147

A H1 hemagglutinin of a human influenza A virus with a carbohydrate-modulated receptor binding site and an unusual cleavage site

I. Günther [a], B. Glatthaar [b], G. Döller [b] and W. Garten [a]

[a] *Institut für Virologie, Philipps-Universität Marburg, Marburg, Germany and* [b] *Abteilung für Medizinische Virologie und Epidemiologie der Viruskrankheiten, Institut für Hygiene der Eberhard Karls-Universität Tübingen, Tübingen, Germany*

Summary

Two receptor binding variants of the influenza virus A/Tübingen/12/85 (H1N1) were separated by their different plaque formation in MDCK cells. Hemagglutination of variant I was restricted to red blood cells of guinea pigs, whereas variant II also hemagglutinated chicken cells. The variants differed also in their ability to bind to $\alpha 2,6$-linked sialic acid. Evidence is presented that this difference is determined by a complex carbohydrate side chain at asparagine$_{131}$ near the receptor binding site which is absent in variant II. With both variants, the arginine found at the cleavage site of all other human isolates analyzed so far was replaced by lysine.

Influenza A virus variant; H1 hemagglutinin; Receptor binding site; Cleavage site

Introduction

Influenza viruses are genetically labile as a result of frequent mutations during virus replication under natural conditions (Parvin et al., 1986). Thus, virus isolates exist as heterogenous populations, of which individual subpopulations can be selected under different cultivation conditions. Variants of the hemagglutinin (HA)

Correspondence to: W. Garten, Institut für Virologie, Philipps-Universität Marburg, Marburg, Germany.

are particularly relevant in this respect, because this glycoprotein interacts with cell membranes at the beginning of infection and plays an essential role as receptor determinant for the virus before triggering penetration of the nucleocapsid into the cell (Klenk and Rott, 1988). Therefore, host changes are often accompanied by changes in receptor specificity caused by an altered receptor binding site (RBS). Adaptation of the original virus isolate (O phase) to a new host by selection of spontaneous virus variants (D phase) with different receptor binding characteristics was reported first by Burnet and Bull in 1943. Since that time a large number of influenza virus variants with different receptor binding capacities have been isolated by passages in different host cells or in the presence of RBS specific antibodies (Cohen and Biddle, 1960; Crecelius et al., 1984; Daniels et al., 1987; Gitelman et al., 1986; Katz and Webster, 1988; Katz et al., 1990; Oxford et al., 1987, 1990; Robertson et al., 1985, 1987, 1991; Schild et al., 1983), by using various serum hemagglutination inhibitors (Rogers et al., 1983), or by binding to an artificial receptor (Rogers et al., 1985). All these changes are due to HA mutations.

Influenza viruses from clinical samples are routinely propagated in embryonated eggs or Madin-Darby canine kidney (MDCK) cells to gain material for immunological and molecular analyses. A change in hemagglutination properties was observed during an epidemic, when human influenza virus A/Tübingen/12/85 (H1N1) isolated from clinical material was passaged in MDCK cells. The first passage in MDCK cells agglutinated only guinea pig red blood cells, but not chicken cells. After further passages in MDCK cells, a variant (variant II) overgrew the original virus (variant I), which agglutinated chicken as well as guinea pig erythrocytes. This phenomenon, demonstrating that the change in hemagglutination properties occurred in a single host cell line within few replication cycles (Döller et al., 1987), reminded of the O-D phase change (Burnet and Bull, 1943).

In the present study we show that the mutation responsible for the change in receptor binding specificity consists in the loss of a carbohydrate side chain that is located in close proximity to the receptor binding site.

Materials and methods

Viruses and cells

The following influenza A viruses have been used: WSN/33, PR/8/33, Tübingen/12/85, Tübingen/60/85, Tübingen/61/85, Tübingen/75/85, Tübingen/76/85, Tübingen/5/89, Tübingen/6/89, Tübingen/21/89, Tübingen/25/89, Hannover/5/89, Hannover/8/89, Hannover/9/89 (all H1N1), and Chick/Germany/N/49 (H10N7) (Virus N). All viruses were propagated in MDCK cells. Trypsin (5 μg per ml) was added to Dulbecco's medium for multiple virus replication in MDCK cells. Virus N and the PR8 strain were also propagated in embryonated eggs. Virus purification was done by sucrose gradient ultracentrifugation as described before (Klenk et al., 1972). Virus was radioactively labelled

by addition of [^{3}H]glucosamine (36 Ci/mmol, 1 mCi/ml) into Dulbecco's medium, in which glucose was replaced by 10 mM fructose (Scholtissek et al., 1975).

Virus assays

Plaque-, hemagglutination-, and hemagglutination inhibition tests were carried out by standard procedures. Plaque assays were performed in MDCK cell cultures in the presence of 5 μg trypsin per 1 ml overlaying medium. Hemagglutination was done in microtiter plates using guinea pig or chick erythrocytes at 0.5% concentrations in phosphate-buffered saline (PBS) at 4°C.

Modification of influenza virus cell receptor

The method for enzymatic modification of chicken erythrocyte oligosaccharides includes de- and resialylation according to the instructions by Schultze and co-workers (1990). Briefly, sialic acid was removed from glycoconjugates by treatment of 10% erythrocytes in 100 mM NaCl, 20 mM CaCl$_2$ and 50 mM MES, pH 6.5, with neuraminidase from *Vibrio cholerae* (EC 3.2.1.18) (0.6 units/ml; Behringwerke, Marburg, Germany) at 37°C for 30 min. After two washes with PBS, the neuraminidase-treated erythrocytes were incubated in 1 ml PBS, 1% BSA, with 280 μg cytidine 5'-monophosphate activated N-acetylneuraminic acid (Sigma, Deisenhofen, Germany) and 2,3-sialyltransferase (EC 2.4.99.4) (3 milliunits; Boehringer, Mannheim, Germany) or 2,6-sialyltransferase (EC 2.4.99. 1) (0.03 units; Boehringer, Mannheim) at 37°C for 3 h.

