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PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice

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Abstract

A single amino acid substitution, from glutamic acid to lysine at position 627 of the PB2 protein, converts a nonlethal H5N1 influenza A virus isolated from a human to a lethal virus in mice. In contrast to the nonlethal virus, which replicates only in respiratory organs, the lethal isolate replicates in a variety of organs, producing systemic infection. Despite a clear difference in virulence and organ tropism between the two viruses, it remains unknown whether the dissimilarity is a result of differences in cell tropism or the reduced replicative ability of the nonlethal virus in mouse cells in general. To determine how this single amino acid change affects virulence and organ tropism in mice, we investigated the growth kinetics of the two H5N1 viruses both in vitro and in vivo. The identity of the PB2 amino acid at position 627 did not appreciably affect viral replicative efficiency in chicken embryo fibroblasts and a quail cell line; however, viruses with lysine at this position instead of glutamic acid grew better in the different mouse cells tested. When the effect of this substitution was investigated in mice, all of the test viruses showed the same cell tropism, but infection by viruses containing lysine at position 627 spread more rapidly than those viruses containing glutamic acid at this position. Further analysis showed a difference in local immune responses: neutrophil infiltration in lungs infected with viruses containing lysine at position 627 persisted longer than that associated with viruses lacking a glutamic acid substitution. Our data indicate that the amino acid at position 627 of the PB2 protein determines the efficiency of viral replication in mouse (not avian) cells, but not tropism among cells in different mouse organs. The presence of lysine leads to more aggressive viral replication, overwhelming the host's defense mechanisms and resulting in high mortality rates in mice.

Keywords: PB2; Lysine; H5N1 influenza A virus

Introduction

In 1997, H5N1 avian influenza viruses were transmitted to humans, resulting in six deaths among 18 persons infected (Claas et al., 1998; Subbarao et al., 1998). In 2003, another H5N1 virus also killed one of two persons infected (Wuethrich, 2003). These outbreaks demonstrated direct avian-to-human transmission of H5N1 viruses and the potential for avian viruses to directly infect humans and cause fatal disease (Claas et al., 1998; Subbarao et al., 1998; Wuethrich, 2003). Viruses isolated from patients in the 1997 outbreak were divided into two groups based on their virulence in mice: virulent viruses, which caused lethal systemic infection characterized by an LD_{50} (dose required to kill 50% of animals) of less than 0.3 plaque-forming units (pfu), and avirulent viruses, which were nonlethal even at a dose of 10^4 pfu (Gao et al., 1999; Katz et al., 2000). A reverse genetics approach identified a single amino acid substitution, Glu-to-Lys, at position 627 in the PB2 protein as being responsible for this difference in virulence (Hatta et al., 2001). The exact same mutation was found in an H7N7 virus from a person who died due to severe pneumonia but not in those isolated from birds or from persons with conjunctivitis during an outbreak in Europe in 2003 (Fouchier and Osterhaus, 2003). The

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Table 1
Virus spread in intranasally infected mice[a]

Virus (background of virus)	Amino acid at 627	LD_{50} (pfu)[b]	Virus titers (log_{10} PFU/g) in				Viral antigen distribution							
			Days p.i.	Nasal turbinate	Lung	Brain	Respiratory organ	Days post inoculation						
								1	2	3	4	5	6	7
HK483RG (HK483)	Lys	1.7	3[b]	3.4 ± 0.6	7.1 ± 0.6	1.4, 1.4	nasal T (R)[c]	–[d]	–	++	++	++	+++	+++
							nasal T (O)[e]	–	–	–	–	+	+++	+++
			6[b]	5.4 ± 1.3	6.1 ± 0.1	3.9 ± 1.0	trachea, bronchus	–	+	+++	+++	+++	+++	+++
							alveolus	+	++	++	+++	+++	+++	+++
HK3PB2-627E (HK483)	Glu	2.3×10^3	3	<[f]	5.1 ± 0.2	<	nasal T (R)	–	–	–	–	++	–	++
							nasal T (O)	–	–	–	–	–	–	–
			6	<	6.8 ± 0.2	<	trachea, bronchus	–	+	++	+++	+++	+++	++
							alveolus	–	+	++	++	++	++	+
HK486RG (HK486)	Glu	4.6×10^4	3	<	5.3 ± 0.3	<	nasal T (R)	–	–	–	–	–	–	–
							nasal T (O)	–	–	–	–	–	–	–
			6	5.3	6.5 ± 0.2	<	trachea, bronchus	–	–	+	++	++	++	+++
							alveolus	–	–	+	++	++	++	++
HK6PB2-627K (HK486)	Lys	5.8	3	<	7.2 ± 0.2	<	nasal T (R)	–	–	–	–	–	++	–
							nasal T (O)	–	–	–	–	++	+++	–
			5	6.6 ± 0.8	7.7 ± 0.1	6.6 ± 0.2	trachea, bronchus		+	++	+++	+++	+++	++
							alveolus	–	+	++	++	+++	+++	+++

