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Antigenic Drift in the Hemagglutinin of the Hong Kong Influenza Subtype: Correlation of Amino Acid Changes with Alterations in Viral Antigenicity

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The nucleotide sequence of the gene coding for the large subunit of influenza virus hemagglutinin (HA1) was determined for strains A/NT/60/68, A/Eng/878/69, and A/Qu/7/70, three early isolates of the Hong Kong subtype. Sequences were obtained by the dideoxy chain termination method, using reverse transcriptase to synthesize partial DNA copies of the RNA gene. HA1 amino acid sequences predicted from the gene sequences were compared with published data for strains A/Aichi/2/68 and A/Vic/3/75. Compared with earlier strains, the HA1s of A/Eng/878/69 and A/Qu/7/70 each contained three amino acid changes. Some of these were also found in A/Vic/3/75, but some were unique to the particular strain. When all of the strains were titrated with a panel of monoclonal antibodies directed against A/NT/60/68, alterations in viral antigenicity could be correlated with particular amino acid changes. The existence of multiple pathways for viral evolution during antigenic drift is discussed.

Influenza virus continues to escape control by vaccination because of periodic changes in its antigenic character. These antigenic changes result from alterations in the primary structure of the two surface proteins, hemagglutinin (HA) and neuraminidase. Antigenic shift, associated with the appearance of a new viral subtype, occurs when the virus acquires a new HA gene or neuraminidase gene or both, resulting in a radical change in its surface proteins. Antigenic drift is associated with smaller, progressive changes in antigenicity within a subtype, primarily due to changes in viral HA (25). All of the antigenic activity of HA appears to reside in HA1, the large subunit of the protein (4, 5, 10). Changes in some sections of HA1 during antigenic drift have been analyzed by making comparisons of peptide maps and partial amino acid sequences for several viral strains (12, 13, 16).

More complete information on changes occurring in the HA molecules during viral evolution has come from an examination of the viral gene coding for HA. Several groups have copied into DNA and cloned that segment of the influenza RNA genome coding for HA and determined its nucleotide sequence (2, 9, 15, 17). Comparisons made in this way for some naturally occurring influenza strains have demonstrated that, even among strains isolated only a few years apart, the number of amino acid differences observed can be so great that identifying those likely to be important in altering viral antigenicity becomes impossible (3).

For this reason, we compared strains isolated early in the evolution of the Hong Kong (H3N2) influenza subtype, which first arose in 1968. Among the HAs from these strains (A/NT/60/68 [NT68], A/Aichi/2/68 [Aichi68], A/Eng/878/69 [Eng69], and A/Qu/7/70 [Qu70]), we found relatively few amino acid differences, some of which were unique for each strain. Our results confirm earlier suggestions that multiple pathways may exist for the development of antigenic variants within a viral subtype (6, 28). By screening the strains with a panel of monoclonal antibodies prepared against NT68, we attempted to correlate particular amino acid changes with alterations in viral antigenicity and to identify the most important changes for each strain.

MATERIALS AND METHODS

Purification of virus and viral RNA. Influenza strains Eng69 and Qu70 (kindly supplied by W. G. Laver) and Aichi68, A/Vic/3/75 (Vic75), and NT68 and its derivatives 29C, 34C, and 375/17 (obtained from S. Fazekas de St. Groth and C. Hannoun [7]) were grown and purified, and the RNA was extracted as described previously (20).

Preparation and characterization of cloned HA gene copies. The synthesis of double-stranded DNA (dsDNA) copies of the HA gene, their insertion into pBR322, and cloning in *Escherichia coli* RRI have been described previously for strains A/Mem/102/72 and A/NT/60/68/29C (21). Cloning in *E. coli* χ1776 and characterization of a partial copy of the HA gene from NT68 have also been described previously

(22). All recombinant DNA experiments were carried out under CII and EK1 or EK2 containment conditions, as prescribed by the Recombinant DNA Committee of the Australian Academy of Science. The nucleotide sequences of cloned dsDNA copies of HA genes were determined by the method of Maxam and Gilbert (14). Sequence data were stored and analyzed with computer programs devised by Staden (23, 24), adapted for our system by C. Bucholtz and A. Reisner.