Digestion with endoglycosidase F and proteases

Endoglycosidase F (endo F) (EC 3.2. 1.96) was used to remove N-linked carbohydrate side chains from viral glycoproteins. Radioactively labelled virus (about 40 HAU) was incubated in 50 mM phosphate buffer, pH 7.0, containing 0.05% SDS, in the presence of 0.2% mercaptoethanol with 0.015 units endo F at 37°C for 16 h (total volume of 10 μl). For proteolytic cleavage, radioactively labelled virus (80 HAU) was incubated at 37°C for 30 min with 0.01 units trypsin (EC 3.4.21.4) in PBS, 0.01 units endoproteinase Arg-C (EC 3.4.21.40) in 0.1 M potassium phosphate buffer, pH 8.0, or 0.04 units endoproteinase Lys-C in 25 mM Tris-HCl–1 mM EDTA buffer, pH 8.5. All enzymes were from Boehringer, Mannheim, Germany. The enzyme reactions were stopped by addition of 10 μl sample buffer for gel electrophoresis with or without mercaptoethanol. HA was electrophoretically separated on polyacrylamide gels under reducing conditions and visualized by fluorography.

Immunotechniques

Antibodies were raised against purified influenza virus A/PR/8/34 and against its glycoproteins, which were solubilized with n-octylglucoside and separated by

ultracentrifugation from the nucleocapsid (Kohama et al., 1981), and then used for repeated subcutaneous injections in rabbits. Western blotting were done as described before (Kuroda et al., 1986).

Genomic sequencing

vRNA and mRNA were isolated using standard procedures and sequenced by the primer extension method as described before (Feldmann et al., 1988) using reverse transcriptase (EC 2.7.7.49) (Boehringer, Mannheim, Germany) and the following oligonucleotides: TAAAAAACCAACCAAAATG (1); TGAGGACAG-TCACAACG (182); AATCATGGTCCTACATTG (304); TGTTACCCAGGGTA-TTTC (351); TCATGGCCCAAACACA (447); TTTTACAGAAATTTGCTA (513); CTAACATAGAAGACCA (631); CAATAATATTTGAGGCAA (799); AT-GAATGTGACGCAAAGTG (901); TCCAGAATGTACACC (958); GGAGAGT-GCCCA (984); GGTTATATCATCATCAG (1131); GGATTTCTGGACATTTGG (1323); CTGGACATTTGGACATAT (1329); TATGACTATCCAAAATAT (1533); ATGGGAGTCTATCAG (1605); ATGGTAGCCTATAC (106R); TTC-GAGTACTGTGTCAAC (146R); GGTGAATCTCCTGTTATA (716R); GAAAGCATACCATGG (848R); ATGATGTAACCATACCAT (1142R); CTG-GTCTCAGATGCATAT (1739R). Numbers refer to the 5′ ends of homologous position within the H1 gene, R is added to primers, which are complementary to mRNS. By using the primer pairs 19/1142R and 799/1739R cDNA of HA gene fragments were obtained, cloned in vector pUC19 (Feldmann et al., 1988) and sequenced on the plasmid by using the T7 polymerase sequencing kit (Pharmacia, Freiburg, Germany). The nucleotide sequence of the influenza virus A/Tübingen/12/85 (H1N1) is submitted to the EMBL Gene bank.

Molecular modelling

This was done with an Evans and Sutherland computer using H3 coordinates deposited in the Brookhaven Protein Data Bank and the software program 'what if version 3.7' kindly provided by Gerrit Vriend EMBL, Heidelberg, Germany.

Results

From the mixed virus population of a clinical sample, receptor binding variants were isolated by the differential plaque formation in MDCK cells in the presence of trypsin. After 3 plaque passages, 2 plaque variants were obtained: variant I formed fuzzy plaques with a size of 1.5 ± 0.4 mm in diameter 80 h p.i. and agglutinated erythrocytes from guinea pigs but not from chicken. Variant II formed clear plaques with a size of 2.8 ± 0.4 mm in diameter 48 h p.i. and agglutinated erythrocytes from chicken and guinea pigs. Variant I, specific for guinea pig erythrocytes alone, was not stable during further passages in MDCK cells, since variant II outgrew variant I in the second passage. Thus, for enrichment

TABLE 1

Agglutination of native and modified erythrocytes by influenza A viruses of the subtype H1

Virus	HA titer (HA units/ml) Erythrocytes from:				
	Guinea pig untreated	Human [1] untreated	Chick untreated	Chick resialylated	
				$Sa\alpha2,3$	$Sa\alpha2,6$
Tübingen/12/85 variant I	64	64	0	0	0
Tübingen/12/85 variant II	128	64	64	0	64
PR/8/34	256	512	128	16	64
WSN/33	256	256	256	0	128

[1] Different blood (group A, AB and O) cells were tested.

of variant I at large scale, plaque-purified virus was needed for inoculation of MDCK cell cultures. The descendant virus of each petri dish was analysed for hemagglutination with both erythrocyte species to exclude variant II.