[a] Balb/c mice were intranasally infected with virus, and the presence of viral antigens was determined daily by immunohistochemistry.
[b] Data from previous report (Hatta et al., 2001).
[c] Respiratory region of nasal turbinate.
[d] The frequencies of stained cells are scored as: +++, widely distributed; ++, patchy; +, rare; –, not detected.
[e] Olfactory region of nasal turbinate.
[f] Less than $10^{1.3}$ pfu/g.

contribution of this mutation to the enhanced virulence of influenza A virus (lethal systemic infection vs. nonlethal respiratory infection) has remained poorly understood. Specifically, it is unknown whether the PB2 mutation affects viral tropism among cells in different mouse organs or viral growth in mouse cells in general. This information is critical for selecting appropriate strategies for elucidating the mechanism by which avian influenza A viruses exhibit high virulence in mammals due to the PB2 mutation.

To gain insight into the role of PB2 in influenza virulence and host range restriction, we performed in vitro and in vivo studies using four viruses (Table 1); virulent A/Hong Kong/483/97 (HK483RG; H5N1), its avirulent mutant with a Lys-to-Glu substitution at position 627 in PB2 (HK3PB2-627E), avirulent A/Hong Kong/486/97 (HK486RG; H5N1), and its virulent mutant with a Glu-to-Lys substitution at this position (HK6PB2-627K).

Results

Avirulent and virulent H5N1 Hong Kong viruses replicate equally well in avian cells, although the latter replicate more efficiently in mouse cells

To evaluate the replicative potential of the different H5N1 viruses, we tested their growth kinetics in cultured cells. In primary chicken embryonic fibroblasts (CEF) and a quail fibrosarcoma cell line (QT6), all four viruses grew to high titers (>10^7 pfu/ml) without any appreciable difference in their growth kinetics (Figs. 1A, B). In Madin–Darby canine kidney cells (MDCK) cells (Fig. 1C), both PB2 mutants (HK3PB2-627E and HK6PB2-627K) grew to higher titers than their parents, but the difference was less than 1.5 log among the four viruses. By contrast, virulent HK483RG and HK6PB2-627K viruses replicated more efficiently in cultured mouse astroctyes and LA-4 mouse lung adenoma cells than did avirulent HK486RG and HK3PB2-627E viruses (Figs. 1D, E). A similar result was obtained with NIH-3T3 mouse fibroblasts (data not shown). These findings suggested that the Glu-to-Lys substitution at position 627 of PB2 increases the replicative efficiency of viruses in mouse cells.

A single amino acid substitution in the PB2 protein of Hong Kong H5N1 viruses promotes wider spread of virus in intranasally infected mice, but does not affect cell tropism in respiratory organs

To further explain how a single amino acid change in the PB2 protein could account for increased virulence in vivo (i.e., more than a 3-log difference in the LD_{50} dose; Hatta et al., 2001), we intranasally infected mice with 100 pfu of virus. The extent of viral replication in nasal turbinate was not directly correlated with the PB2 amino acid residues at position 627 (Table 1). However, viruses with glutamic acid at position 627 in PB2 (HK3PB2-627E and HK486RG) were localized to respiratory organs and spread slowly, whereas those with lysine at this position (HK483RG and

