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Studies on the adaptation of influenza viruses to MDCK cells

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The amino acid sequences and biological properties of the haemagglutinin of three variants of the influenza virus X-31 (H3N2) selected for their capacity to grow in MDCK cells are reported. In two variants, amino acid substitutions at HA_1 residues 8 and 144 correlated with the loss of a site for glycosylation and specific changes in antigenicity, respectively. In all three variants substitution of an arginine residue for histidine at HA_1 position 17 was correlated with increased pH optima of haemolysis. The importance of this substitution for cleavage of the haemagglutinin precursor required to produce infectious virus is discussed in relation to the three-dimensional structure of X-31 haemagglutinin.

Key words: influenza virus haemagglutinin/proteolytic cleavage/haemolysis/amino acid sequences

Introduction

Adaptation to a novel host, which presumably allows the selective survival of appropriate mutants in the new environment, has been observed to result in alterations of a number of viral characteristics. With influenza viruses several changes of biological properties linked to adaptation have been described including attenuation of the virus for its original host, increased or decreased susceptibility to non-specific inhibitors (see Fenner and Cairns, 1959), alteration in the capacity to agglutinate different red cells which is expressed by the change in the O (original) to the D (derivative) phase of the virus (Burnet and Bull, 1943), and selection of antigenic variants without immunological pressure (see Francis, 1959). Little is known about the molecular mechanisms underlying these consequences of adaptation, but in a number of cases the haemagglutinin glycoprotein of the virus membranes appears to be involved. Here we report changes in the properties of X-31 influenza virus haemagglutinin during adaptation of egg-grown virus to growth in canine cells.

Results

Formation of infectious virus after serial passages of X-31 virus in MDCK cells

The X-31 influenza virus, like other egg-adapted viruses of the H3 subtype isolated from humans, replicates poorly in cells in culture. In the MDCK cells used here indistinct plaque formation was observed if trypsin was not present in the agar overlay. If plaques were produced at all, they were hardly visible and could be counted only with difficulty. Correspondingly only little infectious virus was found to be produced under single (30 p.f.u./cell) or multiple (10^{-3} p.f.u./cell) multiplication cycle conditions. In the presence of trypsin, plaques were clearly visible with diameters ranging from 1 to 2 mm. Under these conditions the plaque number increased at least 100-fold resulting in a p.f.u./HA ratio of $\sim 10^6$.

Enhancement of virus growth and plaque formation could be achieved when the virus was passaged serially in MDCK cells without trypsin. Cells were infected with < 1 p.f.u./cell of virus grown in embryonated eggs and were incubated in each passage for 48–72 h at 37°C until reasonable HA titres were found in the medium. The medium used for the following passages was diluted 10- to 1000-fold. After 7–10 passages plaque tests were performed in MDCK cells without trypsin. If enhanced plaque formation was observed, large plaques were picked and purified by further plaque passages. The progeny of such plaques were found to grow identically under multiple cycle conditions independently of whether trypsin was present in the medium or not. Similarly, plaque sizes and plaque numbers did not increase significantly when trypsin was added to the agar overlay. The capacity of these variants to grow in infectious form seems to be restricted, however, to MDCK or other dog (greyhound) cells. They

