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Genetic and antigenic variation in the haemagglutinin of recently circulating human influenza A (H_3N_2) viruses in the United Kingdom

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Summary. Variation in the haemagglutinin (HA) gene of influenza A (H_3N_2) viruses isolated in the U. K. and abroad from 1992–1994, was determined by nucleotide sequencing of the HA1 domain of the HA gene. Viruses isolated in the U.K. early in the 1992–93 season were from the A/Beijing/353/89 lineage and were replaced later that season by viruses from the A/Beijing/32/92 lineage. Viruses from the new lineage continued to be isolated during the 1993–94 season, but were heterogeneous. Most of these isolates were more closely related to an A/Beijing/32/92 variant, A Hong Kong/23/92, but could be distinguished into three groups by serology (of which one group was circulating during the previous season) and four groups based on sequence variation in the HA gene. However, phylogenetic analysis of antigenically-distinct isolates showed that the HA gene is evolving along one lineage. Sequence analysis identified mainstream, subgroup and strain specific amino acid substitutions. There was a broad correlation between the observed amino acid changes and the antigenic sites of the HA. The results of this study highlight the value of regular molecular analysis of circulating viruses.

Introduction

Molecular analysis of sequence changes in influenza viral genes is a powerful tool for determining the extent and nature of genomic variation. Influenza A and B viruses both undergo gradual antigenic variation, as a result of point mutations, referred to as antigenic drift. Type A viruses also experience, although less frequently, major changes resulting from gene reassortment, known as antigenic shift. These antigenic changes in the two surface antigens, the HA and the neuraminidase (NA), enables the virus to evade the immune response of the host and to cause repeated infections. Continual surveillance of circulating viruses is necessary for the selection of suitable vaccine strains. This is particularly important for matching the influenza A (H_3N_2) vaccine component, since viruses of this

subtype have often been associated with excess mortality in the elderly, including those in high-risk groups for influenza. Since the pandemic of 1968, which was caused by viruses with the H_3 HA, influenza A (H_3N_2) viruses have continued to circulate and show antigenic drift [2, 23, 28].

During the 1992–93 winter, influenza B virus predominated worldwide. Low influenza activity was reported in most countries [29]. Of the 408 isolates examined from the U.K. during this season, 21% (86 isolates) of the strains were influenza A (H_3N_2) subtype (Table 1). A/Beijing/353/89-like viruses, which had been circulating in the previous winter, reappeared at the beginning of the '92 winter season but were soon replaced by variants represented by A/Beijing/32/92 and A/Hong Kong/23/92. A single isolate similar to a variant isolated in Spain, A/Madrid/G252/93, which showed significant antigenic drift from A/Beijing/353/89, was also detected [5].

During the 1993–94 season, influenza A (H_3N_2) subtype predominated worldwide causing moderate epidemics [30]. Of the 526 influenza isolates examined from the U.K., 515 (98%) were identified as A (H_3N_2) subtype (Table 1). Although most isolates were shown by haemagglutination inhibition (HI) assay to be similar to A/Beijing/32/92 and A/Hong Kong/32/92, more detailed antigenic analysis of fifteen representative isolates showed that three different variants were circulating concurrently.

In this study, we have sequenced the HA genes of antigenically distinct influenza A (H_3N_2) viruses, using a combination of PCR and cycle sequencing. The pattern of evolution of the HA genes of isolates obtained during 1992–93 and 1993–94 in the U.K. and abroad, was investigated by comparison of their HA sequences and antigenic characteristics to vaccine and reference strains.

Materials and methods

Viruses

Influenza A (H_3N_2) viruses examined in this study are listed in Table 2. Seven viruses (Beij/89, HK/34/90, Beij/92, HK/23/92, Shdong/93, Gdong/25/93 and E/284/93) were grown in the allantoic cavity of 10 day-old embryonated eggs. The other viruses were grown in either MDCK or RMK tissue cultures [4]. Infected fluids were then harvested and stored at −70 °C.

Antigenic analysis

HI tests were performed as previously described [4] using post-infection ferret sera treated with receptor-destroying enzyme. HI tests were carried out using 8 HA units of virus and 0.5% v/v turkey red blood cells. A virus which shows a four-fold or less reactivity with earlier antigens or antisera, or both, has significant antigenic drift.

Primers

Oligonucleotide primers were synthesised on a 392 DNA/RNA Synthesiser (Applied Biosystems). For amplification of the HA1 domain of the HA gene, two pairs of primers were used in a nested PCR. In the first round of amplification primers AH_3G 5′d (AAGCAGGGGATAATTCTATT) and AH_3H 5′d (ATGCCTGAAACCGTACCAAC) were used, corresponding to nucleotide positions 6 and 1145 in the vRNA and cDNA

sequence respectively. In the second round, primer AH_3G was again used, with $AH_3I5'd$ (TCCCTCCCAACCATTTTCTA) which corresponds to nucleotide position 1115 in the cDNA sequence.