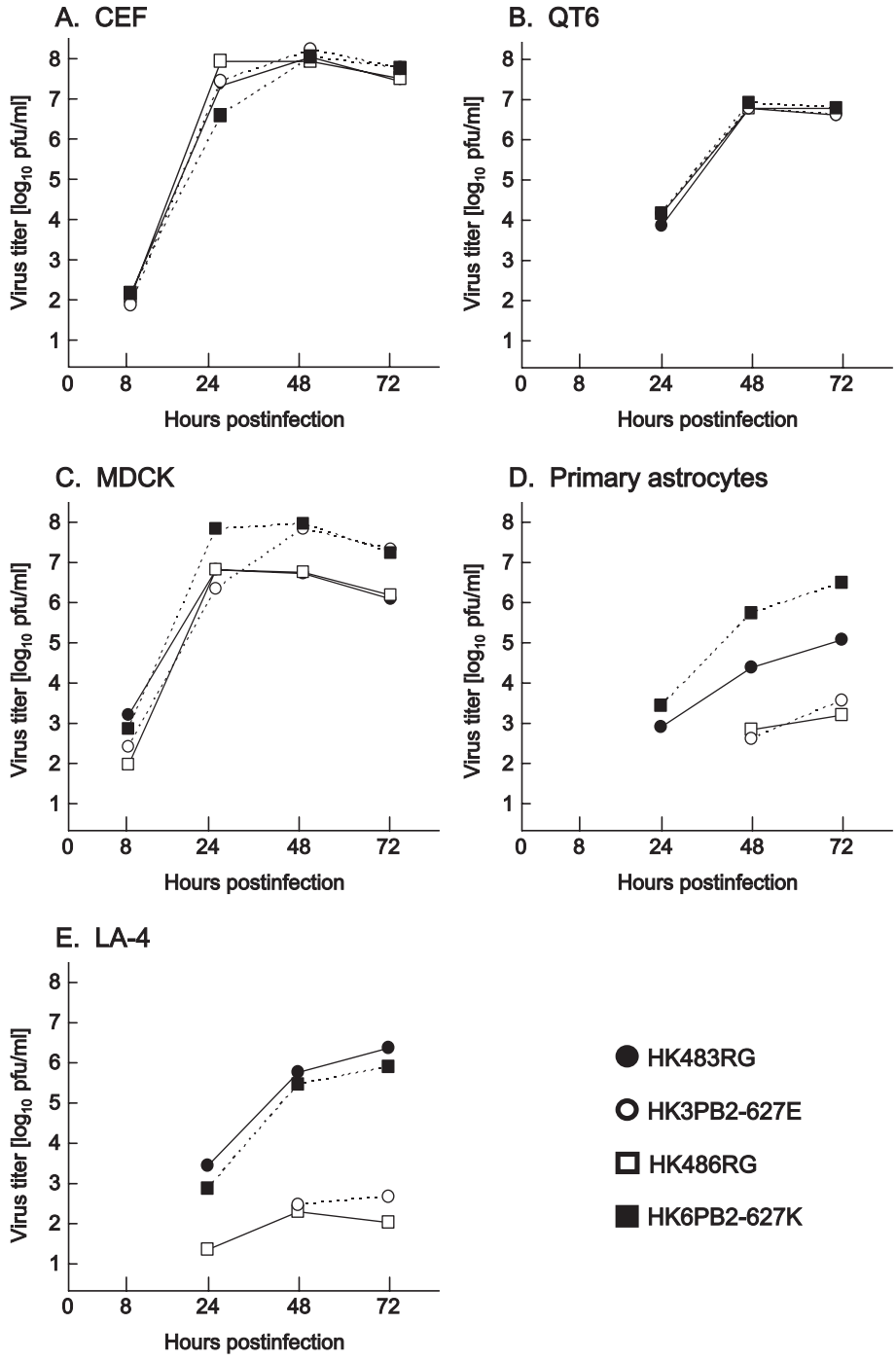

Fig. 1. Difference in the growth kinetics of Hong Kong H5N1 viruses in cultured cells. CEFs (A), QT6 (B), MDCK cells (C), primary mouse astrocytes (D), or LA-4 mouse lung adenoma cells (E) were infected with HK483RG (●), HK3PB2-627E (○), HK486RG (□), or HK6PB2-627K (■) at an MOI of 10^{-4}, and virus titers in supernatants were determined with MDCK cells. The results represent three experiments.

HK6PB2-627K) spread rapidly and were widely distributed (Table 1). All viruses showed similar cell tropism in these organs; nasal, tracheal, bronchial, and alveolar epithelia were infected. Interestingly, in the alveolar wall of HK483RG- and HK6PB2-627K-infected mice, infiltrating neutrophils were seen continuously during the experimental period, although lymphocytes were not recruited until day 5 postinfection (p.i.). In HK3PB2-627E- and HK486RG-infected mice, neutrophil recruitment was transient (to day 3 p.i.) followed by extensive lymphocyte infiltration (Fig. 2). Late in infection with virulent HK483RG and HK6PB2-627K, viral antigens were widely distributed among the nonrespiratory organs: nerve tissues (including ganglia), lymphatic organs, liver, heart, and fatty tissue (Table 2).

