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Antigenicity and evolution amongst recent influenza viruses of H1N1 subtype

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ABSTRACT

The sequence of the HA1 subunit region of the haemagglutinin gene of influenza A/USSR/90/77, and A/Brazil/11/78, A/Lackland/3/78, A/England/333/80 and A/India/6263/80 was determined by dideoxy-sequencing methods using total virion RNA and specific oligonucleotide primers for reverse transcriptase. These 1977-1980 strains share a minimum of 85% amino acid sequence homology with influenza A/PR/8/34. Most of the surface amino acid substitutions which occurred during the evolution of A/PR/8/34 to A/USSR/90/77 and subsequently in the 1978-1980 strains are located in the 4 antigenic sites previously defined by an analysis of laboratory-selected mutants of A/PR/8/34. We deduce an evolutionary pathway for the 1977-80 strains and suggest their different epidemic properties may be a consequence of only a few amino acid changes.

INTRODUCTION

Influenza remains an important cause of morbidity and death in Man, principally because of the ability of the surface haemagglutinin to undergo extensive antigenic variation (1). We can distinguish major changes in antigenicity - termed "antigenic shift" - or minor ones, termed "antigenic drift". The former correlate with major influenza pandemics such as the 1968 Hong Kong flu or the 1977 Russian flu, the latter with more minor epidemics that arise every 2 or 3 years or so between pandemics. A comparative sequence analysis of series of antigenically drifted strains of the H3 haemagglutinin subtype (e.g. ref 2) clearly shows that variation occurs by the gradual accumulation of point mutations causing single amino acid substitutions. It appears that these cluster in 4 antigenic sites on the surface of the globular head of the haemagglutinin (3,4) but amino acid substitutions are by no means confined solely to these relatively discrete sites.

Our own studies on antigenicity have been confined to the earlier 1934 influenza strain A/PR/8/34 of H1 subtype (5). From a study of an extensive

set of antigenic mutants of the virus constructed in the laboratory, we also defined 4 antigenic sites, although their location differed in detail from those of the H3 subtype (5,6). We further argued that these sites were in all probability "immunodominant regions" superimposed on an antigenic continuum, which possibly included much of the surface of the globular head of the molecule. But these results were subject to the criticism that they were based on the immune response of mice to laboratory-selected virus mutants and no field strains had been analysed.

We now present the first study on the complete sequence analysis of the antigenic region of a series of antigenically drifted H1 subtype strains. We selected for this purpose the re-emerged H1N1 'Russian flu' A/USSR/90/77 and 4 subsequent variants, A/Brazil/11/78, A/Lackland/3/78, A/England/333/80 and A/India/6263/80. (These strains are abbreviated as USSR/77, Braz/78, Lack/78, Eng/80 and Ind/80.) From the results we derive an evolutionary pathway and discuss the observed amino acid substitutions with reference to the previously characterized antigenic sites in A/PR/8/34 (abbreviated subsequently as PR8/34). In summary, most, but not all, of the amino acid substitutions which lie on the surface of the haemagglutinin are located at or close to previously defined antigenic sites. A comparison of the 1977-80 strains showed that here also most, but not all, amino acid substitutions involved residues at or close to antigenic sites.

RESULTS

Comparative nucleotide sequence analysis of the haemagglutinin gene of 5 recent (1977-1980) influenza strains of H1N1 subtype

Figure 1 shows the nucleotide sequence established for the region encoding the HA1 domain of the haemagglutinin gene of the influenza virus strains USSR/77, Lack/78, Braz/78, Eng/80 and Ind/80. This was determined by the dideoxy-chain termination sequencing method (7) using 4 synthetic oligonucleotide primers to prime cDNA synthesis directly from total virion RNA (see ref. 5 and Methods). Sequence analysis was confined to the region of the genome which encodes the HA1 subunit since the major antigenic determinants of the molecule occur in this domain (8,9). The sequences of Lack/78, Braz/78, Eng/80 and Ind/80 are identical to that of USSR/77 except where changed nucleotides are indicated in Figure 1. Because of technical difficulties in the sequencing method, certain residues could only be assigned with a 75% probability, i.e. USSR/77: residues 239, 434, 575; Braz/78: residue 506; Eng/80: residues 387, 724, 742, 1048; and Ind/80:

