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ANTIGENIC VARIATION IN THE INFLUENZA A (HONG KONG) VIRUSES

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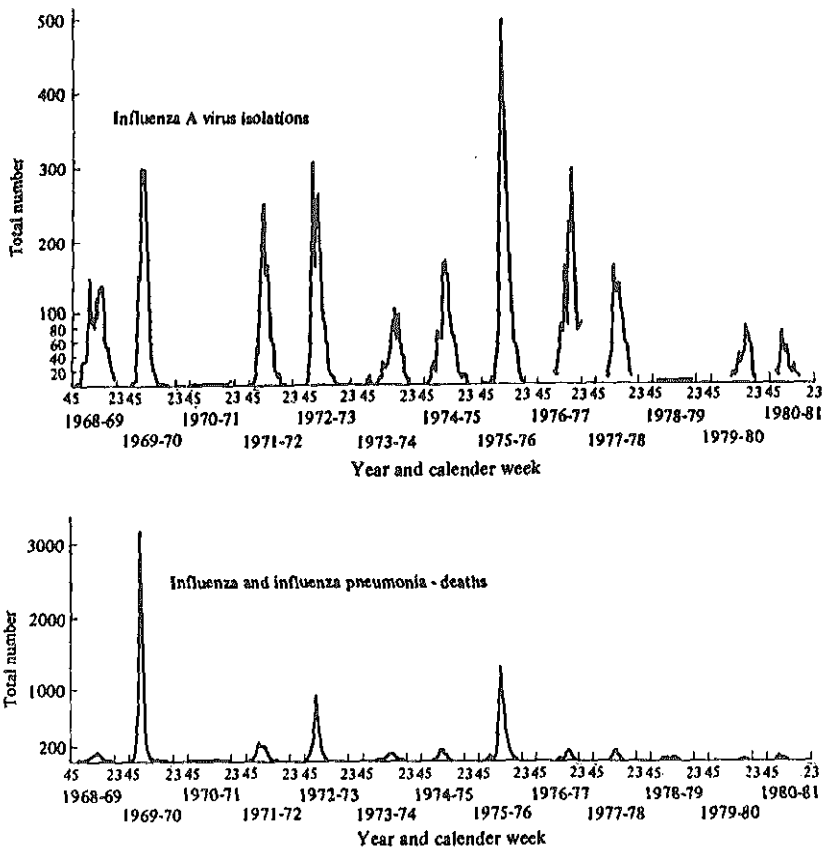
Of the biological properties of influenza viruses which influence the frequent recurrence of influenza, antigenic variation is the best defined genetically and has received the most attention in research. The antigens of importance are the virus membrane glycoproteins, the haemagglutinin and the neuraminidase, and differences in the antigenic properties of these components of different viruses form the basis of the division of influenza A viruses into subtypes (WHO memorandum, 1980). Although the antigenicities of both haemagglutinin and neuraminidase are known to change independently it is generally considered that variation in the former is more important since antibodies against the haemagglutinin neutralize virus infectivity (Webster & Laver, 1975). As a consequence in the last few years a considerable amount of information has been obtained on the structure of the haemagglutinins of a number of viruses in attempts to understand the molecular basis of the antigenic differences between viruses of different subtypes and of the variation which occurs within subtypes often referred to as antigenic drift. In the latter case particular attention has been focused on the haemagglutinins of viruses of the H3N2, 'Hong Kong', subtype and this is a review of information accumulated on antigenic drift in these viruses which were isolated between 1968 and 1980. It contains summaries of the antigenic and primary structure differences between the haemagglutinins and considers the consequences of these variations with reference to the three-dimensional structure of a 1968 haemagglutinin and in terms of the survival of the Hong Kong viruses during this period in the human population.

ANTIGENIC VARIATION

Analyses of antigenic differences in haemagglutinins are routinely made in haemagglutination-inhibition tests using post-infection fer-

Table 1. *Antigenic analysis by haemagglutination-inhibition tests of representative Hong Kong viruses isolated between 1968 and 1981*