Variants I and II differed in their receptor specificity, as shown in Table 1. Red blood cells from guinea pigs bound all viruses analyzed, whereas untreated chicken cells agglutinated all H1 viruses except for variant I. To further elucidate the receptor specificity, desialylated chicken cells were reconstituted by sialic acid (SA) either in $\alpha2,3$- or in $\alpha2,6$ linkage and then used for virus agglutination. In contrast to variant I, variant II recognizes sialic acid in $\alpha2,6$ linkage as do strains PR/8 and WSN. The ability of variant II to utilize $SA\alpha2,6Gal$ sequences agrees with previous findings for other H1 isolates since 1977, which all have this receptor specificity (Rogers and D'Souza, 1989). On the other hand, since variant I did not bind to either of the resialylated erythrocytes used here, its exact receptor specificity is still unknown.

Because of their different receptor specificities, both variants had to be expected to have structurally distinguishable HAs. Therefore, the variants were first analyzed by electrophoresis on polyacrylamide gels. The subunit HA_1 of variant I migrates more slowly than that of variant II (Fig. 1A). The shift in molecular weight of about 3000 Dalton might be caused by one carbohydrate side chain. Complete removal of carbohydrate by the treatment with endoglycosidase F resulted in HA_1 subunits (designated as HA_1^-), which are identical in size, indicating that the carbohydrate is responsible for the molecular weight shift (Fig. 1B).

To localize the attachment site for the additional carbohydrate on HA of variant I, the HAs of both variants were compared by RNA sequencing. As only up to 98% of the primary structure could be characterized by the primer extension sequencing method, the residual sequences were determined by cloning and sequencing the HA gene fragments comprising the nucleotides 22–1142 and 815–1737 for the variant II, and 20–547 and 106–1123 for the variant I, respectively. The total nucleotide sequences of both HAs differ in 4 nucleotides of which three are silent mutations at the positions 442 (A–G), 452 (G–A), and 1210 (C–T). The non-silent mutation in the variant II HA gene is located in position 469, where
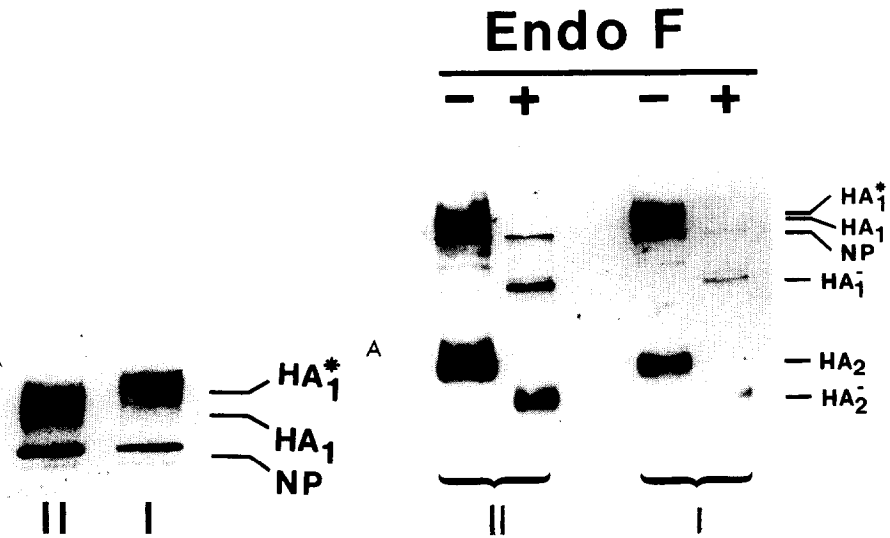
Fig. 1. Western blot of influenza A/Tübingen/12/85 virus variants. Purified viruses obtained from MDCK cell cultures in the presence of trypsin (5 μg/ml) were either treated or not with endo F and electrophoretically separated on 12% (A) and 17.5% (B) acrylamide gels, respectively, transferred onto nitrocellulose and immunostained after incubation with rabbit antiserum raised against glycoproteins of PR8 virus. HA_1^* is correlated with variant I (I), HA_1 with variant II (II). HA_2 migrated out of the 12% gel. The deglycosylated forms are designated as HA_1^- and HA_2^-.

cytidine of the variant I is replaced by uridine. This exchange leads to isoleucine in the amino acid position 133 of the amino acid sequence of variant HA (Fig. 2). The HA of the variant I possesses a threonine residue at that position, which provides a N-glycosylation signal in combination with the asparagine at position 131. Thus, the HA of the variant II has lost that N-glycosylation site. Both variants contain 6 glycosylation sites at the amino acid positions 21, 33, 95, 158, and 289 on HA_1 and 154 on HA_2 not affected by the change of receptor specificity (Fig. 2). The asparagine at position 20 is not glycosylated (Waterfield et al., 1980), because of sterical hindrance of the carbohydrate at asparagine$_{21}$.

