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Antigenic drift in type A influenza virus: Peptide mapping and antigenic analysis of A/PR/8/34 (HON1) variants selected with monoclonal antibodies

(antigenic variants/hemagglutinin/peptide maps/amino acid changes)

W. G. LAVER*, W. GERHARD†, R. G. WEBSTER‡, M. E. FRANKEL†, AND G. M. AIR*

*Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia; †The Wistar Institute of Anatomy and Biology, 36th Street at Spruce, Philadelphia, Pennsylvania 19104; and ‡Division of Virology, St Jude Children's Research Hospital, P.O. Box 318, Memphis, Tennessee 38101

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ABSTRACT **Variants of A/PR/8/34 (HON1) influenza virus, having hemagglutinin molecules with probably a single altered antigenic determinant, were isolated by growing the virus in the presence of the monoclonal hybridoma antibody PEG-1. The variants were analyzed by peptide mapping and characterized antigenically by using PEG-1 and four other monoclonal hybridoma antibodies to PR8 hemagglutinin. Peptide maps of the large hemagglutinin polypeptide, HA1, from 8 out of 10 variants showed a single changed peptide. This peptide from two of the variants was analyzed, and in each case a serine residue in the wild-type hemagglutinin was replaced by leucine in the variant. Although these eight variants showed identical peptide maps, one could be discriminated antigenically from the others with one of the hybridomas. (The peptide maps represented about one-third of the HA1 molecule.) Of the other two variants, one gave the same HA1 map as the wild type, but could be distinguished antigenically from wild-type virus by two of the hybridomas. The other was unique, and could be distinguished, both antigenically and by peptide mapping, from the other variants.**

Since a large number of the variants selected with PEG-1 showed the same peptide change, it is likely that this alteration in amino acid sequence (serine to leucine) was responsible for the inability of the variants to bind PEG-1 monoclonal antibody. We do not know, however, whether the changed amino acids were located within the antigenic sites or whether the change occurred somewhere else in the hemagglutinin molecule and altered the determinants through conformational changes.

Two kinds of antigenic variation occur in influenza type A virus: antigenic drift and major antigenic shifts. The latter probably involves recombination of human and animal influenza viruses (1) and represents a different phenomenon from that of antigenic drift.

Antigenic drift, which involves gradual changes in the surface antigens of influenza virus, is thought to be the result of the selection by an immune population of mutant virus particles having altered antigenic determinants and therefore possessing a growth advantage in the presence of antibody (2–4). The precise mechanism of antigenic drift is not known, but it has been shown that antigenic mutants isolated *in vitro* by selection with antibody have changes in the amino acid sequence of the polypeptides of the hemagglutinin (HA) molecules (5).

Peptide mapping experiments have also shown that changes in amino acid sequence of the polypeptide chains of the HA occur during natural antigenic drift (6), and HA molecules from viruses of different subtypes differ greatly in amino acid sequence (7, 8). It is likely that many of these changes are unrelated to the antigenic differences between the HAs, so that analysis of natural variants may 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 HA 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 molecules. These variants are not neutralized by the antibody, have a selective growth advantage, and their HA molecules can be isolated and analyzed.

The HA "spikes" of influenza virus are glycoprotein molecules of about 235,000 M_r. The molecules are trimers of HA1 + HA2 polypeptides (9), the latter possessing hydrophobic regions which serve to attach the HA to the lipid envelope of the virus. Little information is available concerning the number, nature, and location of the antigenic determinants on the HA molecules, or the way in which these undergo variation during antigenic drift. Two groups of antigenic determinants on the HA, strain-specific and common or crossreacting, have been described (6, 10). Observations by electron microscopy of antibody molecules bound to isolated HA molecules showed that both specific and crossreacting determinants are located on the side of the HA spike, just below its tip. No antibody molecules appeared to bind anywhere else on the HA (11).

Gerhard (12), by using monoclonal antibodies to A/PR/8/34 (HON1) (referred to as PR8) HA, was able to distinguish 40–50 (groups of) antigenic determinants on the HA, but whether these were discrete sites on the molecule or overlapping domains was not known.