Five primers were used to sequence the HA1 domain of the HA gene. The primers AH_3B and AH_3C were originally described by Zhang and Evans [32]. Primers AH_3G 5'd (AAGCAGGGGATAATTCTATT), AH_3B 5'd (AGCAAAGCTTTCAGCAACTG) and AH_3J 5'd (CAAGCCCCTTTCAAAACGTAA) corresponding to nucleotide positions 6, 351 and 953 were used to sequence the vRNA strand.

Primers $AH_3C5'd$(GCTTCCATTTGGAGTGATGC) and $AH_3I5'd$ (TCCCTCCCA-ACCATTTTCTA) corresponding to nucleotides 941 and 1115 were used to sequence the cDNA strand.

Viral RNA extraction and cDNA synthesis

Viral RNA was extracted from 50 µl infected tissue culture fluid or egg fluid by the guanidinium thiocyanate method described by Boom et al. [1]. The RNA (22.2 µl) was then used to prepare cDNA. The reaction mix (17.8 µl) contained 1 × PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.01% Tween 20), 5 mM $MgCl_2$, 1 mM of each dNTP, 25 ng (1 nmol) random primer $(pdN)_6$ (Pharmacia), 1.6 U RNasin (Promega) and 200U M-MLV reverse transriptase (Gibco BRL). The reaction was incubated at room temperature for 10 minutes and then at 37 °C for 45 min. Samples were then heated to 100 °C for 5 min and cooled on ice.

PCR amplification

The HA1 domain of the HA gene was amplified and sequenced, since the HA1 polypeptide forms the antibody-binding and receptor-binding sites on the HA molecule [25, 27]. For the primary PCR, 20 µl cDNA was amplified in a 100 µl volume containing 1 × PCR buffer, 2 mM $MgCl_2$ (final concentration), 5 pmol each outer primer and 1.5 U Taq polymerase and overlaid with mineral oil. Samples were subjected to 1 cycle of denaturation for 2 min at 95 °C and 25 cycles of 1 min at 94 °C, 1 min at 50 °C and 3 min at 72 °C. Two µl of the primary PCR product was then amplified in a 50 µl volume containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each inner primer and 1.25 U Taq polymerase. Samples were overlaid with mineral oil and subjected to 1 cycle for 2 min at 95 °C, followed by 25 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C.

Sequencing and phylogenetic analysis

Following amplification, samples were run on a 1% agarose gel and the band containing the PCR product excised. DNA was then purified on a silica matrix (Geneclean II, Stratech). After precipitation with ethanol, the DNA from each PCR mix was resuspended in 20 µl H_2O and 10 µl used per sequencing reaction.

Nucleotide sequences were then determined using the Taq Dye Deoxy Terminator Cycle Sequencing kit and the 373A DNA Sequencing System (Applied Biosystems). Primers were used at 3.2 pmol per 550 ng template DNA, per reaction. The nucleotide sequences for the isolates described in this study are available from GenBank, under accession numbers Z46391 to Z46417.

Phylogenetic analysis was performed following the alignment of sequences with the program ClustalV [9]. Distances between pairs of sequences were then estimated using the DNADIST or PROTDIST programs from the PHYLIP package (Version 3.5, [8]). Phylogenetic trees were then constructed from the distance matrix data using the FITCH program.

Results

Haemagglutination inhibition assays

The first two influenza AH_3N_2 viruses (E/1/93 and Sc/2/93) isolated in January and February of 1993, were antigenically similar to the vaccine strain for that season, Beij/89 (Table 1). The third strain isolated in March 1993 (A/England/40/93) proved to be similar to a new variant, Beij/92. All subsequent influenza AH_3N_2 isolates examined during the 1992–93 season were similar to Beij/92, except one virus, which was antigenically related to a variant isolated in Spain, Mad/252/93.

The 515 influenza AH_3N_2 viruses isolated in the U.K. during the 1993–94 influenza season were analysed by HI assays (Table 1). 512 of the isolates were similar to Beij/92 and 3 isolates were similar to the variant isolated during the previous season, Mad/252/93. However, more detailed antigenic analysis of 15 representative isolates, (13 Beij/92-like and 2 Mad/252/93-like, Table 2), revealed that these viruses could be distinguished into three different antigenic groupings (subgroups I–III, Table 3). Of the fifteen selected isolates, eleven could be grouped into subgroup I, represented by Sc/160/93 and HK/1/94 (Table 3). These viruses reacted well with the Beij/92 and HK/23/92 antisera, but ferret antisera raised to Sc/160/93 and HK/1/94 reacted poorly to Beij/92 and HK/23/92 viruses. Sc/160/93 and HK/1/94 viruses could be distinguished from each other by cross-reaction with antiserum to the first virus to be isolated from the Beij/92 lineage, HK/34/90.

Four isolates formed a second subgroup-II, represented in Table 3 by Sc/142/93. Subgroup II viruses were antigenically more closely related to HK/23/92 and Shdong/93, than to Beij/92, although their antisera showed a four-fold decrease in reactivity with both HK/23/92 and Shdong/93 viruses.