By using the primer extension method and T7 sequencing on the cloned gene fragments of both variants, lysine was surprisingly found to be the single amino acid, which connects HA_1 with HA_2 (Fig. 2). We were interested to see whether other H1 viruses, isolated at the same time, also contain lysine at their HA cleavage sites. Therefore, additional virus isolates which we obtained from Tübingen and Hannover in the same time course between 1985 and 1989, were analyzed at that critical site of HA on the level of vRNA and cDNA. However, only the Tübingen/12/85 isolate contained lysine at the HA cleavage site. All HAs of the other human influenza virus isolates analyzed so far, had a single

```
                                10 ↓ HA₁-START           20
M K A K L L V L L C A L T A T D A ˙D T I C I G Y H A │N N S T│
                    30                  40                  50
D T V D T V L E K │N V T│V T H S V N L L E D S H N G K L C R
              60                  70                  80
L K G I A P L Q L G K C S I A G ₩ I L G N P E C E S L F S T
              90                  100                 110
K S ₩ S Y I A E T P N S E │N G T│C Y P G Y F A D Y E E L R E
              120                 130       I
Q L S S V S S F E R F E I F P K E S S ₩ P K H ┌N V T┐K G V T
        140                 150                 160
A S C S H K G K S S F Y R N L L ₩ L T E K │N G S│Y P N L S K
        170                 180                 190
S Y V N N K E K E V L V L ₩ G V H H P S N I E D Q R T I Y R
        200                 210                 220
K E N A Y V S V V S S H Y N R R F T P E I A K R P K V R D Q
        230                 240                 250
E G R I N Y Y ₩ T L L E P G D T I I F E A N G N L I A P ₩ Y
        260                 270                 280
A F A L S R G F G S G I I T S N A S M D E C D A K C Q T P Q
        290                 300                 310
G A I │N S S│L P F Q N V H P V T I G E C P K Y V R S A K L R
        320                      ↓ HA₂-START            10
M V T G L R N I P S I Q S K̲ G L F G A I A G F I E G G ₩ T G
        20                  30                  40
M I D G ₩ Y G Y H H Q N E Q G S G Y A A D G K S T Q N A I N
        50                  60                  70
G I T N K V N S I I E K M N T Q F T A V G K E F N K L E K R
        80                  90                  100
M E N L N K K V D D G L L D I ₩ T Y N A E L L V L L E N E R
        110                 120                 130
T L D F H D S N V K N L Y E K V K I Q L K N N A K E I G N G
        140                 150                 160
C F E F Y H K C N N E C M E S V K │N G T│Y D Y P K Y S E E S
        170                 180                 190
K L N R E K I D G V K L E S M G V Y Q I L A I Y S T V A S S
        200                 210                 220
L V L L V S L G A I S F ₩ M C S │▨▨▨│L Q C R I C I
```
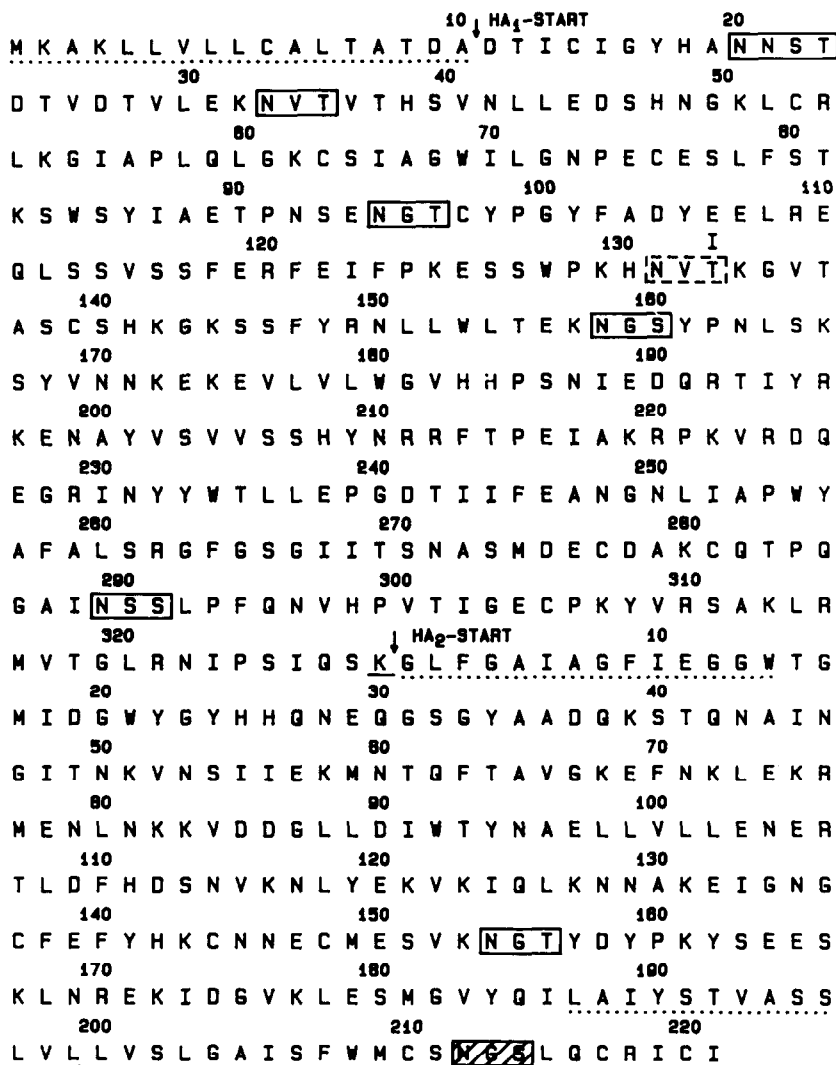
Fig. 2. The amino acid sequence of the hemagglutinin of influenza virus variants A/Tübingen/12/85 (H1). The sequence of the variant I is given in one letter code and numbered according to subtype H1 (Winter et al., 1981). Potential glycosylation sites (sequons) are indicated by boxes; the hatched box on the cytoplasmic domain of HA₂ (amino acids 212–214) is not glycosylated, the dashed box (131–133 on HA₁) contains a sequon only for the variant I, but not for the variant II HA of which the deviated amino acid in position 133 is written. Dotted lines present the hydrophobic domains, i.e. signal peptide (−17 to 10 in HA₁), fusion peptide (1–14 in HA₂) and membrane anchor (187–210). Amino termini of HA₁ and HA₂ are marked by arrows, the lysine (K) in position 329 connecting HA₁ and HA₂ is underlined.

