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The Mechanism of Antigenic Drift in Influenza Virus: Sequence Changes in the Haemagglutinin of Variants Selected with Monoclonal Hybridoma Antibodies [and Discussion]

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The mechanism of antigenic drift in influenza virus: sequence changes in the haemagglutinin of variants selected with monoclonal hybridoma antibodies

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Antigenic variants of the A/PR8 (H0N1) and A/Hong Kong/68 (H3N2) strains of influenza virus were isolated after a single passage of these viruses in the presence of monoclonal hybridoma antibodies to the haemagglutinin.

Hyperimmune rabbit antisera reacted (in haemagglutination-inhibition tests) to high titre with both wild-type and variant viruses, but the monoclonal antibodies, which reacted with the wild-type virus to titres of the order of $1/10^5$ did not react at all (or to very low titre) with the variants that they selected.

This suggests that the changes occurring in the monoclonal variants are restricted to a single antigenic site out of many on the haemagglutinin molecule.

Amino acid analysis of the soluble tryptic peptides from the haemagglutinin 'spikes' of wild-type and variant viruses suggest that the dramatic loss in the ability of the variants to bind the monoclonal antibody used in their selection is associated with a single change in the amino acid sequence of the large haemagglutinin polypeptide, HA_1.

For PR8 virus, eight out of ten variants selected with one monoclonal antibody showed the same sequence change of serine to leucine in the HA_1 polypeptide. The change in the other two variants was not determined. No sequence data on PR8 haemagglutinin are available, so the experiments were continued with a Hong Kong (H3N2) strain where much of the sequence of HA_1 and HA_2 is known.

Three different monoclonal hybridoma antibodies to A/Mem/1/71 (H3N2) haemagglutinin were used to select a total of ten variants of this virus.

Variants selected with one monoclonal antibody were not recognized by the other two monoclonal antibodies as being different from wild-type virus, suggesting that the three antibodies bound to different sites on the surface of the haemagglutinin molecules. Each of the variants occurred with a frequency of about 1 in 10^5 in the wild-type virus.

One group of our variants selected with H14/A2 monoclonal antibody showed the same antigenic properties and the same sequence change (asparagine to lysine) in the N-terminal half of HA_1. Of three variants selected with H14/A20, two showed a different change at a locus also in the N-terminal region of HA_1 (a proline was replaced by serine in one variant and by leucine in the other).

Of the other three variants (selected with H14/A21 monoclonal antibody) one showed a change in HA_1 of serine to tyrosine. This change occurred in residue number 37 of cyanogen bromide fragment 2 (CN2). In the other two variants the change in HA_1 has not been determined, but in these a tryptic peptide comprising residues 49–56 of CN2 was missing.

The tryptic peptides of the HA_1 polypeptide, showing changes in the variants selected with monoclonal antibodies, were also found to undergo sequence changes in

naturally occurring Hong Kong variants isolated from man. In each case, however, the sequence changes in the monoclonal variants were different from those in the field strains.

No changes were found in the HA_2 polypeptide from any of the variants.

Introduction

Antigenic variation in the haemagglutinin 'spikes' of influenza virus is a major obstacle to the development of an effective vaccine against influenza. This variation is of two kinds, a slow gradual series of changes over a period of several years, termed antigenic drift, and sudden and complete changes – the major antigenic shifts – in which 'new' influenza viruses suddenly appear in the human population with haemagglutinin and sometimes neuraminidase antigens that are antigenically quite unrelated to those of viruses infecting man just before the new virus appeared.

These two kinds of variation seem to be caused by entirely different molecular mechanisms (for a review see Webster & Laver 1975). This article will consider the mechanism of drift.

Antigenic drift is thought to be due to the selection, by an immune population, of mutant virus particles having altered antigenic determinants and which therefore possess a growth advantage in the presence of antibody.

Peptide mapping experiments have shown that changes in amino acid sequence of the polypeptide chains of the haemagglutinin occur during natural antigenic drift and antigenic mutants isolated *in vitro* by selection with antibody have also been found to have changes in the amino acid sequence of the polypeptides of the haemagglutinin (HA) molecules (Laver & Webster 1968; Moss & Underwood 1978). Haemagglutinin molecules from viruses of different subtypes differ greatly in amino acid sequence (Ward & Dopheide 1979; Waterfield *et al.* 1979) but it is likely that many of these changes are unrelated to the antigenic differences between the haemagglutinins. Analysis of natural variants may therefore not reflect the differences in antigenic determinants. By using monoclonal antibodies, we have been able to select variant viruses in which the changes in sequence of the haemagglutinin polypeptides have a better chance of being restricted to those affecting the determinant recognized by that particular monoclonal antibody, changing it in such a way that it can no longer 'fit' the corresponding combining site on the antibody. These viruses have a selective growth advantage, and their haemagglutinin molecules can be isolated and analysed.

Gerhard (1978), using monoclonal antibodies to A/PR8/34 (H0N1) haemagglutinin, was able to distinguish 40–50 (groups of) antigenic determinants on the haemagglutinin, but whether these were discrete sites or overlapping domains is not known.

Hybridomas producing monoclonal antibodies to haemagglutinin molecules from A/PR8/34 (H0N1) and A/Hong Kong/68 (H3N2) have been used to select antigenic variants of these viruses (Gerhard & Webster 1978).

We have isolated haemagglutinin molecules from the wild-type and variant viruses and determined the changes in amino acid sequence associated with the altered capacity of the haemagglutinin to combine with antibody.

This paper describes sequence changes found in some of these variants.

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Materials and methods

Antiviral antibodies

The production of continuous cell lines (hybridomas) secreting anti-viral antibodies has been described (Koprowski *et al.* 1977). Hybridoma cell cultures were maintained in Dulbecco's modified Eagle's minimum essential medium containing 75 g/l foetal calf serum, 75 g/l agamma horse serum (Flow Laboratories, Rockville, Maryland), 50 μg/ml gentamycin and 2 mM glutamine. The hybridoma cell lines producing antibodies to the haemagglutinin and used for the selection of virus variants were grown in ascitic form in BALB/c mice.