	A/HK/1/68	A/Eng/878/69	A/HK/107/71	A/Eng/42/72	A/PC/1/73	A/Scot/840/74	A/Vic/3/75	A/Tok/1/75	A/Eng/864/75	A/Vic/112/76	A/Tex/1/77	A/BK/1/79	A/BK/2/79	A/Belg/2/81
A/Hong Kong/1/68	2560	640	640	160	320	160	40	80	160	40	40	<40	<40	<40
A/England/878/69	1280	1280	640	160	320	80	80	80	80	80	<40	<40	<40	<40
A/Hong Kong/107/71	640	640	2560	320	320	80	160	160	80	160	40	40	<40	<40
A/England/42/72	640	320	160	2560	640	640	160	160	160	80	80	160	80	80
A/Pt. Chalmers/1/73	320	160	320	640	1280	640	160	160	80	80	160	160	160	80
A/Scotland/840/74	160	160	160	640	320	2560	320	320	160	160	160	160	80	80
A/Victoria/3/75	80	80	160	320	320	640	1280	320	160	640	160	40	40	80
A/Tokyo/1/75	40	80	<40	160	320	320	320	5120	320	320	320	160	160	160
A/England/864/75	<40	<40	40	320	320	160	160	640	2560	320	1280	1280	320	320
A/Victoria/112/76	40	<40	40	80	80	160	80	160	320	1280	320	320	160	160
A/Texas/1/77	<40	<40	<40	320	160	160	80	640	1280	320	2560	1280	640	640
A/Bangkok/1/79	<40	<40	<40	80	160	80	160	640	640	480	320	2560	640	480
A/Bangkok/2/79	<40	<40	<40	40	80	40	80	320	320	160	320	640	5120	320
A/Belgium/2/81	<40	<40	<40	40	40	40	80	320	640	320	1280	1280	320	2560



Figs. 1 & 2. The results of influenza surveillance in the United Kingdom from 1968–81. The graphs were drawn from information provided by the Communicable Disease Surveillance Centre of the Public Health Laboratory Service, Colindale, London.

ret antisera which are highly strain specific. The results in Table 1 are presented as examples of the type of information available for the Hong Kong viruses and give an indication of the degree of difference between selected viruses observed in influenza surveillance studies. The viruses in the Table were in the main the most frequently isolated in the particular year although for example in 1974 viruses like A/Port Chalmers/1/73 and not A/Scotland/864/74 were more common and in 1976 viruses like A/Victoria/3/75 were more frequently isolated than A/Victoria/112/76. In addition it may be noted that evidence of the co-circulation of different viruses was frequently obtained and this was observed most strikingly in 1975

with the viruses A/England/864/75, A/Victoria/3/75 and A/Tokyo/1/75.

The numbers of Hong Kong viruses isolated each year in the United Kingdom since 1968 are shown in Fig. 1 to indicate variations in the level of influenza activity from year to year and as an index of the impact of influenza in different years figures for mortality due to influenza in this period are shown in Fig. 2. Clearly the numbers of isolates may vary considerably from year to year even though viruses of apparently significant antigenic difference are detected and this may be a reflection of differences in the viruses unrelated to their antigenicity. Moreover in certain years the number of viruses isolated is disproportionate to the amount of disease indicated by the mortality figures, e.g. in 1976–77. However, although since 1975 there appears to have been little influenza in the United Kingdom, and this coincides with experience in the majority of countries, it contrasts with the situation in North America where in the USA 1980–81 was the most serious year for influenza since 1968. The reason for this difference from country to country is not known but since the viruses isolated in all countries were antigenically similar these observations would seem to indicate differences in immunity in the respective populations.

Nevertheless with these reservations it can be concluded that Hong Kong influenza viruses have circulated nearly every year since 1968 and that internationally since the initial epidemics of 1968 and 1969 there have been at least two others, in 1972 and 1975, and that these were caused by viruses (A/England/42/72 and A/Victoria/3/75) which were antigenically distinct from those previously identified.

HAEMAGGLUTININ STRUCTURE

The haemagglutinin of the 1968 Hong Kong virus A/Aichi/2/68 (X–31, Kilbourne, 1969) has a molecular weight of about 225 000 and is a trimer of identical subunits each consisting of two polypeptide chains, HA_1 and HA_2 (Wiley, Skehel & Waterfield, 1977). HA_1 contains 328 amino acids and HA_2 221 and the two polypeptides are linked in each subunit by a single disulphide bond between residues 14 of HA_1 and 137 of HA_2. The amino acid sequences of both chains are known (Verhoeyen *et al.*, 1980; Ward & Dopheide, 1980) and are summarized in Fig 3. Both polypeptides are glycosylated: at six sites in HA_1, at asparagine residues 8, 22, 38,

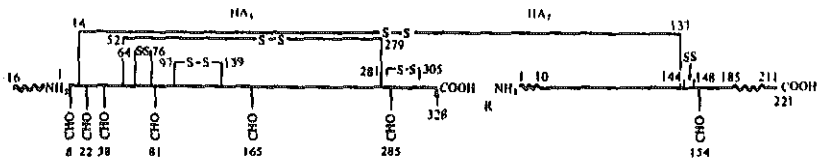


Fig. 3. A summary of the primary structure of the haemagglutinin subunit.

