AMINO ACID CHANGES IN THE HEMAGGLUTININ AND MATRIX PROTEINS OF INFLUENZA A (H2) VIRUSES ADAPTED TO MICE

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Summary. – Mouse-adapted (MA) variants of human and avian influenza A (H2) viruses were generated and characterized with respect to acquisition of virulence in mice. From the nucleotide sequence the amino acid sequence was deduced. The HA1 subunit of the hemagglutinin (HA) contained three amino acid substitutions in the A/black duck/New Jersey/1580/78-MA variant (Glu216→Asp, Lys307→Arg, and Thr318→Ile) and two substitutions in the A/JapanxBellamy/57-MA variant (Lys25→Thr and Ser203→Phe). In the M1 protein, there were two substitutions in the A/black duck/New Jersey/1580/78-MA variant (Asn30→Asp and Gln214→His) and a single substitution in the A/JapanxBellamy/57-MA variant (Met179→Lys). The M2 protein amino acid sequences of the parental virus and the MA variants differed by a single identical mutation (Asn93→Ser). The localization and atomic distances of the observed mutations on the three-dimensional (3D) structure of the HA protein were analyzed for influenza H2 viruses. The obtained results were similar to those published earlier on H1, H3 and H5 subtypes. The amino acid changes in the HA protein could be divided into two groups. In one group the substitutions were situated at the top of the molecule, while in the other group they were clustered in the stem area at the interface region between three HA monomers. The analysis revealed that the substitutions observed in the MA variants probably increase the flexibility of the HA molecule and/or weaken the interactions between monomers or subunits in the HA trimer. The relationships of the observed amino acid changes in the HA and M proteins to the biological properties of the respective viruses and possible mechanisms involved in the acquisition of viral virulence are discussed.

Key words: influenza A viruses; H2 subtype; adaptation to mice; hemagglutinin (H2); M1 protein; M2 protein; amino acid sequence mutations

Introduction

Interspecies transmission of avian influenza A viruses is considered to be one of the most likely explanations for the appearance of a new pandemic human influenza virus (Claas *et al.*, 1998; Webster *et al.*, 1992). Infection of mice with influenza viruses have proven to be a useful tool to study these naturally occurring events in experimental conditions. The questions of viral pathogenesis, mechanisms of virus adaptation and acquisition of virulence for a new host have been addressed in this way (Anders *et al.*, 1994; Govorkova and Smirnov, 1997; Reading *et al.*, 1997; Sweet and Smith, 1980).

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Abbreviations: 3D = three-dimensional; EID = embryo infectious dose; HA = hemagglutinin, hemagglutination; i.n. = intranasal(ly); M/M1 = M gene/matrix protein; MA = mouse adapted; MEM = Eagle's Minimum Essential Medium; MLD = mouse lethal dose; MDCK = Madin-Darby canine kidney; NA = neuraminidase; nt = nucleotide; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RNP = ribonucleoprotein; p.i. = post infection; RT = reverse transcription

Genetic studies indicated that the adaptation of influenza viruses to mice resulted in the selection of variants with increased virulence as a result of acquisition of mutations in individual viral genes that affected different functions (Brown, 1990; Brown and Bailly, 1999; Hartley *et al.*, 1992, 1997). Although virulence is multigenically determined, the majority of authors underline the key role of the surface glycoprotein, HA in controlling virulence of the virus for mice (Brown, 1990; Gitelman *et al.*, 1986; Kaverin *et al.*, 1989; Smeenk *et al.*, 1996). Changes in the HA gene that have been identified for MA variants are associated with alterations of different functions of the HA. The general pathways of adaptation are: loss of the glycosylation site(s) at the top of the HA molecule, an altered pH optimum of fusion of the HA, and changes in receptor-binding specificity and in β-inhibitor sensitivity (Hartley *et al.*, 1992, 1997; Shilov and Sinitsyn, 1994; Ward, 1997).

However, the involvement of other viral genes in the molecular basis of virulence cannot be excluded. It is considered that matrix (M1) gene performs an essential role in controlling virulent functions of influenza viruses for mice. Sequence analysis identified changes at amino acid positions 41, 139 and 227 in the M1 protein, which might be involved in the increased virulence of influenza viruses for mice (Smeenk and Brown, 1994; Ward, 1995). The importance of the polymerase complex in the acquisition of virulence during the adaptation of influenza viruses to mice was observed in several studies (Brown and Bailly, 1999; Kaverin *et al.*, 1989; Smeenk and Brown, 1994). A single mutation in the globular head of the neuraminidase (NA) resulted in the loss of a glycosylation site and in a greater yield of the MA variant, most likely by improving virus release from infected cells (Brown and Bailly, 1999).