Viruses

Influenza viruses A/PR8/34 (H0N1) and A/Memphis/1/71 (H3N2) were used for the production of variants. (In the latter case, a recombinant having A/BEL/42 (H0N1) neuraminidase was used.) These are abbreviated in the text to PR8 and MEM/71 (MEM/71 virus has not been distinguished antigenically from A/Hong Kong/68 virus). The production of variants was done as described previously (Gerhard & Webster 1978). Briefly, monoclonal hybridoma antibody plus cloned parent virus were incubated together for 30 min at 20 °C. This mixture was inoculated into embryonated hens' eggs 11 days old. The viruses that grew in the presence of most concentrated monoclonal antibodies were harvested and 'cloned' twice at limiting dilutions in embryonated hens' eggs 10 days old.

All viruses were purified by adsorption to and elution from chicken erythrocytes, followed by differential centrifugation and sedimentation through a sucrose gradient (10–40% sucrose, 0.15 M NaCl) (Laver 1969). Haemagglutination (HA) titrations and haemagglutination-inhibition (HI) tests were done as described (Fazekas de St Groth & Webster 1966).

Estimation of the frequency of occurrence of antigenic variants in wild-type virus

Allantoic fluid from embryonated eggs infected with cloned A/Mem/1/71 virus and having an infectivity titre of $10^{8.37}$ e.i.d.$_{50}$ was diluted 1:10 and then serially in 0.5 $\log_{10}$ steps. Monoclonal hybridoma antibody with an HI titre of $1/10^5$ was added to each dilution of virus (antibody dilution 1:10) and the mixtures were inoculated into groups of five embryonated eggs. Infectivity titres were calculated according to Spearman-Kärber (Dougherty 1964).

Viruses which grew at the end point were shown to be variants in HI tests with monoclonal antibodies.

Isolation of the haemagglutinin molecules and separation of the HA_1 and HA_2 polypeptides

The virus particles were disrupted with sodium dodecyl sulphate (SDS) and the haemagglutinin molecules were isolated by electrophoresis of the SDS-disrupted virus particles on cellulose acetate strips (Laver 1964). The haemagglutinin molecules were dissolved in saturated guanidine hydrochloride solution containing dithiothreitol, and the heavy and light polypeptides (HA_1 and HA_2 were separated by centrifugation on a guanidine hydrochloride density gradient (Laver 1971)).

Peptide maps

The separated HA_1 and HA_2 polypeptides were *S*-carboxymethylated (Air & Thompson 1969), dialysed against distilled water and then precipitated by the addition of three volumes of ethanol.

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The HA_1 and HA_2 polypeptides were digested with trypsin (TPCK-treated, Worthington Biochemicals) and the tryptic peptides soluble at pH 6.5 were mapped by two-dimensional electrophoresis and chromatography on large sheets of Whatman no. 3 MM paper (Laver *et al.* 1974).

Peptides were located by staining with fluorescamine (10 μg/ml in acetone containing 0.5% pyridine (Udenfriend *et al.* 1972).

Amino acid analysis

Peptides were eluted from the maps with 6 M HCl, hydrolysed in sealed, evacuated tubes for 22 h at 105 °C, and analysed for amino acids by using a Beckman 119CL amino acid analyser. Tryptophan was qualitatively determined by staining duplicate maps with Ehrlich reagent.

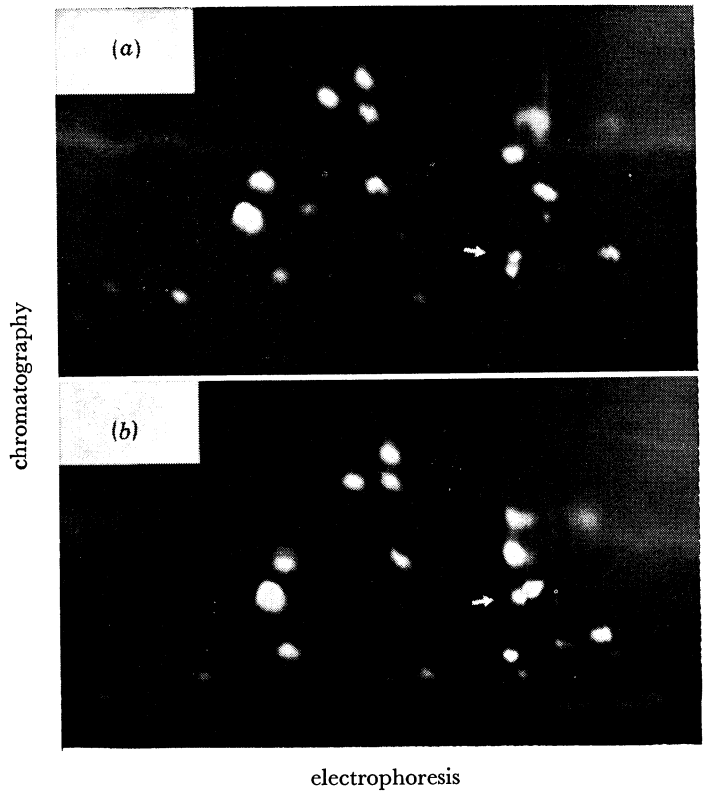

FIGURE 1. Maps of the tryptic peptides (soluble at pH 6.5) from HA_1 of (*a*) wild-type PR8 virus and (*b*) one of the antigenic variants selected with PEG-1 monoclonal hybridoma antibody. The maps were stained with fluorescamine. A single peptide difference among the neutral peptides (arrowed) was seen. In wild-type PR8, this peptide contained one residue each of serine, glutamic acid, proline, glycine, tyrosine and lysine. In the variant, the serine was replaced by leucine. No differences were found in any of the other peptides.

RESULTS

Variants of A/PR8 (H0N1) virus

Figure 1 shows the soluble tryptic peptides from HA_1 of wild-type PR8 virus and one of the antigenic variants selected with PEG-1 monoclonal antibody. A single changed neutral peptide was found, in which a serine residue in the wild-type was replaced by leucine in the variant. Amino acid analysis of the other peptides confirmed that this was the only change that occurred

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in the soluble peptides. Eight out of ten variants selected with PEG-1 monoclonal antibody showed this change in sequence, but its location in the HA_1 molecule is not known. Of the other two variants, one showed an additional neutral peptide on the map and the other showed the same map as the wild-type (Laver *et al.* 1979).