arginine at that position. As a consequence of the unusual HA cleavage site of the A/Tübingen/12/85 isolate, an endoproteinase specific for lysine should activate that influenza virus in vitro, but not· an enzyme specific for arginine. Therefore,
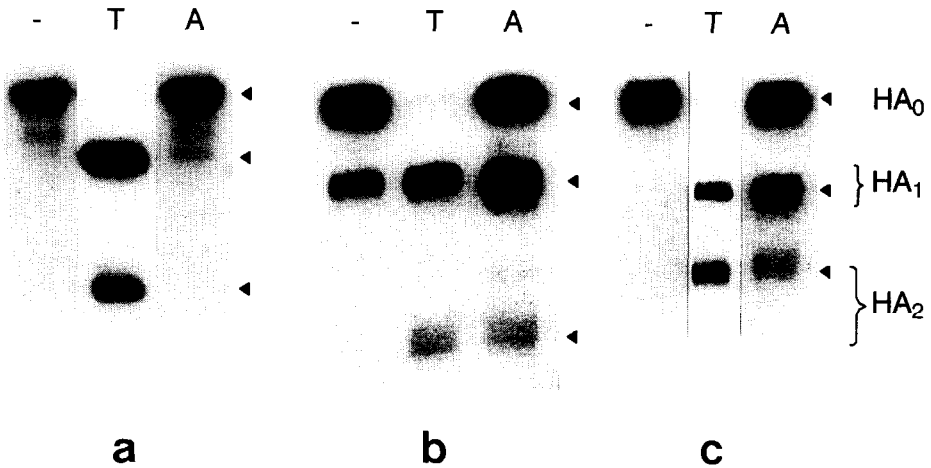
Fig. 3. In vitro cleavability of some influenza A viruses. Influenza viruses A/Tübingen/12/85 (H1N1) (a), A/WSN/33 (H1N1) (b) and Virus N (H10N7) (c) were propagated in MDCK cells and metabolically labelled with [³H]glucosamine. Purified viruses were untreated (–), or treated with trypsin (T) or endoproteinase Arg-C (A), respectively and subjected to SDS-PAGE and fluorography.

HAs of variant II and, as controls, of two other influenza viruses with a corresponding arginine residue at that site were treated with either trypsin or endoproteinase Arg-C from mouse submaxillary gland or endoproteinase Lys-C from *Lysobacter enzymogenes*, respectively, and thereafter analyzed by SDS-PAGE under reducing conditions (Fig. 3). As expected, all viruses were activated by trypsin (Klenk et al., 1975). However, endoproteinase Arg-C did not cleave the HA of the Tübingen isolate, whereas the HAs of the influenza A virus strains WSN and Virus N with a single arginine at that position were cleaved. Endoproteinase Lys-C degraded the HAs totally and was therefore unsuitable for proteolytic activation.

Discussion

In the present study we have characterized 2 variants of a human influenza A (H1N1) isolate that have been obtained after serial passages in MDCK cells. The variants can be distinguished by receptor binding specificities, and this difference is determined by the oligosaccharide at Asn_{131} which due to a mutation at amino acid position 133, is present in variant I and absent in variant II. Because the amino acid in position 133 is part of the RBS (Nobusawa et al., 1991), a change of this amino acid alone may modify receptor specificity. But it is more likely that the main structural alteration, the presence or absence of the carbohydrate side chain at that position, is critical for the shift in receptor specificity. The carbohydrate may either have an indirect effect on folding of the HA polypeptide at the RBS or, more likely, may directly prevent the access of $\alpha 2,6$ bound sialic acid for sterical reasons. From our experiments with resialylated erythrocytes it is not clear how
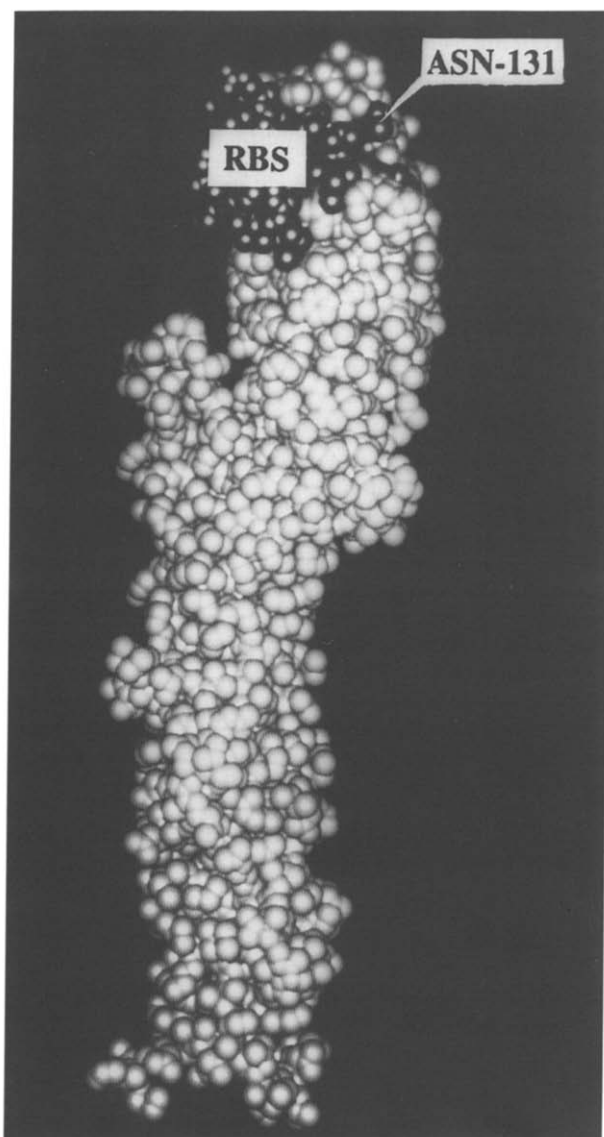
Fig. 4. The relevant mutation at the hemagglutinin receptor binding site of the influenza A/Tübingen/12/85 virus variants. The view of the space-filling model of the H3 subtype HA monomer (Wilson et al., 1981) obtained by molecular modelling (see Material and Methods), shows amino acids contributing to the receptor binding site (RBS) (Weis et al., 1988; Nobusawa et al., 1991). The position of asparagine$_{131}$ (ASN-131) of the variant I is indicated.

