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

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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 α2,6-linked sialic acid. Evidence is presented that this difference is determined by a complex carbohydrate side chain at asparagine131 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)
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 \(^{3}\text{H}\)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 \(\mu\)g 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 \(\mu\)g 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 \(\mu\)1). 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 \(\mu\)l 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: TAAAAACAAACAAAAATG (1); TGGAGACAGTCACAACG (182); AATCATGGTCCTACATTG (304); TGTTACCCAGGTTATTTTCC (351); TCATGGCCCCAACACA (447); TTTTACAGAAATTTGCTA (513); CTAACATAGAAGACCA (631); CAATAATATTTTGGGCAA (799); ATGAAATGACGCAAATGTG (901); TCCAGAATGTACACC (958); GGAGAGTGCACA (984); GGTTATATCTATCATCAG (1131); GGTAGCTGGACATTTGG (1323); CGGAGCTGGAACATAT (1329); 1AIAC1AACCAAAATAT (1533); ATGGAGATCTATCAG (1605); ATGGTAGCTATAC (106R); TTGGAGCTGGAACATAT (146R); GGTGAATCTCCTGTTATA (716R); GAAAGCATACCATGG (848R); ATGATGACCATACCAT (1142R); GTGTAGCAGATGCATAT (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: | Chick resialylated |
|------------------------------------|------------------------|--------------------|--------------------|
|                                    |                        | Guinea pig untreated | Human untreated | Chick untreated | Sαα2,3 | Sαα2,6 |
| 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 α2,3- or in α2,6 linkage and then used for virus agglutination. In contrast to variant I, variant II recognizes sialic acid in α2,6 linkage as do strains PR/8 and WSN. The ability of variant II to utilize SAα2,6Gal 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 HA1 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 HA1 subunits (designated as HA1−), 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+ is correlated with variant I (I), HA1 with variant II (II). HA2 migrated out of the 12% gel. The deglycosylated forms are designated as HA− and HA−−.

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 HA1 and 154 on HA2 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. 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 HA1 with HA2 (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
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 $[^3]$Hglucosamine. 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 α2,6 bound sialic acid for sterical reasons. From our experiments with resialylated erythrocytes it is not clear how
variant I binds to the sialic acid of the receptor. Variant I might recognize $\alpha 2,6$ bound sialic acid if the virus exclusively binds to SA$\alpha 2,6$Gal$\beta 1,3$GalNAc or SA$\alpha 2,6$GalNAc 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 Tubingen 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 Tubingen 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 Tubingen 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, 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.

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