ANTIGENIC DRIFT IN INFLUENZA A VIRUSES

I. Selection and Characterization of Antigenic Variants of A/PR/8/34 [H0N1] Influenza Virus with Monoclonal Antibodies*

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It is well established that the hemagglutinin (HA)[1] molecule of influenza virus is highly variable and frequently exhibits antigenic changes (1–3). In nature the spontaneously arising mutants are likely to be overgrown by the parent virus, unless the mutation provides the variant with a selective growth advantage, such as the ability to replicate in the presence of antibodies that neutralize the parental virus strain. This is thought to be the basis of the minor antigenic changes (drift) that occur continuously in nature in a partially immune host population during interpandemic periods and that sporadically lead to new epidemic virus strains. Experimental support for this mechanism of antigenic drift has been obtained in many previous studies in which antigenic variants have been shown to emerge if influenza virus is grown, in vivo or in vitro, in the presence of subneutralizing doses of anti-viral antiserum (4–8). Antigenic drift, however, is still little understood at the molecular level. It is not known, for example, whether a single amino acid substitution in the antigenic site of the HA is sufficient to cause a detectable antigenic drift or, as suggested by Fazekas de St. Groth (9), to induce a transition from an existing to a subsequent epidemic virus strain.

In the present study, antigenic variants were selected from a cloned preparation of PR8 virus by means of a monoclonal hybridoma antibody (10). Under these experimental conditions, variant viruses could be selected in a single egg passage. The antigenic changes exhibited by these naturally occurring mutants were delineated by means of monoclonal antibodies.

Materials and Methods

Viruses. The influenza virus PR8 (A/PR/8/34 [H0N1]) was grown in embryonated eggs and the infectious allantoic fluid was used as parental virus for the selection of variants. In addition, the following influenza A viruses were used in the radioimmunoassay (RIA) for the determination of the specificity of anti-viral antibodies: SW (A/Swine/31 [HswN1]); WSE (A/WSE/33 [H0N1]); WSN (A/WSN/33 [H0N1]); BH (A/BH/35 [H0N1]); MEL (A/Melbourne/35 [H0N1]); HICK (A/Hickcox/40 [H0N1]); BEL (A/Bellamy/42 [H0N1]); WEISS (A/Weiss/43 [H0N1]); CAM (A/CAM/46 [H1N1]); FM1 (A/FM/1/47 [H1N1]); recombinant viruses: Eq-PR8 (A/equine/Miami/1/63 [Heq2]-

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[1] *Abbreviations used in this paper:* HA, hemagglutinin; H0 and H1, HA of subtypes A0 and A1, respectively; HI, hemagglutination inhibition; IAds, immunoadsorbent; PBS, Dulbecco's phosphate-buffered saline; PEG-1, antibody produced by the hybridoma polyethylene glycol fused cells; RIA, radioimmunoassay; RT, reactivity type; V, variant virus.

A/PR/8/34 [N1]); and JAP-BEL (A/Japan/305/57 [H2]-A/Bellamy/42 [N1]). All viruses were grown in the allantoic cavity of 10-day-old embryonated hen's eggs and were purified by adsorption to and elution from human erythrocytes and by banding in a sucrose gradient as described previously (11).

Monoclonal Anti-HA Antibodies. The monoclonal anti-HA(PR8) antibody produced by the hybridoma PEG-1 (subsequently referred to as PEG-1) has been described in detail elsewhere (10). Clone 6 of the hybridoma mass culture was used. The hybridoma was grown in ascitic form in the peritoneal cavity of BALB/c mice (Charles River Breeding Laboratories, Wilmington, Mass.) pretreated by an i.p. injection of 0.5 ml pristane (Aldrich Chemical Co., Inc., Milwaukee, Wis.).

Secondary monoclonal anti-HA(PR8) antibodies were produced in the splenic fragment culture system according to published methods (11, 12). The antibodies used in the present study were derived from splenic fragment cultures obtained after adoptive transfer of spleen cells from BALB/c mice primed 4–6 mo previously by an i.p. injection of 1250 HA U of PR8. The specificity of the monoclonal antibodies was determined by means of the RIA: an antibody was considered specific if it bound to PR8(H0N1) but not to the hybrid viruses Eq-PR8 (Heq2N1) and JAP-BEL (H2N1) (12).

