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Analyses of the Antigenicity of Influenza Haemagglutinin at the pH Optimum for Virus-mediated Membrane Fusion

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SUMMARY

At the pH optimum for membrane fusion the haemagglutinin glycoprotein (HA) of the influenza virus membrane which is implicated in the fusion activity undergoes a conformational change. We have analysed the effects of this change on the antigenicity of the haemagglutinin by reacting the molecule with monoclonal antibodies of defined specificity. The results obtained indicate that specific changes in antigenicity occur in antigenic sites B and D and are interpreted in terms of the three-dimensional structure of the molecule and the effects of low pH incubation on it. Our results also provide evidence for the antigenic significance of amino acid sequence changes in site B of the HAs of natural isolates and allow clear delineation of this site into two regions.

INTRODUCTION

The initial events in infection by influenza virus are proposed to involve binding of virus to sialic acid residues of glycosylated membrane receptors, endocytosis, and fusion of the membranes of virus and endosome. Virus adsorption has been known for some time to be a property of the haemagglutinin glycoprotein (HA) of the virus membrane and the recent direct implication of HA in membrane fusion (White *et al.*, 1982) confirms previous conclusions from a variety of observations that HA is also involved in this activity (for reviews, see Rott & Klenk, 1977; Compans & Klenk, 1979). *In vitro* membrane fusion and haemolysis mediated by influenza viruses are maximal at about pH 5·0 (Maeda & Ohnishi, 1980; Huang *et al.*, 1981; White *et al.*, 1981), a pH close to that in endosomes, and we have observed that at this pH the conformation of HA differs from that at neutrality. In studies of the structure of bromelain-released HA (BHA), the observed consequences of this low pH structure transition are that the molecule acquires the ability to bind to lipid vesicles, to bind detergent, to aggregate in lipid and detergent-free solutions and it becomes susceptible to proteolysis (Skehel *et al.*, 1982). We report here the results of further studies on the structure of haemagglutinin in the low pH conformation which indicate that the antigenicity of the molecule is specifically modified in the antigenic sites designated B and D (see Wiley *et al.*, 1981).

METHODS

Viruses and haemagglutinins. The X-31 (H3N2) recombinant influenza virus was used (Kilbourne, 1969). Virus was grown in hens' eggs or in primary chick embryo fibroblasts when virus containing uncleaved haemagglutinin was required. Antigenic variants of X-31 were obtained by mixing equal volumes of allantoic fluid containing virus and undiluted ascitic fluid containing monoclonal antibodies, and using the mixture as inoculum. Variants obtained were cloned by limit dilution. Virus purification was as described previously (Skehel & Schild, 1971). Haemagglutinin (BHA) was isolated from purified viruses by digestion with the protease bromelain and purified as described previously (Brand & Skehel, 1972). BHA 'tops' containing amino acids 28 to 328 of HA_1 were prepared from BHA incubated at pH 4·8 for 10 min at 20 °C and then digested with trypsin (BHA : DCC Sigma trypsin; 50 : 1, w/w) for 20 min at 20 °C, pH 7·2. BHA 'tops' were purified from the digestion products by sucrose density gradient centrifugation (100000 *g*, at 20 °C for 16 h in 2·5 to 20% sucrose in phosphate-buffered saline pH 7·2) as described previously (Skehel *et al.*, 1982).

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Haemagglutination-inhibition (HI) tests. These were performed by standard methods (World Health Organization Expert Committee on Influenza, 1953) using anti-haemagglutinin monoclonal antibodies and 5% turkey red blood cells. In the HI blocking tests BHA or BHA 'tops' 20 μg/ml was incubated with dilutions of antibodies for 60 min. Virus (8 HA units) was added for 30 min before addition of red blood cells.

Monoclonal antibody production. BALB/c mice were immunized by intraperitoneal injection of 8000 HA units of purified virus. Eight weeks later they received 32000 HA units of virus intraperitoneally and intravenously. Three days later their spleens were removed and the cells fused with SP2/O-Ag14 myeloma cells (Schulman *et al.*, 1978) as described by Kohler & Milstein (1975, 1976). Hybrid cell culture conditions were based on those described by Fazekas de St. Groth & Scheidegger (1980).