A third subgroup consisting of two viruses (subgroup III), were antigenically very different from subgroups I and II. subgroup III isolates were most closely

Table 1. Influenza isolates examined from the United Kingdom during the 1992-93 and 1993-94 seasons

Season	Number of isolates[a]	A (H_3N_2) Subtype[b]	A (H_3N_2) Variants[c]
1992-93	408	86	2 Beij/89-like
			83 Beij/92-like
			1 Mad/252/93-like
1993-94	526	515	O Beij/89-like
			512 Beij/92-like
			3 Mad/252/93-like

[a]Total number of influenza isolates examined from the United Kingdom during the period January–July 1993, and August 1993–July 1994

[b]Number of isolates identified as influenza A (H_3N_2) subtype

[c]Number of A (H_3N_2) isolates characterised by HI, using antisera to the viruses Beij/89 and Beij/92, as similar to the variants Beij/89, Beij/92 and Mad/252/93

Table 2. Influenza A (H_3N_2) viruses sequenced in this study

	Virus	Abbreviation	Date[a]
Reference strains	A/Beijing/353/89	Beij/89	–[b]
	A/Hong Kong/34/90	HK/34/90	–
	A/Beijing/32/92	Beij/92	–
	A/Hong Kong/23/92	HK/23/92	28/7/92
	A/Madrid/G252/93	Mad/252/93	15/2/93
	A/Shangdong/9/93	Shdong/93	–
1992–93 isolates	A/England/1/93	E/1/93	8/1/93
	A/Scotland/2/93	Sc/2/93	29/1/93
	A/England/247/93	E/247/93	14/5/93
1993–94 isolates	A/Guangdong/25/93	Gdong/25/93	–
	A/England/284/93	E/284/93	17/9/93
	A/Scotland/142/93	Sc/142/93	23/9/93
	A/England/269/93	E/269/93	6/10/93
	A/England/286/93	E/286/93	25/10/93
	A/Scotland/160/93	Sc/160/93	26/10/93
	A/England/289/93	E/289/93	27/10/93
	A/England/328/93	E/328/93	31/10/93
	A/England/346/93	E/346/93	2/11/93
	A/England/347/93	E/347/93	3/11/93
	A/Scotland/173/93	Sc/173/93	5/11/93
	A/Scotland/174/93	Sc/174/93	6/11/93
	A/England/471/93	E/471/93	23/11/93
	A/England/7/94	E/7/94	18/12/93
	A/England/67/94	E/67/94	21/6/94
	A/Hong Kong/1/94	HK/1/94	23/6/94
	A/Hong Kong/2/94	HK/2/94	25/6/94
	A/England/68/94	E/68/94	4/7/94

[a]Date throat swab collected
[b]Exact date not known

related to a reference virus (Mad/252/93) isolated in Spain during the previous season. They showed some cross-reactivity with the 1992–93 vaccine strain, Beij/89, but reacted poorly with antisera to viruses from the Beij/92-lineage (Table 3).

Lastly, the antigenic characteristics of a new variant, Gdong/25/93 which had been isolated in China at the end of 1993, were determined. Gdong/25/93 showed significant antigenic drift from Beij/92, HK/23/92 and Shdong/93.

Sequence and phylogenetic analysis

Amino acid differences in the HA1 domain of the HA gene of all the viruses in Table 2, are shown in comparison to the majority sequence in Fig. 1. All the viruses differed from Beij/89 at nucleotides 557 (T→G) and 578 (A→G), resulting in an amino acid change at position 186 (Ile to Ser) and position 193 (Asn to Ser).

Table 3. Hemagglutination inhibition reactions of selected influenza A (H_3N_2) viruses

Virus	Designated antigenic subgroup	Post infection ferret sera									
		Beij/89	HK/34/90	Beij/92	HK/23/92	Shdong/93	Sc/160/93	HK/1/94	Sc/142/93	Mad/252/93	Gdong/25/93
Beij/89		*1280*	<[d]	40	40	40	40	40	80	40	<
HK/34/90		160	*640*	1280	320	320	160	80	640	<	<
Beij/92		80	320	*1280*	640	640	80	80	640	<	40
HK/23/92		80	80	640	*1280*	640	320	640	1280	40	160
Shdong/93		40	80	640	640	*1280*	320	640	1280	40	80
Sc/160/93[a]	I	160	80	1280	1280	640	*2560*	2560	2560	320	2560
HK/1/94[a]	I	160	<	1280	1280	640	1280	*5120*	2560	320	2560
Sc/142/93[b]	II	80	160	320	1280	1280	320	320	*5120*	80	160
Mad/252/93[c]	III	640	<	160	160	320	320	1280	320	*2560*	320
Gdong/25/93		40	<	160	640	320	640	1280	640	160	*2560*

[a]Sc/160/93 and HK/1/94 represent isolates in subgroup 1 (E/269/93, E/286/93, E/328/93, E/347/93, Sc/160/93, Sc/173/93, Sc/174/94, E/67/94, E/68/94, HK/1/94 and HK/2/94)