The current understanding of structural and functional contributions of the individual viral genes and proteins in the molecular basis of virulence is incomplete. One explanation for that is the limited number of genetically characterized MA variants of viruses. Earlier we have generated and characterized morphological, biological and antigenic properties of some MA variants of human and avian influenza A (H2) viruses (Lipatov *et al.*, 1995, 1996; Govorkova and Smirnov, 1997). In the present study we characterized virulence and growth capacities of these MA variants in different host systems. We also determined and compared the amino acid sequences of the HA and M proteins of the parental viruses and their MA variants, as these proteins had shown to play a pivotal roles in acquisition of virulence for mouse lung (Brown, 1990; Hartley *et al.*, 1997; Smeenk *et al.*, 1996). Sequence information available from GeneBank was included for comparison to perform molecular analysis of the structural changes in the HA.

Materials and Methods

Parental viruses. The avian influenza virus A/black duck/New Jersey/1580/78 (H2N3) (A/dk/NJ/78) and the reassortant prepared from human influenza viruses A/Japan/305/57 and A/Bellamy/42 (H2N1) (A/JapxBell/57) were kindly provided by Dr. R.G.Webster, St. Jude Children's Research Hospital, Memphis, TN, USA. The viruses were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs at 37°C for 48 hrs.

Preparation of MA variants. The MA variants of the influenza viruses A/dk/NJ/78 and A/JapxBell/57 were prepared by 23 and 34 subsequent lung-to-lung passages respectively. Outbred 4–6-week-old albino mice were inoculated intranasally (i.n.) with 50 µl of virus-containing allantoic fluid after light anesthesia with ether. For the 1st passage, an undiluted allantoic fluid was used, while for subsequent passages, a 10% lung suspension in phosphate-buffered saline pH 7.4 (PBS) with antibiotics was used. The second and further passages were carried out 48 hrs after inoculation of the mice.

Virus growth in mouse lungs and embryonated chicken eggs. After each passage, lung suspensions from 4 mice were separately titrated in the hemagglutination (HA) assay with 0.5% suspension of chicken red blood cells. The embryonic infectious dose (EID_{50}) was determined by the standard method. The mouse lethal dose (MLD_{50}) values were measured by infecting groups of 4 mice with serial 10-fold dilutions of virus 10 days post infection (p.i.). The virulence properties for each MA variant were calculated as -log (EID_{50}/MLD_{50}) (Rudneva *et al.*, 1986).

Plaque assay of virus preparations was performed on monolayers of Madin-Darby canine kidney (MDCK) cells in the presence of TPCK trypsin (2.5 µg/ml, Wortington Diagnostics, Freehold, NJ, USA). MDCK cells were cultured in Eagle's Minimum Essential Medium (MEM) with 5% of newborn calf serum. Plaque titers were expressed as log (PFU/ml).

RNA extraction, polymerase chain reaction (PCR) and nucleotide sequencing. Viral RNA was extracted from virus-containing allantoic fluid with the Rneasy Mini Kit (Qiagen, Santa Clarita, CA, USA). cDNA was synthesized from the viral RNA template using a synthetic deoxynucleotide primer (5′-AGCAAAAGCAGG-3′). The cDNA was amplified by reverse transcription–PCR (RT-PCR) as described previously (Shu *et al.*, 1993). The RT-PCR for the HA gene was carried out using the following primers: 5′-GGTTATACCATAGACAACC-3′ (H2-12) and 5′-GTGGATTCTTTGTCTGCTGC-3′ (H2-1185R), which were complementary to nucleotides (nt) 12–31 and 1166–1185, respectively. The RT-PCR for the M gene was carried out using the following primers: 5′-TAGATATTGAAAGATG-3′ (M8L) and 5′-TGCTGGGAGTCAGCAATCTG-3′ (M-482R) or 5′-GAAACAAGGTAGTTTTTTACTC-3′ (M-1023R), which were complementary to nt 8–27, 463–482, and 1002–1023, respectively. The PCR products were purified with the Jet quick PCR purification Spin Kit (Genomed) and were subjected to sequence analysis using a Dye-Terminator Cycle Sequencing Ready Reactions Kit containing Ampli-Taq DNA polymerase FS (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). For each virus PCR preparation HA1 and M genes were completely sequenced using forward and reverse primers. Replicate RT-PCR from