Determination of the HA RNA gene sequence by the Sanger chain termination method. Restriction fragments prepared from plasmids containing cloned dsDNA copies of HA genes from influenza strains A/NT/60/68/29C and A/Mem/102/72 (21, 22) were used to prime the copying of viral genome RNA by reverse transcriptase at specific sites on the HA gene (Table 1). (Reverse transcriptase was the generous gift of J. W. Beard.) The conditions used were similar to those described previously (3). The dsDNA primer fragment (1 to 2 pmol) was mixed with total influenza virus genome RNA (4.5 pmol) either polyadenylated (1, 20) or unadenylated. The RNA and primer (in 5 μl of water) were sealed in a glass capillary, heated at 90 to 100°C for 1 min, and then cooled on ice. Only the plus strand of the DNA fragment anneals to the negative-stranded HA virion RNA. The presence of the other minus DNA strand and the remaining influenza gene segments is inconsequential. The primer-template mixture was distributed among four 5-μl reaction mixtures and used as a substrate for partial complementary DNA (cDNA) synthesis by reverse transcriptase in the presence of the chain-terminating dideoxynucleoside triphosphates (18). Reaction conditions were as described previously (1), except unlabeled deoxynucleoside triphosphates and ^{32}P-labeled deoxynucleoside triphosphate were used at concentrations of 40 and 10 μm, respectively. The concentration of dideoxynucleoside triphosphates was increased accordingly. Incubations were carried out for 10 min at 42 or 46°C. Subsequent treatment of the samples and conditions for electrophoretic separation of DNA products were as described previously (3). Under these reaction conditions, cDNA species synthesized by reverse transcriptase as a result of self-priming by unadenylated influenza virion RNA (22) did not interfere with the analysis of cDNA products synthesized from the priming site provided by the restriction fragment.

Preparation of a panel of monoclonal antibodies against NT68. Hybrid cell lines producing antibodies to HA of NT68 were selected after fusion of myeloma cells with immune spleen cells, using Sendai virus or polyethylene glycol 1500 (11, 19). Hemagglutination and hemagglutination inhibition titers were determined as described previously (8).

RESULTS

Confirmation of the HA gene sequence for NT68. The derivation of the nucleotide sequence coding for the mature HA protein from NT68 was reported previously (3). Most of the sequence was obtained from a cloned partial copy of the gene, missing ~700 bases from the 3′ end of the gene. The remaining residues were obtained from the RNA gene as described above and previously (3). This composite sequence differed at base 1039 from that of laboratory variants derived from NT68 (3). The C → T base change predicted that amino acid 321 should be glutamine in NT68 but arginine in the other strains, a difference not revealed by peptide mapping (16). Therefore, we checked the NT68 sequence directly from the gene for this region and found that the RNA molecules had a C rather than a U residue at base 1039 (data not shown). This suggests that the cloned NT68 gene copy, containing T, represented a variant present in the viral population or that reverse transcriptase had miscopied a base during dsDNA synthesis. The revised sequence for the HA1 coding region of the NT68 HA gene is shown in Fig. 1.

HA gene sequences for Eng69 and Qu70. Nucleotide sequences for the HA1 coding region of the HA genes from Eng69 and Qu70 were determined by the dideoxy chain termination method (18), using restriction fragments from cloned HA gene copies to prime the synthesis of partial DNA copies of the HA (RNA) gene, as described above. An example of the results obtained after separation of the reaction products by polyacrylamide gel electrophoresis is shown in Fig. 2. The sequences were obtained from multiple priming experiments for each region of the gene. Artifact bands occasionally appeared in a sequence (generally bands at a particular

TABLE 1. *Restriction fragments used as primers for the sequencing of HA gene RNA*

Boundary restriction site of primer fragments		Length of primer (base pairs)	Priming site (base no.)[a]
Remote from priming site	At priming site		
*Mbo*II	*Hae*III	32	76
*Hae*III	*Mbo*I[b]	97	172
*Alu*I[c]	*Hin*fI	39	250
*Hin*fI	*Ava*II	49	296
*Ava*II	*Ava*II	39	332
*Hin*dIII	*Ava*II	152	504
*Hin*cII[c]	*Ava*I	95	633
*Ava*I	*Hin*fI	52	682
*Hin*fI	*Ava*II[c]	54	733
*Ava*II[c]	*Hpa*II	62	792
*Hpa*II	*Hha*I	67	859
*Hha*I	*Mbo*I[b]	116	976

[a] Bases are numbered from the 3′ end of the HA gene.