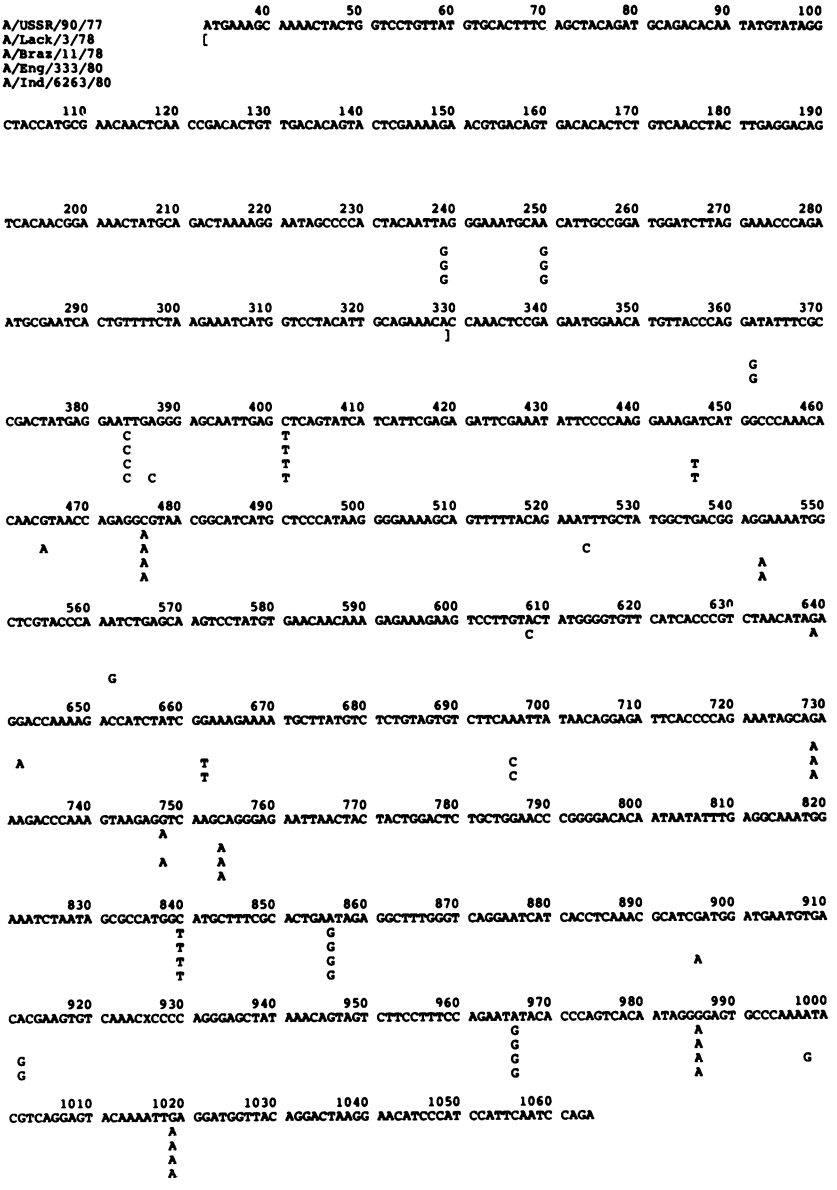

Figure 1 The nucleotide sequence of the HA1 region of the haemagglutinin gene of A/USSR/90/77 compared with that of A/Lack/3/78, A/Braz/11/78, A/Eng/333/80 and A/Ind/6263/80. Only where the 1978-1980 strains differ from USSR/77 is the sequence change indicated. The sequences are presented in mRNA sense, and are numbered by aligning with the RNA segment encoding the haemagglutinin of A/PR/8/34 (11). (See text for further explanation of symbols.)