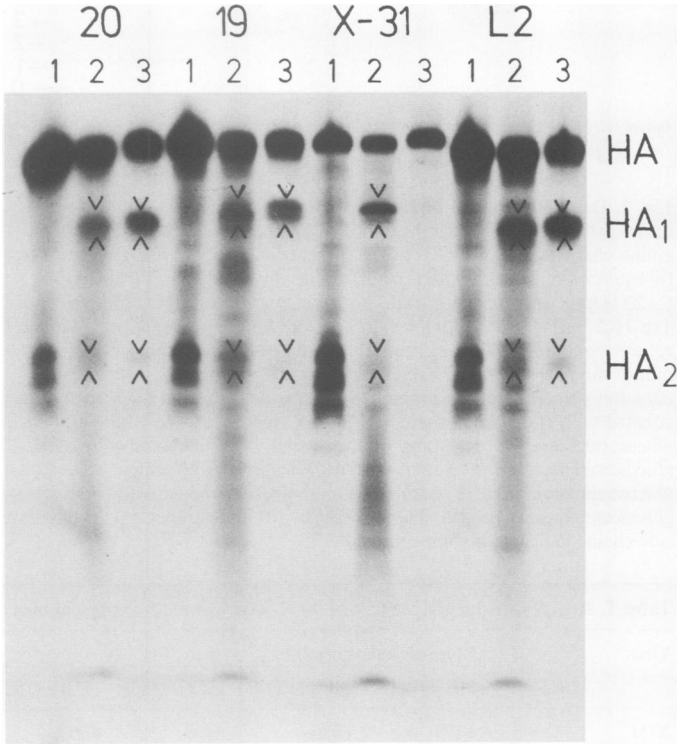

Fig. 1. Glycoproteins of X-31 virus and its MDCK cell-adapted variants 20, L2 and 19. Cells were pulse-labelled 4 h after infection with [^{3}H]mannose and cell lysates were analysed by polyacrylamide gel electrophoresis. **(1)** 30 min pulse, **(2)** 30 min pulse, cell lysates were incubated for 30 min with 10 μg trypsin per ml at 37°C prior to gel electrophoresis, **(3)** 30 min pulse followed by a 4 h chase period with unlabelled mannose.

were unable to plaque in CV1, MDBK or chicken embryo cells without trypsin, although they are produced efficiently in the embryonated egg.

Processing of the haemagglutinin after adaptation

Since proteolytic cleavage of the precursor haemagglutinin molecule into the disulfide-linked polypeptides HA_1 and HA_2 is a precondition for influenza virus infectivity (Klenk *et al.*, 1975, 1977; Lazarowitz and Choppin, 1975), it was expected that during adaptation viruses would have been selected with haemagglutinins susceptible to proteolytic activation in the MDCK cells. The results of analyses of glycopolypeptide synthesis in infected MDCK cells are shown in Figure 1. The haemagglutinin of the unadapted egg-grown X-31 virus is found exclusively in the uncleaved form after a 30 min pulse with [^{3}H]mannose and a 4 h chase period. Even after a 20 h labelling period only small amounts of HA_1 and HA_2 could be detected. Obviously after trypsin treatment the haemagglutinin was cleaved. In contrast, cells infected with adapted viruses always contained cleaved haemagglutinin. *In vivo* cleavage was at least as effective as *in vitro* cleavage with trypsin. It can also be seen in Figure 1 that haemagglutinins of the L2 and 20 variants exhibited significant differences in their apparent mol. wt. compared with the original X-31 virus and the variant 19, and similar differences were also found in the relative mobility of the haemagglutinin polypeptides HA_1 of these variants.

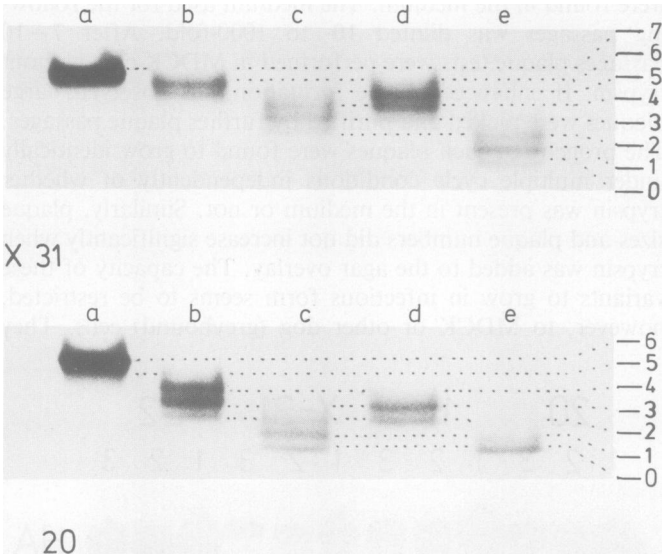

Fig. 2. Determination of the number of carbohydrate side chains on the haemagglutinin of X-31 and variant 20. Virus labelled with [^{3}H]glucosamine was grown in CE cells in the presence of MdN (2 mM) and purified by equilibrium centrifugation in sucrose density gradients. Virus samples (~20 HAU) were incubated with endo-glucosaminidase H (Miles) in 20 μl Tris-HCl buffer (0.1 M, pH 6.8) at 37°C and analysed by polyacrylamide gel electrophoresis. Virus samples that had been denatured by boiling for 2 min in the presence of 0.2% SDS and 1% mercaptoethanol prior to incubation with endo-glucosaminidase H were also analysed **(a)** Virus not incubated with endo-glucosaminidase H. **(b)** Virus incubated with endo-glucosaminidase H for 15 min. **(c)** Denatured virus incubated with endo-glucosaminidase H for 15 min. **(d)** Virus incubated with endo-glucosaminidase H for 3 h. **(e)** Denatured virus incubated with endo-glucosaminidase H for 3 h. The HA bands with the number of carbohydrate side chains indicated are shown.