81, 165 and 285; and at one site in HA_2, asparagine 154. In virus particles the molecule is associated with the lipid membrane by a hydrophobic region near the carboxyl terminus of HA_2 between residues 185 and 210; the projecting portion of the molecule consisting of the HA_1 polypeptides and the HA_2 (residues 1–175) polypeptides, of molecular weight about 210 000 is released as a soluble glycoprotein, BHA, by digesting viruses with bromelain. X-ray crystallographic analyses of BHA crystals indicate that the amino terminus of HA_1 is also near the lipid membrane of the virus. The HA_1 chain extends from the base of the molecule through a fibrous stem into a peripheral β-structure-rich region, and then returns to the fibrous region and terminates about 30 Å from the virus membrane. The most prominent features of the part of the subunit composed of HA_2 residues are two antiparallel α-helices, one 29 Å long which proceeds distally from the membrane end of the molecule to connect through an extended chain with the other helix which stretches 76 Å back towards the membrane. A stereo drawing of a tracing of the α-carbon atoms of a subunit is shown in Fig. 4 together with a schematic diagram. Details of the structure have been published (Wilson, Skehel & Wiley, 1981).

VARIATIONS IN STRUCTURE

Extensive immunochemical information has not been reported for the haemagglutinin; that which is available indicates that strain-specific antibody binding sites are located on the HA_1 polypeptide chain. Of the isolated polypeptides, only HA_1 was observed to react in immunodiffusion tests with antibodies against the complete molecule (Brand & Skehel, 1972); following cyanogen bromide cleavage, antibody binding activity was found to be restricted to the 168 residue amino terminal fragment of HA_1 (Jackson *et al.*, 1979);

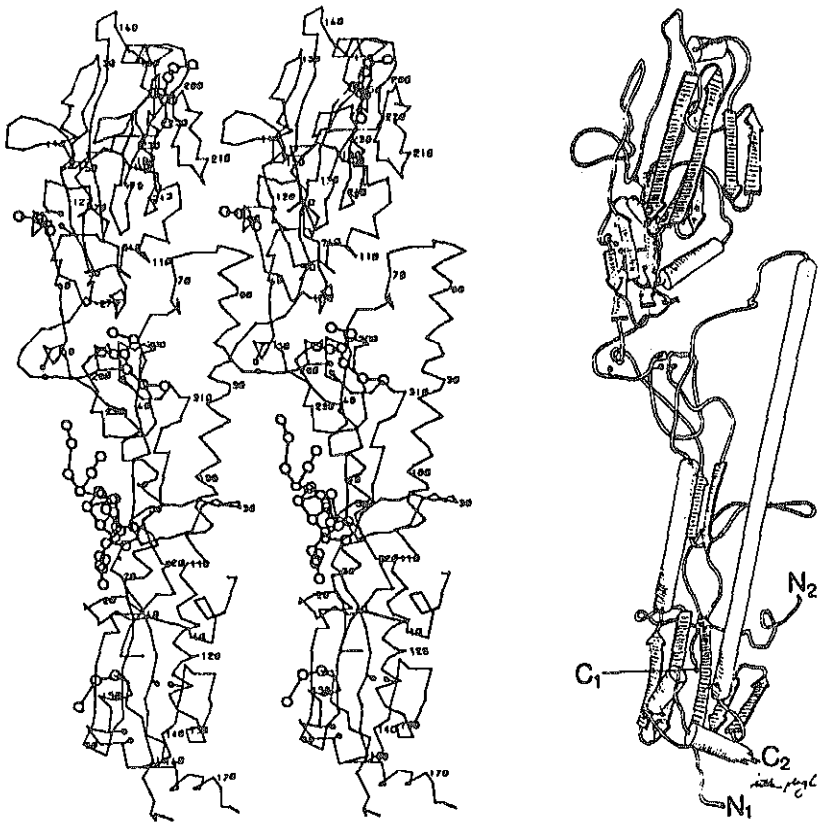


Fig. 4. A stereo drawing of the α-carbon tracing of a haemagglutinin subunit and on the right a schematic diagram indicating the terminal amino acids. o——o indicates carbohydrate side chains.

