CHARACTERIZATION OF ADAPTATION OF AN AVIAN INFLUENZA A (H5N2) VIRUS TO A MAMMALIAN HOST

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Summary. – We have used the mouse model to monitor the acquisition of virulence of a non-pathogenic influenza A virus upon adaptation to a new mammalian host. An avian strain, A/Mallard duck/Pennsylvania/10218/84 (H5N2) (Mld/PA/84) was adapted to mice by 23 serial lung-to-lung passages until a highly virulent mouse-adapted (MA) variant (Mld/PA/84-MA) emerged. This MA variant was characterized and compared to the parental strain as well as some of its intermediate passage variants. MA variant caused bronchopneumonia in mice with a high mortality rate (the virulence of Mld/PA/84-MA measured as log (EID_{50}/LD_{50}) was 1.75), while the parental, avirulent strain Mld/PA/84 did not cause illness and mortality in mice (log (EID_{50}/LD_{50}) was 7.25). Hemagglutination-inhibition (HAI) test with a set of hemagglutinin- (HA) specific monoclonal antibodies (MAbs) revealed antigenic differences between the parental strain and MA variant. Mld/PA/84-MA reacted with HA-specific MAbs in higher titers than the parental strain. The HA genes of the parental strain Mld/PA/84, the 1st, 3rd, 8th, and 15th intermediate passage variants, and Mld/PA/84-MA were sequenced. Three amino acid changes at positions 203, 273 and 320 were determined in the HA of MA variant. The first of them, Leu→Pro (320), appeared in the HA stem region at the 8th passage. Two other in the HA1 globular region (Ser→Phe (203) and Glu→Gly (273)) appeared at the 15th passage. All of these substitutions were associated with the increase of viral infectivity for mouse lungs and changes in the HA antigenicity. The potential role of these changes in HA with respect to the process of viral interspecies transmission and acquisition of virulence for new host is discussed.

Key words: avian influenza virus; H5N2; mammalian host; adaptation

Introduction

Influenza A viruses have many hosts in nature. Besides humans, a large variety of animals, e.g. pigs, horses, sea mammals, and birds, can be infected. Phylogenetic studies have revealed that wild aquatic birds are the primordial source of all influenza A viruses of avian and mammalian species (Webster *et al.*, 1992). These studies have also shown that the two most recent influenza pandemics, the 1957 Asian and the 1968 Hong Kong ones, were the result of a reassortment event between avian and human influenza viruses. The result is a so-called *antigenic shift*, which means an event during which the HA of an avian influenza virus is introduced in the human population (Murphy and Webster, 1990). For the 1918 Spanish epidemic virus there is no evidence for reassortment preceding the introduction into humans (Taubenberger *et al.*,1997; Reid *et al.*, 1999).

An influenza A (H5N1) virus strain was isolated from a little child in May 1997 in Hong Kong (de Jong *et al.*, 1997). The genetic characterization of this strain has revealed that all its genes were phylogenetically related to highly

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Abbreviations: aa = amino acid; HA = hemagglutinin; HAI = hemagglutination-inhibition; HAU = HA unit; i.n. = intranasal; MA = mouse-adapted; MAb = monoclonal antibody; MDCK = Madin Darby Canine Kidney; MOI = multiplicity of infection; PAGE = polyacrylamide gel electrophoresis; PBS = phosphate-buffered saline; p.i. = post infection; RT-PCR = reverse transcription–polymerase chain reaction