[b]Sc/142/93 represents isolates in subgroup II (Sc/142/93, E/289/93, E/346/93 and E/471/93)

[c]Mad/252/93 represents isolates in subgroup III (E/284/93 and E/7/94)

[d]<40

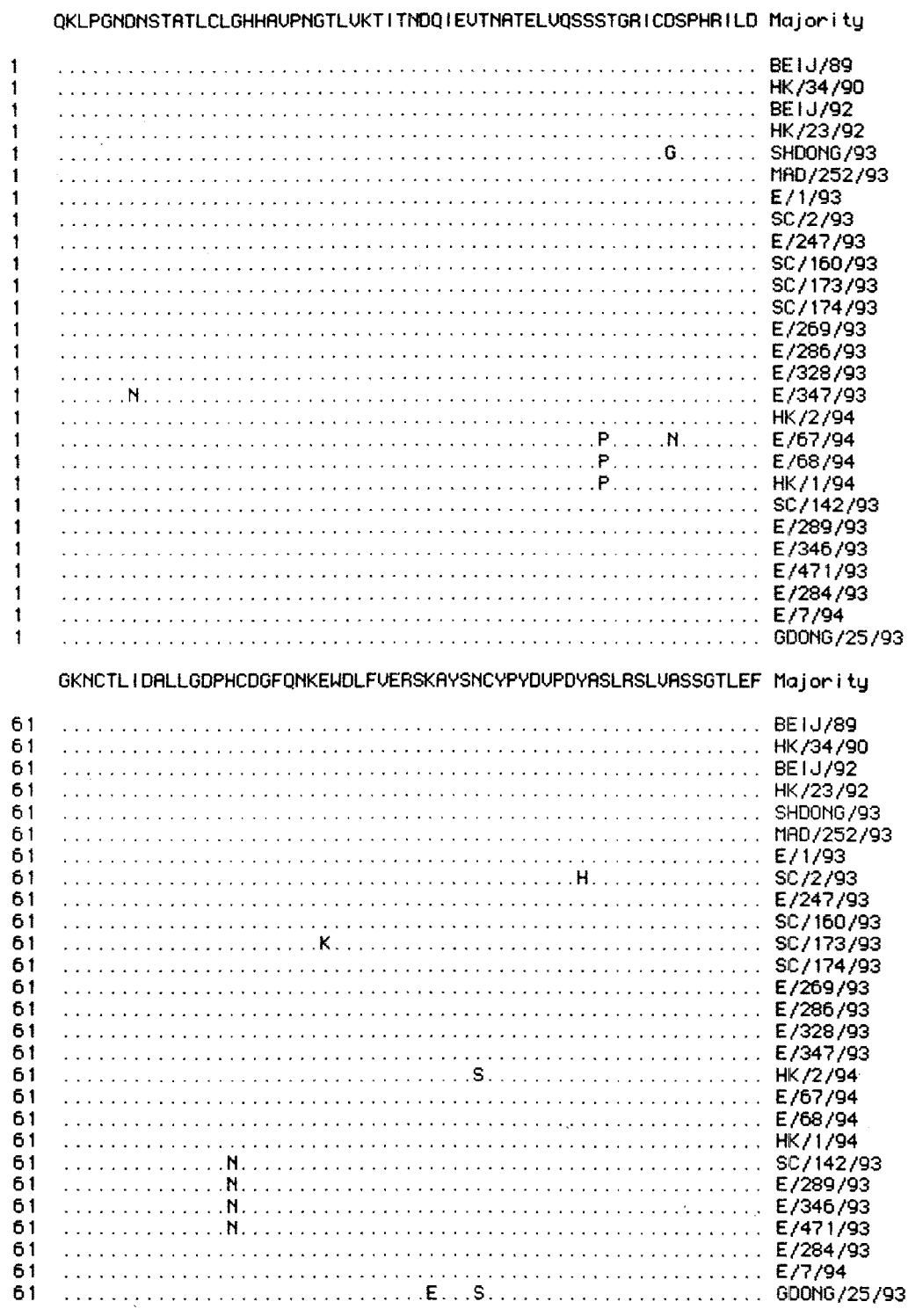

Fig. 1 (*continued*)

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    INEDFNWTGVAQDGKSYACKRGSVNSFFSRLNWLHKLEYKYPALNVTMPNNGKFDKLYIW Majority

121 ..........S.E.........K..........ES.......................... BEIJ/89
121 ..............G.......................S.................V..... HK/34/90
121 ..............G.......................S..................... BEIJ/92
121 ..............G.............................................. HK/23/92
121 ..............G.............................................. SHDONG/93
121 ........................K..................................... MAD/252/93
121 ............N.D.........K..........ES..............D........ E/1/93
121 ............N.D.........K..........ES..............D........ SC/2/93
121 ..................S..................................... E/247/93
121 ..................................................... SC/160/93
121 ..................................................... SC/173/93
121 ..................................................... SC/174/93
121 ..................................................... E/269/93
121 ..................................................... E/286/93
121 ..................................................... E/328/93
121 ..................................................... E/347/93
121 ..................................................... HK/2/94
121 ...N..............T..................................... E/67/94
121 ...N..................................................... E/68/94
121 ...N..................................................... HK/1/94
121 T.............G..................................... SC/142/93
121 T.............G..................................... E/289/93
121 T.............G..................................... E/346/93
121 T.............G..................................... E/471/93
121 ........................K..................................... E/284/93
121 ........................K..................................... E/7/94
121 ..................................................... GDONG/25/93