[b] Fragments containing *Mbo*I sites were obtained by digestion of a plasmid containing a dsDNA copy of the HA gene from A/Mem/102/72, grown in the *dam* host *E. coli* 2230.

[c] Restriction sites present in clones of 29C but not in A/Mem/102/72.

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Base No.                                                                A.A.No.

  78 CAA GAC CTT CCA GGA AAT GAC AAC AAC ACA GCA ACG CTG TGC CTG GGA CAT CAT GCG GTG
     gln asp leu pro gly asn asp asn asn thr ala thr leu cys leu gly his his ala val   20

 138 CCA AAC GGA ACA CTA GTG AAA ACA ATC ACA GAT GAT CAG ATT GAA GTG ACT AAT GCT ACT
     pro asn gly thr leu val lys thr ile thr asp asp gln ile glu val thr asn ala thr   40

 198 GAG CTA GTT CAG AGC TCC TCA ACG GGG AAA ATA TGC AAC AAT CCT CAT CGA ATC CTT GAT
     glu leu val gln ser ser ser thr gly lys ile cys asn asn pro his arg ile leu asp   60

 258 GGA ATA GAC TGC ACA CTG ATA GAT GCT CTA TTG GGG GAC CCT CAT TGT GAT GTT TTT CAA
     gly ile asp cys thr leu ile asp ala leu leu gly asp pro his cys asp val phe gln   80

 318 AAT GAG ACA TGG GAC CTT TTC GTT GAA CGC AGC AAA GCT TTC AGC AAC TGT TAC CCT TAT
     asn glu thr trp asp leu phe val glu arg ser lys ala phe ser asn cys tyr pro tyr  100

 378 GAT GTG CCA GAT TAT GCC TCC CTT AGG TCA CTA GTT GCC TCG TCA GGC ACT CTG GAG TTT
     asp val pro asp tyr ala ser leu arg ser leu val ala ser ser gly thr leu glu phe  120

 438 ATC ACT GAG GGT TTC ACT TGG ACT GGG GTC ACT CAG AAT GGG GGA AGC AAT GCT TGC AAA
     ile thr glu gly phe thr trp thr gly val thr gln asn gly gly ser asn ala cys lys  140

 498 AGG GGA CCT GGT AGC GGT TTT TTC AGT AGA CTG AAC TGG TTG ACC AAA TCA GGA AGC ACA
     arg gly pro gly ser gly phe phe ser arg leu asn trp leu thr lys ser gly ser thr  160

 558 TAT CCA GTG CTG AAC GTG ACT ATG CCA AAC AAT GAC AAT TTT GAC AAA CTA TAC ATT TGG
     tyr pro val leu asn val thr met pro asn asn asp asn phe asp lys leu tyr ile trp  180

 618 GGG GTT CAC CAC CCG AGC ACG AAC CAA GAA CAA ACC AGC CTG TAT GTT CAA GCA TCA GGG
     gly val his his pro ser thr asn gln glu gln thr ser leu tyr val gln ala ser gly  200

 678 AGA GTC ACA GTC TCT ACC AGG AGA AGC CAG CAA ACT ATA ATC CCG AAT ATC GGG TCC AGA
     arg val thr val ser thr arg arg ser gln gln thr ile ile pro asn ile gly ser arg  220

 738 CCC TGG GTA AGG GGT CTG TCT AGT AGA ATA AGC ATC TAT TGG ACA ATA GTT AAG CCG GGA
     pro trp val arg gly leu ser ser arg ile ser ile tyr trp thr ile val lys pro gly  240

 798 GAC GTA CTG GTA ATT AAT AGT AAT GGG AAC CTA ATC GCT CCT CGG GGT TAT TTC AAA ATG
     asp val leu val ile asn ser asn gly asn leu ile ala pro arg gly tyr phe lys met  260