Because these differences in electrophoretic mobility suggest alterations in glycosylation patterns, we have determined the number of the carbohydrate side chains of the haemagglutinins of X-31 virus and its variants 19, 20 and L2. The procedure involves growth of the virus in the presence of the carbohydrate side chain trimming inhibitor methyldeoxynojirimycin (MdN) (Saunier *et al.*, 1982), followed by controlled removal of the resulting mannose-rich oligosaccharides with endo-N-acetyl-glucosaminidase H (Klenk and Schwarz, in preparation). When virus was labelled with [^{3}H]glucosamine, eight glycopolypeptide bands were obtained from the X-31 virus and the variant 19, while variants 20 and L2 yielded only seven bands (Figure 2), indicating that haemagglutinins of the latter variants contain only six carbohydrate side chains. These results taken together with the data shown in Figure 1 demonstrate that in variants 20 and L2 the HA_1 polypeptides have one less oligosaccharide side chain.

Passage of influenza viruses in different cells has been reported to lead to changes in antigenicity (e.g., Francis, 1959; Schild *et al.*, 1983). The antigenic properties of the haemagglutinin of the MDCK-adapted variants were therefore analysed using monoclonal and polyclonal antibody preparations. The results presented in Table I indicate that differences could be detected between variants 20 and L2 and the original X-31 virus and variant 19. The differences in the reactivities with the monoclonal antibodies were restricted to those antibodies which recognize amino acids in the prominent loop designated site A (Wiley *et al.*, 1981).

Amino acid sequences of the haemagglutinins of adapted viruses

To analyse the structural basis of the observations on haemagglutinin biosynthesis, the nucleotide sequences of the genes for the haemagglutinins of the variants were determined and their haemagglutinin amino acid sequences deduced. As summarized in Table II only three amino acid substitutions were detected in the MDCK cell-adapted variants two of which were restricted to variants 20 and L2. Of these two substitutions, residue 144 has been shown before to be antigenically important in X-31 virus haemagglutinin (Laver *et al.*, 1979; Daniels *et al.*, 1983) and the change observed here is also accompanied by a change in antigenicity as judged by the results shown in Table I. The second change in amino acid se-

Table I. Analysis of the antigenicity of X-31 virus variant haemagglutinins

Virus	Monoclonal antibodies						Post-infection antibodies from	
	HC3 (144)	HC113 (145)	HC68 (193)	HC19 (157)	HC45 (63)	HC159 (143)	Ferrets	Chicken
X-31	6400	6400	6400	6400	3200	6400	1280	512
19	6400	6400	6400	6400	3200	6400	1280	512
20	100	< 100	6400	6400	3200	< 100	160	32
2	100	< 100	6400	6400	3200	< 100	160	32

Haemagglutination inhibition reactions were measured by standard procedures. The results presented are the reciprocals of the highest dilutions of the antibodies at which haemagglutination was inhibited. Monoclonal antibodies are numbered according to the culture number, e.g., HC3 and HC159. The numbers in parentheses indicate the residues substituted in variants selected by the particular antibody.

Table II. Amino acid sequence changes in X-31 virus variant haemagglutinins

Virus	Codon change	Amino acid change
19	126–128 CAT - CGT	$HA_1$17 H - R
20 and L2	126–128 CAT - CGT	$HA_1$17 H - R
	99–101 AAC - AAA	$HA_1$8 N - K
	507–509 GGT - GAT	$HA_1$144 G - D