Table 1. Growth characteristics and virulence of influenza A (H2) MA variants

Virus		Growth characteristics			
		Chicken embryo (-log EID_{50})	MDCK (log PFU/ml)	Mouse lungs (–log MLD_{50})	Virulence (-log (EID_{50}/MLD_{50}))
A/JapxBell/57	Parental	6.0	6.08	NP	>6.0
(H2N1)	MA variant	7.75	7.3	4.0	3.75
A/dk/NJ/78	Parental	6.3	6.45	NP	>6.3
(H2N3)	MA variant	7.75	7.7	4.75	3.0

NP = non-pathogenic for mice.

independently produced RNA preparations gave identical sequence results. The samples were electrophoresed, detected and analyzed by the PE/ABI Model 377 DNA sequencer (Perkin-Elmer, Applied Biosystems). The sequences determined in this study are available under GenBank Acc. Nos. AF231354 – AF231357 (HA gene) and AF231358 – AF231361 (M gene).

Analysis of the HA and M amino acid sequences. Assembly of sequencing contigs and translation of nucleotide sequences into amino acid sequences were performed with the Vector NTI program (Informax Inc.). Additional sequences of the HA and M proteins were obtained from GenBank and were studied with GeneDoc 2.3 software. Multiple sequence analysis was done using the H3 numbering system in accordance with the alignment of Nobusawa *et al.* (1991). A 3D structure of the HA monomer indicating the localization of the amino acid substitutions was generated using the RasWin Molecular Graphics program, version 2.6 for Windows (R. Sayle, Glaxo Wellcome Research and Development Stevenage, Hertfordshire, U.K.).

Possible location of the changed amino acids on the HA molecule was analyzed on a model of the complex between the HA of influenza X-31 virus and 3′-sialyllactose in the 3D space of HA molecule (Weis *et al.*, 1988). The location of these amino acids was determined using the H3 numbering system and assuming that the structure of the HA molecule is similar in different subtypes of influenza A viruses (Both *et al.*, 1983; Caton *et al.*, 1983). The distances between the atoms from HA1 and HA2 subunits and other monomers were estimated using the programs RESSEL and SHOWSR, kindly provided by Dr. M.D. Shenderovich, Institute of Organic Synthesis, Riga, Latvia (Matrosovich *et al.*, 1993).

Results

Growth characteristics and virulence of the influenza A (H2) MA variants

Initial i.n. inoculation of the human A/JapxBell/57 and avian A/dk/NJ/78 parental influenza viruses did not result in detection of infectious virus in mouse lungs. Multiple lung-to-lung passages were undertaken for adaptation of these viruses to mouse lungs. Low yields of infectious virus (-log EID_{50} of 1.5–2.0) were observed after 4–5 passages, and additional passages were required to obtain a more pathogenic virus. To examine the growth capacity and virulence of the MA variants, we determined their titers in embryonated chicken eggs, MDCK cells and mouse lungs (Table 1). In comparison with parental viruses the lethal dose (-log EID_{50}) values were by 1.75 higher for influenza A/JapxBell/57-MA variant and by 1.45 higher for influenza A/dk/NJ/78-MA variant. The yield of infectious virus determined by plaque assay in MDCK cells increased for both influenza MA variants during serial passages through mouse lungs. There were slight differences in virulence values (-log (EID_{50}/MLD_{50})) between the two investigated MA variants, the avian influenza A/dk/NJ/78-MA variant was more virulent than the human A/JapxBell/57-MA variant (3.0 and 3.75, respectively). Biological properties of these MA variants and characteristics of their HA antigen changes were described in more detail earlier (Lipatov *et al.*, 1995, 1996; Govorkova and Smirnov, 1997). However, the molecular basis of these changes was not studied. In this study we determined and analyzed the amino acid sequences of the HA, M1 and M2 proteins, which may be of importance for the functional changes in the individual viral proteins following adaptation to mice.

Increased flexibility of the HA molecule in the influenza A (H2) MA variants

The sequence of the HA1 subunit of influenza A/dk/NJ/78-MA variant was shown to differ from that of the parental virus by 4 nucleotides, which resulted in 3 amino acid substitutions (Glu216→Asp, Lys307→Arg, and Thr318→Ile). For the HA1 of MA variant of influenza A/JapxBell/57 virus 2 nucleotide changes were found, resulting in two amino acid substitutions at positions 25 (Lys→Thr) and 203 (Ser→Phe).