A number of techniques may be used to characterize antigenic determinants on protein molecules. One is to isolate and characterize antigenic fragments of the protein. Preliminary attempts to do this with influenza virus HA have not been successful. One approach we have taken, and which is described in this paper, is to select antigenic variants of influenza virus under pressure of monoclonal antibody to the HA and determine the changes in amino acid sequence of the HA polypeptides associated with the altered capacity of the molecule to combine with antibody.

A previous paper (13) describes the selection with monoclonal antibodies and immunological characterization of antigenic variants of PR8 influenza virus. The present paper describes the selection of additional variants and examination, by peptide mapping, of amino acid sequence changes in their HA polypeptides.

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Abbreviations: HA, hemagglutinin; RIA, radioimmunoassay.

MATERIALS AND METHODS

Antiviral Antibodies. The production of continuous cell lines (hybridomas) secreting antiviral antibodies has been described (14). Hybridoma cell cultures were maintained in Dulbecco's modified Eagle's minimal essential medium containing 7.5% fetal calf serum, 7.5% agamma horse serum (Flow Laboratories, Rockville, MD), gentamycin at 50 μg/ml, and 2 mM glutamine. The hybridoma (PEG-1) cell line producing antibodies to PR8 HA used for selection of virus variants was grown in ascitic form in BALB/c mice.

Viruses. Influenza virus PR8, obtained from E. D. Kilbourne, Mount Sinai Hospital, New York, was used for the production of variants. The HA of this particular isolate of PR8 was stable in sodium dodecyl sulfate and therefore was well suited for peptide mapping experiments. The production of variants was done as described (13). Briefly, monoclonal hybridoma antibody (PEG-1) plus cloned parent virus (PR8) were incubated together for 30 min at 20°C. The mixture was inoculated into 11-day-old embryonated hens' eggs. The viruses that grew in the presence of the most concentrated monoclonal antibodies were harvested and "cloned" twice at limiting dilutions in 10-day-old embryonated hens' eggs. The viruses isolated from 12 separate eggs were designated V1 through V12. All viruses were purified by adsorption to and elution from chicken erythrocytes and subsequent differential centrifugation and sedimentation through a sucrose gradient (10–40% sucrose/0.15 M NaCl) as described (15). Virus preparations were quantitated by hemagglutination titration as described (16).

Radioimmunoassay. The radioimmunoassay (RIA) used in these studies has been described (17). A stationary phase immunoadsorbent [virus-coated to the bottom of the wells of polyvinyl microtiter plates (Cooke Engineering, Alexandria, VA)] was incubated with dilutions of antibody. The wells were washed and then incubated with ^{125}I-labeled rabbit anti-mouse F(ab')$_2$. After a final washing procedure the wells were separated from the plate and assayed for radioactivity in a γ counter. Treatment of the data for binding constant measurements is described elsewhere (17).

Isolation of the HA Molecules and Separation of the HA1 and HA2 Polypeptides. The virus particles were disrupted with sodium dodecyl sulfate and the HA molecules were isolated by electrophoresis of the sodium dodecyl sulfate-disrupted virus particles on cellulose acetate strips (18). The HA molecules were dissolved in saturated guanidine hydrochloride solution containing dithiothreitol, and the heavy and light polypeptides (HA1 and HA2) were separated by centrifugation on a guanidine hydrochloride/dithiothreitol density gradient (18).

Peptide Maps. Fractions from the guanidine hydrochloride gradients containing the HA1 polypeptide were pooled and dialyzed against saline, and the protein was precipitated by the addition of 5 vol of ethanol. The fractions containing HA2 were dialyzed, and the insoluble light chain was recovered by centrifugation. The proteins were then digested with trypsin, 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 by the method of Laver (19) as modified by Laver *et al.* (6). Peptides were located by staining with ninhydrin or, if the peptides were to be eluted for analysis, with fluorescamine [10 μg/ml in acetone containing 0.5% pyridine (20)].

Amino Acid Analysis. Peptides were eluted from the maps with 10% pyridine, filtered, dried, hydrolyzed with 6 M HCl for 22 hr at 105°C, and analyzed for amino acids by using a Beckman 119CL amino acid analyzer.