TABLE 1. CROSS-REACTIONS OF THE ANTIGENIC VARIANTS WITH THE MONOCLONAL ANTIBODIES USED FOR THEIR SELECTION

(HI titres† of the monoclonal antibodies with the antigenic variants of A/Mem/1/71 virus.)

monoclonal antibody to A/Mem/1/71(H)	wild-type	variants selected with H14/A2				variants selected with H14/A20			variants selected with H14/A21		
		V1	V2	V3	V4	V1	V2	V3	V1	V2	V3
H14/A2	4.1	2.4	2.4	2.4	2.4	4.1	4.1	4.1	4.1	4.1	4.1
H14/A20	4.8	4.8	4.8	4.8	4.8	<	<	<	4.8	4.8	4.8
H14/A21	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	<	<	<

<, Less than 1.7.
† Expressed as decadic logarithms.

TABLE 2. CROSS-REACTIONS OF THE ANTIGENIC VARIANTS WITH A PANEL OF MONOCLONAL ANTIBODIES TO A/MEM/1/71 HAEMAGGLUTININ

(HI titres† of monoclonal antibodies and the variants.)

monoclonal antibody preparation	wild-type	variants selected with H14/A2				variants selected with H14/A20			variants selected with H14/A21		
		V1	V2	V3	V4	V1	V2	V3	V1	V2	V3
Mem 25/1	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	<	<	<
Mem 196/4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	<	<	<
H14/B18	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	2.5	<	2.5
Mem 200/2	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	4.4	3.2	4.4
HK 30/2	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.4	4.4	4.4
Mem 93/1	4.1	4.1	4.1	4.1	4.1	<	<	<	4.1	4.1	4.1
27/2	5.0	5.0	5.0	5.0	5.0	5.0	<	<	5.0	5.0	5.0
126/2	4.4	4.4	4.4	4.4	4.4	4.4	<	<	4.4	4.4	4.4
212/1	5.1	5.1	5.1	5.1	5.1	5.1	2.6	<	5.1	5.1	5.1
123/4	4.7	4.7	4.7	4.7	4.7	4.7	3.8	3.0	4.7	4.7	4.7
12 other monoclonal antibody preparations	+	+	+	+	+	+	+	+	+	+	+

+, Titres for wild-type and variants were identical.
<, Less than 1.7.
† Expressed as decadic logarithms.

Antigenic properties of variants of A/Mem/1/71 (H3N2) virus

Three different monoclonal hybridoma antibodies to A/Mem/1/71 haemagglutinin, designated H14/A2, H14/A20 and H14/A21, were used to select antigenic variants of this virus. The reduction in the ability of the variants to react with the antibody used for their selection was dramatic (table 1). Haemagglutination-inhibition titres of the monoclonal antibody H14/A21, for example, were $1/10^5$ for wild-type virus and less than 1/50 for the variants isolated after a single passage in embryonated eggs. Variants selected with one monoclonal antibody were inhibited in HI tests to the same titre as wild-type virus when tested with the other two monoclonal antibodies, suggesting that the sites recognized by these three monoclonal antibodies are different and independent regions on the haemagglutinin molecule.

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Other monoclonal antibodies to A/Mem/1/71 haemagglutinin were, however, able to discriminate between some of the variants selected with the same monoclonal antibody (table 2). This may be because a conformational change induced by an amino acid substitution affecting one antigenic site can also affect others, or because some amino acids may be part of two (or more) sites.

This suggests that not all of the antigenic sites on the haemagglutinin are discrete but that considerable overlapping of some sites may occur.

TABLE 3. FREQUENCY OF ANTIGENIC VARIANTS IN WILD-TYPE VIRUS

infectivity titre (decadic logarithms) of A/Mem/1/71 virus in the presence of:

	monoclonal hybridoma antibodies			
normal mouse ascites fluid	H14/A2	H14/A20	H14/A21	mixture of H14/A2 and H14/A20†
7.37	2.33	2.37	2.33	<

<, No infectious particles detected.

† Other combinations of the three monoclonal antibodies also neutralized every virus particle in the preparation of wild-type Mem/71.

Frequency of occurrence of antigenic variants in the wild-type virus

The proportion of variant virus particles in a population of wild-type A/Mem/1/71 virus having alterations in a single antigenic site was estimated by measuring the infectivity of wild-type virus in the presence of excess amounts of each of the monoclonal antibodies.

The results (table 3) showed that wild-type Mem/71 virus which had an infectivity titre of $10^{7.37}$ e.i.d.$_{50}$, when assayed in the presence of normal mouse ascites fluid, had a titre of $10^{2.3-2.4}$ e.i.d.$_{50}$ in the presence of the monoclonal antibodies. In the presence of mixtures of the monoclonal antibodies the infectivity decreased to undetectable levels.

These results suggest that the proportion of variant virus particles in the preparation of Mem/71 virus that had alterations in the antigenic sites recognized by these three particular monoclonal antibodies was of the order of 1 in 10^5 in each case.

Sequence changes in the haemagglutinin polypeptides of the Mem/71 variants

The dramatic change in antigenic activity of the variants which enabled them to escape completely from the antibody used for their selection was associated with changes in sequence of the large polypeptide (HA_1) of their haemagglutinin 'spikes'. In seven out of ten variants a single amino acid change was found in the soluble tryptic peptides. (In the other three variants the change has not yet been characterized.)

Sequence changes in HA_1 of variants selected with the monoclonal antibodies

(i) *H14/A2 variants*

Peptide maps of the four variants selected with this antibody showed a single peptide difference from the wild-type (figure 2). Peptide 11 on the wild-type map was missing and a new peptide appeared on the variant map. Peptide 11 from wild-type Mem/71 contained histidine, arginine, CM-cysteine, two asparagine residues (Asn rather than Asp from the mobility of peptide 11), proline and isoleucine. The new peptide from the variant had the same composition, except that an asparagine residue was replaced by lysine. The composition of wild-type

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peptide 11 is the same as a peptide of known sequence, Ile-Cys-Asn-Asn-Pro-His-Arg located in the N-terminal cyanogen bromide fragment, CN1, of A/Mem/102/72 HA_1 (C. W. Ward & T. A. Dopheide, unpublished). Because the new peptide was not split by trypsin, it is likely that it was the asparagine residue adjacent to the proline that was replaced by lysine in the variant.