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                                    10        20        30        40        50  -     60
A/PR/8/34              MKANLLVLLCALAAADADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCRLKGIAPLQLGKCN
A/USSR/77                 K        S T

   70       - 80        90    -   100       110       120     ---  130       -140       150
IAGWLLGNPECDPLLPVRSWSYIVETPNSENGICYPGDFIDYEELREQLSSVSSFERFEIFKESSWPNHNTTKGVTAACSHAGKSSFYRNLLWL
   I       ES FSKK     A        T    Y A                          R   K  V R    S   K

   160       170       180       190       200       210       220       230       240
TEKEGSYPKLKNSYVNKKGKEVLVLWGIHHPSNSKDQQNIYQNENAYVSVVTSNYNRRFTPEIAERPKVRDQAGRMNYYWTLLKPGDTIIFEAN
  EN     N SK    N E        V     IE  KT  RK        S                    G    I       E

250       260-      270       280       290       300       310       320
GNLIAPRYAFALSRGFGSGIITSNASMHECNTKCQTPLGAINSSLPFQNIHPVTIGECPKYVRSAKLRMVTGLRNIPSIQS
      WH    N               D  D      Q                          T
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Figure 2 Comparison of the amino acid sequence of the HA1 domain of the haemagglutinin of A/USSR/90/77 with A/PR/8/34 (Cambridge). For USSR/77 only residues which differ from A/PR/8/34 are marked. Residues are numbered with reference to the protein sequence alignment of A/Aichi/2/68 and A/PR/8/34 (11) to facilitate the location of amino acids on the three-dimensional structure of the molecule of H3 subtype (12). Where additional residues occur in A/PR/8/34 relative to A/Aichi/2/68, these are indicated by dashes (-) and are numbered according to the preceding residue number and a subscript, e.g. 125c, 260a.

residues 225, 434, 829. Residue 926 (X) was ambiguous in all strains. Residues 36-46 could not be determined using this approach and were established instead by sequencing short cDNA copies of the virion RNA by the chemical degradation method (see Materials and Methods)(10). Furthermore, nucleotides 34-329 of Lack/78 were not determined as oligonucleotide primer I (see Methods) failed to prime cDNA synthesis in this strain. Despite these ambiguities, we estimate that the sequences presented here have an accuracy of greater than 99%.

Comparison of the amino acid sequence of A/USSR/90/77 and A/PR/8/34

In Figure 2 we compare the amino acid sequence deduced from the nucleotide sequence for USSR/77 with that previously found in PR8/34 (Cambridge strain) for the HA1 domain region (11). The numbering system of amino acids is designed to facilitate alignment with the sequence of A/Aichi/2/68 and the location of amino acids on the three-dimensional structure of its haemagglutinin (see legend to Figure 2). No insertions or deletions resulting in changes in alignment occur between these two strains themselves, but there are 45 amino acid substitutions. Three of these, at positions 8, 6 and at -4, occur in the "signal peptide" domain and are absent in the definitive haemagglutinin molecule. The overall amino acid homology is 87%.

Table I lists the 48 nucleotide differences and the corresponding 42

amino acid substitutions between the definitive HA1 domain of the haemagglutinin of PR8/34 and USSR/77. (We exclude those occurring in the signal peptide region, see above, and also silent nucleotide changes.) By comparing the positions of these changes with the location of the previously established antigenic sites, Sa and Sb, Ca and Cb in PR8/34 (5), we deduce that 22 (52%) amino acid changes occur in or close to antigenic sites. If we exclude amino acid changes "buried" within the three-dimensional structure and assuming that the three-dimensional structure of the haemagglutinin of PR8/34 and USSR/77 is not significantly different, 69% of the surface residues map to antigenic sites. Furthermore, changes have occurred in all four of the proposed antigenic sites. Most of the remaining 20 amino acid substitutions represent conservative changes which are buried in the molecule and presumably reflect polymorphisms which are of neutral selective advantage (12). However, nine residue changes which occur at the surface of the protein cannot be assigned to the previously determined antigenic sites of the molecule (Table I). These changes may indicate regions of the molecule which can be involved in antibody contact, but which are distinct from the immunodominant sites described earlier. It is nonetheless clear that during evolution to the USSR/77 virus strain, mutations on the surface of the molecule occurred mainly but not exclusively within the dominant antigenic sites, and that point mutations have occurred at all four sites. It should be noted that, although the haemagglutinin structure was determined for the H3 subtype, the close correlation between antigenic and structural properties of the H1 and H3 subtypes suggests that the H3 subtype structure may be used to determine the relative position of amino acids in the H1 subtype haemagglutinin (3,5), and for this reason all amino acid residues referred to here have been aligned to the H3 subtype haemagglutinin sequence (11).