RESULTS

Antigenic Analysis of Variant Viruses. Variants 1–12 were examined for antigenic changes by analyzing the reactivity of these viruses with a panel of 18 monoclonal antibodies. These antibodies, whose specificities are directed against the viral HA molecule, have been shown to comprise a reactivity matrix capable of distinguishing the same number of closely related HA molecules as a panel of 95 randomly selected monoclonal anti-HA antibodies produced in the splenic fragment system (13).

Table 1 shows the reaction of five hybridoma antibodies with the parent and 12 variant viruses as observed by RIA. Of the remaining 13 antibodies, 10, although specific for the HA molecule, were unable to distinguish between parent and variant viruses. The remaining three antibodies displayed reactivity patterns represented by hybridomas in Table 1.

It is evident that all 12 viruses selected from wild-type PR8 in the presence of PEG-1 hybridoma antibody represent antigenic variants of the parental virus because none of the variants reacted with PEG-1 in the RIA. Additional discrimination between variants was accomplished (*i*) by hybridoma H3/4C4, which can distinguish V3, V9, and V12 from the other variants; (*ii*) by H2/6A5, which further distinguishes V9 from V3 and V12; and (*iii*) by H9/B20, which discriminates V3 from V12. Thus, the panel of hybridoma antibodies included in the analysis allowed unequivocal delineation of four types of antigenic variation expressed by the HA molecule of the PR8 variants—V3, V9, V12, and the large group of apparently identical variants (V1, V2, V4, V5, V6, V7, V8, V10, and V11).

The binding constants for the reaction of hybridoma antibodies H2/6A5 and H3/4C4 with the parent virus and with variants 1–12 are shown in Table 2. Once again, it can be seen that the monoclonal antibody H2/6A5 reacts with variants 1, 2, and 4–11 with approximately the same binding energy, whereas it is unable to bind to variant 3 or 12. Hybridoma antibody H3/4C4, on the other hand, binds to variants 3, 9, and 12 only, thereby implying a difference in variants 1, 2, 4, 5, 6, 7, 8, 10, and 11 from variants 3, 9, and 12.

Peptide Maps. HA molecules from wild-type PR8 virus and some of the antigenic variants selected with monoclonal antibody were examined in peptide mapping experiments for alterations in amino acid sequence. The HA1 and HA2 polypeptides were mapped separately. Maps of the tryptic pep-

Table 1. Antigenic analysis of a series of variants

Hybridoma antibodies	PR/8/34	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
PEG-1	6.55	0	0	0	0	0	0	0	0	0	0	0	0.65
H3/4C4	6.58	<1	<1	5.56	<1	<1	<1	<1	<1	4.98	<1	<1	6.43
H2/6A5	3.47	3.13	3.26	0	3.28	2.40	2.58	2.54	2.63	2.62	2.41	2.50	0
H9/B20	6.12	7.11	6.18	0	6.07	5.83	5.92	5.45	5.61	5.77	5.04	5.55	4.58
H2/4B1	6.79	6.89	6.83	6.24	6.82	6.89	6.82	6.88	6.82	6.87	6.72	6.88	6.88

The data are expressed in $\log_2$ [100 cpm_{bound}virus X/cpm_{bound}PR8].

Table 2. Binding constants for the interaction of hybridoma antibodies with PR8 and 12 variants

Viruses	Binding constant, M^{-1} H2/6A5	H3/4C4
Parent	$9.37 \pm 0.12 \times 10^8$	$8.32 \pm 0.57 \times 10^{10}$
V1	$6.96 \pm 0.07 \times 10^8$	$<10^6$
V2	$6.14 \pm 0.05 \times 10^8$	$<10^6$
V3	$<10^6$	$2.29 \pm 0.03 \times 10^9$
V4	$5.22 \pm 0.07 \times 10^8$	$<10^6$
V5	$4.53 \pm 0.07 \times 10^8$	$<10^6$
V6	$4.82 \pm 0.09 \times 10^8$	$<10^6$
V7	$5.12 \pm 0.11 \times 10^8$	$<10^8$
V8	$5.41 \pm 0.20 \times 10^8$	$<10^6$
V9	$5.34 \pm 0.04 \times 10^8$	$2.86 \pm 0.07 \times 10^9$
V10	$5.50 \pm 0.12 \times 10^8$	$<10^6$
V11	$5.53 \pm 0.11 \times 10^8$	$<10^6$
V12	$<10^6$	$1.53 \pm 0.02 \times 10^9$