    GVHHPSTDSDQTSLYVRASGRVTVSTKRSQQTVIPNIGSRPWVRGLSSRISIYWTIVKPG Majority

181 .....I..RE..N.......................................... BEIJ/89
181 ........R.....................................Q..... HK/34/90
181 ........R.....................T.....Q..... BEIJ/92
181 ..........................T.....Q..... HK/23/92
181 ..........................T.....Q..... SHDONG/93
181 ..........................K.....Q..... MAD/252/93
181 ........RE.....I..................................... E/1/93
181 ........RE.....I..................................... SC/2/93
181 ..................................................... E/247/93
181 ..................................F.................. SC/160/93
181 ..................................F.................. SC/173/93
181 ..................................F.................. SC/174/93
181 ..................................F.................. E/269/93
181 ..................................F.................. E/286/93
181 ..................................F.................. E/328/93
181 ..................................F.................. E/347/93
181 ..................I.................................... HK/2/94
181 ..................D..Y.................. E/67/94
181 ..................D..Y.................. E/68/94
181 ..................D..Y.................. HK/1/94
181 ..............K.....T.................. SC/142/93
181 ..................T.................. E/289/93
181 ........R.....T.................. E/346/93
181 ..............K.....T.....R..... E/471/93
181 ..................F.....R..... E/284/93
181 ..................F.................. E/7/94
181 .....................Q..... GDONG/25/93
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Fig. 1 (*continued*)