The potential carbohydrate attachment sites (13) located at amino acid residues 20, 33, 131, 271 and 289 in PR8/34 are all conserved in USSR/77. However, three additional potential attachment sites occur at residues 94a, 158 and 163 (Table I).

Amino acid sequences of recent H1N1 subtype virus strains

Table II lists the 15 amino acid substitutions in the amino acid sequences of the haemagglutinins of the recent H1N1 strains which have occurred during evolution from 1977 to 1980. When these substitutions are compared with the antigenic map of PR8/34, we see that eight mutations occur in or close to the Sa, Sb or Ca sites, although none are found at the

Table I Location of nucleotide and amino acid changes occurring between A/PR/8/34 and A/USSR/90/77

Nucleotide Change[a]	Position	Amino acid Change	Position	Antigenic site[c]	Location on molecule[b]	Nucleotide Change	Position	Amino acid Change	Position	Antigenic site	Location on molecule
C - A	264	L - I	70	-	buried	T - G	572	N - K	166	Sa	surface
C - A	287	D - E	77	?Cb	surface	G - C	587	K - N	171	?Ca	surface
C - T	288	P - S	77a	?Cb	surface	G - A	592	G - E	173	Ca	surface
C - T	294	L - F	79	Cb	surface	A - G	618	I - V	182	-	buried
C - T	297	P - S	80	?Cb	surface	G - T	637	S - I	188	-	surface
G - A T - A	300 301	V - K	81	Cb	surface	A - G	639	K - E	189	?Sb	surface
G - A	304	R - K	82	Cb	surface	C - A	648	Q - K	192	Sb	surface
T - C	322	V - A	88	-	buried	A - C	652	N - T	193	Sb	surface
T - C	349	I - T[d]	96	-	surface	A - G	661	Q - R	196	Sb	surface
G - T	363	D - Y	101	-	buried	T - A	665	N - K	197	?Sb	surface
A - G T - C	369 370	I - A	103	-	buried	A - T	690	T - S	206		buried
C - A	446	S - R	125c	?Sa	surface	A - G	748	D - G	225	Ca	surface
C - A	458	N - K	129	Sa	surface	G - T	764	M - I	230	-	buried
A - G C - T	465 466	T - V	132	-	surface	A - G	786	K - E	238	-	surface
A - G	472	K - R	134	-	surface	A - T	837	R - W	255	-	?surface
G - T	486	A - S	138	-	buried	T - C	840	Y - H	256	-	buried
G - A C - A	498 499	A - K	142	?Ca	surface	G - A	856	S - N	260a	-	buried
A - G	543	K - E	157	?Sa/Sb	surface	C - G	900	H - D	275	-	surface
G - A G - T	546 548	E - N[d]	158	Sa	surface	A - G	909	N - D	278	-	surface
G - T	563	K - N[d]	163	Sa	surface	T - A	931	L - Q	285	-	surface
A - G A - C	568 569	K - S	165	Sa	surface	G - A	1011	A - T	312	-	surface

Cb site. Three surface mutations occur in regions other than the defined antigenic sites at residues 132, 190 and 279. The one at residue 190 occurs at a position previously proposed to be part of the receptor binding site (12). The four remaining changed residues are apparently buried in the protein.