Antisera. Hyperimmune antisera to isolated HA(PR8) subunits and to intact viruses were prepared in rabbits and in goats (13, 14).

Serological Tests. RIA was performed as described previously (12) except for the viral immunoadsorbent (IAds) which was prepared according to the method of Rosenthal et al. (15). Briefly, the purified virus (roughly 25 HA U) in 25 μl Dulbecco's phosphate-buffered saline was added to individual wells of round bottom polyvinyl plates (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.). The virus sample was dried overnight and fixed for 5 min with methanol at room temperature, then residual methanol was rinsed off. 15-μl replicate samples containing 2.5–5 ng of monoclonal antibody were added to each well and incubated for 90 min at room temperature. The wells were washed three times and the amount of antibody bound to the various IAds was quantitated by means of ^{125}I-labeled rabbit anti-mouse F(ab')$_2$ or goat antisera (Meloy Laboratories Inc., Springfield, Va.) specific for the predominant isotype of the given monoclonal antibody. The reactivity type (RT) was determined as follows (12): the amount of antibody in the test sample that bound to the parental virus PR8 was defined as 100%. Positive binding indicates that greater than or equal to 10% of the antibody in the test sample bound to variant or heterologous virus; negative binding indicates that less than 10% was bound. Each assay was performed in duplicate.

HA titrations and hemagglutinin-inhibition (HI) tests were done as previously described (16). The antisera were treated with receptor-destroying enzyme and the dilutions of antiserum were allowed to interact with antigen for 60 min at 20°C before the addition of chicken erythrocytes.

Virus neutralization tests were performed in embryonated eggs as outlined below for the selection of variants except that 50 egg infective doses of virus were incubated with various dilutions of anti-viral antibodies.

Selection of Virus Variants. 0.5-ml dilutions of PEG-1 ascitic fluid were mixed with undiluted A/PR8 virus (0.05 ml high infectivity allantoic fluid) and incubated at 20°C for 30 min. The virus-antibody mixtures were injected into groups of 10 embryonated hen's eggs (11 days) and incubated at 35°C for 2 days. The inoculated eggs were harvested individually and tested for the presence of influenza virus by HA titration. The virus yields from the individual eggs were tested in HI assays with the monoclonal antibody preparation to A/PR8; those viruses that were inhibited 10-fold less efficiently or were not inhibited at all by the monoclonal antibody preparation were cloned twice at limiting dilution in embryonated hen's eggs.

Results

Selection of PR8-Variants with Monoclonal Antibodies Produced by PEG-1. A cloned parental PR8 virus preparation was made by two consecutive growth passages of PR8 at limit dilution in embryonated hen's eggs in the absence of anti-viral antibodies. As shown in Table I, PEG-1 was effective at inhibiting hemagglutination by and infectivity of the parental virus preparation.

TABLE I
Interaction of Parent and Variant Viruses with PEG-1

Virus	Neutralization of infectivity (log 2)	HI titer (log 2)	RIA ng Antibody binding
PR8 (Parent)	7.87	14.33	65
PR8-V2	<3.0	<5.95	<0.1
PR8-V3	<3.0	9.27 (−5.06)	0.9 (−6.20)
PR8-V4	<3.0	8.27 (−6.06)	1.0 (−6.04)
PR8-V6	<3.0	10.64 (−3.69)	2.8 (−4.55)
PR8-V7	<3.0	<5.32	<0.1
PR8-V8	<3.0	<5.32	<0.1
PR8-V9	<3.0	<5.32	<0.1

PEG-1 antibody was assayed in neutralization test, HI test, and RIA for interaction with the indicated viruses. The tests were performed as outlined in Materials and Methods. Neutralization and HI titer refer to undiluted PEG-1 ascitic fluid. The concentration of antibody determined in the RIA refers to a 15-μl sample of PEG-1 diluted 1/250. Entries in parentheses: log 2 (heterologous interaction)-log 2 (homologous interaction).

Virus harvested individually from eight eggs after incubation of the parental PR8 virus inoculum with undiluted PEG-1 ascitic fluid was cloned twice at limit dilution in embryonated eggs in the absence of PEG-1. These virus preparations were designated PR8-V2 through PR8-V9 (V, variant). PR8-V5 was not used in these experiments. Compared to the parental PR8, these viruses exhibited no detectable or a much smaller reactivity with PEG-1 in HI test, neutralization test, and RIA, thus indicating that they represented variants of the parental PR8 (Table I).