and haemagglutination-inhibition activity by a variety of monoclonal antibodies was found to be blocked by a large fragment of HA_1, residues 28–328, obtained by tryptic digestion of haemagglutinin after incubation at pH 5.0 (unpublished). These findings are consistent with the observations of limited variation in the amino acid sequences of the HA_2 components since only four amino acid differences at residues 2, 132, 150 and 212 were detected between the haemagglutinins of 1968 and 1975 viruses (Min Jou *et al.*, 1980; Verhoeyen *et al.*, 1980). In contrast, the data in Table 2 indicate that in the same haemagglutinins there were 21 amino acid substitutions in the HA_1 components. This Table also contains the amino acid sequence of the HA_1 polypeptides of eight Hong Kong viruses

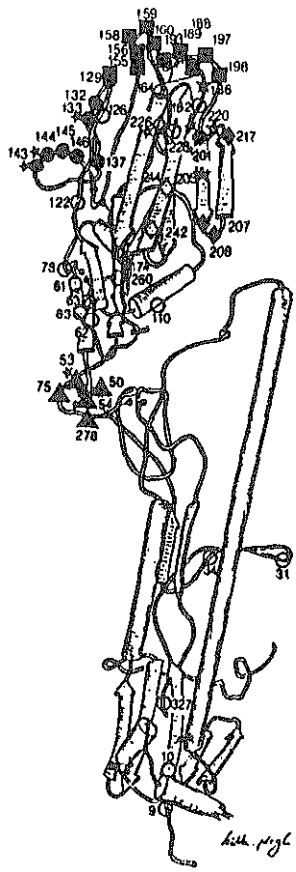


Fig. 5. Schematic diagram of a subunit of the 1968 haemagglutinin showing the positions of amino acid substitutions observed in the haemagglutinin sequence of A/Bangkok/1/79. Stars indicate the positions of amino acid substitutions observed in the haemagglutinins of antigenic variants selected using monoclonal antibodies. Amino acid sequence data communicated by G. W. Both and M. J. Sleigh and from Webster & Laver (1980) and Laver *et al.* (1979).

considered in Table 1 and indicates that in comparison with the 1968 haemagglutinin the number of amino acid substitutions has increased from 12 in 1972 to 21 in 1975 and 33 in 1979 (Sleigh, Both, Underwood & Bender, 1981). The locations of these substitutions are not randomly distributed throughout the polypeptide; there appear to be three main regions of variation between residues 50 and 63, 122 and 160, and 182 and 201, and two long sequences of conserved amino acids between residues 84 and 121, and 279 and 326. The consequences of the changes in amino acid side chain size, charge, or both size and charge for the structure of the molecule

Table 2. *Amino acid sequences of HA_1 polypeptides from respresentativ*

	10	20	30	40	50
X-31	QDLPGNDNSTATLCLGHHAVPNGTLVKTITDDQIEVTNATELVQSSSTGKICNNPH
A/England/878/69			N		
A/Hong Kong/107/71	K			T	
A/England/42/72			N		
A/Memphis/102/72	F		N		
A/Pt. Chalmers/1/73			N		
A/Victoria/3/75			N		
A/Texas/1/77	N		N		R DS
A/Bangkok/1/79	N		N		R DS

	130	140	150	160	170
X-31	ITEGFTWTGVTQNGGSNACKRGPGSGFFSRLNWLTKSGSTYPVLNVTMPNNDNFD
A/England/878/69					
A/Hong Kong/107/71	E E	D		R	
A/England/42/72	N	D	Y		
A/Memphis/102/72	N	D	Y		
A/Pt. Chalmers/1/73	N	D	Y	A	
A/Victoria/3/75	N N	S D	Y	Q	S
A/Texas/1/77	N N	Y DNS	Y E		G
A/Bangkok/1/79	N N	S Y DNS	YE E K		G

	250	260	270	280	290
X-31	DVLVINSNGNLIAPRGYFKMRTGKSSIMRSDAPIDTCISECITPNGSIPNDKPFQNVN
A/England/878/69					
A/Hong Kong/107/71					
A/England/42/72	I		G		
A/memphis/102/72	I		G		
A/Pt. Chalmers/1/73	I		G		
A/Victoria/3/75	I		G	S	
A/Texas/1/77	I L	I	G	S	
A/Bangkok/1/79	I L	I	G	S	

Data from Sleigh, Both, Underwood & Bender (1981), and communicated by M. J. Sleigh and G. W. Both.

have been considered before (Wiley, Wilson & Skehel, 1981). The substitution of threonine 83 in A/Victoria/3/75 haemagglutinin removes the carbohydrate attachment site at asparagine 81 and the aspartic to asparagine change at residue 63 creates another site for glycosylation.