pathogenic avian influenza viruses circulating in poultry in the same period (Claas *et al.*, 1998; Subbarao *et al.*, 1998). This was the first reported case of transmission of an avian influenza A virus directly to man that caused a respiratory disease. Earlier attempts to infect experimentally humans with avian influenza A viruses did not result in their efficient replication (Beare and Webster, 1991) and therefore, the transmission of the influenza A (H5N1) virus was primarily considered an incident. However, later that year, additional seventeen confirmed cases of influenza A (H5N1) virus infection of humans were reported with, including the first case, a total of six fatalities (Yuen *et al.*, 1998). Fortunately, the avian-like H5N1 viruses did not spread efficiently between humans, but they managed to replicate efficiently in this new (mammalian) host. Efficient interspecies transmission of influenza viruses is the result of a combination of appropriate genetic viral, cellular and environmental factors. Despite extensive nucleotide sequence analysis of both the avian and human influenza A (H5N1) virus strains isolated in Hong Kong, no specific alterations could be related to the adaptation of the avian strain to the new host (Suarez *et al.*, 1998; Bender *et al.*, 1999). However, because of differences in the mutation rates of individual gene segments, an avian-avian reassortment may have preceded the interspecies transmission (Zhou *et al.*, 1999).

To get more information about the process of interspecies transmission and adaptation to a new host, a mammalian model system has been used. Influenza viruses do not cause natural infection in mice, but they can be adapted, to replication in mouse lungs. These animals are commonly used as a model to study nonspecific and specific mechanisms of host defense. Pathogenesis of virus-induced bronchopneumonia has appeared to be similar in humans and mice (Sweet and Smith, 1980). This model system has previously been used to study adaptation of human influenza A viruses to mice and the genetic basis of the acquired virulence (for review see Ward, 1997). In MA human influenza viruses, changes in the HA gene during adaptation play a key role in growth in mouse lungs and acquisition of virulence (Rudneva *et al.*, 1986; Kaverin *et al.*, 1989; Brown, 1990; Smeenk and Brown, 1994; Smeenk *et al.*, 1996; Hartley *et al.*, 1997).

We have previously published the adaptation of avian influenza strains to the mouse (Lipatov *et al.*, 1995, 1996; Govorkova and Smirnov, 1997). In the present study, we have used the mouse model to monitor the acquisition of virulence of a non-pathogenic influenza A virus upon adaptation to a new (mammalian) host. To mimic the situation of an antigenic shift we decided to adapt an avian influenza A virus to a mammalian host. Because avian H5 influenza A viruses obviously manage to transmit and replicate in a mammalian host, we have selected a strain of this subtype for our studies. An avian H5N2 strain was adapted to mice by serial lung-to-lung passages until a highly virulent MA variant emerged. The latter was characterized and compared to the parental strain as well as some of the intermediate passage variants. The HA genes of the parental strain, four passage variants, and MA variant were sequenced. The potential role of the changes which occurred during the process of interspecies transmission in the acquisition of virulence are discussed.

Materials and Methods

Viruses. The avian influenza virus strain (parental strain) A/Mallard duck/Pennsylvania/10218/84 (H5N2) (Mld/PA/84) (Rohm *et al.*, 1995) was kindly provided by Dr. R.G. Webster, St. Jude Children's Research Hospital, Memphis, TN, USA. The virus was propagated in the allantoic cavity of 10-day-old embryonated chicken eggs at 37°C for two days. The allantoic fluid was collected and used for inoculation of mice. The MA variant (Mld/PA/84-MA) was prepared by 23 subsequent lung-to-lung passages of the parental strain. Three to four-week-old albino mice (four mice for each passage) were infected by intranasal (i.n.) inoculation of 50 µl of allantoic fluid under light ether anaesthesia. The mice were sacrificed 48 hrs post infection (p.i.), and subsequently a 10% suspension of their lungs was prepared in phosphate-buffered saline (PBS) with antibiotics. After centrifugation at 11,000 rpm for 2 mins, the supernatant was used for the next passage. The 1st (Mld/PA/84-1st), 3rd (Mld/PA/84-3rd), 8th (Mld/PA/84-8th), 15th (Mld/PA/84-15th), and 23rd (Mld/PA/84-MA) MA lung passage variants were isolated from the lung suspensions by one passage in embryonated chicken eggs. The resulting allantoic fluids were used in the experiments.