Antigenic Relationship between Parental Virus and Variants. An antigenic comparison between parental PR8 virus and its variants was done in HI tests using several antisera raised in vivo against the parental virus (Table II). In contrast to PEG-1 (Table I), these antisera cross-reacted extensively with all variants and, thus, could not provide clear-cut evidence for antigenic differences between variants and parental virus. Similarly, the antisera to variants 2, 3, and 4, respectively, were unable to distinguish the homologous variant from the parental virus or from the other variants.

Taken together, the data of Tables I and II indicate that differences exist among these viruses, but they are probably recognized only by a small fraction of the anti-viral antibodies present in the heterogeneous antisera. Therefore, the antigenic relationship between parental and variant viruses was further delineated by means of monoclonal anti-HA antibodies produced in vitro in the splenic fragment system. Due to the relatively small quantities of antibody available, this analysis could be performed only in the RIA. The RT of a monoclonal antibody in the RIA was determined as described in Materials and Methods.

As shown in Table III, 81 of the 95 randomly selected monoclonal anti-HA(PR8 parent) antibodies reacted in the RIA with all variants. 14 antibodies provided evidence for antigenic differences between the HA of parent and variant viruses: 11 of the 14 delineated a determinant present on the HA of all viruses except V3 and V4; two antibodies delineated a determinant present only on the parent, V3, V4, and V6; and one delineated a determinant present only

TABLE II

Antigenic Relationship between Parental and Variant Influenza Viruses in HI Tests

Virus	Goat-anti* PR8 parent (isolated HA)	Rabbit-anti‡ PR8 parent (isolated HA)	BALB/c-anti§ PR8 parent (whole virus)	Murine-anti‖ PR8 parent (whole virus)	Rabbit-anti¶ variant 2 (whole virus)	Rabbit-anti¶ variant 3 (whole virus)	Rabbit-anti¶ variant 4 (whole virus)
PR8 Parent	13.55	14.92	12.87	12.37	13.55	14.21	14.21
PR8-V2	14.55	15.61	11.87	12.47	14.61	15.52	15.52
PR8-V3	13.29	14.36	12.82	12.87	13.29	14.14	14.43
PR8-V4	13.29	14.43	11.87	12.62	13.29	14.14	14.21
PR8-V6	13.43	14.55	12.27	12.67	13.55	13.87	14.05
PR8-V7	14.14	15.52	11.27	12.27	14.55	15.14	15.01
PR8-V8	13.67	15.32	11.67	12.17	13.67	14.61	14.61
PR8-V9	13.29	15.21	12.02	12.07	14.14	14.21	14.21

Figure gives the reciprocal of the dilution inhibiting three out of four hemagglutinating doses of the above viruses.

* Hyperimmune goat antisera to the isolated HA molecule.

‡ Hyperimmune rabbit antisera to the isolated HA molecule.

§ Pooled serum from BALB/c mice obtained 8 days after first boost with PR8 parental virus.

‖ Pooled serum from DBA, C57BL/10, and A mice obtained 8 days after first boost with PR8 parental virus.

¶ Hyperimmune rabbit antisera to intact purified virus.

TABLE III

Comparison between Parental Virus and Variants by Means of Monoclonal Antibodies in the RIA

Number of antibody clones	PR8 Parent	RT exhibited in RIA*							Minimum number of distinct clonotypes‡
		V2	V3	V4	V6	V7	V8	V9	
81§	+	+	+	+	+	+	+	+	46
11§	+	+	−	−	+	+	+	+	7
2§	+	−	+	+	+	−	−	−	1
1§	+	−	−	−	+	−	−	−	1
(PEG-1, cl 6‖)	+	−	−	−	−	−	−	−	1

* Antibodies were tested for binding to indicated viruses in the RIA. Negative (−) reactivity indicates that less than 10% of the antibody in the test sample bound to the given virus. See also Materials and Methods for the definition of the RT.

‡ Number of antibodies that could be further distinguished on the basis of their interaction in the RIA with the influenza viruses SW, WSN, WSE, BH, MEL, HICK, WEISS, BEL, CAM, and FM1.

§ Anti-HA (PR8) antibody clones produced in the splenic fragment system.