Estimates of the significance of these changes in amino acid sequence for antigenic variation are presently the main approach to defining the antigenically important regions of the molecule. They have been considerably assisted by the results of experiments involving analyses of the haemagglutinins of antigenic mutants selected by growth of viruses in the presence of monoclonal antibodies (Laver *et al.*, 1979; Webster & Laver, 1980; Moss, Underwood, Bender & Whittaker, 1980). These results indicate

Hong Kong viruses

that amino acid substitutions which affect the binding of different groups of antibodies are located at amino acid residue 54, between residues 133 and 144 and between residues 201 and 226. At least amino acid substitutions in these regions of the haemagglutinins of the viruses shown in Table 2 may, therefore, be implicated in antigenic variation.

Further assessment of the involvement of different regions of the haemagglutinin in antibody binding comes from consideration of the positions of amino acid substitutions in the three-dimensional structure of the molecule. This was discussed recently for Hong Kong viruses up to A/Victoria/3/75 (Wiley *et al.*, 1981) and in Fig. 5 this sort of analysis is extended to the A/Bangkok/1/79 virus by illustrating the sites of amino acid substitution in this haemaggluti-

nin in comparison with the X-31 1968 haemagglutinin for which the structure is known. In agreement with observations made with the earlier isolates of the subtype, the amino acid substitutions are clustered in four main regions: from amino acids 122 to 146 including the protruding loop from residues 139 to 146; at the top of the molecule, residues 155–160 of a loop structure, and in the region containing α-helix 188–193 between residues 188 and 198; in the vicinity of the disulphide bond between residues 52 and 277 and, contrasting with these three areas which are exposed on the haemagglutinin surface, in an interface region between subunits which includes residues 201–220. The suggested importance of these regions in antigenic variation is again consistent with the locations of the amino acid substitutions, mentioned above, which were observed in the haemagglutinins of antigenic variants selected using monoclonal and avid antibodies. In these cases haemagglutination-inhibition and neutralization of virus by the antibodies were prevented by amino acid substitutions at, for example, residues 143, 186, 54 and 205.

The relationship between amino acid substitutions in these regions, antibody binding and the neutralization of virus infectivity, has not been determined. The proximity of the first two areas described to the proposed sialic acid binding pocket (Wilson *et al.*, 1981) suggests that antibody binding in these areas would directly prevent interaction of the haemagglutinin with cellular receptors by steric hindrance. Since the monoclonal antibodies used in the selection of all the antigenic variants considered inhibit haemagglutination, it is also possible that binding of antibody at the sites influenced by amino acid substitution in the last two areas described also effects virus neutralization by preventing interaction with the sialic acid-containing receptors. Alternatively such antibodies may primarily influence the participation of the haemagglutinin in the membrane fusion activity of the virus; such alternative mechanisms of neutralization, however, remain to be established.

What is the contribution of these changes in haemagglutinin structure to the survival of Hong Kong influenza viruses in the human population since their introduction in 1968? On the basis that survival necessitates infection of non-immune individuals it is achieved by viruses infecting the newly born exclusively or by viruses with haemagglutinins sufficiently novel antigenically to allow re-infections as well as primary infections. Obviously the Hong Kong influenza viruses followed the latter course, as did influenza

viruses of the H_1 and H_2 subtypes before, between 1918 and 1957 and 1957 and 1968 respectively. Assessment of the extent of the variation in haemagglutinin structure required to allow re-infection can be made by examining the haemagglutinins of the viruses which were able to cause the second and third world-wide epidemics of this period in 1972 and 1975. As discussed in detail previously (Wiley *et al.*, 1981) amino acid substitutions in all four of the regions of the haemagglutinin molecule described above occur in the haemagglutinins of both A/England/42/72 and A/Victoria/3/75 viruses. Whether all of these changes are required for a virus to achieve the potential to re-infect or whether changes in structure at certain antigenic sites are more important than at others is not known. It is possible that analyses of the human immune response to influenza infections will resolve these questions but at present it can only be concluded that since binding of antibodies at sites influenced by amino acid substitutions in any one of the four regions prevents infection, modification in all four regions will be required to avoid neutralization and ensure virus survival.

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