Determination of viral growth in mouse lungs and virulence test. Mouse lung suspensions obtained from each passage were titrated (HA titers) in a HA assay with a 0.5% suspension of chicken red blood cells. Infectivity (EID_{50}) titers of the 1st, 3rd, 8th, 12th, 15th, 19th, 21st and 23rd passages were determined in embryonated chicken eggs by a standard method. The virulence test was performed as described earlier (Rudneva *et al.*, 1986) and the log (EID_{50}/LD_{50}) was used as a measure of virulence. To determine the LD_{50}, four animals in each group were infected by i.n. ino-culation of 50 µl of ten-fold dilutions of Mld/PA/84 and Mld/PA/84-MA. The LD_{50} was calculated after an observation period of 15 days.

HAI test was carried out by a standard procedure. Four HAU of viruses and a 0.5% suspension of chicken red blood cells were used. The parental strain Mld/PA/84, the 1st, 3rd, 8th, and 15th lung passage variants, and Mld/PA/84-MA were analyzed. The HAI reactivity of a set of MAbs against influenza virus A/Chicken/Pennsylvania/1370/83 (H5N2) (Ck/Pen/83), generously provided by Dr. R.G. Webster, and polyclonal antisera against influenza viruses A/Tern/South Africa/63 (H5N3) (Te/SA/63) and A/Hong Kong/156/97 (H5N1) (HK/97) were examined. In addition, the HI activities of a non-infected mouse serum and a non-infected mouse lung extract were determined. β inhibitors, mannose-binding lectins were isolated from a mouse serum (Anders *et al.*, 1990; Reading *et al.*, 1997). A mouse lung extract was obtained by sonication of lungs in PBS and was used as a source of a inhibitors

nsitive to neuraminidase (Križanová and Ráthová, 1969; Smeenk *et al.*, 1996). HAI titers (reactivities) were expressed as reciprocals of the highest dilutions of MAbs , sera or extracts that resulted in HAI.

Radiolabeling and polyacrylamide gel electrophoresis (PAGE). The labeling of virus-specific proteins was performed as described (Lipatov *et al.*, 1995). Briefly, confluent monolayers of Madin Darby Canine Kidney (MDCK) cells grown in Dulbecco's Modified Eagle's medium were infected with virus-containing allantoic fluid at a multiplicity of infection (MOI) of approximately 15 PFU/cell. At 4.5 hrs p.i. the medium was removed and the cells were washed twice with RPMI medium without methionine. Then, 50 μCi of [^{35}S]methionine in the same medium was added to the cells for 1 hr. Afterwards, the cells were washed twice with STE buffer pH 7.4, lysed in a reducing buffer, and subjected to PAGE (15% gel) in a standard buffer system (Laemmli, 1970).

Nucleotide sequencing. The HA genes of Mld/PA/84, Mld/PA/84-1st, Mld/PA/84-3rd, Mld/PA/84-8th, Mld/PA/84-15th, and Mld/PA/84-MA were subjected to nucleotide sequencing. Extraction of RNA and its amplification by reverse transcription–polymerase chain reaction (RT-PCR) were carried out as described (Claas *et al.*, 1993). In brief, RNA was extracted from virus-containing allantoic fluid using a guanidinium isothiocyanate solution and collected by precipitation with isopropanol. After reverse transcription, viral RNA was amplified using oligonucleotide primers that were selected from a consensus sequence retrieved from the GenBank. The amplified products were subjected to nucleotide sequencing using the Thermo Sequenase™ Dye Terminator Cycle Sequencing Pre-mix Kit (Amersham Life Sciences) and an Applied Biosystems 377 Sequencer. The sequences of the Mld/PA/84 and Mld/PA/84-MA HA genes were submitted to the GenBank (Acc. Nos. AF100180 and AF100179, respectively). Nucleotide sequences were converted to amino acid sequences and aligned using the Wisconsin GCG Package. For HA polypeptides, the H3 numbering system of Wilson *et al.* (1981) was used.