 858 CGC ACT GGG AAA AGC TCA ATA ATG AGG TCA GAT GCA CCT ATT GAT ACC TGT ATT TCT GAA
     arg thr gly lys ser ser ile met arg ser asp ala pro ile asp thr cys ile ser glu  280

 918 TGC ATC ACT CCA AAT GGA AGC ATT CCC AAT GAC AAG CCC TTT CAA AAC GTA AAC AAG ATC
     cys ile thr pro asn gly ser ile pro asn asp lys pro phe gln asn val asn lys ile  300

 978 ACA TAT GGA GCA TGC CCC AAG TAT GTT AAG CAA AAC ACC CTG AAG TTG GCA ACA GGG ATG
     thr tyr gly ala cys pro lys tyr val lys gln asn thr leu lys leu ala thr gly met  320

1038 CGG AAT GTA CCA GAG AAA CAA ACT
     arg asn val pro glu lys gln thr
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FIG. 1. *Nucleotide sequence of cDNA transcribed from the HA RNA gene of NT68 and the amino acid sequence predicted from it. The nucleic acid sequence is shown from base 78, the first base in the codon for the N-terminal Gln residue of Hong Kong influenza HA (26, 27). Positions of silent base changes seen in Eng69 or Qu70 are indicated (▼), and codons where alterations in HA1 in these strains result in amino acid changes are underlined.*

position in all four channels of the sequencing gel [Fig. 2]). Although the uncertainty could generally be resolved by repeating the experiment, some doubt remains as to the identity of bases 891 and 1014 in Eng69 and Qu70. Base 370 is also in doubt in these strains and NT68. However, it is the second base of the codon for a tyrosine residue which forms part of a Cys-Tyr-Pro tripeptide conserved in HA1 of all strains of the influenza subtypes so far examined (2, 9, 15, 17, 26). A change at this position, therefore, seems unlikely.

Table 2 summarizes the positions where a change in the base sequence in these strains, compared with the sequence for NT68, leads to an altered amino acid in the HA1 protein. These

positions are also marked on the HA1 nucleotide sequence for NT68 shown in Fig. 1. Table 3 shows the locations of silent base changes in Eng69 and Qu70 compared with NT68. Included for comparison in Table 2 are data for Aichi68 (26; C. W. Ward and T. A. A. Dopheide, personal communication) and Vic75 (15).

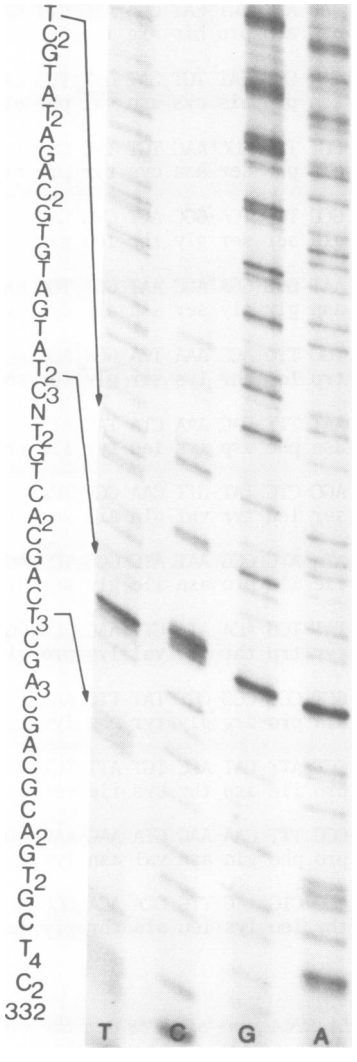

FIG. 2. *Nucleotide sequence beyond base 332 of the HA gene of Eng69. The HinfI-AvaII, 49-base-pair fragment from a cloned HA gene copy from strain 29C (Table 1) was used to prime the copying of the HA gene of Eng69 by reverse transcriptase, as described in the text. Products from the reactions, each containing one of the four chain-terminating dideoxynucleoside triphosphates, A, G, C, and T, were separated by electrophoresis on an 8% polyacrylamide thin gel containing 7 M urea. The autoradiogram of the gel shows a case in which the identity of a base at one position on the gel could not be determined (base 370).*

Amino acid changes in HA1 of early isolates of the Hong Kong subtype. All four field isolates Aichi68, Eng69, Qu70, and Vic75 differ from NT68 at residues 9 and 31 (Table 2). Apart from these, there are no common changes from NT68 among the three early strains, although some of the changes (e.g., at residues 63 and 144 in Eng69 and at residue 78 in Aichi68) also occur in Vic75, which contains 16 amino acid changes from NT68 in addition to those shown in Table 2 (15). Some of the amino acid changes for Eng69 and Qu70 have been described previously (12, 16).