Each of the four variants selected with H14/A2 antibody showed the same change in peptide 11.

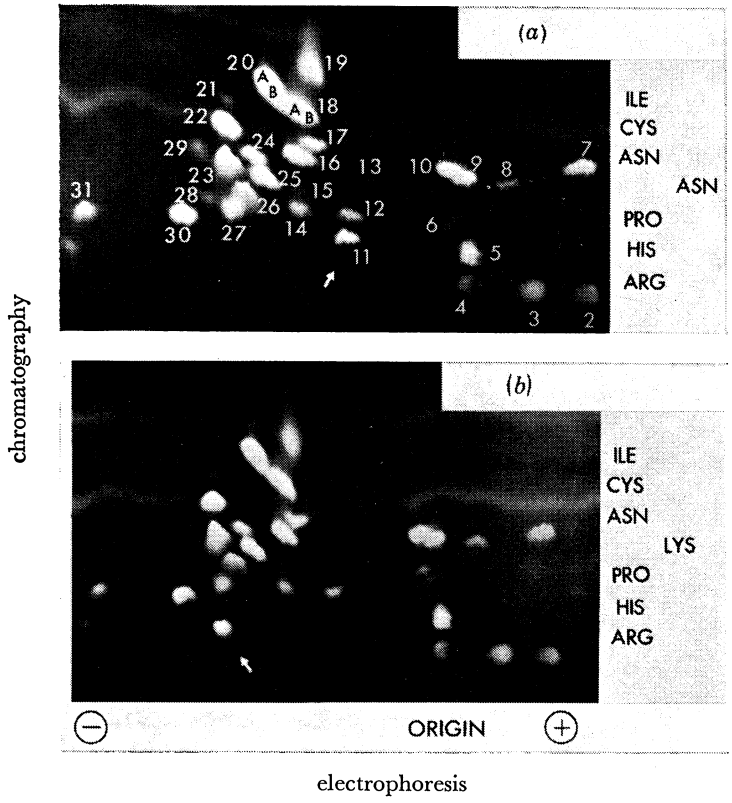

FIGURE 2. Maps of the tryptic peptides (soluble at pH 6.5) from HA_1 of (*a*) wild-type Mem/71 virus and (*b*) one of the four variants, variant 3, selected with H14/A2 monoclonal hybridoma antibody. The maps were stained with fluorescamine. A single peptide difference (peptide 11, arrowed) was seen on the maps. The sequence of this peptide from the wild-type and variant is shown. The other three variants selected with H14/A2 monoclonal antibody showed the same change (asparagine to lysine) in peptide 11. No differences were found in any of the other peptides.

(ii) *H14/A20 variants*

Of the three variants isolated with H14/A20 monoclonal antibody, one (V1) grew very poorly and the change in this variant has not yet been determined. The other two variants (V2 and V3) were analysed and each showed a change in the sequence of peptide 17 (numbering of the peptides for wild-type Mem/71 is shown in figure 2).

In wild-type Mem/71, peptide 17 is assumed to have the sequence Gly-Pro-Gly-Ser-Gly-Phe-Phe-Ser-Arg since it has the same composition except for one residue (position 3) as a peptide of known sequence, Gly-Pro-Asp-Ser-Gly-Phe-Phe-Ser-Arg, located in the N-terminal cyanogen bromide fragment, CN1, of the related field strain A/Mem/102/72 (Ward & Dopheide, unpublished).

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In variant 2 the proline in peptide 17 was replaced by serine while in variant 3 the proline was replaced by leucine.

(iii) *H14/A21 variants*

Three variants were analysed. Variant 1 showed a change in peptide 16, which differs only slightly in composition (arginine instead of lysine) from a peptide of known sequence Val-Thr-Val-Ser-Thr-Lys found at position 34–39 in the second cyanogen bromide fragment, CN2, of A/Mem/102/72 HA_1 (Ward & Dopheide 1979).

In variant 1, the serine at position 37 was replaced by tyrosine. In variants 2 and 3 this change did not occur and peptide 16 had the same composition as in the wild-type. Instead, peptide 23 (and an associated cleavage product, peptide 24) did not appear on the peptide maps of these variants. Peptide 23 in Mem/71 has the same composition as a peptide of known sequence, Ile-Gly-Ser-Arg-Pro-Trp-Val-Arg, which occupies residues 49–56 in cyanogen bromide fragment 2 (CN2) of A/Mem/102/72 HA_1 (Ward & Dopheide 1979). This peptide is situated only 11 residues away from residue 37 of CN2, which changed from serine to tyrosine in variant 1, but so far the sequence change responsible for the disappearance of peptide 23 (and 24) from the maps of variants 2 and 3 has not been determined.

H14/A2 VARIANTS	1	Ile	Cys	Asn	Lys	Pro	His	Arg
	2	Ile	Cys	Asn	Lys	Pro	His	Arg
	3	Ile	Cys	Asn	Lys	Pro	His	Arg
	4	Ile	Cys	Asn	Lys	Pro	His	Arg
FIELD STRAINS HK/68		Ile	Cys	Asn	Asn	Pro	His	Arg
MEM/71		Ile	Cys	Asn	Asn	Pro	His	Arg
ENG/72		Ile	Cys	Asn	Asn	Pro	His	Arg
MEM/72		Ile	Cys	Asn	Asn	Pro	His	Arg
PC/73		Ile	Cys	Asn	Asn	Pro	His	Arg
VIC/75		Ile	Cys	Asp	Asn	Pro	His	Arg
TEX/77		Ile	Cys	Asp	Ser	Pro	His	Arg

FIGURE 3. The amino acid sequence of peptide 11 (figure 2) from HA_1 of Mem/71 virus, the four antigenic variants selected with H14/A2 monoclonal antibody and naturally occurring antigenic variants (field strains) of A/Hong Kong/68 virus. The sequence of peptide 11 was determined for the A/Mem/102/72 strain. The sequence of this peptide from the other viruses was deduced from composition data.