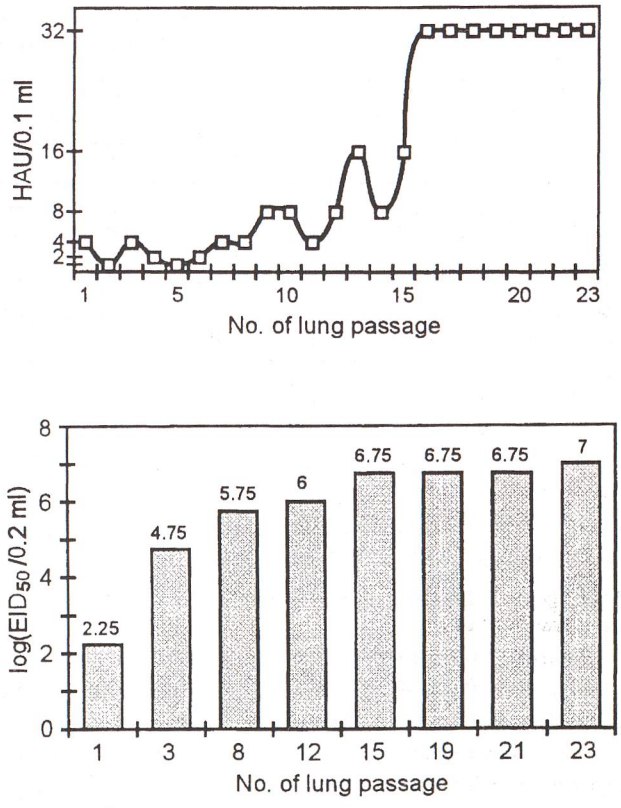

Fig. 1
Yields of the viruses replicating in mouse lungs during the adaptation process

Results

Effect of mouse lungs passaging on EID_{50} and LD_{50} titers

During adaptation of the avian influenza A (H5N2) virus strain Mld/PA/84 to mice, its virulence increased, and its MA variant (Mld/PA/84-MA), which was highly virulent for mice was obtained after 23 serial lung-to-lung passages. The HA titers of the virus isolated from the lungs over the passages and the viral infectivity (EID_{50}) titers were determined (Fig. 1). Limited amounts of infectious virus were found in the lungs after the 1st passage. A major increase in the EID_{50} titer and thus in the amount of infectious viruses reproducing in the lungs could be observed between the 3rd and the 15th passage, reaching log EID_{50} of 6.75. The HA titer, however, was relatively low. Additional passages were undertaken to stabilize the selected mutants with growth potential in mouse lungs and to increase their EID_{50} and HA titers. The MA variant isolated from the 23rd lung passage grew to high EID_{50} titers in embryonated chicken eggs and mouse lungs. The log LD_{50} for mice was 6.0 and the log EID_{50} was 7.75. The parental, avirulent strain Mld/PA/84 did not cause illness and mortality in mice and had log (EID_{50}/LD_{50}) of 7.25. For the virulent variant Mld/PA/84-MA, this virulence index was 1.75. From these data it can be concluded that the serial lung passaging of an avian influenza A (H5N2) virus in mice has propagated a virus with a highly virulent phenotype in mice, i.e. the avian virus has adapted to new (mammalian) host.

Comparison of antigenic properties of Mld/PA/84, Mld/PA/84-MA, and some lung passage variants

A comparative antigenic analysis of the parental avian strain Mld/PA/84 and its MA variant Mld/PA/84-MA was carried out by HAI test using a set of HA-specific MAbs against Ck/PA/83 (H5N2) strain and polyclonal antisera against strains Te/SA/63 (H5N3) and HK/97 (H5N1) (all strains of influenza A virus, Table 1). Significant (more than 4-fold) differences in HAI titers were observed with MAbs