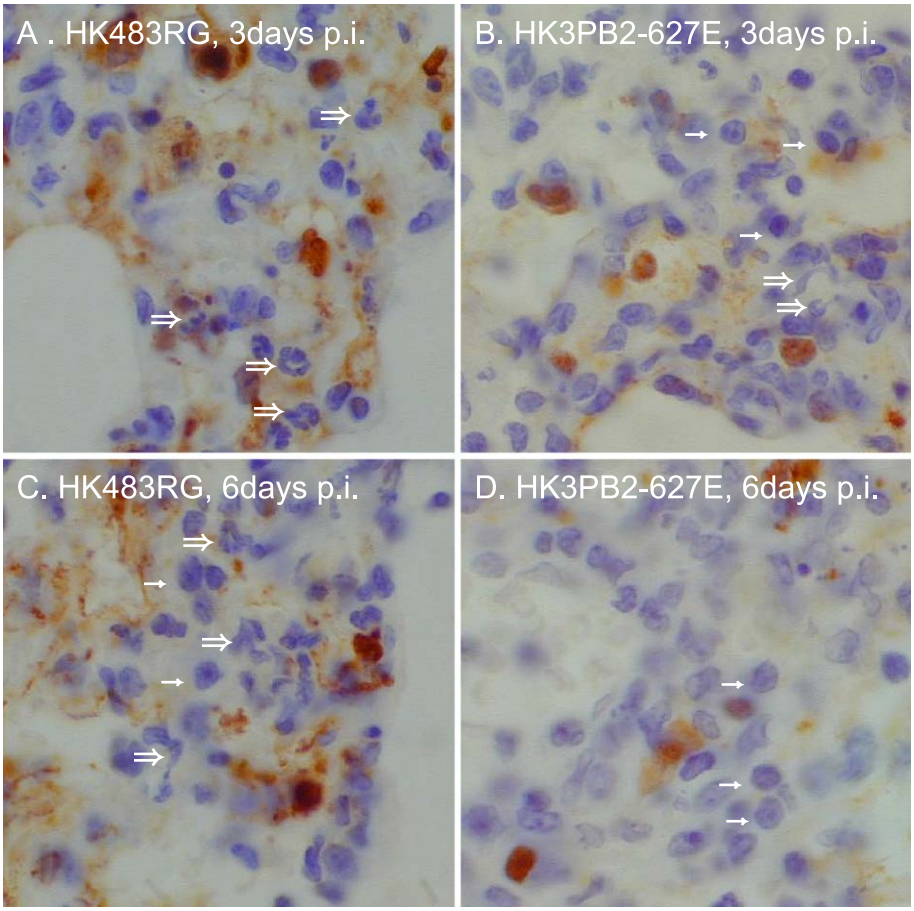

Fig. 2. Histologic changes in the lungs of mice intranasally infected with the virulent HK483RG virus or the avirulent HK3PB2-627E virus. Prominent differences were observed in the pattern of neutrophil (□) and lymphocyte (□) recruitment: in mice infected with HK483RG (A, C), persistent infiltration of neutrophils was characteristic during the entire experimental period (from days 2 to 7), while the lungs of mice intranasally infected with avirulent HK3PB2-627E (B,D) showed transient neutrophilic inflammation followed by rapid infiltration of lymphocytes by day 3 p.i.

By contrast, HK3PB2-627E and HK486 viruses were limited to the respiratory organs and to some ganglia associated with respiratory organs (vagoglossopharyngeal ganglia and peribronchial autonomic ganglia). Electron microscopy demonstrated the production of progeny viruses in these ganglia (Fig. 3).

These data demonstrate the same cell tropism in the tissue of respiratory organs (bronchiolar and alveolar epithelia) for all tested viruses in intranasally infected mice. However, infection with viruses containing lysine at position 627 of PB2 (HK483RG and HK6PB2-627K) spread faster along the respiratory tree than did those containing glutamic acid at this position (HK486RG and HK3PB2-627E).

The single amino acid change in PB2 that promoted virulence does not affect CNS cell tropism

Upon intranasal inoculation, virulent but not avirulent Hong Kong H5N1 viruses were recovered from the CNS (Gao et al., 1999; Hatta et al., 2001; Katz et al., 2000; Lipatov et al., 2003). To determine whether this discrepancy in organ tropism reflects cell tropism due to the single amino acid alteration in PB2 at position 627, we inoculated the viruses directly into mouse brain. Mice intracerebrally infected with virulent HK483RG or HK6PB2-627K virus were lethargic at 4 days p.i. and died by 6 days p.i., whereas these symptoms were absent in mice infected with avirulent HK486RG or HK3PB2-627E virus as well as in mock-infected animals. In mice infected with virulent HK483RG or HK6PB2-627K, viral antigen-positive cells were distributed throughout the parenchyma during later infection, whereas in the brains of mice infected with avirulent HK486RG or HK3PB2-627E, they showed only a limited distribution even late in infection (Fig. 4). Although initially the types of antigen-positive cells differed between virulent and avirulent viruses (fibroblasts in pia mater in days 1 to 2 p.i. vs. ependymal/choroidal cells around ventricules in day 3 p.i.), viral antigens were eventually found in all cell types within nervous tissue: neurons, glial cells, ependymal and choroidal cells, and fibroblasts (Fig. 5).

