapted human influenza viruses do not contain highly cleavable HAs and are not neurotropic. Virulent avian influenza viruses, on the other hand, do possess highly cleavable HAs and are neurotropic in birds, but do not replicate in mammals at all. Thus, H7N7 equine influenza viruses in mice offer a system with which to study the mechanisms of viral pathogenesis, including neurotropism.

I thank Krisna Wells and Michelle Hill for excellent technical assistance and John Gilbert for writing assistance.

This work was supported by Public Health Service grant AI-29599 from the National Institute of Allergy and Infectious Diseases, Cancer Center Support (CORE) grant CA-21765, and American Lebanese-Syrian Associated Charities.

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