‖ Hybridoma antibody used for selection of variant viruses.

on the parent and V6. Furthermore, a unique determinant of the parent HA was detected by the PEG-1 antibody.

Since all antibodies that expressed a given RT could represent a single clonotype, i.e., a single species of antibody combining sites, the mere number of antibody clones exhibiting any given RT cannot provide, per se, a measure of

the extent of antigenic relatedness. Thus, to estimate the minimum number of different antibody combining sites included in the analysis, all antibodies were further tested for their reaction with the following 10 heterologous viruses: SW, WSN, WSE, BH, MEL, HICK, WEISS, BEL, CAM, and FM1. This analysis indicated that the 81 antibodies that cross-reacted with all variants comprised a minimum of 46 different clonotypes, each characterized by a unique pattern of reaction with the panel of the above mentioned heterologous viruses (Table III, last column). Thus, under the experimental conditions of the RIA, at least 46 distinct antibody combining sites were unable to recognize a difference between the HA of parental virus and that of its variants. Similarly, at least seven clonotypes delineated the determinant present on the HA of all viruses except V3 and V4. In contrast, the other three determinants seemed to be recognized by only a single clonotype.

The Ability of Strain-Specific and Cross-Reactive Antibodies to Recognize the Antigenic Change on the Variants. Two major groups of antigenic determinants can be distinguished, operationally, on the viral HA: determinants that are characteristic for a given virus strain (strain-specific) and determinants that are shared by two or more virus strains (common) (12, 17–20). Recently, Laver et al. (17) speculated that the two groups of determinants might be subject to independent antigenic variation. In the present study, PR8 variants were selected on the basis of an antigenic change that decreased their reactivity with PEG-1, an antibody that is directed against a strain-specific HA(PR8) determinant. Thus, if the above hypothesis were correct, the antigenic change on the PR8 variant would be recognized exclusively by antibodies to the strain-specific determinants but not by antibodies to the common determinants. The splenic antibodies were, therefore, grouped with regard to the HA determinant recognized; antibodies that did not cross-react with any of the 10 heterologous viruses included in the analysis were assumed to be directed against one of the PR8 strain-specific determinants. Antibodies to common determinants (i.e., those shared in a cross-reactive form by PR8 and one or several heterologous viruses) were further subdivided into slightly cross-reactive (those that cross-reacted in the RIA with PR8 and three heterologous viruses) and highly cross-reactive (those that cross-reacted in the RIA with PR8 and more than three heterologous viruses) antibodies.

The results of this analysis are in partial agreement with the above hypothesis (Table IV). Thus, 41% of the strain-specific antibodies but none of the highly cross-reactive antibodies recognized the antigenic change on the variants. However, some slightly cross-reactive antibodies were also able to delineate the antigenic change, though less frequently than strain-specific antibodies.

Antigenic Relationship between Variants and Standard Virus Strains of the A0 Subtype. The analysis described in the previous section of all antibody clones for their reactivity to heterologous viruses of the A0 and A1 subtypes could shed some light on the question of whether the antigenic drift that occurred in the PR8 variants was in the direction of existing laboratory-maintained virus strains. Complete or partial correlation of the reactivity of the various antibodies with a variant and a heterologous virus would indicate identity with or drift in the direction of the given virus strain.

Table V shows that roughly 50% of the monoclonal antibody preparations

TABLE IV

Relationship between Cross-Reactivity of Monoclonal Antibodies and Recognition of Antigenic Change on PR8 Variants

Reactivity of antibodies* (number of cross-reacting heterologous viruses)	Number of antibodies	
	Total	Recognizing antigenic change on variants
HA(PR8)-Specific	17	7 (41%)
Slightly cross-reactive (1-3)	35	7 (20%)
Highly cross-reactive (4-10)	43	0

* The reactivity of the splenic monoclonal antibodies was based on their interaction in the RIA with PR8, SW, WSN, WSE, BH, MEL, HICK, WEISS, BEL, CAM, and FM1 (Table III legend). HA(PR8)-specific antibodies reacted exclusively with PR8. Slightly cross-reactive antibodies reacted with PR8 and 1-3 heterologous viruses; highly cross-reactive antibodies reacted with PR8 and 4-10 heterologous viruses.