Table 1. HAI test of the parental Mld/PA/84 strain, its MA variant and intermediate lung passage variants with monoclonal antibodies, polyclonal antisera and nonspecific inhibitors

Viruses	MAbs								Polyclonal antisera against		Nonspecific inhibitors from	
	CP22	CP28	CP34	CP46	CP52	CP55	CP58	CP79	Tc/SA/63	HK/97	Lungs	Serum
Mld/PA/84	100	<100	200	6400	<100	12800	12800	25600	5120	1280	32	64
Mld/PA/84-1st	200	<100	100	3200	<100	6400	6400	12800	nd	nd	32	64
Mld/PA/84-3rd	100	<100	<100	6400	<100	12800	6400	12800	nd	nd	64	64
Mld/PA/84-8th	400	<100	<100	25600	<100	6400	6400	12800	nd	nd	64	64
Mld/PA/84-15th	6400	100	800	102400	<100	51200	25600	51200	nd	nd	64	64
Mld/PA/84-MA	12800	200	3200	≥102400	<100	51200	51200	≥102400	10240	2560	64	64

HI titers (reactivities) expressed as reciprocals of highest positive dilutions of MAbs, antisera, lung extract or normal mouse serum.
nd = not done.

CP22, CP34 and CP46. For the rest of MAbs no differences were observed. MAb CP52 did not react with any of the viruses. No significant differences between the polyclonal antisera were observed.

Analysis of antigenic properties of the lung passage variants Mld/PA/84-1st, Mld/PA/84-3rd, Mld/PA/84-8th, and Mld/PA/84-15th with the MAbs directed against Ck/PA/83 revealed that variants from up to the 3rd passage were not significantly distinct from the parental strain. The 8th passage variant was distinguishable by at least a 4-fold difference in its reactivity with MAbs CP22 and CP46; it reacted in higher dilutions than the parental strain. The inhibitory activity of these MAbs and CP79 was increasing in correlation with virus adaptation. The reactivity of the MAbs with Mld/PA/84-15th resembled that with the MA variant; therefore, these two viruses were antigenically closely related. When comparing the parental strain and its MA variant, more than 4-fold differences in HAI activity were observed with MAbs CP22, CP34, and CP46, whereas MAbs CP55, CP58, and CP79 showed only 4-fold differences. MAbCP52 did not react with any of the viruses.

Serial passaging in mouse lungs did not only result in acquisition of virulence for mice but also in changes in the antigenicity of viral HA. These changes appeared between the 8th and 15th passage. The reactions with polyclonal antisera did not reveal significant changes.

Resistance to α and β inhibitors of hemagglutination

In the course of adaptation of Mld/PA/84 to a new host and mutation/selection of Mld/PA/84-MA, no significant differences could be observed in the resistance of these viruses to non-specific α and β inhibitors of hemagglutination (Table 1). A lung extract contains macrophageal a inhibitors that are sialic acid-containing analogues of the receptor. A normal mouse serum contains heat-sensitive β inhibitors which are mannose-binding lectins that inhibit hemagglutination and neutralize virus infectivity by binding to the carbohydrate attached to the tip of the HA spike. In this way, the binding of the virus to the receptor is prevented (Anders *et al.*, 1990; Reading *et al.*, 1997). These results allow to assume that in this case the virus adaptation to mice lungs does not result in changes in the receptor-binding activity of HA of Mld/PA/84 H5 strain.

Mobility of viral proteins

Mld/PA/84 and Mld/PA/84-MA were labeled by [^{35}S]methionine and analyzed by PAGE (Fig. 2). No differences were observed in the mobility of viral proteins originating from the avian parental strain and its MA variant. PAGE of purified virus proteins collected from allantoic fluid by centrifugation also did not reveal any differences in protein patterns of these two viruses. Similarly, a density scanning of the stained PAGE profiles did not reveal any changes in the cleavability of their HAs (data not shown). Obviously, despite significant distinctions in biological and antigenic properties, the MA variant did not differ clearly in physical-chemical properties of viral proteins as compared to those of the parental strain.