Evolution of recent H1N1 subtype strains

An evolutionary pathway for the recent H1N1 subtype strains isolated from 1977-1980 can be postulated on the basis of the deduced amino acid changes (Figure 3). The epidemic strains are all shown as dead ends, the mainline being maintained through the postulated intermediates X_0, X_1, X_2 and X_3. An alternative direct lineage between strains, which would exclude these intermediates, seems less likely on general natural selection theory and also because such a pathway would require that those mutations which are unique to each individual strain (e.g. an alteration at 189 for Lack/78) undergo specific reversion during evolution to later strains. In Figure 3 the altered amino acid residues are noted by number (cross refer to Figure 2 and Table II) and where a residue is part of an antigenic site the residue number is underlined. It is clear that each virus strain has acquired a point mutation in at least one and usually two of the antigenic sites defined for PR8/34 when compared to its nearest related virus strain (Figure 3 and Table II). Thus, evolution from USSR/77 to Lack/78 involved changes at two antigenic sites by mutation at residue 189 (probable Sb site) and at residue 225 (Ca site). Evolution from USSR/77 to Braz/78 involved changes at residues 219 and 227 which are both superficial residues lying close to residues identified as part of the Ca site. Changes at residue 157 (Sa/Sb site) and 125c (probable Sa site) and at residue 208 (probable Ca site) characterize the differences of both Eng/80 and Ind/80 from previous

Table I

a The changes are shown relative to the sequence of PR8/34; thus USSR/77 has an A at nucleotide 264 rather than a C found at that position in PR8/34. The corresponding amino acid (at residue 70) has therefore changed from L to I. Silent nucleotide changes are omitted.

b The location of each residue on the three-dimensional structure, whether surface or buried, is indicated (see ref. 12).

c This identifies antigenic residues by the sites (5). A (?) indicates residues which lie immediately adjacent to antigenic residues in the three-dimensional structure and are tentatively assigned to the sites shown.

d indicates amino acid changes which create a potential carbohydrate attachment sequences NXS/T

Table II Location of nucleotide and amino acid changes occurring between A/USSR/90/77 and subsequent field strains.

Nucleotide[a] Change	Position	Amino acid[a] Change	Position	Virus strain[b]	Antigenic site[d]	Location on molecule[c]
A - G	250	N - S	65	BEI	-	buried
A - T	446	R - S	125c	EI	?Sa	surface
G - A	465	V - I	132	B	-	surface
G - A	543	E - K	157	EI	?Sa/Sb	surface
A - G	562	N - S[e]	163	E	Sa	surface
G - A	639	E - K	189	L	?Sb	surface
G - A	642	D - N	190	I	-	?surface
A - C	696	N - H	208	EI	?Ca	surface
G - A	729	E - K	219	BEI	?Ca	surface
G - A	748	G - D	225	L I	Ca	surface
C - A	754	A - E	227	BEI	?Ca	surface
C - T	840	H - Y	256	LBEI	-	buried
A - G	856	N - S	260a	LBEI	-	?buried
A - G	912	T - A	279	EI	-	surface
A - G	966	I - V	297	LBEI	-	buried

a The nucleotide and amino acid changes are shown relative to the USSR/77 sequence; thus, virus strains L, B, E and I have G at nucleotide 966 rather than an A found at that position in USSR/77. The corresponding amino acid (at residue 297) has therefore undergone change from I to V. Silent nucleotide changes are omitted.

b L, B, E and I are further abbreviations for Lack/78, Braz/78, Eng/80 and Ind/80 respectively.

c indicates the location whether buried or surface on the three-dimensional structure.

d see footnote c to Table I.

e a mutation which results in a loss of a potential carbohydrate attachment site.

strains. Ind/80 differs from Eng/80 at three residues: residue 225 (Ca site), residue 163 (Sa site, which results in the loss of a carbohydrate attachment site in Eng/80) and at residue 190. This latter residue although superficial and close to the Sb site points inwards towards the molecule in the three-dimensional structure (12). Although this residue is unlikely to make direct contact with antibody molecules, this mutation may nevertheless affect antibody binding to adjacent superficial residues.