The binding constants (mean ± SEM) were determined by RIA as described (17).

tides (soluble at pH 6.5) from the small HA polypeptide, HA2, of wild-type PR8 virus and variants 1 and 2 are shown in Fig. 1; maps of the large polypeptide, HA1, from the same viruses are shown in Fig. 2. Fig. 3 gives the numbering of the HA1 peptides. Maps of the HA2 polypeptide from wild-type PR8 virus and the variants were identical, but a single peptide difference was seen on the maps of the HA1 polypeptide.

The difference involved a change of one of the neutral peptides (peptide 5) from lower to higher R_F during chromatography. This change is more clearly seen in Fig. 4, which

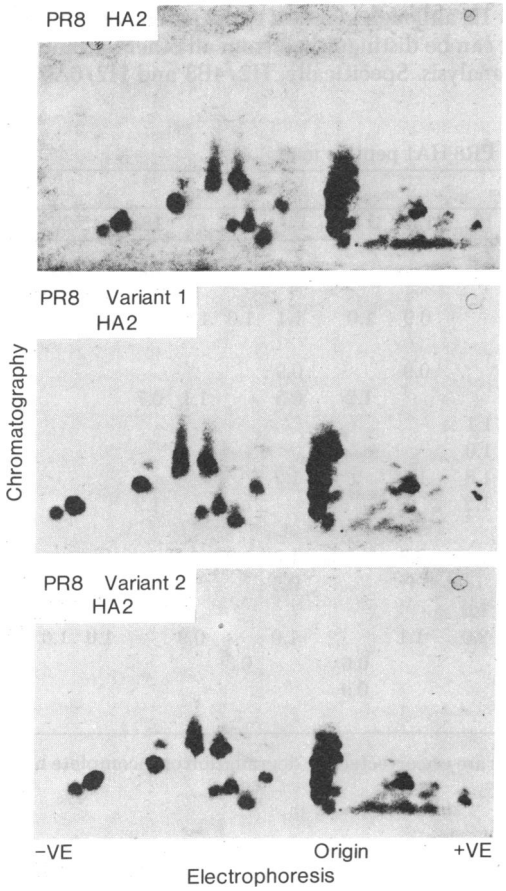

FIG. 1. Maps of the tryptic peptides (soluble at pH 6.5) from HA2 of wild-type PR8 virus and variants 1 and 2. The maps were stained with ninhydrin. No differences between the maps can be seen.

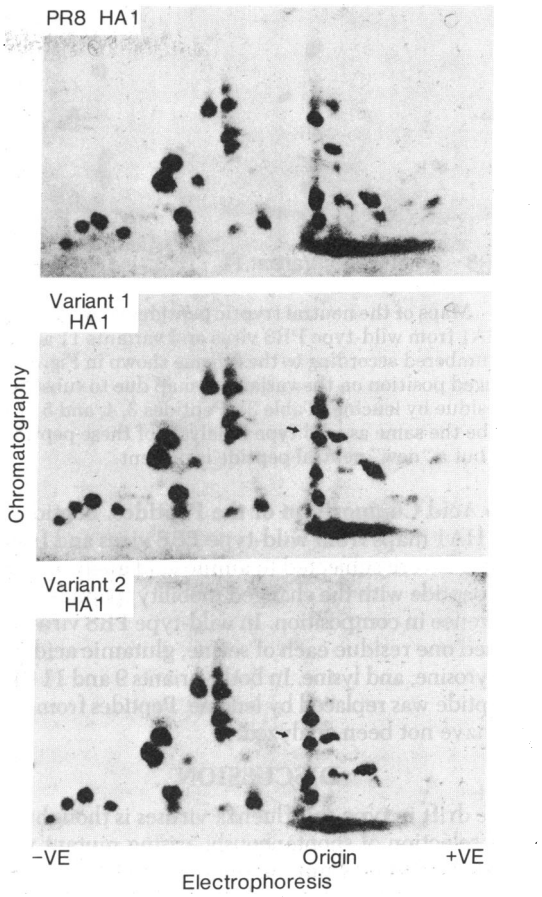

FIG. 2. Maps of the tryptic peptides (soluble at pH 6.5) from HA1 of wild-type PR8 virus and variants 1 and 2. The maps were stained with ninhydrin. A single peptide difference (arrow) between the wild-type and variant maps can be seen.

shows the neutral peptides from more lightly loaded maps of HA1 from wild-type PR8 virus and two of the variants.