Titration of field isolates with monoclonal antibodies raised against NT68. Although the number of HA1 amino acid differences among the early Hong Kong field isolates NT68, Aichi68, Eng69, and Qu70 is small, the changes are not clustered, but scattered through the HA1 region (Table 2). Presumably, some of the changes are not antigenically significant and therefore are less important to an analysis of antigenic drift in the subtype. In an attempt to assess the antigenic significance of the observed amino acid changes, we measured the influence of the HA1 amino acid substitutions in different strains on the binding to whole virus of HA-specific monoclonal antibodies. Assistance in interpreting these data comes from two strains, originally isolated in the laboratory by their ability to grow in the presence of antibody directed against NT68 (6, 7) and differing from it by single HA1 amino acid substitutions. One of the variants, 375/17, differs from NT68 only by a change from glycine to aspartic acid at residue 144 in HA1 (Both and Sleigh, unpublished data), a change also seen in Eng69 and Vic75. The variant 29C has, in addition to the change at residue 144, the Leu → Gln change at residue 226 also seen in Qu70 (2, 3, 16).

Figure 3 shows the HA inhibition titers for a panel of HA-specific monoclonal antibodies, using the virus strain NT68 and its two variants 375/17 and 29C, as well as the field isolates Aichi68, Eng69, Qu70, and Vic75. Each block on the histogram shows the amount of a particular antibody needed to inhibit the hemagglutinating activity of the virus strain, relative to the titer for NT68, which is adjusted to zero. That is, a positive value indicates that more antibody is needed to inhibit hemagglutination by a particular strain than by NT68, whereas a negative value indicates that less antibody is required. Results are shown for 71 of the 125 antibodies tested.

Antibodies whose titration values were influenced by changes at residues 144 and 226 were

TABLE 2. *Nucleotide sequence and amino acid changes in HA1*

Strain	2[a] (81)[b]	9 (103)	31 (168)	63 (264)	78 (310)	81 (318)	129 (463)	144 (508)	158 (550)	226 (754)
NT68	GAC Asp	AAC Asn	GAU Asp	GAC Asp	GUU Val	AAU Asn	GGG Gly	GGU Gly[c]	GGA Gly	CUG Leu
Aichi68[d]	**Tyr**[e]	**Ser**	**Asn**		**Gly**				**Glu**	
Eng69		AGU **Ser**	AAU **Asn**	AAC **Asn**		GAU **Asp**		GAU **Asp**		
Qu70		AGC **Ser**	AAU **Asn**		GGU **Gly**		GAG **Glu**			CAG **Gln**
Vic75[f]		AGC **Ser**	AAU **Asn**	AAC **Asn**	GGA **Gly**			GAU **Asp**		

[a] Amino acid number. Amino acids are numbered from the N-terminal Gln of the mature HA protein (26, 27).

[b] Base number is given within parentheses. Nucleotides at which alterations occur are numbered from the 3′ end of the gene but given in the cDNA sense.

[c] When the sequences of two separate preparations of RNA in this region were determined, one sample appeared completely as GGU, and the other appeared as a mixture of GGU and GAU, in agreement with peptide mapping data (16).

[d] Amino acid sequence data of Ward and Dopheide (26).

[e] Boldfaced type indicates a change in amino acid compared with NT68. Only codons and amino acids differing from NT68 are shown.

[f] Sequence data of Min Jou et al. (15). The HA1 from Vic75 contains 16 additional amino acid differences from NT68.

identified with the two NT68 variants, 375/17 and 29C. A significant change was arbitrarily taken to be 1.0 or more on the logarithmic scale shown in Fig. 3. Group B of Fig. 3 shows all of the antibodies whose titration value, and hence presumably ability to bind to 375/17, differed significantly from that of NT68. Most of these antibodies responded similarly with 29C, which also contains the Gly → Asp change at residue 144. Group D contains all of the other antibodies whose titration value was significantly altered in 29C, i.e., due to the Leu → Gln change at residue 226.