To determine if the avirulent viruses replicating in brain had acquired mutations enabling their growth in this organ,

Table 2
Extent of virus spread in intranasally infected mice[a]

Tissue	Viral antigen distribution in organs and tissues of mice infected with[b]			
	HK483RG	HK3PB2-627E	HK486RG	HK6PB2-627K
Brain	+++	–	–	+++
Cranial ganglia	+++	+	+	+++
Spinal cord	+++	–	–	+++
Ganglia[c]	+++	±	–	+++
Lungs	+++	++	++	+++
Nasal turbinate	+++	++	–	+
Liver	++	–	–	++
Spleen	±	–	–	±
Lymph node	±	–	–	±
Kidney	–	–	–	–
Heart	++	–	–	++
Teeth[d]	++	–	–	–
Skin	++	–	–	–
Fatty tissue	+++	–	–	+++
Intestine[e]	–	–	–	+

[a] Balb/c mice were intranasally infected with virus, and organs collected on days 1–7 p.i. were analyzed for viral antigen positivity by immunohistochemical methods. This table summarizes antigen distribution throughout the experimental period.
[b] +++, many; ++, some; +, sporadic; ±, transient detection; –, not detected.
[c] Autonomic ganglia.
[d] Viral antigens were detected in cells of dental pulp.
[e] Viral antigens were detected in Auerbach's ganglia.

we sequenced HK486RG or HK3PB2-627E virus in brain samples harvested on day 7 after intracerebral inoculation. Sequence analysis revealed that among eight isolates from the brains of mice inoculated with HK3PB2-627E, two harbored a Glu-to-Lys substitution at position 627 of PB2, two had an Asp-to-Asn substitution at position 701, while the remaining four lacked mutations. None of the six isolates from brain inoculated with HK486RG strain had amino acid substitutions in PB2. Thus, although growth in mouse brain clearly selects for influenza A variants with a mutation enhancing the replicative potential, viruses with glutamic acid at position 627 in PB2 are still able to replicate in this organ, but spread more slowly than virulent viruses.

Discussion

Here, we show that the increased virulence of Hong Kong H5N1 viruses with a Glu-to-Lys substitution at position 627 of PB2 is related to a difference in replicative efficiency, but not cell tropism. Although the H5N1 viruses tested in this study did not differ in their growth in MDCK cells, the PB2 amino acid at position 627 affects the replicative efficiency of an avian–human reassortant, but not that of its parent influenza A viruses in these cells, further emphasizing the importance of this amino acid residue in host range restriction of these viruses (Subbarao et al., 1993). Because H5N1 viruses differing in this amino acid had similar growth kinetics in avian cells, but not in mouse cells, we conclude that the primary effect of the Glu-to-Lys substitution is mediated through interactions with host proteins. The more rapid growth of PB2 mutant viruses appears to overwhelm host defense mechanisms, which otherwise limit infection to respiratory organs.

How does the PB2 amino acid substitution at position 627 affect viral replication in mice? The PB2 regions involved in interactions with PB1 protein (amino acids 51–259), cap binding (amino acids 552–565, 363, 404, and 633–650), transcription (amino acids 660–759), nuclear localization

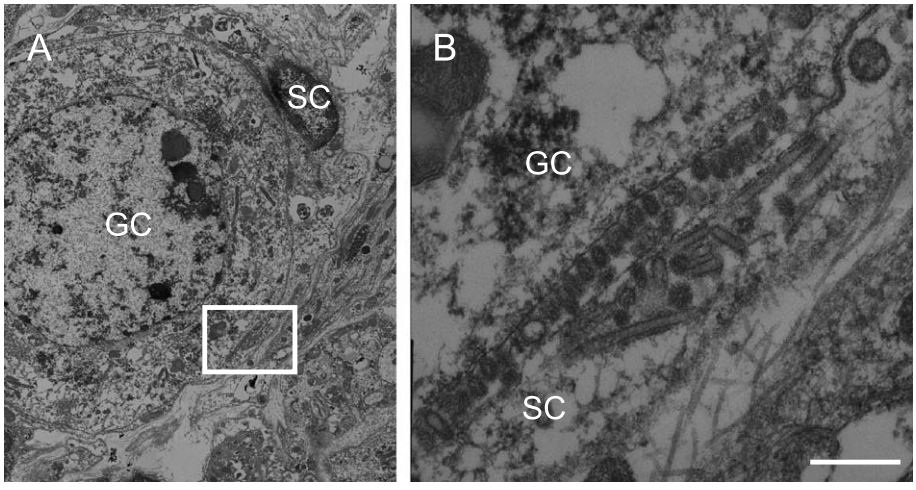

Fig. 3. Viral replication in autonomic ganglia. Autonomic ganglia of a mouse intranasally infected with HK6PB2-627K virus were processed for electron microscopy at 6 days p.i. (A) Ganglion cells surrounded by satellite cells (SC). (B) High magnification of square area (inset) in panel A. Numerous budding virions are apparent on the surface of the ganglion cell (GC). Scale bar = 500 nm.