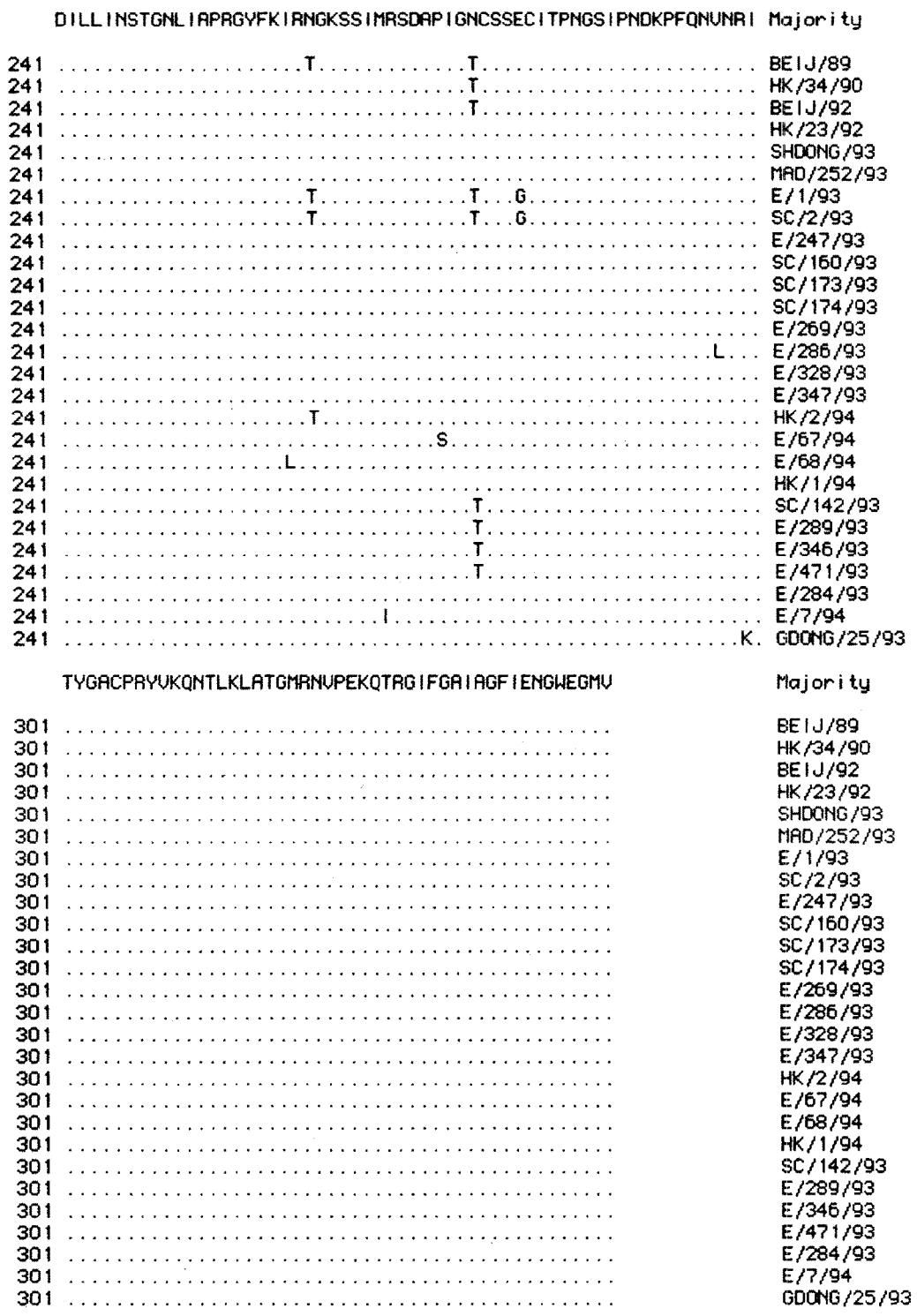

Fig. 1. Comparison of amino acid changes in the HA1 region of the HA gene of the influenza A (H_3N_2) viruses in Table 1. Sequences were aligned using the clustal method (Megalign, version 1.03: [21]). Only differences from the majority sequence are shown

The possession of isoleucine at position 186 or lysine at 193 has previously been shown to result from adaptation of the virus to growth in eggs [13, 15]. The isoleucine at amino acid 186 in the egg-derived Beij/89 virus may therefore be the result of host-mediated selection and so was omitted from the phylogenetic analysis since it has been demonstrated that the relationships between HA sequences are not affected by ignoring host-mediated changes [6]. Nucleotide and amino acid phylogenies were compared using software programs based on different algorithms. The topology of the tree was the same when either maximum likelihood (Phylip, version 3.5: [8, 14], maximum parsimony (PAUP version 3.1) or neighbor-joining algorithms (Megalign, version 1.03: [21] were used to construct the tree. Only the tree based on amino acid differences using Phylip is shown (Fig. 2).

Two viruses isolated in the U.K. early in the 1992–93 influenza season, E/1/93 and Sc/2/93, had sequences closely related to the 1992–93 vaccine strain Beij/89 (Fig. 1). They appear as a branch on the Beij/89-lineage of the tree (Fig. 2). Viruses isolated later in the season, including the virus E/247/93 analysed in this

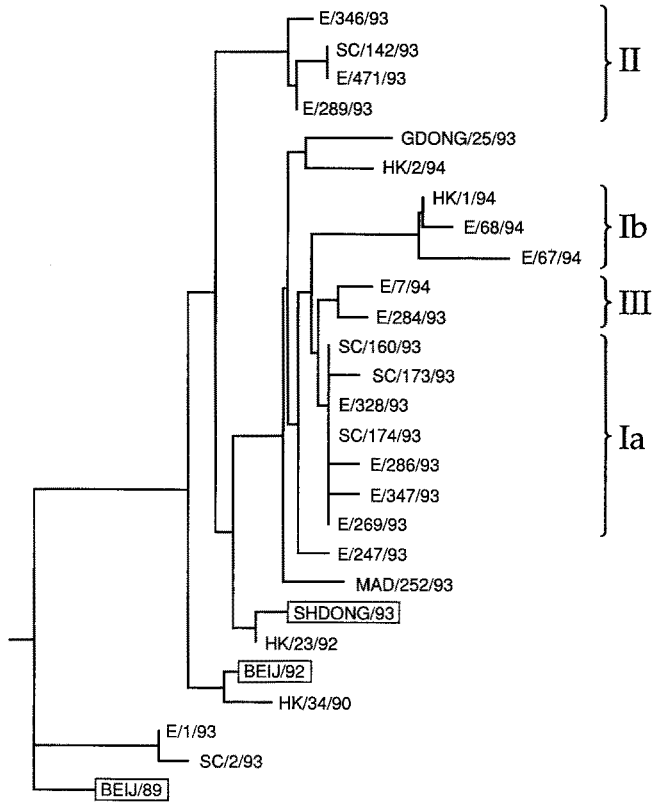

Fig. 2. Phylogenetic tree of the HA1 domains of the HA gene of the influenza A (H_3N_2) viruses in Table 1, based on amino acid differences. The tree is unrooted and was constructed using the Phylip suite of programs [8]. Branch lengths (horizontal lines) are proportional to the number of amino acid changes. Vaccine strains are boxed and brackets indicate the subgroups described in the text

study, had greater sequence similarity to the Beij/92 group of viruses than to Beij/89 (Fig. 1). E/247/93 was more closely related to HK/23/92 than to Beij/92, having 7 amino acid changes compared to Beij/92 and only four in comparison to HK/23/92.