Sequence changes in the monoclonal variants compared with changes in field strains

Similar experiments to those described above have been done by using the following naturally occurring antigenic variants of A/Hong Kong/68 virus: A/England/42/72, A/Memphis/102/72, A/Port Chalmers/73, A/Victoria/75 and A/Texas/1/77. We have found that the peptides from HA_1 of the monoclonal variants, having the sequence changes described, are also peptides that show changes in the field strains but in each case the amino acids which change are different for the monoclonal variants and the field strains. Thus, the asparagine residue at position 4 in peptide 11 of Hong Kong/68 virus, which changed to lysine in the four H14/A2 variants, remained unchanged in the field strains until 1977 when, in Texas/77, it changed to serine. The asparagine at position 3 in peptide 11 of Hong Kong/68 changed to aspartic acid in Victoria/75. In peptide 17, changes occurred in every one of the field strains examined, but the change at position 2 (proline to serine or leucine) found in the H14/A20 variants was never

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found in any of the field strains. In peptide 16, changes also occurred, but the residue at position 4 in Hong Kong/68, which changed from serine to tyrosine in H14/A21 variant 1, did not change in any of the field strains examined.

These results are shown in figures 3–5.

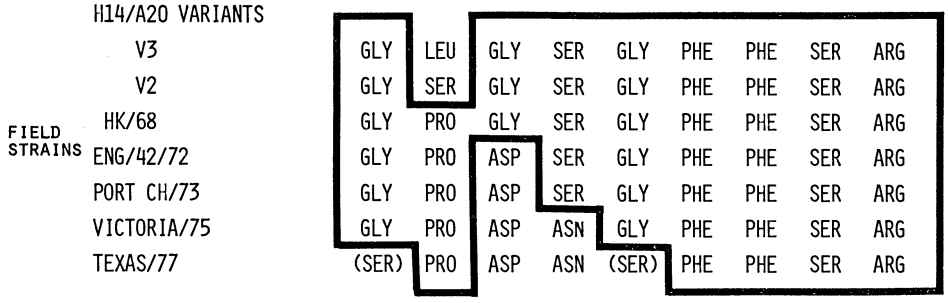

FIGURE 4. The amino acid sequence of peptide 17 (figure 2) from HA_1 of variants 2 and 3 of Mem/71 virus selected with the monoclonal hybridoma antibody, H14/A20 and naturally occurring antigenic variants (field strains) of A/Hong Kong/68 virus. The sequence of peptide 17 was determined for the A/Mem/102/72 strain. The sequence of this peptide from the other viruses was deduced from composition data. It is not known which of the two glycine residues in Victoria/75 was replaced by serine in Texas/77.

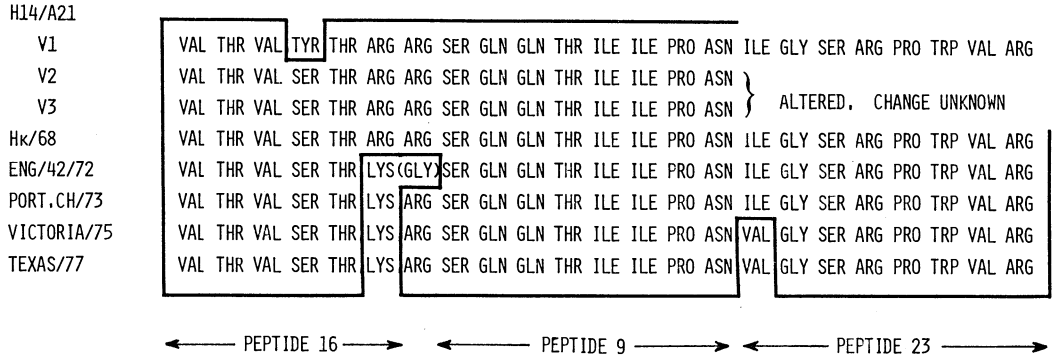

FIGURE 5. The amino acid sequence of peptides 16, 9 and 23 (figure 2) from HA_1 of the variants of Mem/71 virus selected with H14/A21 monoclonal antibody and naturally occurring antigenic variants (field strains) of A/Hong Kong/68 virus. The sequence of these peptides was determined for the A/Mem/102/72 strain. Sequences for the other strains were deduced from composition data. Peptide 23 was missing from the maps of HA_1 from variants 2 and 3, but the change in sequence responsible for the disappearance of this peptide is not known.

No sequence changes found in the HA_2 polypeptide

The soluble tryptic peptides from the HA_2 polypeptide of A/Mem/1/71 virus and antigenic variants of this virus selected with A H14/A2, H14/A20 and H14/A21 monoclonal antibodies were mapped and analysed in the same way as the HA_1 peptides.

No differences were found between the wild-type and variant HA_2 soluble peptides.

DISCUSSION

Antigenic variation in the haemagglutinin 'spikes' of influenza viruses is a major obstacle in the development of an effective vaccine against the disease. The aims of the work described here are to determine the number of different antigenic sites on the haemagglutinin 'spikes' of

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influenza virus, the chemical structure of these sites and the way in which they change during antigenic drift.

We use the term 'antigenic site' in the sense in which it is used by Atassi & Smith (1978). An antigenic site comprises those amino acids that are involved in reaction with antibody. These amino acids may, of course, be widely separated in the primary structure of the polypeptide chain, but are brought into close association by folding of the protein.

In those proteins (myoglobin and lysozyme) for which the antigenic structure has been defined (Atassi 1977; Atassi & Lee 1978), the antigenic sites have been found to be small in size and to have precise boundaries. In lysozyme, three sites could be distinguished. Each site contained five or six amino acids that were close together on the surface of the molecule, but which were often very distant in the primary sequence.

Little information is available concerning the number, nature and location of the antigenic sites on the haemagglutinin molecules. Two groups of antigenic determinants on the haemagglutinin, strain-specific and common or cross-reacting, have been described (Laver *et al.* 1974; Virelizier *et al.* 1974).

Observations in the electron microscope of antibody molecules bound to isolated haemagglutinin molecules showed both specific and cross-reacting determinants to be located on the side of the haemagglutinin spike, just below its tip. No antibody molecules appeared to bind anywhere else on the haemagglutinin (Wrigley *et al.* 1977).