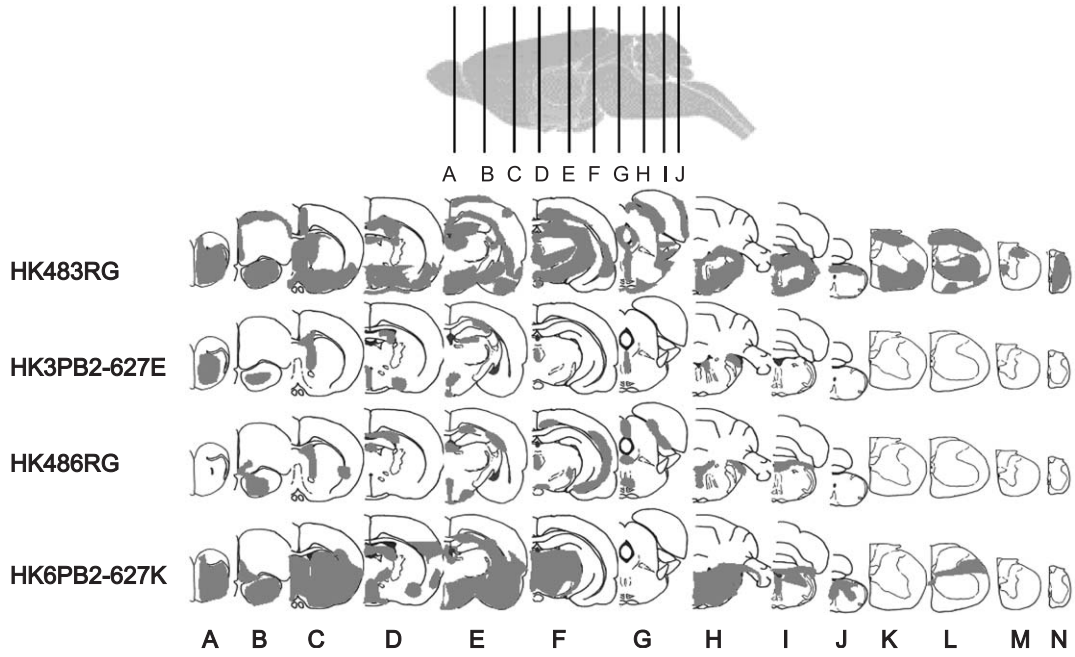

Fig. 4. Viral antigen distribution in the brains of mice intracerebrally infected with highly pathogenic HK483RG or HK6PB2-627K viruses (at 6 days p.i.) or less pathogenic HK486RG or HK3PB2-627E (at 7 days p.i.) viruses. In contrast to the restricted infection of periventricular areas in mice infected with avirulent HK486RG or HK3PB2-627E (containing glutamic acid at position 627 of PB2), viral antigens as detected by immunohistochemistry were widely distributed in brain parenchyma, including spinal cord, in mice infected with virulent HK483RG or HK6PB2-627K (containing lysine at position 627 of PB2). Dark areas indicate the presence of viral antigen-positive cells. (A–J) Coronary sectioning of mouse brain. (K–N) Coronary sectioning of spinal cord at the cervical, thoracic, lumbar, and sacral sites.