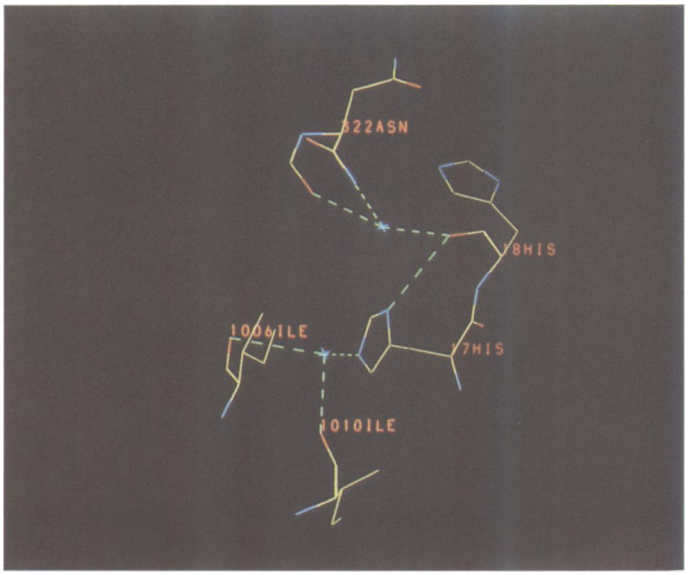

Fig. 3. The environment of residue 17 of the HA_1 polypeptide of X-31 haemagglutinin. Histidine 17 of HA_1 forms hydrogen bonds with residues 6, 10, 12 and 14 in the amino-terminal peptide of HA_2 and with residues 321 and 322 of HA_1, in some cases *via* water molecules marked as X in the figure. The nitrogen NE_2 of histidine 17 is located 10 Å from the HA_2 terminal amino group. Substitution of arginine for histidine 17 as reported here would disrupt the contacts to both HA_1 and HA_2 and introduce a positive charge within < 10 Å of the amino terminus of HA_2. In the absence of an X-ray structure for the mutant haemagglutinin the precise consequences of this change are not known but it will probably destabilize the location of the amino-terminal region of HA_2 at neutral pH raising the pH at which haemolysis occurs and it may affect the structure of the HA_1 C terminus - HA_2 N terminus region of the haemagglutinin precursor which is the site of proteolytic processing.

quence involves substitution of a lysine residue for an asparagine at HA_1 position 8 and results in the loss of a site for attachment of a carbohydrate side chain which is known to be glycosylated in the haemagglutinin of the wild-type X-31 virus. The third mutation involving the substitution of a histidine in HA_1 position 17 by an arginine was the only change common to all three variants.

Discussion

These results establish that adaptation of X-31 virus to growth in MDCK cells coincides with the introduction of a number of changes in the structure of the haemagglutinin. Firstly, precursor haemagglutinin is cleaved to produce functionally active molecules containing HA_1 and HA_2 polypeptides. This is not an unexpected result given the information available on the requirement for haemagglutinin cleavage in infectivity (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975) and membrane fusion (Huang *et al.*, 1980; White *et al.*, 1981). It served to reinforce these observations and, because of the precise coincidence between virus growth and haemagglutinin cleavability, suggests that this process is the major limiting step in adaptation.

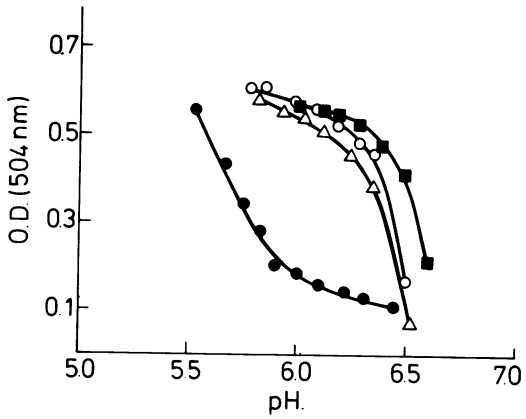

Fig. 4. Variation in the haemolytic activity of X-31 variants as a function of pH. Viruses were adsorbed to human type O erythrocytes at 4°C and were subsequently incubated at 37°C for 30 min at the pH indicated. Haemoglobin content of cell supernatants was assayed spectrophotometrically at 504 nm. ●–● X-31 virus, ○–○, △–△, ■–■ variants 19, L2 and 20, respectively.