Further analysis of the role of these mutations in structural and possible functional changes was done in comparison with the localization of the amino acids in the proposed 3D

Table 2. Amino acid substitutions in the HA1 of MA variants as compared to parental influeza A (H2) viruses and characteristic of HA1 of an influenza A (H3N2) virus

A/black duck/New Jersey/1580/78 (H2N3)			X-31 (H3N2)		
Amino acid substitution	Contacted amino acids[a]	Possible bond	Amino acid position	Contacted amino acids[a]	Possible bond
216 Glu→Asp	184 His***		216 Asn*	184 His***	
	220 Arg***			220 Arg***	
	212 Arg*(other mono-mer omer of HA1 subunit)	I.B.		212 Thr*	H.B.
307 Lys**→Arg	295 His*	H.B.	307 Lys**	295 Gln*	
	60 Asn***			60 Asn***	
	(HA2 subunit)			(HA2 subunit)	
	92 Trp***			92 Trp***	
	(HA2 subunit)			(HA2 subunit)	
318 Thr***→Ile	38 His*	H.B.	318 Thr***	38 Asn*	H.B.
	40 Lys			40 Thr*	H.I
	48 Ile**			48 Ile**	
	(HA2 subunit)			(HA2 subunit)	
	52 Val**			52 Leu	
	(HA2 subunit)			(HA2 subunit)	
A/Japan x Bellamy/57 (H2N1)			X-31 (H3N2)		
25 Lys→Thr	33 Asn (CHO)		25 Leu*	33 Gln	H.B.
	34 Val			34 Ile	H.I.
	35 Thr			35 Glu	H.I.
	315 Val			315 Lys	
203 Ser→Phe	246 Glu	H.B.	203 Thr	246 Asn	H.B.
	212 Arg			212 Thr	H.B.

H.B. = hydrogen bond; I.B. = ionic bond; H.I. = hydrophobic interaction; CHO = carbohydrate chain.
*Amino acids conserved for the investigated subtype; **Amino acids, characteristic for the majority of HA subtypes; ***Amino acids conserved in all HA subtypes.
[a]Contacted amino acids are those for which critical distance between Van-der-Waals spheres is less than 1.5 A.

structure of the HA of influenza X-31 (H3N2) virus (Weis *et al.*, 1988). Our analysis revealed that for X-31 virus the nearest neighbours for Asn216 were His184, Arg220 from the same monomer and Thr212 from another monomer. His184 and Arg220 are highly conserved throughout all the HA subtypes and thus may play an essential role in stabilization of the HA structure. The formation of the hydrogen bond between the hydroxyl group of the Thr212 from one monomer and the Oδ1 of Asn216 from another monomer can also be ivolved in stabilization of the HA structure. A similar analysis performed for the HA molecule of the influenza A/dk/NJ/78 virus demonstrated that the viruses of the H2 subtype possess predominantly Glu216 and Arg212 as the nearest connecting amino acids from another monomer of the HA1 subunit. We predicted that these two amino acids form an ionic bond connecting two monomers of the HA1 subunit. The adaptation of the influenza A/dk/NJ/78 virus to mice resulted in the replacement of Glu216 by a shorter Asp, a change that can weaken the ionic bond between these amino acids (Table 2).

The HA of the A/dk/NJ/78-MA variant also differed from the parental virus by a Lys to Arg change at position 307. Considering the X-31 virus HA structure it can be suggested that there might be a hydrogen bond between O of Lys307 and N of Gln295. For the influenza A viruses of the H2 subtype, Lys307 can form a similar hydrogen bond with His295. In addition, Lys307 interacts with Asn60 and Trp92 of the HA2 subunit, which are highly conserved for the majority of the HA subtypes. Moreover, in close proximity of Lys307 there is Cys305, which forms a cystein bridge with Cys281. The S-S bond between the two cysteins is considered to play an essential role in stabilizing the structure of the HA molecule. Lys307 is conserved in the majority of influenza viruses and its replacement by Arg in influenza A/dk/NJ/78-MA variant enlarges the frame at this site and may alter the flexibility of the HA more significantly than in variable regions.