Phylogenetic analysis of selected viruses isolated from October 1993–July 1994, which were distinguished by HI assays into three antigenic subgroups (I–III), revealed that the HA sequences of these viruses clustered into four subgroups (Ia, Ib, II & III), on the Beij/92-lineage of the tree (Fig. 2). The largest antigenic subgroup (subgroup I) could be further distinguished into subgroups Ia and Ib, by sequence analysis. Viruses from all four subgroups were more closely related to HK/23/92 than Beij/92. They all showed an amino acid change from Beij/92 and HK/23/92 at position 226 (Gln to Leu, or in E/284/93, Gln to Arg). Amino acid changes from Beij/92 and HK/23/92 at positions 135 and 214 were common to viruses in subgroups Ia, Ib and III. Changes shared within a subgroup were found at position 219 in subgroup Ia, residues 47, 124, 216 and 219 in subgroup Ib and at position 145 in subgroup III. Two isolates, HK/2/94 and Gdong/25/93, shared the mainstream substitutions at positions 135 and 214 with subgroups Ia, Ib and III, but they retained serine at position 219. These two viruses therefore formed a separate branch on the tree (Fig. 2). None of the amino acid changes shared by viruses in subgroup II (positions 75, 121 and 276) were found in the other subgroups. The 1994–95 vaccine strain, Shdong/93, clustered on the same branch as HK/23/92, having only one amino acid change from HK/23/92 at residue 53.

The phylogenetic tree constructed on the basis of nucleotide differences showed the same relationships between viruses except for the virus E/269/93 (data not shown). On comparison of nucleotide differences, this virus appeared to cluster not in subgroup Ia, but in subgroup III.

Discussion

A phylogenetic tree based on nucleotide changes in the HA of influenza A (H_3N_2) viruses, isolated between 1968–92, has previously been described [7]. Influenza A(H_3N_2) viruses diverged into two lineages between 1989–90. Viruses from both lineages co-circulated during the period 1990–92. The sequence analysis in this study of influenza A (H_3N_2) viruses isolated between 1992–94, has provided information on the recent pathway of evolution of the HA.

The majority of viruses isolated during the 1993–94 influenza season were shown to be genetically and antigenically derived from the Beij/92-lineage. However, although the HA genes of subgroup III viruses clustered on the Beij/92-lineage on the phylogenetic tree (Fig. 2), subgroup III viruses reacted poorly with antisera to the Beij/92-group of viruses. They showed some cross-reactivity with the Beij/89 antiserum and examination of the amino acid sequences revealed that the antigenicity of subgroup III isolates could be explained by the presence of lysine at position 145 both in these viruses and in Beij/89: this position (Fig. 3) is located on the antigenic site A of the HA molecule [25, 27]. All of the other

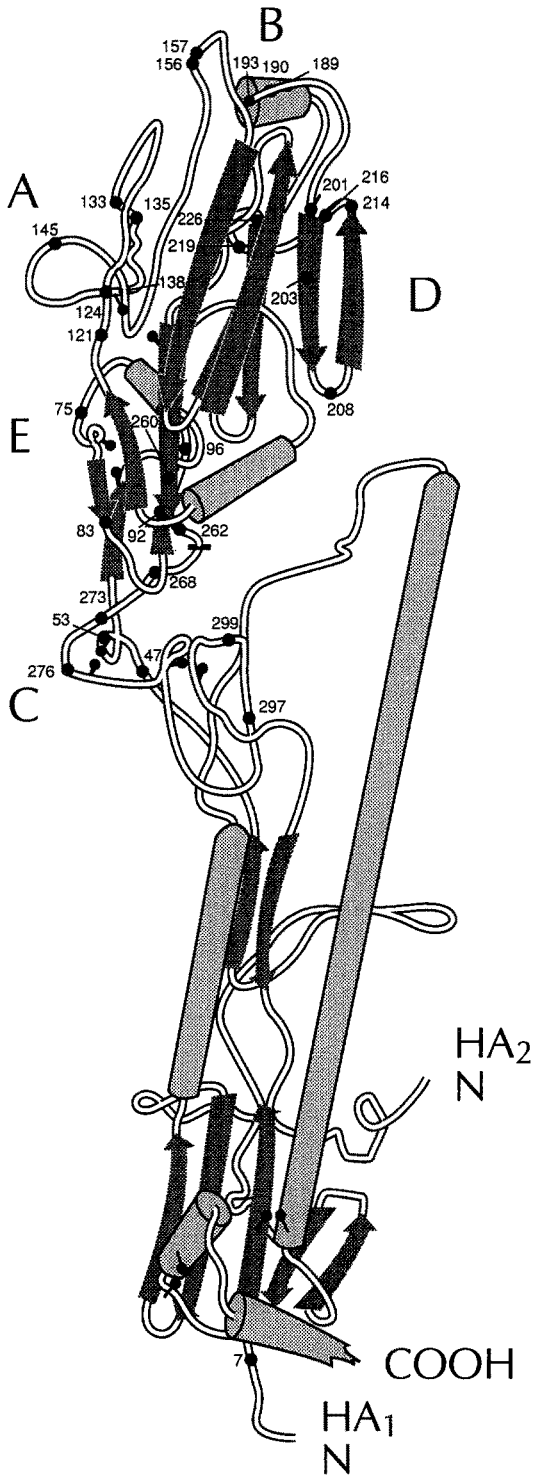

Fig. 3. Drawing of the HA monomer (based on the Hong Kong 1968 virus HA). ● indicate the location of amino acid changes in viruses in the subgroups Ia, Ib, II & III described in the text. *A-E* are antigenic sites described by Wiley et al. [2, 26]

viruses on the Beij/92-lineage had asparagine substituted at position 145. The molecular analysis therefore confirmed that viruses from the Beij/89-lineage were not detected by HI after February 1993. This finding highlights the ability of sequence analysis to define along which lineage evolution is proceeding.