Sequence analysis

The results of comparison of the amino acid (aa) sequences of the H5 HA of Mld/PA/84, Mld/PA/84-MA, and intermediate lung passage variants are presented in Table 2. In the HA1 subunit of the Mld/PA/84-MA variant, three amino acid substitutions have accumulated. First a mutation at aa 320 (Leu→Pro) appeared at the 8th passage. Two other mutations at aa 203 (Ser→Phe) and 273 (Glu→Gly) appeared at the 15th passage. In the three-dimensional configuration of the H3 HA, aa 320 is located in the stem region, while aa 203 and 273 are located in the globular domain (Fig. 3).

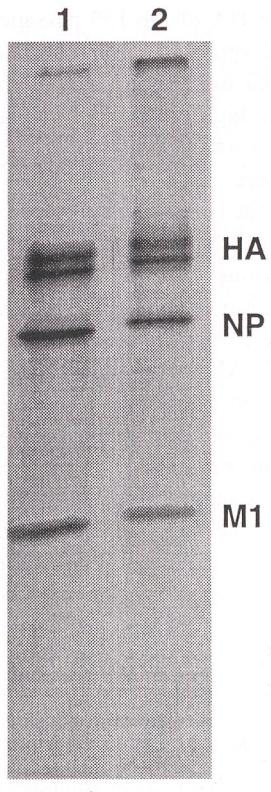

Fig. 2
PAGE of virus-specific proteins labeled with [^{35}S]methionine in MDCK cells
The cells labeled at 4.5–5 hrs p.i. Mld/PA/84 (lane 1); Mld/PA/84-MA (lane 2).

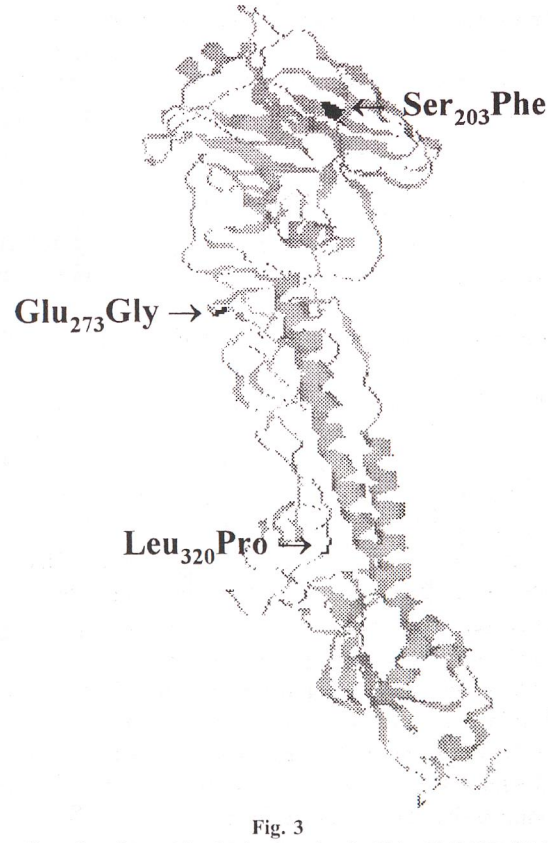

Fig. 3
Location of amino acid substitutions in the HA of Mld/PA/84-MA variant
Three-dimensional structure of HA. Numbering of amino acid positions according to HA of H3 subtype (Wilson *et al.*, 1981).