TABLE V

Relationship between Reactivity of Antibody Clones with Variants and Reactivity with Heterologous Virus Strains

Splenic antibodies included in analysis		Antibodies reacting in RIA with:								
		V2*	V3*	V6*	WSE	BH	MEL	HICK	WEISS	BEL
95‡		92 (98%)	83 (87%)	95 (100%)	46 (48%)	49 (52%)	45 (47%)	31 (33%)	43 (45%)	26 (27%)
71§	E				41.9	44.6	41.0	28.2	39.1	23.7
	0:				46	48	42	29	43	23

* V2 represents V2, V7, V8, and V9; V3 represents V3 and V4. The splenic antibodies could not further differentiate the variants within each group under the conditions of the RIA.

‡ The 95 splenic antibodies include those that react exclusively with PR8 and those that crossreact in the RIA with a heterologous virus strain.

§ 71 splenic antibodies that react with PR8-V3 and one or several heterologous viruses. The expected number (E) of clones reacting with the indicated heterologous virus is calculated from the total number of cross-reacting clones assuming that there is no correlation between reactivity with V3 and a heterologous virus. 0 = observed number of clones reacting with V3 and indicated heterologous virus.

cross-reacted in the RIA with WSE, BH, MEL, or WEISS. On the other hand, the least cross-reactive of the PR8 variants (V3, V4) reacted with 87% and the most cross-reactive (V6) with 100% of the splenic antibodies. This indicated that all variants remained antigenically very closely related to the PR8 parental virus. Some drift toward the antigenicity of WSE, BH, and WEISS may have occurred in V3 and V4 since antibodies reacting with the latter variants reacted slightly more often than expected with WSE, BH, and WEISS. However, the difference was not significant in the χ^2 test.

Discussion

Influenza virus can produce many different types of variants (21). Two types of variation in the HA molecule may provide a mutant virus with a selective

growth advantage over the parental virus if the selection of variants is based on the neutralization of the parent virus by anti-HA antibodies. A mutation may occur that leaves the antigenicity of the HA unchanged but increase its avidity for the receptors of the host cell (22). Consequently, antibodies would exhibit a decreased activity against such adsorptive mutants in assays where antibodies have to compete with the binding of the virus to cell receptors as in the neutralization or the HI test. Antigenic analyses performed in the RIA, however, are virtually independent of this type of variation except for the possibility that an adsorptive mutation would lead to a considerable underestimation of the virus concentration used in the RIA (which is based on the HA titer of the virus). However, it does not seem possible that the variants used in the present study are adsorptive mutants since the various viral immunoadsorbents were able to bind equal amounts of most antibodies tested. Therefore, since each type of cross-reaction exhibited by a monoclonal antibody in the RIA defines a distinct group of antigenic determinants, each novel type of cross-reaction, thus, delineates a distinct antigenic mutation.

Analysis of the seven viruses (V2, V3, V4, V6, V7, V8, V9) selected with PEG-1 showed that all represented antigenic variants of the parental virus. Furthermore, the seven variants exhibited three distinct antigenic changes, one exemplified by the variant group, V2, V7, V8, and V9, one by the group V3 and V4, and one by V6. This is obviously a minimum estimate of the number of distinct antigenic mutations expressed by the variants, since some additional antigenic differences among the variants might have escaped detection as a result of the limited number of antibodies included in the analysis. Furthermore, the RIA as performed and scored in the present study cannot provide an accurate measure of the affinity of each variant-antibody interaction (12). Notion of the latter would be required, however, to prove unequivocally the identity of a given antigenic change expressed on the variants.

Antigenic differences between the variants and the parental virus were minimal. For instance, only 12 of the 95 monoclonal splenic antibodies were able to detect the antigenic change exhibited by V3 and V4 in the RIA and only PEG-1 recognized the variation exhibited by V6. These findings are further corroborated by the fact that heterogeneous anti-HA antisera could hardly discriminate between the variants and the parental virus. This suggests that none of the mutations, although they must have resulted from at least one amino acid substitution, would have been epidemiologically relevant, i.e., none of the variants would have escaped rapid neutralization in a host population with pre-existing immunity to the parental virus. This conclusion is also supported by results of the comparison of the variants with formerly epidemic virus strains of the A0 subtype: all variants were antigenically much more closely related to PR8 (year of original isolation, 1934) than to BH (1935), MEL (1935) or any of the later isolates of the H0N1 era.