Significance of the observed amino acids changes

None of the amino acid differences observed in the four subgroups would affect the structure of the haemagglutinin—by disruption of disulphide bonds (at residues 14, 52, 64, 76, 97, 139, 277, 281 and 305) [26, 28], or of the glycosylation sites at residues 8, 22, 38, 63, 126, 165, 246 and 285 (Fig. 1). However, the glycosylation site at position 276 is lost in the subgroup II viruses. There appeared to be a broad correlation between the observed amino acid differences and the previously described antigenic sites of the haemagglutinin, shown in Fig. 3 [2, 25, 28].

Egg-mediated variation

Studies on clinical samples and laboratory isolates have identified substitutions in the HA of egg-selected A (H_3N_2) variants at several residues including 145 (Asn-Lys), 156 (Glu-Lys), 186 (Ser-Ile), 193 (Asn-Lys) and 226 (Leu-Gln) [10–12, 15, 20]. Specific egg-selected amino acid changes may also be selected due to immune pressure [19]. Substitutions at residues 145, 156, 193 and 226, which were seen in some of the viruses in this study, appeared to be associated with antigenic drift in these viruses, since these substitutions were present in both egg and MDCK- or RMK-cell derived viruses. Substitution of isoleucine at position 186 was only present in the egg-derived Beij/89 virus. This correlates with a previous report that this amino acid change is exclusively a result of egg adaptation [15].

Vaccines

Following antigenic characterization of the majority of viruses isolated in the U.K. during 1993–94 which suggested they were more closely related to HK/23/92 than to Beij/92, the influenza A (H_3N_2) vaccine strain was updated from Beij/92 to a HK/23/92-like virus – i.e. Shdong/93 – for the 1994–95 vaccine. Viruses in subgroup Ib were isolated after the selection of Shdong/93 as the vaccine strain. These viruses had at least 7 amino acid differences from Shdong/93. Subgroup Ib isolates had differences in sites A (Gln to Lys at 135; Asp to Asn at 124), site C (Ser to Pro at 47) and site D (Thr to Ile at 214; Asn to Gln at 216; Ser to Tyr at 219; Glu to Leu at 226). The Ser to Pro substitution (residue 47), is involved in a β-sheet [28], but it is unclear whether it is likely to have a significant effect on this structure. Two of the viruses in subgroup Ib, E/67/94 and E/68/94, were isolated in the U.K. during June and July of 1994. Subgroup Ib-like variants have survived since closely-related viruses have subsequently been isolated in the U.K. during the 1994–95 season. Although the occurrence of "herald" strains has previously been described for influenza A (H_1N_1) viruses [16, 17], subgroup Ib viruses did not become epidemiologically important during the 1994–95 season.

The Gdong/25/93 virus had the common difference at site A (Gly to Lys at 135) and one difference at site D (Thr to Ile at 214), but also two differences close to site E (Lys to Gln at 92; Asn to Ser at 96). It is not obvious how these changes

might effect the properties of this virus, but the changes at positions 96, 135 and 226 may affect the receptor pocket interaction [26]. Also, if residues 92 and 96 are considered part of site E, this virus fulfills the criteria of Wilson and Cox [28], that new drifts of antigenic importance generally have four or more amino acid substitutions located in two or more of the antigenic sites. The influenza A (H_3N_2) strain chosen for the 1995–96 vaccine is a virus antigenically similar to Gdong/93, A/Johannesburg/33/94 [31]. More detailed predictions of the effects of the amino acid differences described herein will require computer based minimization and molecular modelling, using packages such as INSIGHT and QUANTA. This will be investigated in future studies. Further investigations, such as recent studies by Bullough et al. [3], will provide insights into the effects of conformational changes on the structure and function of the HA.

It is therefore apparent that influenza A (H_3N_2) viruses have continued to show antigenic drift in the HA gene. The emergence of new variants, such as Gdong/25/93, underlines the importance of influenza virus surveillance. The continuing analysis of circulating viruses is therefore still required to aid the selection of the most suitable vaccine strain. Cycle sequencing of PCR products provides a rapid method of sequence analysis, without the need for culture of virus in the laboratory. Furthermore, phylogenetic analysis is able to define the exact relationships between influenza variants and provides information on the route of evolution. Although not all variants are epidemiologically important, the collation of data from temporally and geographically distinct influenza viruses may eventually allow the prediction of patterns of HA evolution, and hence improve vaccine strain selection.

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