The HA1 polypeptides from 10 of the 12 variants were mapped, and 8 showed the same alteration in the same peptide. One variant (V3) did not show a change in this peptide, but the maps of HA1 from V3 showed that an additional neutral peptide was present (Fig. 4). Peptide maps of HA1 from another variant (V12) were indistinguishable from those of HA1 of wild-type PR8 virus.

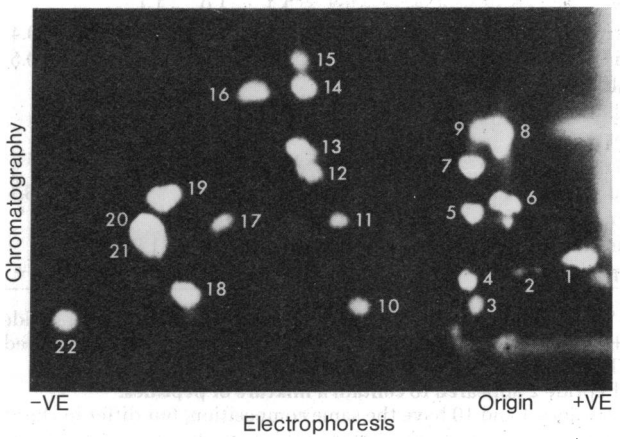

FIG. 3. Maps of the tryptic peptides (soluble at pH 6.5) from HA1 of variant 9. Peptides were stained with fluorescamine and eluted for analysis. The amino acid composition of the numbered peptides is given in Table 3.

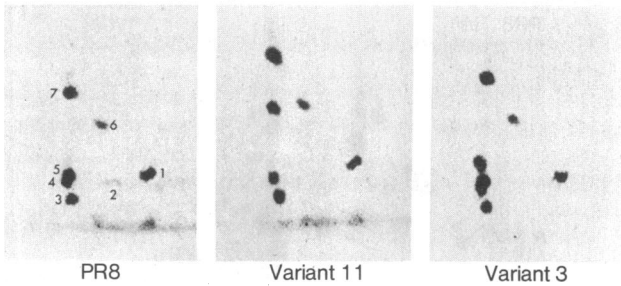

FIG. 4. Maps of the neutral tryptic peptides from lightly loaded maps of HA1 from wild-type PR8 virus and variants 11 and 3. Peptides are numbered according to the scheme shown in Fig. 3. Peptide 5 has changed position on the variant 11 map due to substitution of a serine residue by leucine (Table 3). Peptides 3, 4, and 5 of variant 3 seem to be the same as wild type (analyses of these peptides were not done) but a "new" neutral peptide is present.

Amino Acid Composition of the Peptides. Peptides eluted from the HA1 maps from wild-type PR8 virus and from variants 9 and 11 were subjected to amino acid analysis (Table 3). Only the peptide with the changed mobility (peptide 5) showed any difference in composition. In wild-type PR8 virus, peptide 5 contained one residue each of serine, glutamic acid, proline, glycine, tyrosine, and lysine. In both variants 9 and 11 the serine in this peptide was replaced by leucine. Peptides from the other variants have not been analyzed.

DISCUSSION

Antigenic drift in type A influenza viruses is thought to result from the selection of spontaneously arising mutant viruses in the presence of a host immune response. In the present study, 12 mutant viruses have been selected by growing cloned PR8 virus in embryonated hens' eggs in the presence of the monoclonal anti-HA antibody PEG-1. Since PEG-1 antibody is able to neutralize the parental PR8 virus, mutants must have a modification of the HA determinant recognized by PEG-1 (namely, the PEG-1 epitope) in order to escape neutralization. The present study attempts to relate the antigenic changes in the PEG-1 epitope to structural changes in the HA polypeptides by comparing the variant viruses in antigenic analyses and in peptide mapping experiments.