Nucleotide sequence analyses. Sequences were determined using the dideoxynucleotide chain-terminating procedure of Sanger *et al.* (1977), as used previously in influenza virus RNA studies by, for example, Air (1981), Both & Sleigh (1981) and Caton *et al.* (1982). Each 10 μl reaction contained 0·05 M-Tris–HCl pH 8·3, 0·012 M-MgCl, 0·02 M-dithiothreitol, 0·0004 M each of dATP, dCTP, dGTP and dTTP, 7 μg virus RNA, 3 units of human placental RNase inhibitor (Bethesda Research Laboratories), 5 units of reverse transcriptase (Life Sciences, St Petersburg, Fla., U.S.A.), and either ddATP, ddCTP, ddGTP or ddTTP, 0·00025 M. After 120 min at 42 °C the products of the reaction were analysed on polyacrylamide gels containing 8% acrylamide. Reactions were primed using ^{32}P-5′-labelled oligodeoxynucleotides prepared as described by Patel *et al.* (1982) and purified by ion exchange high pressure liquid chromatography (Partisil SAX 10-50). The primers used, numbered according to the sequence of X-31 haemagglutinin cDNA (Verhoeyen *et al.*, 1980), were as follows: 5-AAAGCAGGGG-14; 191-TGCTACTGAGCT-202; 345-CGCAGCAAAG-354; 493-GCAAAAGGGG-502; 623-TCACCACCCG-632; 777-TGGACAATAG-786. Sequences of the HA_1 regions of all variants were read with no difficulty except for nucleotides 113 to 121, which in all cases gave an unreadable sequence. This region which encodes amino acids 12 to 15 is conserved throughout the H3 subtype and contains the universally conserved cysteine at position 14. We therefore assume the variants described here to be unchanged in this region.

RESULTS

Determination of monoclonal antibody specificity

The specificities of anti-haemagglutinin monoclonal antibodies were determined by sequencing the RNAs of antigenic variants selected by growing X-31 virus in the presence of the individual antibodies. The primer extension procedure used is described in Methods, and the results obtained are shown in Table 1.

Antigenicity of HA in the pH 5·0 conformation and of HA 'tops'

On incubation at pH 5·0 X-31 BHA is aggregated (pH 5·0 BHA). In this conformation BHA is susceptible to tryptic digestion and the products obtained are readily separated into aggregate and soluble fractions by sucrose density gradient centrifugation. The aggregate contains the HA_2 glycopolypeptide disulphide linked to the 27 residue amino-terminal glycopeptide of HA_1 and the soluble fraction contains the glycopolypeptide residues 28 to 328 of HA_1 (Skehel *et al.*, 1982). The soluble fraction of sedimentation coefficient about 2S is designated HA 'tops'. A secondary cleavage of this glycopolypeptide at residue 224 is also observed but the two products remain covalently linked by disulphide bonds (Fig. 1).

HI blocking tests

The results of experiments in which HA 'tops' and BHA preparations were used to block the HI activities of monoclonal antibodies are shown in Table 2. The regions of the molecule recognized by the individual antibodies, as judged by amino acid substitutions in the HAs of antigenic variants, are indicated as A, B, C, D and E as before (Wiley *et al.*, 1981). Antibodies HC3 and HC45, which recognize amino acid residues 144 and 63 in sites A and E respectively, appear to bind to HA 'tops' and BHA in the pH 5·0 conformation, pH 5·0 BHA. Similarly, antibody H14/A2 which recognizes amino acid 54 (Laver *et al.*, 1980) in site C also reacts with HA 'tops' and pH 5·0 BHA. These results indicate that sites A, C and E are not irreversibly affected antigenically in pH 5·0 BHA either before or after tryptic digestion. Sites B and D, on the other hand, are modified by exposure to low pH. Antibodies HC31, HC263 and HC67 (site B) and antibody H14/A21-3 (site D) which inhibit haemagglutination irrespective of the