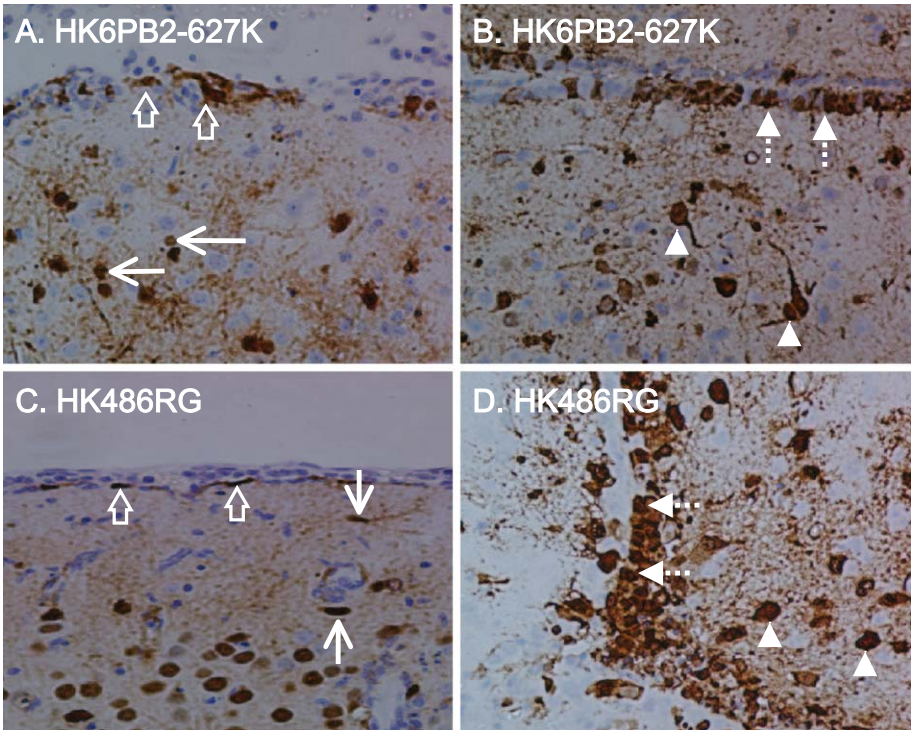

Fig. 5. Cell tropism in the brains of mice intracerebrally infected with a virulent or avirulent strain. Balb/c mice were intracerebrally infected with virulent HK6PB2-627K (A; cortical area beneath the pia mater, B; periventricules) or avirulent HK486RG (C; cortical area beneath the pia mater, D; periventricules) virus. The organs were obtained every day during 6 (virulent strains) or 7 (avirulent strains) days p.i.; the viral antigen distributions were examined by immunohistology. Both viruses showed the same cell tropism in brain, that is, viral antigen-positive cells (brown) were detected in neurons (▾), glial cells (↓), ependyma and choroidal cells (⋯▶), and fibroblasts (⇨).

(amino acids 449–495, and 736–739), and heat shock protein (HSP) 90 (amino acid 1–515) have been mapped (Fechter et al., 2003; Gonzalez et al., 1996; Hatta et al., 2000; Li et al., 2001; Masunaga et al., 1999; Momose et al., 2002; Ohtsu et al., 2002; Perales et al., 1996). Although residue 627 is not included among these sites, we suggest that it influences the interaction of PB2 with host factors and thus RNA synthesis (as suggested by Crescenzo-Chaigne et al., 2002) in mouse cells. Because overexpression of PB2 abrogates the antiviral activity of interferon-induced Mx1 (Huang et al., 1992; Stranden et al., 1993), this host protein would appear to offer an attractive candidate target. However, most of the laboratory mouse strains including Balb/c used in this study lack functional Mx1 protein (Jin et al., 1998). Thus, the host proteins mediating viral replicative efficiency through interactions with PB2 remain to be determined.

The PB2s of all authentic human influenza A viruses examined thus far (35 strains) possess lysine at position 627, while those of their progenitor avian viruses (Wright and Webster, 2001) examined thus far (106 strains) all contain glutamic acid at this position. Our previous (Hatta et al., 2001) and current studies demonstrate that the amino acid residue at this site is one of the major determinants of influenza virus replicative potential in mice. Interestingly, virulence in ferrets is not affected by this amino acid residue (Zitzow et al., 2002). In fact, ferrets are highly susceptible to both human and avian influenza viruses. Thus, the mouse appears to offer a useful model with which to study the host range restriction imposed by the PB2 protein.

Neutrophil infiltration persisted longer in the lung of mice infected with virulent viruses, while lymphocytes replaced neutrophils in mouse lungs soon after infection with avirulent viruses (Fig. 2). Oxygen radicals produced by neutrophils and phagocytes are important pathogenic factors in influenza virus-induced pneumonia in mice (Oda et al., 1989). Hence, long-lasting inflammation with neutrophil infiltration may contribute to the outcome of infection through production of oxygen radicals. Thus, our data suggest that virulent influenza A viruses become lethal by overwhelming host immune responses or restricting the type of response elicited.

In conclusion, we demonstrated that replication of influenza A viruses in avian cells is not affected whether the amino acid residue at position 627 is glutamic acid or lysine. The Glu-to-Lys mutation at this position in the avian virus PB2 does not affect viral tropism among cells in different mouse organs, but enhances its ability to support efficient viral replication in mouse cells in general. Thus, cellular components affecting viral virulence and organ tropism with regard to the PB2 627 residue are not cell type-specific in mice, rather commonly exist in this animal species. This information is essential in selecting strategies for elucidating the mechanism by which avian influenza A viruses gain the potential for high replicative ability in mammalian cells.