Secondly, in comparison with the original X-31 virus, MDCK cell-adapted variants contained three different amino acid residues at positions 8, 17 and 144 of HA_1. The change of glycine 144 to aspartic acid is known to change the antigenicity of viruses of the H3 subtype (Wiley *et al.*, 1981) and similar antigenic changes in haemagglutinins of the MDCK cell-adapted viruses, L2 and 20, were observed here using defined monoclonal antibodies and post-infection antisera. The substitution at position 8 of HA_1 results in the loss of a carbohydrate attachment site and our data show clearly that an oligosaccharide is missing from the haemagglutinins 20 and L2 in which this change occurs. The oligosaccharide side chain at this position is sulphated (Compans and Pinter, 1975) and is responsible for the host antigen activity of haemagglutinins of viruses of the H3 subtype grown in hens' eggs (Ward *et al.*, 1981). The significance of either of these changes in amino acid sequence to the processes involved in adaptation is not known. As noted above (Schild *et al.*, 1983), changes in the antigenicity of the haemagglutinins of influenza B viruses on passage in different cells have been detected using monoclonal antibodies, and the results presented here establish amino acid 144 as an antigenically important residue subject to such modification during passage of virus of the H3 subtype in MDCK cells. It is, however, clear that neither of these amino acid substitutions are crucial for the adaptation to growth in MDCK cells, since they were not observed in the variant 19.

The only mutation which was found in all three variants was the replacement of histidine by arginine at position 17 of HA_1. This residue is located ~35 Å from the virus membrane in close proximity to HA_2 residue 6 and the amino terminus of HA_2 (Figure 3). A change at this position has been recorded in a mutant of X-31 virus selected for an increased pH optimum of haemolysis (Daniels *et al.*, in preparation) and a similar change in haemolytic activity was observed for the viruses studied here (Figure 4). It is noteworthy that all viruses including A/PR/8/34 (H1N1), A/FM/1/47 (H1N1), A/turkey/Oregon/71 (H7N3) and A/seal/Mass/1/80 (H7N7), when adapted to growth in MDCK cells, contain cleaved haemagglutinins and have similarly been observed to acquire elevated pH optima for haemolysis (data not shown). It is likely that the elevated pH optimum of haemolysis which accompanies the substitution of an arginine residue at HA_1 17

reflects a decrease in the stability of the haemagglutinin at this position. It is also possible that as a consequence of this decreased stability cleavage of the haemagglutinin precursors of the adapted viruses is facilitated. These possibilities are presently being assessed directly in biochemical experiments using viruses containing specifically modified haemagglutinins.

Materials and methods

Viruses and cells

The A/PR/8/34 x A/Aichi/2/68 recombinant virus X-31 (H3N2, Baez *et al.*, 1980) grown in 11-day-old hens eggs was used. Adaptation experiments were done in Madin Darby canine kidney (MDCK) cells. In some experiments MDBK, monkey kidney CV1, greyhound kidney cells (No. 03-280) and primary cultures of chick embryo cells overlayed with reinforced Eagle's medium without serum (REM) were used. Virus growth and plaque assays were done in the absence or presence of trypsin (10 μg/ml) in the medium as described (Klenk *et al.*, 1975).

Haemagglutination, haemagglutination inhibition tests and determination of haemolytic activities were performed according to standard procedures.

Polyacrylamide gel electrophoresis

Monolayers of cells on 2.5 cm plastic Petri dishes were labelled by adding [^{3}H]mannose (30 μCi/culture) for the times indicated. When the pulse was followed by a chase, the radioactive medium was removed and the cells were washed three times with REM and incubated in this medium. At the end of the incubation period the cells were washed three times with cold phosphate-buffered saline (PBS) and lysed. For gel electrophoresis, samples were boiled for 2 min with SDS (2%) and mercaptoethanol (2%). Slab gels (Laemmli, 1970) containing 15% acrylamide and 0.2% bisacrylamide were analysed by fluorography (Bonner and Laskey, 1974).

Nucleotide sequence analyses

Virus purification and extraction of RNA was as described before (Hay *et al.*, 1977) as was the procedure for RNA sequence determination by the chain terminating method of Sanger *et al.* (1977) using synthetic primers of reverse transcription (Daniels *et al.*, 1983). The oligonucleotide primers used were complementary in sequence to nucleotides 5–15; 191–202; 367–378; 627–638; 801–810; 1025–1034; 1134–1144; 1330–1340; 1614–1628 of the X-31 virus RNA gene for haemagglutinin (Verhoeyen *et al.*, 1980).

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