Once the influence of these two particular amino acid changes had been identified, the effect of the remaining amino acid changes in the field strains could be assessed. Other groups of antibodies were identified because of a distinctive response by one or more of the field strains. For example, group A contains antibodies for which a similar response was seen with all four strains, relative to NT68. Allowance must be made for the lower base line for titration figures of Eng69 compared with other strains (see below). Group C contains all of the antibodies apart from those already contained in groups A and B whose hemagglutination inhibition titration value for Eng69 was significantly different from that with NT68. Group E contains antibodies binding less strongly to Qu70 than to other strains. The most common pattern observed was that of antibodies in group F. More than half of the antibodies tested and most of those not shown in the diagram fall into this group.

TABLE 3. *Silent base changes in the sequence coding for mature HA1*[a]

Base no.	NT68	Eng69	Qu70
104	C	T	—[b]
119	C	—	T
134	G	—	A
143	C	—	T
281	T	C	—
314	T	C	—
620	G	—	A
734	C	—	T
764	A	—	G
791	G	A	—
884	G	—	A
1022	G	A	—

[a] The changes are those seen in the DNA copy of virion RNA, i.e., in the mRNA sense. Bases are numbered from the 3′ end of the HA gene, as in Table 2.

[b] —, Base was the same as in NT68.

DISCUSSION

Sequences coding for the HA1 region of the HA gene from influenza strains Eng69, Qu70, and NT68 were obtained by generating partial cDNA copies of the RNA gene, as described above. The method is rapid and provides the