Thr318 is highly conserved among all influenza virus subtypes as well. There are 4 amino acids in the HA of the influenza X-31 virus, which are situated in close proximity

to this amino acid (Asn38 and Thr40 from the HA1 subunit, and Ile48 and Leu52 from the HA2 subunit). Our analysis revealed that the hydroxyl group of Thr318 of the influenza X-31 virus HA formed a hydrogen bond with the Nδ2 of Asn38. Besides, the methyl group of Thr318 entered hydrophobic interactions with the metyl group of Thr40, and this amino acid interacted with the hydrophobic Ile48 and Leu52 of the HA2 subunit. It can be expected that for influenza H2 viruses similar hydrogen bond connects Thr318 to His38, and that the chain of Lys40, Ile48 and Val52 of the HA2 subunit participates in hydrophobic interactions (Table 2). The replacement of Thr by Ile in the MA variant broke the hydrogen bond and deformed the frame of this site due to the large size of the latter amino acid.

The analysis of the distances between the atoms in the crystallographic representation of the HA of the influenza X-31 virus revealed that the O and the hydroxyl group of Thr203 could form a hydrogen bond with the N of Asn246 and the hydroxyl group of Thr212. Three monomers were in close contact with each other in that part of the HA molecule, and because of that Thr212 was able to form a hydrogen bond with Asn216 of the other monomer of the HA1 subunit. A similar analysis for the MA variant of influenza A/JapxBell/57 virus showed that the change of a small Ser into a bulky Phe at position 203 could destroy this bond and weaken the interactions between HA monomers (Table 2).

In the influenza X-31 virus, Leu25 of the HA1 subunit is in close contact with four amino acids (Table 2). The methyl group of Leu25 has hydrophobic interactions with Glu35 and Ile34, and forms a hydrogen bond with Gln33. Following these observations, we propose that similar interactions may take place for the influenza A/JapxBell/57 virus. The adaptation of influenza A/JapxBell/57 virus to mouse lungs resulted in substitution of the charged, bulky Lys by a small, neutral Thr. This replacement could affect the HA structure and could be especially dramatic because the Asn33 of the A/JapxBell/57 virus is glycosylated.

Thus, the analysis showed that the amino acid substitutions in the HA1 subunit of the HA gene of the two influenza H2 MA variants probably affect interactions, which stabilize the structure of the HA. As a result the flexibility of the HA molecule could increase.

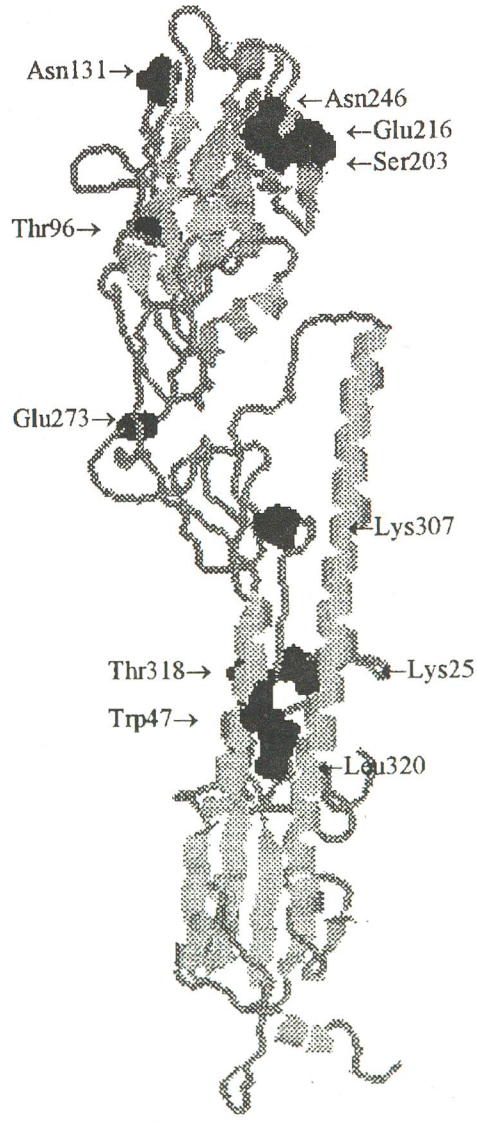

Fig. 1
3D structure of HA monomer showing positions of amino acid substitutions determined for influenza A virus MA variants
The figure was generated with the RasWin Molecular Graphics program, version 2.6 for Windows (R. Sayle, Glaxo Wellcome Research and Development Stevenage). The H3 numbering system in accordance with the aligment of Nobusawa *et al.* (1991) was used. Amino acids are shown for parental influenza viruses and are marked in black.