Variants 1, 2, 4, 5, 6, 7, 8, 10, and 11 appear to be identical by the two techniques. All variants in this group possess the same reactivity type versus the panel of hybridomas, all bind to hybridoma antibody H2/6A5 with equal binding energy, and all show the same changed peptide (peptide 5 in Fig. 3 and Table 3) in the peptide maps. A serine in this peptide from wild-type PR8 virus was replaced by leucine in variant 11. This peptide from the other variants in this group has not been analyzed. From the position of the peptide on the maps, however, it is likely that the same alteration in amino acid sequence of HA1 occurred in each case. No peptide differences could be seen on the HA2 maps; but only HA2 peptides from variants 1 and 2 were mapped, and none of the HA2 peptides was analyzed.

V9 shows the same change from wild type in the peptide maps and antigenic analyses as V11—i.e., the replacement of serine by leucine in peptide 5. However, V9 can be distinguished from V11 (and other variants) by hybridoma RIA analysis. H3/4C4, which cannot bind to variants 1, 2, 4, 5, 6, 7, 8, 10, and 11, can bind to V9. This suggests that V9 has a second amino acid substitution in a peptide that was insoluble and was not included in the mapping, because the amino acid composition of the other soluble tryptic peptides of V9 is the same as V11 and wild type. The mapped peptides contained a total of about 107 amino acids and therefore represent only one-third of the HA1 molecule.

Variant 12, although identical to the parent virus by peptide mapping, can be distinguished from all other variants by hybridoma analysis. Specifically, H2/4B3 and H2/6A5 cannot

Table 3. Composition of peptides from wild-type PR8 HA1 peptide map

Amino acid	Peptide number[a]																				
	1	2[b]	3	4[c]	5[d]	6[e]	7	8[f]	9[f]	10[c]	11	12	13[g]	14	15[g]	16	17	18	19	20	21	22
Lys				0.9	1.0		0.9	0.9	0.8	0.8	0.9	0.9						1.1		1.0		1.0
His																						
Arg	0.9		1.0	1.0						1.5			0.9	1.0	1.1	1.0	1.0		1.0		0.9	
Asp			0.8			0.8		1.2	1.0		1.8											
Thr				1.0				1.3	1.2	1.0			0.9		0.9							
Ser	3.6				1.0	2.0		0.8			1.0			1.2	0.5		1.1	0.7				
Glu	2.8		1.0	2.5	0.8	1.1	1.1	1.6	1.2	2.2		1.1										
Pro				2.2	1.1	1.0	1.1			2.0		1.0										
Gly			1.1		1.0				0.4			1.8	1.1		1.2							1.0
Ala			1.0	1.2					0.5	1.1		1.2						1.2				
Half Cys																						
Val	1.0										1.7		0.9		1.0	1.0					1.1	
Met					-								+		0.5							
Ile				1.0		2.1	0.9			0.9		1.0										
Leu	1.1							3.2	3.1			2.0	1.1		1.0		0.9		1.0	1.0		
Tyr					0.8									0.6		0.3						
Phe	1.0			0.7			1.8			1.0	0.8			0.9								
Trp[h]	+							+	+													

The number of residues of each amino acid found in each peptide is shown. Values are uncorrected for degradation or incomplete hydrolysis.
"+" indicates that the amino acid was present but not quantitated.
[a] The numbering of the peptides is shown in Fig. 3.
[b] Peptide 2 appeared to contain a mixture of peptides.
[c] Peptides 4 and 10 have the same composition, but differ in charge.
[d] In variants 9 and 11 the serine in this peptide was replaced by leucine.
[e] Peptide 6 contains no basic amino acids and may be the COOH-terminal peptide of HA1.
[f] Peptides 8 and 9 are probably two halves of a single spot.
[g] Peptides 13 and 15 are the same, the difference in position being due to partial oxidation of the methionine.
[h] Peptides containing tryptophan were detected by staining with Ehrlich reagent.

bind to V12, whereas they do bind to the wild-type virus. Either an unmapped insoluble peptide yielded the antigenic variation, or the amino acid substitution, although sufficient for antigenic metamorphosis, was insufficient for altering a peptide to distinguish it in peptide maps. Peptides on the V12 HA1 maps were not analyzed, and V12 HA2 was not mapped.