variant I binds to the sialic acid of the receptor. Variant I might recognize α2,6 bound sialic acid if the virus exclusively binds to SAα2,6Galβ1,3GalNAc or SAα2,6GalNAc groups which are not formed by the sialyltransferase from rat liver

used in these experiments. Thus, the receptor could not be determined by the resialylation experiments. Alternatively, a low binding affinity to the linkage types analyzed here can also explain why the receptor specificity was not detected. The third possibility, that variant I virus binds neither to 2,3- nor 2,6-linked sialic acid cannot be ruled out, either.

Several groups have demonstrated before similar changes of receptor specificity based on single amino acid changes at the RBS, some of which also alter the carbohydrate equipment. Thus, a variant of influenza A/USSR/90/77 (H1N1) virus has been described that differs from the original virus in its receptor binding capacity to blood cells from guinea pigs (Gitelman et al., 1986). This difference was again caused by the carbohydrate side chain responsible for the receptor binding variation analyzed in the present study. A carbohydrate at the same position was also lost during egg-adaptation of an H1N1 virus (Robertson et al., 1991). Furthermore, a host cell mutant of influenza virus A/WSN (H1N1) that had acquired a carbohydrate at asparagine$_{129}$ (Deom et al., 1986), two amino acids away from the carbohydrate attachment site of the Tübingen variant I, showed reduced binding to SAα2,3 and SAα2,6 receptors (Aytay and Schulze, 1991). Changes in carbohydrates following egg adaptation were not only detected with H1 isolates but also with H3 isolates (Katz and Webster, 1988; Wang et al., 1989; Katz et al., 1990) and influenza B variants (Robertson et al., 1985; Oxford et al., 1990). Taking all data together, these findings give strong evidence that the carbohydrates around the RBS play an important role for receptor specificity and determine the host tropism in some cases. Nevertheless, other amino acid substitutions without alterations of carbohydrates exist around the receptor binding domain in many influenza A and B viruses which affect the binding activities and specificities (Both et al., 1983; Rogers et al., 1983a,b; 1985; Naeve et al., 1984; Nobusawa and Nakajima, 1988; Katz and Webster, 1988; Suzuki et al., 1989; Katz et al., 1990; Nobusawa et al., 1991).

On the other hand, changes of carbohydrates appear around the RBS without any obvious change in receptor specificity but may be important for the antigenic drift (Skehel et al., 1984; Seidel et al., 1991). Antigenicity is modulated by a carbohydrate shift from position 131 to 129 in the antigenic epitope Sb in some H1 variants which were selected in the presence of monoclonal antibodies (Yates et al., 1990). Whether such antigenic alterations occurred with the Tübingen variants which show an amino acid exchange in the same epitope was not investigated here, but may be relevant under epidemiological aspects and for considering vaccine production.

The Tübingen isolate contained either a priori a subpopulation of at least two variants which can be distinguished by their receptor specificity and carbohydrate content, or less likely, a variant I was primarily present which rapidly converts to the variant II when the propagation was performed in MDCK cells. Our findings indicate that MDCK cell-derived viruses do not inevitably represent the situation of the influenza-infected individuals, in contrast to the results by Robertson and co-workers (1991) who showed identity of a directly analyzed HA$_1$ coding region of clinical material with that of virus propagated in MDCK cells. Moreover, identifi-

cation of the RBSs of viruses from a clinical specimen and from embryonated hen's egg has been shown (Rajakumar et al., 1990).

Both variants of the A/Tübingen/12/85 strain have an unusual proteolytic activation site in the hemagglutinin that consists of a single lysine. Such a cleavage site has been observed before only with the H14 serotype present in avian isolates (Kawaoka et al., 1990). All other mammalian and apathogenic avian influenza viruses have a single arginine in this position. Arginine has also been found, when we analyzed the cleavage site of other human H1 isolates obtained in 1985 and subsequent years at various places in Germany (data not shown). Since trypsin which has been used in the isolation procedure of the A/Tübingen/12/85 variants does not discriminate between lysine and arginine cleavage sites, it is not likely that there was selection pressure favouring a mutation from arginine to lysine during isolation. Thus, it is reasonable to assume that the lysine cleavage site existed already in the original clinical specimen. The human alveolar system should therefore contain an appropriate protease to activate at lysine. Such an enzyme must also be present in the chick embryo which allows virus replication without substitution of exogenous protease. Whether the chick embryo enzyme is identical with the factor X-like activator of arginine sites described by Gotoh and co-workers (1990) remains to be seen (Ogasawara et al., 1992). However, the data presented here show clearly that, depending on the presence of an arginine or a lysine at the cleavage site, the H1 hemagglutinin discriminates between activating proteases. It will be interesting to see whether such differences have an effect on the pathogenicity and other biological properties of the virus.