DISCUSSION

Evolution of the haemagglutinin

A comparison of the amino acid sequence of the HA1 subunit of the haemagglutinin of USSR/77 with that of PR8/34 indicates a 13% evolutionary

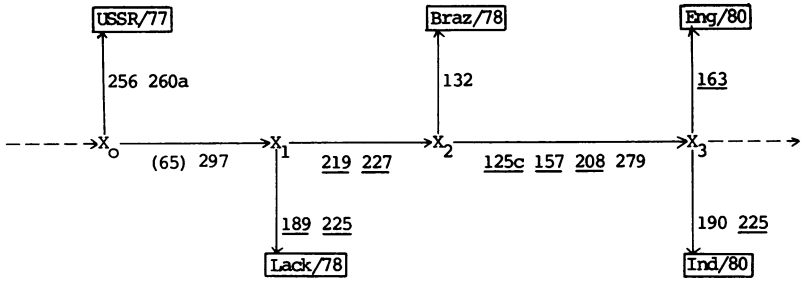

Figure 3 The evolution of recent epidemic H1 subtype virus strains. The numbers refer to the amino acid residues which have changed during the evolution of virus strains. X_0, X_1, X_2 and X_3 are proposed intermediates during the evolutionary pathway (see text). Since residue 65 is unknown for Lack/78, it is bracketed. Antigenic residues are underlined (see also Table I).

drift, in all cases involving single amino acid substitutions. Because of the 'laboratory drift' (14), well documented for the haemagglutinin of PR8/34 (5) and which occurs presumably due to passaging of strains since their isolation, we must regard this figure as approximate. Indeed, a recent independent sequence of the identical USSR/77 strain (15) differed in the amino acid sequence at position 157, despite the fact that there has been less time for laboratory drift of USSR/77 than for PR8/34. It has been shown previously on the basis of serological and limited sequence analysis that USSR/77 is closely related to a virus strain A/FW/1/50 which circulated in 1950 (16-19). The estimate of 13% drift between PR8/34 and USSR/77 corresponds to a rate of drift of approximately 0.8% per year between 1934 and 1950, which is similar to the observed rate of 0.9% per year during the evolution of antigenically drifted variants of the H3 subtype virus (2,20). Influenza viruses of different subtype probably undergo relatively constant rates of evolution during circulation in man.

The proposed evolutionary pathway of the recent field strains from 1977-1980 is shown in Figure 3. But considering evolution of these H1N1 subtype virus strains over the longer period of 1934 to 1980, there are two examples of sequential change of amino acid at a single defined residue; at residue 132 a change from T → V → I occurs between PR8/34, USSR/77 and Braz/78 and at residue 163 a change from K → N → S occurs in PR8/34, USSR/77 and Eng/80. Also, there are four examples, at residues 125c, 157, 189 and 225 of residues which change from PR8/34 to USSR/77 which revert in at least one of the subsequent virus strains. While we cannot formally exclude that these

reversions are "apparent" rather than real, representing alternative evolutionary pathways, we find no evidence for intermediates in any alternative scheme to that of Figure 3 which could support this formal alternative. Indeed, one of the reversions to an amino acid characteristic of PR8/34 at residue 225 appears to have occurred independently in both Lack/78 and Ind/80 (Figs. 2 and 3). Reversions, and sequential evolution, have been previously noted in studies of the evolution of the haemagglutinin among field strains of H3 subtype (2, 21, 22). Both phenomena reflect the scope for variation, which is a fundamental property of the haemagglutinin molecule.

Antigenic variation

Many of the residues found changed between PR8/34 and USSR/77 and within the post-1977 virus strains can be assigned to antigenic sites on the basis of the antigenic map produced for PR8/34 by analysis of antibody-selected viruses with monoclonal antibodies (5). Without this *in vitro* map designations of residues to antigenic sites would not have been possible for these field strains. This observation supports the previous identification of 4 main antigenic sites for PR8/34 and extends it to other H1N1 subtype virus strains. Furthermore, it suggests that the immune recognition of the haemagglutinin in man is similar to that of the mouse system used in model experiments, and supports the view that antibody selection plays a crucial role during evolution of new virus strains in the field.