Discussion

Influenza A virus strains with HA of H5 subtype may be divided in two groups: pathogenic and non-pathogenic for their hosts. The first group includes highly pathogenic avian and avian-like human strains with H5 HA isolated in 1997 in Hong Kong. These viruses replicate systemically in avian species causing illness with extremely high mortality rate (Suarez *et al.*, 1999). Moreover, these highly pathogenic strains with H5 HA capable to cause experimental lethal infection in mice replicate in lungs; some of them systemically infect the brain and other non-respiratory organs without any previous adaptation and, consequently, changes associated with the interspecies barrier crossing (Gao *et al.*, 1999; Lu *et al.*, 1999). The second group is avian non-pathogenic strains with H5 HA. The natural hosts of these strains are aquatic and domestic birds. Viruses replication proceeds in their intestines and do not cause any symptoms of illness (Webster *et al.*, 1992). The experimental transmission and acquisition of virulence for mammalian hosts of non-pathogenic avian influenza A virus strains with H5 HA have not been studied before. Therefore, the avian non-pathogenic influenza A (H5N2) virus Mld/PA/84 strain (Rohm *et al.*, 1995) was chosen for the adaptation to mouse lungs in the present study.

Table 2. Amino acid changes in HA of Mld/PA/84 during adaptation to mice

Viruses	Amino acids at positions		
	203	273	320
Mld/PA/84	Ser	Glu	Leu
Mld/PA/84-1st	Ser	Glu	Leu
Mld/PA/84-3rd	Ser	Glu	Leu
Mld/PA/84-8th	Ser	Glu	**Pro**
Mld/PA/84-15th	**Phe**	**Gly**	**Pro**
Mld/PA/84-MA	**Phe**	**Gly**	**Pro**

Numbering of amino acid positions according to H3 subtype of HA (Wilson *et al.*, 1981).

Adaptation of an influenza A virus to a new host with acquisition of virulence is polycomponent process which requires an appropriate combination of viral, environmental and host factors. In the case of experimental modeling of adaptation, e.g. adaptation of influenza viruses to mice, at least host and environmental factors may be controlled and estimated. Therefore adaptation of influenza viruses to mice is a useful approach for experimental study of viral factors responsible for the interspecies transmission and virulence for new host.

Generally, all of viral genes take part in the interaction of a virus with a new host organism and may be altered and selected. Previous studies with human influenza A virus strains adapted to mice have shown that the HA, M and also PB1 genes are involved in the acquisition of virulence for the mouse lungs (Brown, 1990; Smeenk and Brown, 1994; Rudneva *et al.*, 1986; Kaverin *et al.*, 1989; Ward, 1995; Hartley *et al.*, 1997). However, changes in the HA gene which appear during adaptation to mouse lungs play pivotal role in acquisition or increasing of virulence (Brown, 1990; Smeenk and Brown, 1994; Shilov and Sinitsyn, 1994; Hartley *et al.*, 1997). Therefore in this study, we have investigated adaptation of an avian non-pathogenic influenza A (H5N2) virus strain to the mouse lungs and the changes in the HA that are associated with the interspecies barrier crossing and acquisition of virulence.

The avian influenza A (H5N2) virus Mld/PA/84 strain was adapted to mice by 23 serial lung-to-lung passages and the obtained highly virulent MA variant as well as several passage variants were characterized and compared to the parental strain. As it may be concluded from virus titers, Mld/PA/84 strain initially replicated in the mouse lungs, but it did not cause mortality (log (EID_{50}/LD_{50}) was 7.25) and any symptoms of bronchopneumonia. The increasing of virus titers in mouse lungs was observed during the first 3 lung passages in spite of absence of virulence and changes in the antigenicity and primary structure of HA. These results indicate that the avian non-pathogenic strain of H5 subtype was capable to replicate in mouse lungs during several passages without any changes in HA and acquisition of virulence for the new (mammalian) host. Further increasing of viral infectivity in mouse lungs, appearance of the first specific lesions only at the sites adjacent to the bronchi, and small alterations in the HA antigenicity were detected at the 8th passage. These changes in viral characteristic were, most likely, associated with substitution in HA at aa 320 (Leu→Pro) detected at the 8th passage. During next 7 passages, further enhancement of viral reproduction in mouse lungs, spreading of hemorrhagic lesions, and significant changes in HA antigenicity were observed. The HA of Mld/PA/84-15th was antigenicaly distinct from those of the parental strain and Mld/PA/84-8th. Two additional substitutions at aa 203 (Ser→Phe) and 273 (Glu→Gly) were detected in the HA of the 15th passage variant. This viral characteristic remained conserved during the next 8 passages, which were undertaken to stabilize the selected mutations. The highly virulent MA variant isolated from the 23rd passage was identical to Mld/PA/84-15th in the HA primary structure.