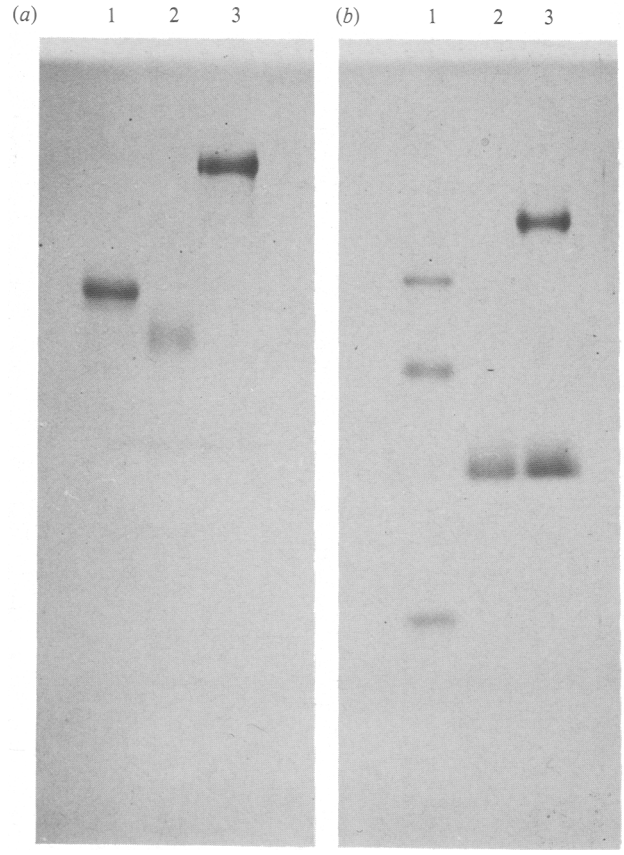

Fig. 1. Polypeptide compositions of haemagglutinin and HA 'tops'. Bromelain-released haemagglutinin (BHA) and the HA 'tops' and aggregate fractions obtained by tryptic digestion of BHA in the pH 5·0 conformation were purified as described in Methods. Samples were boiled in either 0·5% SDS and 8 M-urea or 0·5% SDS, 8 M-urea and 0·05 M-2-mercaptoethanol for 2 min before electrophoresis on polyacrylamide gels containing 16·5% acrylamide for 16 h at 10 V/cm as described previously (Skehel *et al.*, 1982). (*a*) Lane 1, HA 'tops'; lane 2, aggregate; lane 3, BHA, non-reduced samples. (*b*) Lane 1, HA 'tops'; lane 2, aggregate; lane 3, BHA, reduced samples.

Table 1. *Nucleotide and amino acid changes in the HAs of antigenic variants of X-31 selected using different monoclonal antibodies*

Variant	Nucleotide no. and change	Amino acid no. and change
45	264 G → A	63 Asp → Asn
101	264 G → A	63 Asp → Asn
3	508 G → A	144 Gly → Asp
113	511 G → A	145 Ser → Asn
67	543 A → G	156 Lys → Glu
67	656 C → A	193 Ser → Arg
19	547 C → T	157 Ser → Leu
263	550 G → A	158 Gly → Glu
83	655 G → T	193 Ser → Ile
31	670 C → T	198 Ala → Val
221	672 T → C	199 Ser → Pro

presence of HA 'tops' or pH 5·0 BHA clearly fail to combine with these components. However, not all antibodies that recognize amino acids grouped in site B are unable to react with HA 'tops' and pH 5·0 BHA. Antibodies HC19 and HC83 which recognize amino acid residues 157 and 193 are prevented from inhibiting virus haemagglutination by both preparations.

Table 2. *Haemagglutination-inhibition blocking reactions*

	Monoclonal antibody*									
Blocking protein	HC3 (144-A)	HC19 (157-B)	HC31 (198-B)	HC45 (63-E)	HC67 (156 + 193-B)	HC83 (193-B)	HC113 (145-A)	HC263 (158-B)	H14/A2 (54-C)	H14/A21-3 (205-D)
O	10240	10240	5120	5120	5120	5120	10240	2560	100	20480
BHA pH 7·0	320	160	640	320	160	160	80	20	10	2560
BHA pH 5·0	640	320	5120	640	5120	160	640	2560	–†	–
HA 'tops'	320	1280	5120	320	5120	160	640	2560	10	20480

* The specificity of the monoclonal antibodies is given in parentheses by the amino acid recognized (see Table 1) and the antigenic site in which the amino acid is located (sites A to E). The results presented are the reciprocals of the highest dilutions of the antibodies at which haemagglutination was inhibited. Antibodies H14/A2 and H14/A21-3 were given by W. Gerhard and R. Webster, and were characterized in Laver *et al.* (1980).

† –, Not done.

Table 3. *Haemagglutination-inhibition reactions**

	Monoclonal antibody									
Virus	HC3 (144-A)	HC19 (157-B)	HC31 (198-B)	HC45 (63-E)	HC67 (156 + 193-B)	HC101 (63-E)	HC113 (145-A)	HC83 (193-B)	HC263 (158-B)	HC221 (199-B)
X-31 pH 7·0	12800	6400	3200	6400	2560	12800	12800	6400	25600	12800
X-31 pH 5·0	12800	3200	200	6400	20	12800	12800	6400	6400	100
Precursor X-31 pH 7·0	25600	6400	3200	6400	–†	–	–	–	–	–
Precursor X-31 pH 5·0	25600	6400	3200	6400	–	–	–	–	–	–

* HI reactions were by standard procedures. The specificity of the monoclonal antibodies is as given in Table 1. The results presented are the reciprocals of the highest dilutions of the antibodies at which haemagglutination was inhibited.