The three amino acid differences between the most recently studied strains, i.e. Eng/80 and Ind/80, occur at residues 163, 190 and 225 (Table II and Figure 3). It is therefore tempting to speculate that the apparent differences in the epidemiological success of these strains (23) is related to one or more of these amino acid substitutions. Firstly, residue 225 is part of the antigenic site defined as the Ca site. Secondly, the apparent relative success of Eng/80 may result from the change of amino acid at residue 190 in Ind/80. Wilson *et al* (12) have proposed that residue 190 constitutes part of the virus-host cell receptor binding site. Residue 190 is invariant as E (glu) in the H3 subtype, the H2 subtype and in one of the H1N1 subtype strains characterised so far (4, 24, 25), and is D (asp) for the other H1 subtype virus strains characterised except for Ind/80, where it is N (asn) (5, 11). Both E and D are similar charged residues and the isolation of a virus strain which has undergone mutation to an uncharged residue weakens the hypothesis that this residue is involved in receptor binding.

Lastly, the different epidemic properties of Eng/80 and Ind/80 may be due to the mutation observed in the major antigenic site Sa at residue 163 in Eng/80. This mutation abolishes a potential carbohydrate attachment site found in USSR/77 and the other strains studied here. Since it has been suggested previously that carbohydrate attachment modulates the antigenic structure of the haemagglutinin (3,5), it is possible that the loss of carbohydrate at residue 163 during the evolution to Eng/80 acted as a major antigenic change in this strain and conferred a selective advantage for this emerging strain over the other circulating virus strains.

The epidemic properties of individual virus strains are likely to result from a combination of factors affecting the different stages of virus replication. Nonetheless, it is widely accepted that structural changes in the haemagglutinin play a key role in the development of epidemics of influenza in man (1). While it was true for the winter of 1981/82 that Eng/80-type strains appeared to be predominant as compared with Ind/80-like viruses (23), a final assessment of epidemiological "success" must await a study of the further evolution of H1 strains and their sequence analysis.

MATERIALS AND METHODS

Virus purification and preparation of viral RNA

Virus strains were received at WHO Collaborating Centres (London and Atlanta) and were antigenically representative of many individual viral isolates grown either in hens' eggs or in monkey kidney cells. Viral stocks were amplified by growth in eggs followed by the isolation of viral RNA (5). We cannot exclude that a variant of the original isolate was studied here because of its selection during the egg passage, as has been suggested from recent studies in influenza B strains (26).

RNA sequence determination

RNA sequence analysis by the dideoxy-chain termination method was performed essentially as described before (5) using four deoxyoligonucleotide primers d(TAAAAACAACCAAAATG), d(AATCATGGTCCTACATTG), d(TTTTACAGAAATTTGCTA) and d(CAATAATATTTGAGGCAA), designated respectively primers I-IV and complementary to nucleotide residues 19-35, 304-321, 513-530 and 799-816 of the haemagglutinin gene of influenza A/PR/8/34 (11). The reaction was modified slightly in that reaction mixtures were incubated for 15 minutes at 37°C, then mixed with 1 µl of "chase" mixture containing 56mM KCl, 40mM Tris-HCl (pH 8.3), 8mM $MgCl_2$, 16mM β-mercaptoethanol, 1.5mM dATP and 0.55 units of

reverse transcriptase (Life Sciences Inc.) and incubated for a further 15 minutes at 37°C in sealed glass capillaries. Reactions were terminated and the products separated by gel electrophoresis as before (5).

Residues 36-46 were determined by 5' phosphate labelling of primer I using γ-^{32}P-ATP and polynucleotide kinase and then extending this primer in a reaction containing viral RNA, dATP, dCTP, dGTP and ddTTP and reverse transcriptase. Reaction products were separated by electrophoresis on a denaturing 20% polyacrylamide gel and the major radioactive band (28 bases long) was eluted and its sequence determined by chemical degradation (10).

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