Acknowledgements

References

Aytay, S. and Schulze, I.T. (1991) Single amino acid substitutions in the hemagglutinin can alter the host range and receptor binding properties of H1 strains of influenza A virus. J. Virol. 65, 3022–3028.

Both, G.W., Shi, C.H. and Kilbourne, E.D. (1983) Hemagglutinin of swine influenza virus: a single amino acid change pleiotropically affects viral antigenicity and replication. Proc. Natl. Acad. Sci. USA 80, 6996–7000.

Burnet, F.M. and Bull, D.R. (1943) Changes in influenza virus associated with adaptation to passage in chick embryos. Aust. J. Exp. Med. Sci. 21, 55–69.

Cohen, A. and Biddle, F. (1960) The effect of passage in different hosts on the inhibitor sensitivity of an asian influenza virus strain. Virology 11, 458–473.

Concannon, P., Cummings, I.W. and Salser, W.A. (1984) Nucleotide sequence of the influenza virus A/USSR/90/77 hemagglutinin gene. J. Virol. 49, 276–278.

Crecelius, D.M., Deom, C.M. and Schulze, I.T. (1984) Biological properties of a hemagglutinin mutant of influenza virus selected by host cells. Virology 139, 164–177.

Daniels, R.S., Jeffries, S., Yates, P., Schild, C.G., Rogers, G.N., Paulson, J.C., Wharton, S.A., Douglas, A.R., Skehel, J.J. and Wiley, D.C. (1987) The receptor-binding and membrane-fusion properties of influenza virus variants selected using anti-haemagglutinin monoclonal antibodies. EMBO J. 6, 1459–1465.

Deom, C.M., Caton, A.J. and Schulze, I.T. (1986) Host cell-mediated selection of a mutant influenza A virus that has lost a complex oligosaccharide from the tip of the hemagglutinin. Proc. Natl. Acad. Sci. USA 83, 3771–3775.

Döller, P.C., Döller, G. and Gerth, H.-J. (1987) Subtype-specific identification of influenza virus in cell cultures with FITC-labelled egg yolk antibodies. Med. Microbiol. Immunol. 176, 27–35.

Feldmann, H., Kretzschmar, E., Klingeborn, B., Rott, R., Klenk, H.-D. and Garten, W. (1988) The structure of serotype H10 hemagglutinin of influenza A virus: comparison of an apathogenic avian and a mammalian strain pathogenic for mink. Virology, 165, 428–437.

Gitelman, A.K., Kaverin, N.V., Kharitonenkov, I.G., Rudneva, I.A. and Zhdanov, V.M. (1984) Changes in the antigenic specificity of influenza virus haemagglutinin in the course of adaptation to mice. Virology 134, 230–232.

Gotoh, B., Ogasawara, T., Toyoda, T., Inocencio, N.M., Hamaguchi, M. and Nagai, Y. (1990) An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo. EMBO J. 9, 4189–4195.

Katz, J.M. and Webster, R.G. (1988) Antigenic and structural characterization of multiple subpopulations of H3N2 influenza virus from an individual. Virology 165, 446–456.

Katz, J.M., Wang, M. and Webster, R.G. (1990) Direct sequencing of the HA gene of influenza (H3N2) virus in original clinical samples reveals sequence identity with mammalian cell-grown virus. J. Virol. 64, 1808–1811.

Kawaoka, Y., Yamnikova, S., Chambers, T.M., Lvov, D.K. and Webster, R.G. (1990) Molecular characterization of a new hemagglutinin, subtype H 14, of influenza A virus. Virology 179, 759–767.

Klenk, H.-D., Rott, R. and Becht, H. (1972) On the structure of the influenza virus envelope. Virology 47, 579–591.

Klenk, H.-D., Rott, R., Orlich, M. and Blödorn, J. (1975) Activation on influenza A viruses by trypsin treatment. Virology 68, 426–439.

Klenk, H.-D. and Rott, R. (1988) The molecular biology of influenza virus pathogenicity. Adv. Virus Res. 34, 247–281.

Kohama, T., Garten, W. and Klenk, H.-D. (1981) Changes in conformation and charge paralleling proteolytic activation of Newcastle disease virus glycoproteins. Virology 111, 364–376.

Kuroda, K., Hauser, C., Rott, R., Klenk, H.-D. and Doerfler, W. (1986) Expression of the influenza virus hemagglutinin in insect cells by a baculovirus vector. EMBO J. 5, 1359–1365.

Naeve, C.W., Hinshaw, V.S. and Webster, R.G. (1984) Mutations in the hemagglutinin receptor binding site can change the biological properties of an influenza virus. Virology 51, 567–569.

Nobusawa, E. and Nakajima, K. (1988) Amino acid substitution at position 226 of the hemagglutinin molecule of influenza (H1N1) virus affects receptor binding activity but not fusion activity. Virology 167, 8–14.

Nobusawa, E., Aoyama, T., Kato, H., Suzuki, Y., Tateno, Y. and Nakajima, K. (1991) Comparison of complete amino acid sequences and receptor binding properties among 13 serotypes of hemagglutinins of influenza viruses. Virology 182, 475–485.

Ogasawara, T., Gotoh, B., Suzuki, H., Asaka, J., Shimokata, K., Rott, R. and Nagai, Y. (1992) Expression of factor X and its significance for the determination of paramyxovirus tropism in the chick embryo. EMBO J. 11, 467–472.