Antigenic drift from an existing strain to a future epidemic virus strain thus seems to require several amino acid substitutions. This idea agrees with the observation of Laver et al. (17) who demonstrated multiple differences in the peptide maps of the HA of epidemic strains isolated in 1968 and 1972, respectively. However, neither the number nor the location of the amino acid substitutions was determined in these studies, and it is not known whether all

substitutions were in the antigenic sites. The present observations are obviously incompatible with the assumption made by Fazekas de St. Groth (9) that each epidemic strain within a subtype is derived from the preceding epidemic virus strain by a single amino acid substitution in the antigenic area of the HA molecule. Although the possibility has not yet been eliminated, it seems rather unlikely that the degree of variation observed in the PEG-1 epitope of the HA differs fundamentally from the variation of other HA determinants.

It has been estimated by means of monoclonal anti-HA antibodies of murine origin that the antigenic site of the HA is composed of approximately 15–60 individual determinants (23), some of which are strain-specific and others of which are shared by the HA of two or more virus strains. Given the electron microscopic observation that anti-HA antibodies interact only with a part of the hydrophilic portion of the HA molecule (24) measuring roughly 50 × 50 Å and given that the size of an antibody combining site is 15 × 20 Å (25), one must assume that many of the determinants represent overlapping protein structures. This notion is supported by the observation that not only strain-specific but also some cross-reactive antibodies were able to recognize the antigenic changes in the strain-specific PEG-1 epitope. On the other hand, none of the 43 antibodies that cross-reacted with more than three heterologous viruses could recognize the antigenic changes in the PEG-1 epitope. This indicates that the strain-specific determinants can vary independently of the highly cross-reactive (common) determinants (17). Thus, the area of complementarity of the highly cross-reactive antibody combining sites does not comprise the PR8 strain-specific determinants. The present observation does not, however, exclude the possibility that the area of complementarity of the combining site of strain-specific antibodies comprises both strain-specific and cross-reactive determinants, and therefore, does not allow us to conclude that the strain-specific and common determinants are separate entities. The latter could be proven by the demonstration of noncompetitive binding of specific and cross-reactive hybridoma antibodies to the same HA molecule under saturating conditions or by the demonstration that variants selected with cross-reactive antibodies are not recognized by strain-specific antibodies.

The present study selected PR8 variants only on the basis of mutations that modified (among others) the PEG-1 epitope of the parental HA molecule. It remains to be seen whether the same degree of variability also occurs in other HA determinants. This is currently being investigated using anti-HA hybridoma antibodies with other reactivities for selection of variants. It is anticipated that this type of analysis will ultimately provide an estimate of the repertoire of antigenic changes available to a given virus strain such as PR8. Furthermore, it may allow us to accurately trace phylogenetic relationships between virus strains within a subtype through selection of several generations of consecutive variants. And finally, it is likely that a panel of antigenic variants whose primary HA structures differ only by amino acid substitutions related to the antigenic site, may be very useful, in conjunction with amino acid sequencing, in the analysis of the molecular structure of the antigenic area of the HA.

Summary

Antigenic variants of A/PR/8/34 [H0N1] influenza virus were selected after a

single passage of the parent virus in embryonated chicken eggs in the presence of monoclonal antibodies to this virus. The monoclonal antibodies were produced by a hybridoma and were specific for an antigenic determinant on the HA molecule of the parent virus. Seven antigenic variants were analyzed with 95 monoclonal anti-HA antibodies prepared in vitro in the splenic fragment culture system.

Three subgroups of antigenic variants were distinguished. The antigenic changes were primarily recognized by monoclonal antibodies to the strain-specific determinants of the parental hemagglutinin (HA) molecule. Monoclonal antibodies to HA determinants shared (in an identical or cross-reactive form) by parental virus and more than three heterologous viruses of the H0N1 and H1N1 subtypes were unable to recognize the antigenic change on the variants. Similarly, heterogeneous antibody preparations could not differentiate between parental and variant viruses.

The results are compatible with the idea that the HA of PR8 has available a large repertoire of antigenic modifications that may result from single amino acid substitutions, and that antigenic changes can occur in the strain-specific determinants on the HA molecule in the absence of concomitant changes in the cross-reactive HA determinants. The findings suggest that antigenic drift, in order to be epidemiologically significant, probably requires a series of amino acid substitutions in, or close to, the antigenic area on the HA molecule.

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