† –, Not done.

Haemagglutination-inhibition tests

Since haemagglutination activity is not affected by incubating haemagglutinin or virus at pH 5·0 (Skehel *et al.*, 1982) antigenic analyses of HA in the pH 5·0 conformation were also performed directly in HI tests (Table 3), and similar results to those shown in Table 2 were obtained. Virus incubated at pH 5·0 lost the ability to interact with HC31, HC67 and HC263, and was therefore modified in site B. In these tests virus containing uncleaved precursor haemagglutinin was also analysed and it was observed that the antigenicity of these viruses was not modified by incubation at pH 5·0. These observations are consistent with the observed inabilities of uncleaved haemagglutinin to undergo the pH 5·0 structure transition as judged by sensitivity to proteolysis (R. S. Daniels *et al.*, unpublished observations) and to cause cell fusion (White *et al.*, 1981).

DISCUSSION

Conformational changes in the HA of X-31 influenza virus have been observed to occur at pH values corresponding to those optimal for the membrane fusion activity of the virus (Skehel *et al.*, 1982). These changes were monitored by circular dichroism, sedimentation, electron microscopy, and proteolytic susceptibility studies and it was concluded that they did not involve gross secondary structure modifications. The results were consistent with the relative movements at low pH of molecular domains that independently maintain their tertiary structure. The observations presented here on the antigenicity of HA in the low pH conformation are consistent with these conclusions. These results indicate that the structures of antigenic sites A, C and E of X-31 HA (Wiley *et al.*, 1981) are maintained in pH 5·0 HA or more precisely, since antigen conformation may be influenced by antibody interaction, that the structures at these sites are not irreversibly modified at low pH.

The observations that after incubation at pH 5·0 uncleaved precursor HA, which does not undergo the low pH structural transition, reacts with all the antibodies used indicate that changes in the antigenicity of cleaved HA at low pH are not simply a consequence of pH-dependent local changes such as the ionization of groups at the site of antibody interaction. They support the notion that the modifications of antigenic sites B and D result from specific pH 5·0 induced changes in their structure. Additional observations of the structure of pH 5·0 BHA indicate that the HA 'tops' derived from the pH 5·0 HA trimer by tryptic digestion are released as monomers and, therefore, that monomer–monomer contacts in the distal region of the molecule are broken at low pH (Daniels *et al.*, 1983). Interpretation of the antibody interactions reported here is assisted by these results since the antibodies that do not react with either pH 5·0 HA or HA 'tops' recognize amino acids in close proximity to residues in adjacent monomers of the HA trimer. Such antibodies may recognize epitopes which span the monomer–monomer boundary. For example, only 1·5 nm separates residue 198 in one monomer from 198 in another monomer of the trimer, and a single antibody might contact both residues simultaneously. It is perhaps more likely, however, that disorientation of the residues at this site occurs as a result of monomer–monomer contact breakage and the consequent loss of structural constraints unique in the trimer. Whatever the precise explanation, the loss of intermonomer contacts in the distal globular region of HA at low pH could clearly lead to modification of the structure recognized as antigenic in the trimer and to the abolition of binding of these antibodies.

Finally, the antibody-binding sites of HAs of the H3 subtype were initially proposed (Wiley *et al.*, 1981) on the basis of amino acid sequence variations observed in the HAs of viruses isolated between 1968 and 1975, sites A, B, C, D and E, and in five monoclonal antibody-selected variants of A/Memphis/102/72 (Laver *et al.*, 1979, 1980) sites A, C and D. The results presented here from analyses of monoclonal antibody-selected X-31 variants provide the direct proof required for site B. They also indicate that antibodies that bind to site B can be divided into two groups on the basis of their reaction with HA in the pH 5·0 conformation. Thus, antibodies that recognize amino acids 157 and 193, which extend towards site A, react with HA at both pH 7·0 and pH 5·0; antibodies that recognize residues 156, 158 and 198 react only with HA at pH 7·0.

This delineation of sequentially adjacent amino acid residues into different antibody-binding sites and the locations of the sites in the molecule are similar to those described recently by Caton *et al.* (1982) for influenza viruses of the H1 subtype. The sites were designated by these authors as Sa and Sb.

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