Oxford, J.S., Corcoran, T., Knott, R., Bates, J., Bartolomei, O., Major, D., Newman, R.W., Yates, P., Robertson, J., Webster, R.G. and Schild, G.C. (1987) Serological studies with influenza A (H1N1) viruses cultivated in eggs or in a canine kidney cell line (MDCK). Bull. W.H.O. 65, 181–187.

Oxford, J.S., Schild, G.C., Corcoran, T., Newman, R., Major, D., Robertson, J., Bootman, J., Higgins, P., Al-Nakib, W. and Tyrrell, D.A.J. (1990) A host-cell-selected variant of influenza B virus with a single nucleotide substitution in HA affecting a potential glycosylation site was attenuated in virulence for volunteers. Arch. Virol. 110, 37–46.

Parvin, J.D., Moscana, A., Pan, W.T., Leider, J.M. and Palese, P. (1986) Measurement of the mutation rates of animal viruses: influenza A virus and poliovirus type 1. J. Virol. 59, 377–383.

Rajakumar, A., Swierkocz, E. and Schulze, I.T. (1990) Sequence of an influenza virus hemagglutinin determined directly from a clinical sample. Proc. Natl. Acad. Sci. USA 87, 4154–4158.

Robertson, J.S., Naeve, C.W., Webster, R.G., Bootman, J.S., Newman, R. and Schild, G.C. (1985) Alterations in the hemagglutinin associated with adaptation of influenza B virus to growth in eggs. Virology 143, 166–174.

Robertson, J.S., Bootman, R., Newman, R., Oxford, J.S., Daniels, R.S., Webster, R.G. and Schild, G.C. (1987) Structural changes in the haemagglutinin which accompany egg adaptation of an influenza A (H1N1) virus. Virology 160, 31–37.

Robertson, J.S., Nicolson, C., Bootman, J.S., Major, D., Robertson, E.W. and Wood, J.M. (1991) Sequence analysis of the haemagglutinin (HA) of influenza A (H1N1) viruses present in clinical material and comparison with the HA of laboratory-derived virus. J. Gen. Virol. 72, 2671–2677.

Rogers, G.N., Pritchett, T.J., Lane, J.L. and Paulson, J.C. (1983a) Differential sensitivity of human, avian and equine influenza A viruses to a glycoprotein inhibitor of infection: selection of receptor-specific variants. Virology 131, 394–408.

Rogers, G.N., Paulson, J.C., Daniels, R.S., Skehel, J.J., Wilson, I.A. and Wiley, D.C. (1983b) Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature 304, 76–78.

Rogers, G.N., Daniels, R.S., Skehel, J.J., Wiley, D.C., Wang, X., Higa, H.H. and Paulson, J.C. (1985) Host-mediated selection of influenza virus receptor variants. J. Biol. Chem. 260, 7362–7367.

Rogers, G.N. and D'Souza, B.L. (1989) Receptor binding properties of human and animal H1 influenza virus isolates. Virology 173, 317–322.

Schild, G.C., Oxford, J.S., de Jong, J.C. and Webster, R.G. (1983) Evidence for host-cell selection of influenza virus antigenic variants. Nature 303, 706–709.

Scholtissek, C., Rott, R. and Klenk, H.-D. (1975) Two different mechanisms of the inhibition of the multiplication of enveloped viruses by glucosamine. Virology 63, 191–200.

Schultze, B., Gross, H.-J., Brossmer, R., Klenk, H.-D. and Herrler, G. (1990) Hemagglutinating encephalomyelitis virus attaches to N-acetyl-9-o-acetylneuraminic acid-containing receptors on erythrocytes: comparison with bovine coronavirus and influenza C virus. Virus Research 16, 185–194.

Seidel, W., Künkel, F., Geisler, B., Garten, W., Herrmann, B., Döhner, L. and Klenk, H.-D. (1991) Intraepidemic variants of influenza virus H3 hemagglutinin differing in the number of carbohydrate side chains. Arch. Virol. 120, 289–296.

Skehel, J.J., Stevens, D.J., Daniels, R.S., Douglas, A.R., Knossow, M., Wilson, J.A. and Wiley, D.C. (1984) A carbohydrate site chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. Proc. Natl. Acad. Sci. 81, 1779–1783.

Suzuki, Y., Kato, H., Naeve, C.W. and Webster, R.G. (1989) Single amino acid substitution in an antigenic site of influenza virus hemagglutinin can alter the specificity of binding to cell membrane-associated gangliosides. J. Virol. 63, 4298–4302.

Wang, M., Katz J.M. and Webster, R. (1989) Extensive heterogenicity in the hemagglutinin of egg-grown influenza viruses from different patients. Virology 171, 275–279.

Waterfield, M.D., Gething, M.J., Scrace, G. and Skehel, J.J. (1980) The carbohydrate side chains and disulfide bonds of the hemagglutinin of influenza virus A/Japan/305/57 (H2N1) In: Laver, G. and Air, G. (Eds.), Structure and variation in influenza virus. Elsevier, Amsterdam, pp. 11–20.

Weis, W., Brown, J.H., Cusack, S., Paulson, J.C., Skehel, J.J. and Wiley, D.C. (1988) Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. Nature 333, 426–431.

Wilson, I.A., Skehel, J.J. and Wiley, D.C. (1981) Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3A resolution. Nature 289, 366–373.

Winter, G., Fields, S. and Brownlee, G.G. (1981) Nucleotide sequence of the haemagglutinin gene of a human influenza virus H1 subtype. Nature 292, 72–75.

Yates, P.J., Bootman, J.S. and Robertson, J.S. (1990) The antigenic structure of a human influenza A (H1N1) virus isolate grown exclusively in MDCK cells. J. Gen. Virol. 71, 1683–1688.