Gerhard (1978) has produced monoclonal antibodies to the haemagglutinin of PR8 virus *in vitro*. These were able to discriminate 44 (groups of) determinants on the haemagglutinin molecules of a variety of H0N1 and H1N1 virus strains. It is not known whether these different determinants represent discrete areas on the haemagglutinin, or whether they are overlapping domains, but the availability of such monoclonal antibodies means that antigenic variation can now be monitored at the level of individual sites and this may lead to a better understanding of the mechanism of antigenic drift.

By using monoclonal antibodies, we have been able to select variant influenza viruses in which the changes in sequence of the haemagglutinin polypeptides were probably restricted to those affecting the site recognized by that particular monoclonal antibody, changing it in such a way that it could no longer 'fit' the corresponding combining site on the antibody molecules. These variants were not neutralized by the antibody, had a selective growth advantage, and their haemagglutinin molecules were isolated and analysed.

In the first series of experiments, variants of A/PR8 (H0N1) virus were selected by using PEG-1 monoclonal hybridoma antibody. Of ten variants selected with PEG-1, eight showed the same single change of serine to leucine in the sequence of HA_1 (Laver *et al.* 1979).

However, no amino acid sequence data for PR8 haemagglutinin are available and the change could not be assigned to a particular location in the molecule. For this reason we abandoned the PR8 variants and concentrated on variants of A/Hong Kong/68 (H3N2) virus for which extensive sequence data have been obtained for both HA_1 and HA_2 (Ward & Dopheide 1979).

We selected three groups of variants of Hong Kong (H3N2) influenza virus (the Mem/71 strain was used) with monoclonal hybridoma antibodies. Each of these antibodies probably recognized and bound to a single sharply defined site out of many on the surface of the haemagglutinin molecule.

Variants were selected after a single passage in eggs of the wild-type virus mixed with monoclonal antibody.

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The variants occurred with a frequency of about 1 in 10^5 in virus grown in embryonated eggs. Since infected allantoic fluid normally contains about 10^8 e.i.d.$_{50}$/ml, selection of the variants with monoclonal antibody was readily achieved. We also attempted to select variants by using a mixture of two different monoclonal antibodies. No variants could be selected in this way, which was not surprising since the frequency of occurrence of variants with changes in two independent sites would be of the order of 1 in 10^{10}.

The monoclonal antibodies reacted to high titre (in HI tests) with the wild-type virus, but not at all (or to very low titres) with the variants selected by that particular antibody. The reduction in HI titre was of the order of 10^5 fold. Variants selected with one of the monoclonal antibodies, however, reacted to the same titre as wild-type virus with the other two monoclonal antibodies. This suggested that the three monoclonal antibodies recognized three different sites on the surface of the haemagglutinin.

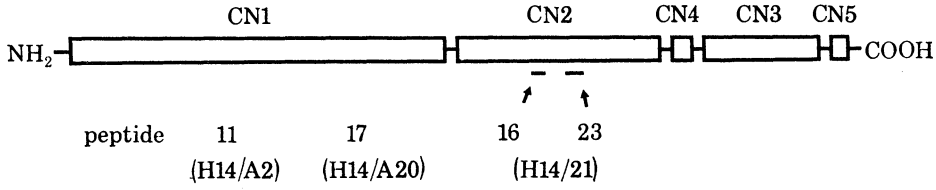

FIGURE 6. Diagram of the HA_1 molecule of A/Mem/102/72 showing the order of the five cyanogen bromide fragments and the location of the peptides in which sequence changes occurred in the variants selected with the three monoclonal antibodies. Peptides 16 and 23 occupy residues 34–39 and 49–56 respectively of CN2. Peptides 11 and 17 occur in CN1, but their exact position is not yet known.

Hyperimmune, heterogeneous antiserum raised in rabbits against wild-type haemagglutinin molecules reacted to high titre with both the monoclonal variants and wild-type virus in HI or immunodiffusion tests (Gerhard & Webster 1978).

Not more than one amino acid change was found when the polypeptides from the haemagglutinin 'spikes' of the variant viruses were analysed, suggesting that the dramatic loss in the ability of the variants to bind the monoclonal antibody used in their selection was associated with a single change in the amino acid sequence of the large haemagglutinin polypeptide, HA_1.

Of the four variants selected with H14/A2 monoclonal antibody, each showed the same sequence change (asparagine to lysine) in a peptide (11) derived from the N-terminal cyanogen bromide fragment (CN1) of HA_1. The precise location of the change will be known when the complete sequence of HA_1 has been determined. Of the three variants selected with H14/A20 monoclonal antibody, one showed a sequence change of proline to serine; in another the same proline was replaced by leucine. These changes occurred in peptide 17, also from CN1. The change in the third variant has not been characterized.

Of the three variants selected with H14/A21 monoclonal antibody, one showed a sequence change of serine to tyrosine in peptide 16. In the other two variants, peptide 23 was altered but the change in sequence is not yet known. Peptide 16 occupies residues 34–39 of cyanogen bromide fragment, CN2, and is separated from peptide 23 (which occupies residues 49–56 of CN2) by a nine-residue peptide that does not change in the monoclonal variants, or in any of the naturally occurring antigenic variants of Hong Kong influenza so far examined. The arrangement of the cyanogen bromide fragments and the location of the changed peptides is shown in figure 6.

We cannot be absolutely certain, however, that only a single change in sequence occurred

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in the haemagglutinin of the monoclonal variants. Only the soluble tryptic peptides were analysed. These contained approximately 250 amino acids which represented 76% of the HA_1 molecule, and undetected changes may have occurred in the insoluble peptides of HA_1 and possibly HA_2.

We have analysed the soluble tryptic peptides of HA_1 from a number of field strains that arose from Hong Kong/68 virus during antigenic drift. It was found that those regions of the haemagglutinin molecule that changed in the variants selected with monoclonal antibodies also changed during natural antigenic drift. In each case, however, the amino acids that changed in the monoclonal variants differed from those that changed in the field strains (figures 3–5).