Materials and methods

Virus preparation

Four H5N1 viruses were previously generated by reverse genetics (Hatta et al., 2001): HK483RG, constructed from plasmids based on the consensus sequence of A/Hong Kong/483/97; HK3PB2-627E, a mutant of HK483RG containing a Lys-to-Glu substitution at position 627 of PB2; HK486RG, constructed from plasmids based on the consensus sequence of A/Hong Kong/486/97; and HK6PB2-627K, a mutant of HK486RG containing a Glu-to-Lys substitution at position 627 of PB2 (Table 1). Stock viruses were made with MDCK cells. All experiments with live Hong Kong H5N1 viruses were performed in a biosafety level 3+ containment laboratory approved for such use by the USDA and CDC.

Cells

293T human embryonic kidney cells, a derivative of 293 cells constitutively expressing the gene for the simian virus 40 T antigen, were maintained in Dulbecco's Modified Eagle Medium (DMEM; glucose concentration, 4.5 g/l) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, and antibiotics. MDCK were grown in Minimum Essential Medium with Eagle salts (EMEM) containing 5% newborn calf serum (NCS), 4 mM L-glutamine, and antibiotics. QT6 quail fibrosarcoma cells (ATCC #CRL-1708) and LA-4 mouse lung adenoma cells (ATCC #CCL-196) were maintained in Ham's F10 supplemented with 10% FBS and 10% tryptose phosphate broth (TPB) and in DMEM (glucose concentration, 4.5 g/l) supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics, respectively. Primary cultures of astrocytes were prepared from the cerebral cortex of newborn mice. After removal of the meninges, brain tissue was forced gently through a nylon sieve (pore size, 80 μm) and plated in multiwell plates at a low cell density in DMEM supplemented with 20 mM glucose, 10% FBS, 2 mM L-glutamine, and 50 μg/ml gentamycin. Ten days later, 90% confluent monolayers were infected with virus. The purity of astrocyte cultures was evaluated by immunostaining with antiserum against glial fibrillary acidic protein (DAKO Japan Inc., Kyoto). CEF were prepared from 10-day-old embryonated eggs and maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 50 μg/ml gentamycin.

Virus growth kinetics in cell culture

Cells were infected with virus at a multiplicity of infection of 10^{-4}, overlaid with DMEM or EMEM and incubated at 37 °C. Aliquots of the supernatants were collected at 8, 24, 48, and 72 h p.i. and titrated with MDCK cells by plaque assay.

Sequencing of viruses recovered from mouse brain

The PB2 genes of viruses recovered from the brains of mice intracerebrally inoculated with avirulent HK486RG or HK3PB2-627E strain were sequenced as follows. Brain homogenates from virus-infected mice were inoculated onto MDCK cells and total RNA was isolated 12 h later. The PB2 genes were amplified by RT-PCR into two fragments 1–1453 and 1278–2341, using two pairs of gene-specific oligonucleotide primers (for the 1–1453 fragment, 5′-AGC AAA AGC AGG TCA ATT ATA TTC AAT ATG A-3′ and 5′-TTA GTG ACA TTT CCG TGC TG-3′; for the 1278-2341 fragment, 5′-TTT GAA TTT CGT AAA CAG AGC AAA TC-3′ and 5′-AGT AGA AAC AAG GTC GTT T-3′) and *PfuUltra* (Stratagene, La Jolla, CA). PCR products were directly sequenced with an autosequencer (Applied Biosystems Inc.).

Pathological examination

Four-week-old female BALB/c mice were intranasally inoculated with 50 μl (100 pfu) of viruses. Mice were euthanized on days 1–7 p.i. and tissues (liver, gall bladder, spleen, kidney, heart, lungs, trachea, nasal turbinate, tongue, esophagus, stomach, small and large intestine, pancreas, thymus, tracheobronchial lymph node, brain, spinal cord, ganglia, fatty tissue, tooth, and skin) were removed and fixed in 10% phosphate-buffered formalin. They were then dehydrated, embedded in paraffin, and cut into 5-μm-thick sections that were stained with standard hematoxylin-and-eosin. For viral antigen detection, sections were processed for immunostaining by the two-step dextran polymer method (DAKO), with a rabbit polyclonal antibody to A/whistling swan/Shimane/83 (H5N3) used as the primary antibody. For electron microscopy, ganglia were immersion-fixed in neutral-buffered formalin and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Tissues were postfixed in 1% osmium tetroxide, rinsed, dehydrated in ethanol and propylenoxide, and embedded in Epon 812 resin. Ultrathin sections were cut, placed on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEM-100CXII).

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