In variant 3, the peptide map contains all of the tryptic peptides of the parent virus plus a new one. It could be argued that such a map would result from a mixture of two virus populations, parent and variant, with the two peptide maps superimposed. However, when the variant was recloned and remapped, identical results were obtained. It thus appears most likely that the new spot on the peptide map of V3 comes from a previously unmapped insoluble peptide.

A comparison of the peptide maps of variants 1–12 by hybridoma RIA analysis yields the following observations: peptide mapping can discriminate three sets of viruses (parent and V12; variants 1, 2, 4, 5, 6, 7, 8, 9, 10, and 11; and V3), and hybridoma RIA analysis can further distinguish variants 9 and 12 as separate and unique. Hence, in variants 9 and 12, changes are probably occurring in the primary sequence of peptides that are not separated by the protocol described here.

As mentioned above, a prerequisite for the selection of variant viruses in the present system is a modification of the PEG-1 epitope of the parental HA molecule that would prevent the PEG-1 antibody from neutralizing the variant virus. We have suggested (13) that variants selected in the presence of monoclonal antibodies may represent in general single point mutants of the parental virus. This idea has been based, first, on the fact that virus variants could be readily selected from a cloned parental virus preparation in a single growth passage in the presence of an over-neutralizing dose of monoclonal antibody; and, second, on the observation that variant viruses invariably differed only very slightly from the parental virus with regard to the overall antigenicity of the HA molecule. The present study is compatible with the above idea, because no variant exhibited a peptide map that differed from the parental map by more than one peptide. However, since at least one peptide involved in the formation of the PEG-1 epitope may not have been mapped in these viruses (i.e., the one that gave rise to the "new" peptide in V3), it cannot be excluded that these variants differ from the parental virus by multiple point mutations.

By the criteria of the techniques used in this work, 9 of the 12 variants appear identical. Although it is possible that this variant occurs at a high frequency, it should be kept in mind that the variants were chosen because of their good growth characteristics, thus skewing selection.

It is possible that the changes seen in the peptide maps bear no relationship to the altered antigenic properties of the variants selected with PEG-1 monoclonal antibody. These changes in sequence may have nothing to do with antigenicity but are, perhaps, associated with an increased growth capacity of the virus in the presence of mouse ascities fluid. This is unlikely, because antigenic variants selected with two other monoclonal hybridoma antibodies (H2/6C4 and H2/4B3) did not show the peptide change found in the PEG-1 variants. Maps of HA1 from the H2/6C4 and H2/4B3 variants were identical to that of HA1 from wild-type PR8 virus, so that any change which occurred must have been in an insoluble peptide.

These results suggest that the changes found in the PEG-1 variants were responsible for the inability of these variants to bind the PEG-1 monoclonal antibody used for their selection.

Since sequence data on the entire HA of PR8 is not available, there seemed little point in determining the sequence of the peptides with altered map positions. However, the analysis used in this work is applicable to similar systems in which such data are available—e.g., A/Jap/305/57 (H2N2) and A/Memphis/102/72 (H3N2) viruses (7, 8, 21). Furthermore, the HA from A/Hong Kong/68 (H3N2) has been crystallized, and x-ray diffraction data are becoming available (22).

This approach should enable us to find out (*i*) the exact changes that occur in the amino acid sequence of the HA polypeptides of different variants selected with the same clone of antibody and of variants selected with different clones and (*ii*) whether the changes in amino acid sequence occur within the antigenic sites or whether they occur somewhere else in the HA molecule and alter the determinants through conformational changes.

Preliminary results have shown that variants of Hong Kong virus selected with monoclonal hybridoma antibodies to Hong Kong HA possess sequence changes. Four variants selected with one monoclonal antibody showed the same change; variants selected with other monoclonal antibodies showed different changes. The changes occurred only in the HA1 polypeptide, and the regions found to vary under pressure of monoclonal antibodies were similar to those found to vary in naturally occurring field strains. These findings will be reported in detail elsewhere.

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