Changes in the HA antigenicity are characteristic for the adaptation of influenza virus to mouse lungs. They were described previously for MA variants of human and avian strains belonging to H1, H2, and H3 subtypes of HA (Gitelman *et al.*, 1984; Lipatov *et al.*, 1995; Govorkova and Smirnov, 1997). Moreover, the antigenic alterations of HA may be observed after several passages in mouse lungs before the virus acquires pathogenicity i.e. ability to kill mice (Gitelman *et al.*, 1984). In the present study, the first small antigenic changes were detected after 8 lung passages and were, most likely, associated with substitution at aa 320 (Leu→Pro) in the HA stem region. We may suppose that this substitution slightly influenced conformation of HA, particularly one of its antigenic sites. A more drastic alteration in the HA antigenicity was observed at the 15th passage. It was probably result of substitution at aa 203 (Ser→Phe) and 273 (Glu→Gly) and was associated with acquisition of high virulence.

One of the mechanisms of interspecies barrier crossing and acquisition of virulence of influenza viruses for mice is host cell-mediated selection of antigenic variants. Another way for acquisition of pathogenicity for mouse lungs was described for human influenza A MA viruses with H1 or H3 HA (Shilov and Sinitsyn, 1994; Hartley *et al.*, 1997; Reading *et al.*, 1997). In this case the adaptation to mice led to loss of glycosylation sites in HA molecule and, consequently, acquisition of resistance to α and β inhibitors of hemagglutination. These inhibitors play role in non-specific mechanism of host defense (Reading *et al.*, 1997). In our present study, no differences between Mld/PA/84 and Mld/PA/84-MA in glycosylation of HA as well as in reactions with the normal mouse serum and lung extract containing α and β inhibitors were observed. This finding also indirectly shows the absence of changes in HA receptor-binding properties. We may conclude that the adaptation of an avian non-pathogenic strain of H5 subtype to mouse lungs and the acquisition of virulence are not related to the acquisition of resistance to non-specific inhibitors of hemagglutination.

Thus, the results of this study characterize the stepwise adaptation of an avian non-pathogenic influenza A (H5N2) virus to a new (mammalian) host and show the changes in antigenicity and primary structure of HA associated with interspecies barrier crossing and acquisition of virulence. The relationship between the stepwise amino acid substitutions, antigenic alterations of HA, and acquisition of virulence is clearly shown. It is difficult to suggest a common

mechanism(s) of influenza virus adaptation to mice. However, we may assume that antigenic changes in HA are most common during the adaptation and the acquisition of resistance to non-specific inhibitors of hemagglutination is most likely restricted among the strains of different HA subtypes. Of course, these changes reflected only a part of possible mechanisms of viral adaptation to new host. It has been reported that amino acid replacements in HA of MA variants of influenza A virus may also influence the optimum pH of HA-mediated fusion (Smeenk *et al.*, 1996; Hartley *et al.*, 1997). Changes in M gene may be also involved in acquisition of virulence for mouse lungs (Smeenk and Brown, 1994; Smeenk *et al.*, 1996). The results of morphological studies on Mld/PA/84 and Mld/PA/84-MA have revealed some differences in virion morphology indicating indirectly the possibility of changes in M gene that controls the morphology and virion particles formation (unpublished results; Smirnov *et al.*, 1991; Roberts *et al.*, 1998). These viral characteristics will be examined in more detail in our future studies that probably allow to explain the mechanisms underlying the interspecies transmission and acquisition of virulence.

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