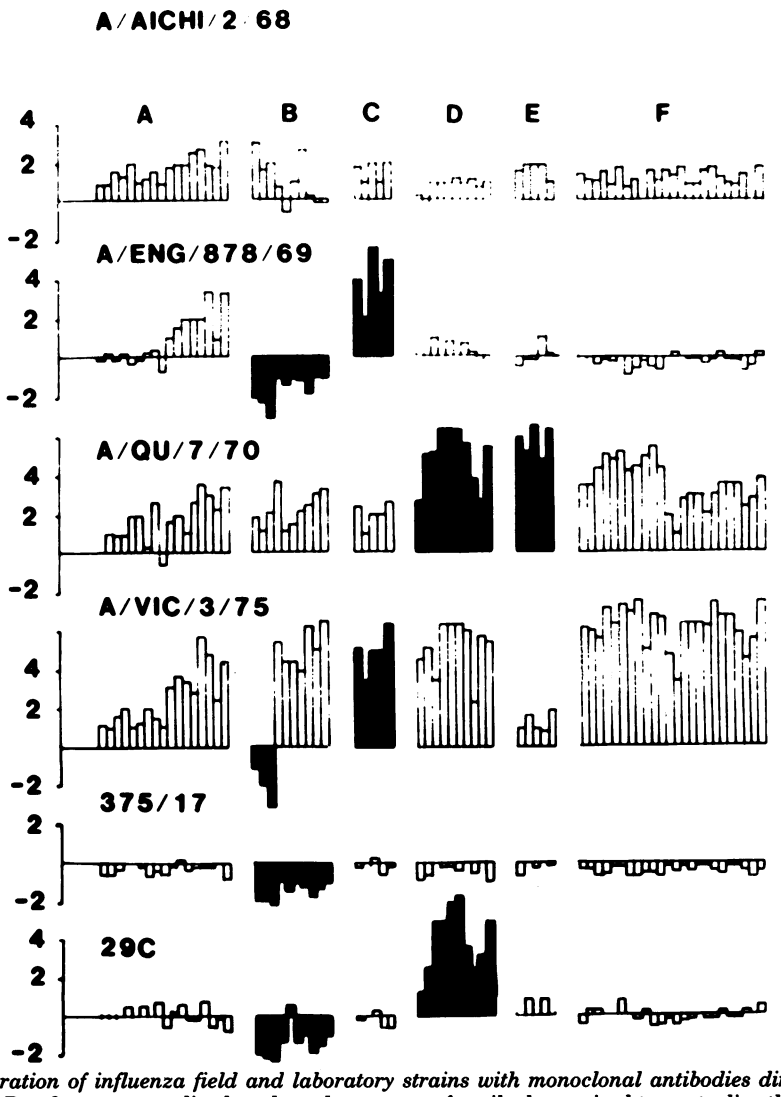

FIG. 3. *Titration of influenza field and laboratory strains with monoclonal antibodies directed against HA of NT68. Results are normalized to show the amount of antibody required to neutralize the hemagglutinating activity of the virus (on a $\log_2$ scale) compared with the amount needed to neutralize NT68. Each column of the histogram represents a different monoclonal antibody. Division of the antibodies into groups is described in the text. Blacked-in columns on the histograms are used to draw attention to strains showing a distinctive response for particular antibody groups.*

sequence of the predominant RNA species present. This provides an advantage in evolutionary studies, since it avoids the risk that a sequence determined from a cloned gene copy will be that of a variant present in the viral population at low frequency. For example, sequences determined from cloned gene copies for strains NT68 and 29C each predicted an HA amino acid sequence which differed at one residue from that determined for the viral population as a whole (see above; 2).

From the sequences determined for the HA genes of Eng69 and Qu70, the amino acid differences between these strains and the early field isolates NT68 and Aichi68 could be identified. Apart from the differences from NT68 common to the other three strains, Aichi68, Eng69, and Qu70 had a small, nonoverlapping set of changes. These provided an opportunity to identify the antigenically significant changes occurring in each strain and to examine the evolutionary pathways involved in influenza virus antigenic drift.

Antigenic significance of amino acid changes in HA1 of influenza field strains. An initial examination of the titration data for

the 71 antibodies shown in Fig. 3 reveals that, whereas Eng69 and the two laboratory mutants 375/17 and 29C had an affinity similar to that of NT68 for the majority of antibodies, Aichi68, Qu70, and Vic75 showed a decreased affinity for almost every antibody tested. Indeed, for Aichi68 this seems to be the major effect observed, with very few of the antibodies otherwise showing a significantly altered titration value. The effect could be attributed to a change in HA1 affecting the reactivity of all antigenic determinants or could be explained by a change in the ability of the virus to adsorb to chicken erythrocytes during the hemagglutination inhibition test.

Such an adsorptive change has been demonstrated for Aichi68 (6) and Vic75 (Underwood, unpublished data). Aichi68, Qu70, and Vic75 have in common the alteration of valine (residue 78) to glycine. Interestingly, the field strain A/PC/1/73, also reported to have altered adsorptive properties (6), contains glycine in this position as well (Both and Sleigh, manuscript in preparation), suggesting that there is a correlation between the change at residue 78 and the alteration in adsorptivity. The effect of this apparent change in adsorptivity should be taken into account when the magnitudes of changes in antibody titration figures for these strains are assessed.

Apart from the change at residue 78, Aichi68 contains the changes at residues 9 and 31 common to all four field isolates and unique changes at residues 2 and 158. Only one antibody (not shown in Fig. 3) had a significantly decreased affinity for Aichi68 compared with other strains, suggesting that these unique changes are unlikely to be antigenically significant. Since residue 2 appears to be located near the viral membrane at the base of the HA spike, it, at least, is unlikely to be in a position to influence HA antigenic sites (D. Wiley, personal communication). Antibodies contained in group A include some showing a decreased affinity for all four viral strains compared with NT68, suggesting that one or both of the changes at residues 9 and 31 may have a small effect on viral antigenicity.