Changes in the M gene in the course of adaptation of influenza A (H2) viruses to mouse lungs

Previous studies have indicated the importance of the M1 protein in the adaptation of influenza virus to mice and the M2 protein in determining the pathogenicity for a new mammalian host (Buckler-White *et al.*, 1986; Smeenk and Brown, 1994; Ward, 1995). Therefore the amino acid sequences of the M1 and M2 proteins of the parental influenza A/JapxBell/57 and A/dk/NJ/78 viruses and their MA variants were determined as well.

Influenza A/JapxBell/57-MA variant showed only a single substitution in the M1 protein at position 179 (Met→Lys). The M1 protein of the influenza A/dk/NJ/78-MA differed from the parental virus at two positions: 30 (Asn→Asp) and 214 (Gln→His). Although nowadays the X-ray data are

available for the N-proximal moiety of the M1 protein (amino acids 2–158) (Sha and Luo, 1997), the analysis of the possible location of this protein on the 3D structure is still difficult. Therefore we undertook a comparative analysis of the amino acid sequence data obtained for these two influenza H2 viruses with sequence data from the available genetic database and used it in a multiple sequence alignment of the M1 protein. There were no differences at 3 amino acid positions (41, 139 and 227, previously described for MA variants) in the M1 protein of the influenza A/JapxBell/57-MA and A/dk/NJ/78-MA variants. The analysis of the amino acid sequence data from GenBank showed that the M1 protein of the influenza A/dk/NJ/78-MA variant was closely related to the M1 of highly pathogenic avian H5 and H7 subtype viruses. The influenza A/JapxBell/57-MA variant was most closely related to A/FM/1/47-MA variant and A/PR/8/34, A/NWS/33 and A/WSN/33 viruses, which were characterized as virulent for mice (Smeenk and Brown, 1994; Webster *et al.*, 1992).

Functional domains of the M1 protein have been characterized earlier. Particular amino acid sequences have mapped as lipid and RNA-protein binding sites involved in zinc binding (Elster *et al.*, 1994; Ye *et al.*, 1987). However, none of the amino acid substitutions in the MA variants of the influenza A/JapxBell/57 and A/dk/NJ/78 viruses were located within functionally important domains of the M1 protein.

For the M2 protein, the same amino acid substitution (Asn93→Ser) was observed in the MA variants for both influenza A (H2) viruses. This mutation was situated in the cytoplasmic tail of the M2 protein.

Discussion

Identification of mutant genes in relation with the altered functions of the proteins has been frequently used as an approach to study the genetic basis and the mechanism of the influenza virus virulence.

At least three different mechanisms of changes in the HA associated with increased mouse lung virulence have been described: loss of potential glycosylation site, a substitution in the HA that could increase the optimum pH of fusion, and a change, which promotes cleavage of the HA (reviewed by Ward, 1997). The first of these mechanisms frequently occurrs during adaptation to mice of "late" human viruses, which had a number of additional glycosylation sites in comparison with avian viruses (Nobusawa *et al.*, 1991). Carbohydrates on the HA protein could affect the virus receptor-binding activity in different ways: either directly by prevention of the attachment of the HA to the cellular receptors, or indirectly by the interaction with inhibitors, which made the receptor-binding site of the HA inaccessible for receptors (Gambaryan *et al.*, 1998). Thus, such mutations at the top of the HA trimer permit the virus to escape neutralization by murine serum and pulmonary lectins (Reading *et al.*, 1997). This mechanism allows to avoide the innate defense of the respiratory tract against influenza and appears to be sufficient for successful growth of the virus in the mouse lungs (Hartley *et al.*, 1997).

Other amino acid substitutions in the HA protein arise during the adaptation of avian influenza viruses and "early" human viruses to mice. These viruses do not contain the carbohydrate chains characteristic for "late" human viruses (carbohydrates at amino acids 94 and 131 on the HA of influenza A/USSR/90/77 virus and carbohydrate at amino acid 246 on the HA of A/Phillipines/2/82 virus). "Classical" glycosylation sites at amino acids 165 and 169, and in the stem region of the HA were not usually lost during the adaptation of influenza viruses to mouse lungs. The avian influenza viruses predominantly possess only one glycosylation site at the membrane-distal end of the HA, and therefore the adaptation to mouse lungs requires another mechanism. Our results demonstrated that an increased flexibility of the HA molecule could be considered as one of the mechanisms involved in the adaptation of avian influenza viruses.