We do not yet know whether the changes in sequence found in HA_1 of the variants selected with monoclonal antibodies actually occur in those five or six amino acids that make up the site recognized by that particular monoclonal antibody. It is possible that the sequence changes occur in a region of the haemagglutinin remote from the antigenic sites and induce conformational changes that alter the sites in such a way that they are no longer recognized by the antibody.

Bromelain-released haemagglutinin molecules have been crystallized and X-ray data are becoming available (Wiley & Skehel 1977), but in the absence of a full X-ray analysis of the HA molecule we have no idea where the changes occurring in the monoclonal variants are located in the three-dimensional structure.

Only one of the sequence changes in HA_1 is known precisely; residue 37 of cyanogen bromide fragment, CN2, changed from serine to tyrosine in variant 1 selected with H14/A21 monoclonal antibody.

Another change occurred close by in CN2 in the two other variants selected with H14/A21 antibody (figure 6). This change (which involved peptide 23) has not yet been characterized.

The peptides where changes occurred in the variants selected with the other two monoclonal antibodies, H14/A2 and H14/A21, are both located within the N-terminal cyanogen bromide fragment (CN1) but the sequence data for this region of HA_1 is incomplete and the exact position of these changes are not known (figure 6).

The three groups of variants selected with the three monoclonal antibodies were clearly different, both antigenically and in the sequence changes found (table 2, figures 3–5). The four variants within one group (H14/A2) could not be distinguished antigenically and each showed the same sequence change (asparagine to lysine). In the other two groups, however, variants within the group did show differences. In the variants selected with H14/A20 monoclonal antibody, V1 was easily distinguished, antigenically, from V2 and V3. These later two variants could also be distinguished antigenically from each other and each showed a different amino acid substitution (proline to serine in V2 and the same proline to leucine in V3). The change in V1 has not been characterized.

Some monoclonal antibodies could also differentiate between variants selected with H14/A21 monoclonal antibody. Antibodies H14/B18 and Mem/200/2 (table 2) not only recognized the three H14/A21 variants as different from the wild-type, but could also discriminate V2 from V1 and V3. No monoclone was found that would differentiate the latter two variants even though the sequence changes associated with them involved different peptides.

Some monoclonal hybridoma antibodies to Mem/71 haemagglutinin, which reacted to high titre with the wild-type virus, also reacted with some of the variants, but to a lower titre.

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These cross-reactions observed between different monoclonal antibodies and the variants suggest that the antigenic sites on the surface of the haemagglutinin may be overlapping, so that antibodies directed against one site will recognize some, but not all, of the amino acids occurring in another.

The variants occurred with a frequency of about 1 in 10^5 in wild-type virus and grew easily in the presence of a single monoclonal antibody. No variants grew in the presence of two different monoclonal antibodies. Natural antigenic drift must occur by the selection of variants in the presence of antibody. Since an immune individual would be expected to produce antibodies against many of the sites on the haemagglutinin molecule, it is difficult to see how any variants which arise in nature escape neutralization.

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Discussion

D. A. J. Tyrrell, F.R.S. Dr Feizi and her colleagues (1979) have recently studied naturally occurring monoclonal antibodies, namely anti-I myeloma proteins, and have shown that these might recognize different parts of the oligosaccharide chain which constituted the antigen. Similarly, a mouse myeloma protein might recognize a few amino acids in an antigenic site, but whereas one might be centred on a particular amino acid and thus be profoundly affected if it were replaced, another might 'see' a different part of the same chain in which the substituted amino acid lay in the periphery, so that it would react with the substituted site, though less well than if it were unsubstituted.

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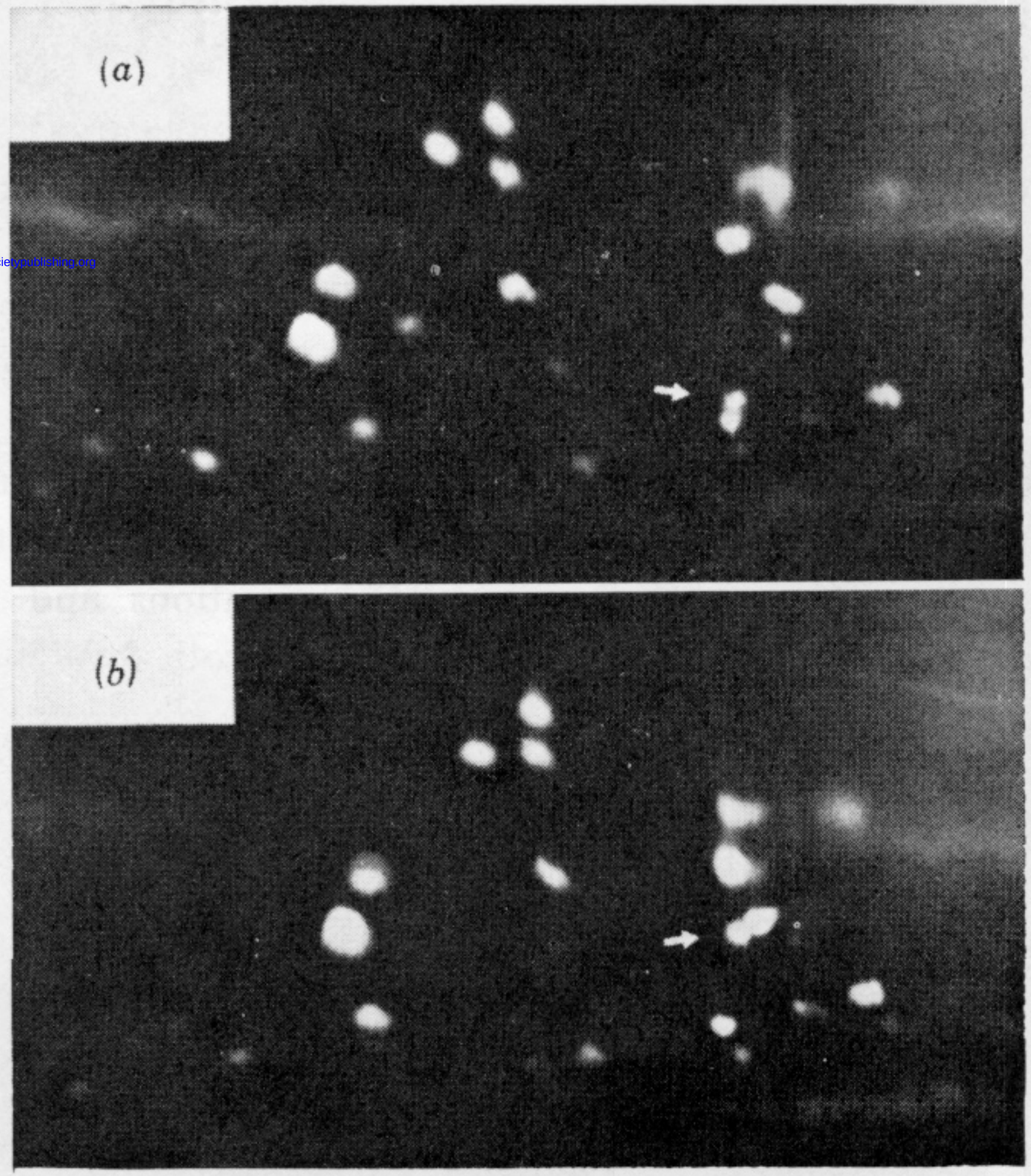