It is clear from the results that for Vic75 the HA antigenic character has been radically altered, with many of the antibodies of the panel apparently unable to bind to the virus at all. This might be expected for such a strain, isolated late in subtype evolution and containing a total of 21 amino acid changes in the HA1 region (15). However, it was surprising to find a similar, although lesser, effect for Qu70, with many antibodies, particularly the very large number contained in group F, showing radically altered titration values. Apart from changes at residues 9, 31, and 78, which Qu70 shares with other strains not showing this altered antigenicity, Qu70 has only two amino acid changes, at positions 129 (Gly → Glu) and 226 (Leu → Gln). Antibodies responding to the change at position 226 were identified with the laboratory strain 29C and are all contained in group D. Group E contains all of the antibodies showing decreased binding only to Qu70, and these may be recognizing the unique Qu70 change at residue 129. In view of the magnitudes of the changes observed and the large numbers of antibodies involved, it seems likely that both of these amino acid changes in Qu70 are antigenically important. However, there seems to be an additional effect, perhaps due to interaction between these changes or between one or both and a change elsewhere in the molecule, leading to the altered binding exemplified by the members of group F. Interestingly, the antibodies apparently affected by the Leu → Gln change at residue 226 also showed decreased binding to Vic75, suggesting that the alteration in antigenicity associated with this change can be achieved just as well by one or more amino acid changes elsewhere in the molecule.

Of the amino acid changes contained in Eng69, the one most easily assessed is at residue 144. Antibodies responding to this change were identified in the laboratory variant 375/17 and are included in group B. Since the NT68 stock used to titrate the antibodies was subsequently found to contain a mixture of Gly and Asp at residue 144 (3, 16), strains containing only Gly (Aichi68 and Qu70) or only Asp (Eng69 and Vic75) showed changes in hemagglutination inhibition values of opposite polarities for group B antibodies, compared with NT68. Only some of the antibodies in group B showed the response predicted for Vic75, which has Asp at residue 144 (15), but for others the effect appears to be masked by changes occurring elsewhere in HA. The antigenic importance of the change at residue 144 has been described and discussed previously (13, 16, 28).

Group C contains the remaining antibodies whose binding was significantly altered in Eng69 and indicates that Eng69 must contain at least one antigenically significant change in addition to that at residue 144. The same group of antibodies also had a decreased affinity for Vic75. In view of the discussion above on group D antibodies, it is impossible to say whether group C antibodies are responding to the change common to Vic75 and Eng69 at residue 63 or to the unique Eng69 change at residue 81. However, apparently at least one of these changes is antigenically significant.

From the monoclonal antibody binding data, we conclude that the most significant change for Aichi68 is in viral adsorptivity, probably due to

the change at residue 78. For Qu70, both unique changes at residues 129 and 226 seem important, with an additional interaction between two or more of the changes leading to a radical alteration in the antigenic properties of the virus. For Eng69 the importance of the change at residue 144 has already been established (13, 16, 28). In addition, one or both of the changes at residues 63 and 81 seem to be antigenically significant.

Pathways for evolution during antigenic drift. It seems likely that at any stage in the evolution of a viral subtype, several different strains may be circulating simultaneously. Strains having the best combination of antigenic and growth characteristics would give rise to new sets of variants which would then be subject to selection by the same criteria. If at any stage more than one virus strain is successful, then progressive evolution in this way should provide branching lines of evolution, with successive viruses within a line acquiring new amino acid changes but retaining the important changes of their predecessors. Advantageous amino acid changes which accumulate in later strains of the subtype may be acquired in a different order on separate evolutionary lines. For example, Qu70 and A/Mem/1/71 both lack the Gly → Asp change at residue 144 which had appeared in Eng69 and exists in other strains after 1972 (see above; 12). Similarly, the Asp → Asn change at position 63 is present in Eng69 and Vic75 but absent from Qu70 and A/Mem/102/72 (22, 26). The antigenically important Gly → Glu change (residue 129) of Qu70 has not been found in any other strain of the Hong Kong subtype but may eventually reappear as the virus continues to evolve.

The four early isolates of the Hong Kong subtype examined here contain largely nonoverlapping sets of amino acid differences from a putative precursor strain. Therefore, they may represent early members of several different evolutionary lines within the subtype. On the other hand, partial amino acid sequences for HA1 of several later Hong Kong isolates suggested that these strains could be arranged in a single evolutionary line (12). Amino acid changes acquired by earlier strains were largely carried through to later strains in this group.

Once complete amino acid sequences for HA1 of more recent members of the Hong Kong subtype are available, it should be possible to decide whether some of the evolutionary lines established early in subtype development die out or whether they all continue to evolve, perhaps extending toward one or more endpoint strains, incorporating the maximum permissible number of advantageous amino acid changes seen in earlier strains.

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