In this study, we analyzed possible location of the substitutions on the 3D structure of the HA that occurred during the adaptation of the influenza A viruses of different subtypes to mice. One group of substitutions consisted of 5 amino acids situated at the top of the HA molecule, and the other group consisted of 4 amino acids clustered in the stem region of the HA, in the interface region of three monomers. Two amino acid changes could not be assigned to these groups, Lys307 of A/dk/NJ/78 and Glu273 of A/dk/PA/84 (H5N2). The amino acid changes at positions 96 and 131 of A/USSR/90/77 (H1N1), 47 of A/FM/1/47(H1N1), 273 of A/dk/PA/84, 25 of A/JapxBell/57, and 318 of A/dk/NJ/78 can be classified as superficial. The changes at positions 246 of A/Phillipines/2/82 (H3N2), 203 and 320 of A/dk/PA/84; 203 of A/JapxBell/57, and 216 and 307 of A/dk/NJ/78 are partly buried, they are more or less masked by surrounding amino acids.

The majority of the observed amino acid changes in the HA led to an increase in the flexibility of the HA molecule. Destabilization of the HA structure could be associated with alteration of HA properties and functions. It is possible to assume that amino acid substitutions in the globular head of the HA trimer may indirectly change the receptor-binding properties of the virus or affect pH of membrane fusion. Previous studies have demonstrated that alterations in pH of fusion might represent a general mechanism for the adaptation of influenza viruses to mouse lungs (Hartley *et al.*, 1997; Smeenk and Brown, 1994; Ward and de Koning-Ward, 1995). Daniels *et al.* (1985) identified amino acid

substitutions that resulted in an elevated pH of fusion. The amino acids that were involved were distributed around the fusion peptide or in the regions where the HA subunits were in contact. The present study shows that mutations at the intermolecular surface of the HA molecules of the MA variants may directly (by an increased flexibility of the trimer) or indirectly (by the contact with amino acids involved in regulation of the fusion pH) affect this important pathway of mouse lung adaptation.

There is no direct evidence that the increased virulence after mouse adaptation is associated with the accessibility of the cleavage site of HA for new host proteases. It is possible to make a suggestion that the increased virulence of influenza viruses for mice following adaptation can be the result of a facilitated cleavage of the HA. The analysis undertaken in the present study may give some structural evidence of the possible role of this mechanism in the adaptation of influenza viruses to mice. An increased flexibility of the HA molecule in the stem region, and weaker intermolecular interactions can relieve the conformational changes of the HA and thus possibly affect the cleavage of the HA.

Amino acid substitutions in the M1 protein have been shown to correlate with acquisition of virulence of influenza viruses to mouse lungs and brain (Smeenk and Brown, 1994; Ward, 1995) and increase the growth rate in other host systems (Klimov *et al.*, 1991; Yasuda *et al.*, 1993). The amino acid changes in the M1 protein determined for influenza A/JapxBell/57 and A/dk/NJ/78 MA variants are not located in the functional domains of the protein (Elster *et al.*, 1994; Ye *et al.*, 1987). However, according to the proposed model of the M1 protein arrangement in the virus particle, the amino acid substitution at position 30 is located in the part of the a-helix that it accessible to tritium bombardment, and the mutations at positions 179 and 214 are located at 2 most exposed regions of the C-proximal part of the M1 protein. The disposition of the secondary structure elements of the M1 protein (Shishkov *et al.*, 1999) allowed us to suggest that the amino acid changes observed in the MA variants of the influenza A/JapxBell/57 and A/dk/NJ/78 viruses occur near outer surface of the membrane. Therefore that part of the M1 protein may interact with the lipid membrane and RNPs. A contact between M1 protein and HA (NA glycoproteins) has not been shown yet, but interactions with RNP (Ye *et al.*, 1987) may indirectly affect the efficiency of maturation of the virus.

The possible role of the M2 protein in the acquisition of virulent properties of influenza viruses for mice has not been described. The amino acid changes in the cytoplasmic tail of M2 may also be involved in interactions with other viral proteins during virus maturation.

The present study confirmed that the virulence of influenza viruses can be considered a polygenic phenomenon. Detailed analysis of sequence data will remain important for the understanding of the molecular basis and mechanisms of viral virulence.

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