FIGURE 1. Maps of the tryptic peptides (soluble at pH 6.5) from HA_1 of (*a*) wild-type PR8 virus and (*b*) one of the antigenic variants selected with PEG-1 monoclonal hybridoma antibody. The maps were stained with fluorescamine. A single peptide difference among the neutral peptides (arrowed) was seen. In wild-type PR8, this peptide contained one residue each of serine, glutamic acid, proline, glycine, tyrosine and lysine. In the variant, the serine was replaced by leucine. No differences were found in any of the other peptides.

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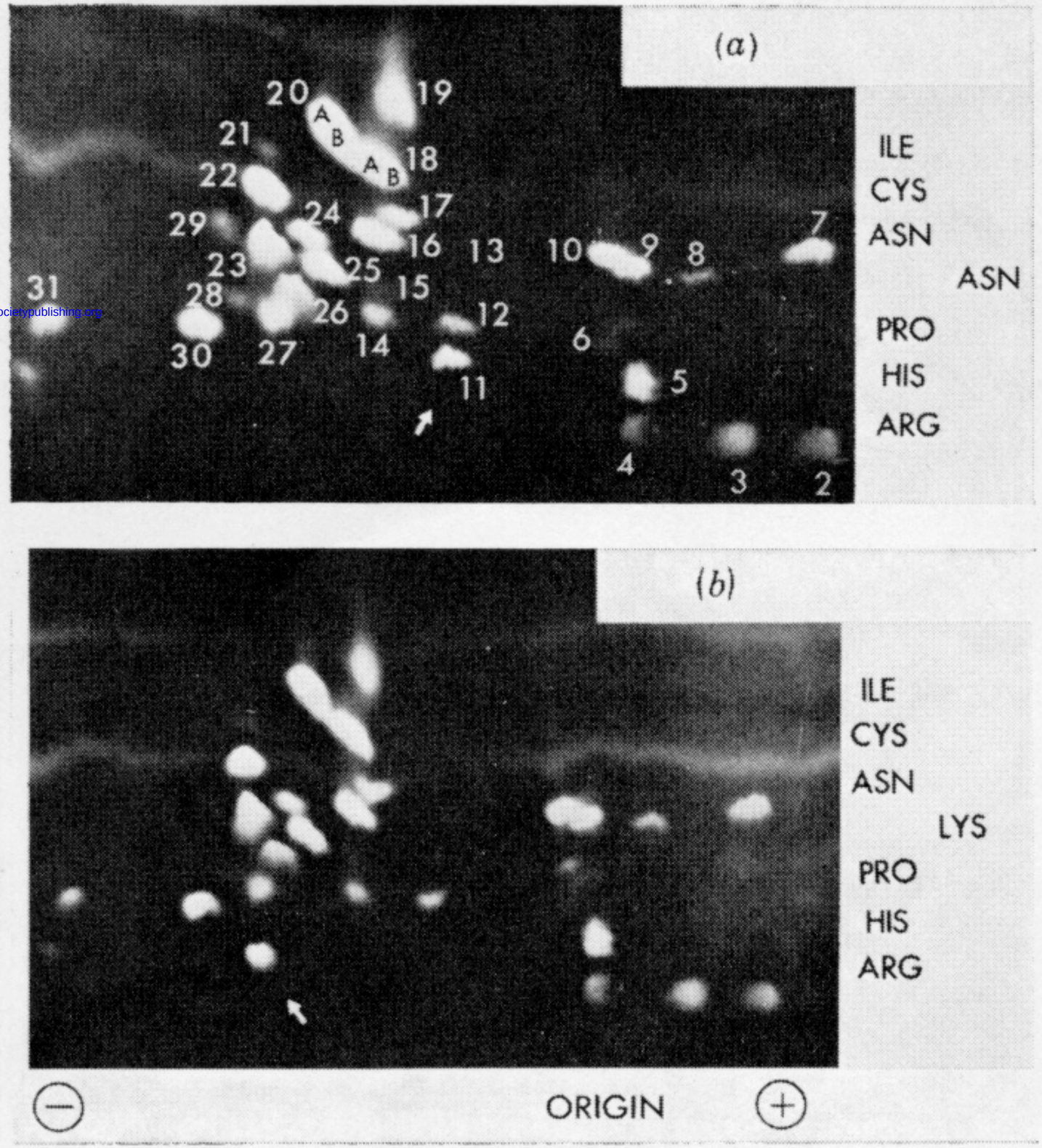

FIGURE 2. Maps of the tryptic peptides (soluble at pH 6.5) from HA_1 of (*a*) wild-type Mem/71 virus and (*b*) one of the four variants, variant 3, selected with H14/A2 monoclonal hybridoma antibody. The maps were stained with fluorescamine. A single peptide difference (peptide 11, arrowed) was seen on the maps. The sequence of this peptide from the wild-type and variant is shown. The other three variants selected with H14/A2 monoclonal antibody showed the same change (asparagine to lysine) in peptide 11. No differences were found in any of the other peptides.

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