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Genetic dimorphism in influenza viruses: Characterization of stably associated hemagglutinin mutants differing in antigenicity and biological properties

(virulence/recombination/RNA/vaccine)

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ABSTRACT **Influenza virus recombinant X-53 produced for use in the 1976 National Immunization Program for swine influenza was found to comprise two types of virions differing in their antigenic, replicative, and plaque-forming characteristics. One type, characteristic of X-53 and designated "L," was relatively low-yielding in chicken embryos, produced small clear plaques in Madin–Darby dog kidney cells, and was selectively inhibited by heterotypic antibody to the A/sw/Cam/39 strain of swine influenza virus. The other, X-53a or "H," was high-yielding in chicken embryos, produced large turbid plaques in dog kidney cells, and was not inhibited by concentrations of A/sw/Cam/39 antisera inhibitory to X-53. It was shown that A/NJ/11/76 (HswN1) virus, from which X-53 was derived, and five other swine influenza virus isolates from humans and pigs were dimorphic mixtures of the two types of virus. Segregation of the hemagglutinin genes of L and H variants by further recombination demonstrated that their different properties were pleiotropic phenotypes of mutation in the hemagglutinin gene. Under selective conditions suppressive to the L mutant, mutation of cloned L to H virus was observed. This observation, as well as the apparent ubiquity of the two mutants in nature, suggests that this is another example of viral dimorphism—the stable association of two allelic mutants. Of special significance is the indication that antigenic variants may be selected by selection for properties other than antigenicity, and therefore may represent mutants with pathogenic effects determined by factors other than lesser modulation by host antibody.**

During the conduct of experiments designed to produce a high-yielding influenza vaccine virus for use in the National Immunization Program of 1976, recombinant X-53 was isolated. By antigenic analysis and RNA gel electrophoresis this virus was shown to have derived genes for the external glycoprotein antigens (hemagglutinin and neuraminidase) from the swine influenza virus A/NJ/11/76 (HswN1) and its 6 remaining genes from A/PR/8/34 (1), the high yield (H) donor strain used in the production of H recombinant vaccine strains (2, 3). After determination that X-53 contained only those swine influenza virus genes required for appropriate immunogenicity, we realized that further enhancement of the intermediate yield of this virus could not be expected from further recombination with A/PR/8/34. Therefore, an attempt was made to increase the yield characteristics of the recombinant by additional recombination of the virus with another high-yielding virus, A/swine/Cambridge/39. In that experiment a control passage of X-53 virus alone in the presence of A/swine/Cambridge/39 antiserum used to suppress the external antigens of that virus selected a virus (X-53a) with enhanced replication characteristics. The studies reported here demonstrate that X-53 contained two populations of recombinants from which X-53a was selected; that X-53 and X-53a, as well as their wild-type antecedents, differ in their hemagglutinins; and that this difference, probably based on a single mutation, is correlated with differences in antigenicity, replication, and biological behavior of the viruses.

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MATERIALS AND METHODS

Viruses. A/NJ/76 (HswN1) strains, numbers 8, 9, 10, and 11, were generously provided by Martin Goldfield as first passage chicken embryo material. A/Wisconsin/56/76 (HswN1) and A/Wisconsin/263/76 (HswN1) viruses were received from Bernard Easterday. A/Cam/39 (HswN1) was donated by Geoffrey Schild; A/PR/8/34 has been used for years in this laboratory as a source of "H" genes in recombination experiments (2, 3).

Antisera. Antisera to the viruses specified were produced by intravenous injection of approximately 3000 hemagglutinating units of virus purified by sucrose gradient centrifugation; a second injection was made after 40 days and final bleedings were at 47 days. Ferret sera, kindly supplied by Alan Kendal of The National Center for Disease Control, was obtained 13–14 days after intranasal infection. Nonspecific inhibitors of hemaglutination were removed by treatment with *Vibrio cholerae* neuraminidase (4).

Assays. Viral hemagglutination titers were determined by either the microtiter (5) or tube dilution method (6). Neuraminidase inhibition tests were performed as described (7). Polyacrylamide gel electrophoresis and extraction of viral RNA was carried out as described by Palese and Schulman (8). Cultivation of virus was done in Madin–Darby canine kidney (MDCK) cells (8) or in 10- to 11-day-old white Leghorn chicken embryos. Limit dilution cloning of virus in the chicken embryo was accomplished by injection of 0.5 log dilutions of virus in six eggs per dilution and successive transfer of allantoic fluid from eggs at the highest dilution positive for hemagglutination.

RESULTS

Derivation of X-53a from X-53 (Intermediate Yield) Recombinant. Selection of a H recombinant after dual infection with parental viruses is expedited by "loading" with genes from the H donor and selection against the external antigens of that virus. This can be facilitated by partial inactivation of infectivity of the other parental virus (2, 3). Therefore, prior to its recombination with the high yielding A/swine/Cambridge/39 virus, recombinant X-53 was subjected to thermal inactivation at 30°C for 42 hr with reduction in infectivity from $10^{-7.5}$ to $10^{-4.5}$ EID_{50} (dilution of virus infecting 50% of the eggs). As illustrated in Table 1, partially inactivated X-53 virus

Abbreviations: MDCK, Madin–Darby canine kidney; H, high yield; L, low yield.

Table 1. Emergence of high titer mutant after passage with A/sw/Cam/39 antiserum during recombination experiments

Group	Viral inoculum (EID_{50})	Hemagglutination titer at passage levels* 1†	2‡	3‡	4§
1	X-53 ($10^{3.5}$) + A/sw/Cam/39 ($10^{4.0}$)	512	1024	512	512
2	X-53 ($10^{3.7}$)	10	128	1048	8192
3	A/sw/Cam/39 ($10^{4.3}$)	128	242	1024	8192

* Reciprocal of dilution at titration end point; geometric mean of four fluids.
† No antiserums.
‡ One-tenth milliliter of a 10^{-1} dilution of allantoic fluid virus from preceding passage inoculated with A/sw/Cam/39 antiserum (R-2008 rabbit serum; 40-day bleeding) at 1:10 dilution. (Note that antiserum at this concentration does not completely neutralize homotypic virus.)
§ Dilution (10^{-7}) passage without antiserum.

Table 2. Emergence of H mutant of A/sw/NJ/11/76 virus during low dilution passage of virus with A/sw/Cam/39 antiserum

Passage	Virus	Dilution	A/sw/Cam/39 antiserum*	Hemagglutination titer†
1a	A/sw/NJ/11/76‡	10^{-1}	0	16
b	A/sw/NJ/11/76‡		+	16
2a	1a	10^{-2}	0	16
b	1b	10^{-2}	+	128
3a	1aa	10^{-2}	0	32
b	1bb	10^{-2}	+	128
2a§	1a	10^{-8}	0	16
b	1b	10^{-8}	0	512

* R-2008 rabbit serum; 40-day bleeding.
† Reciprocal of dilution at titration end point; geometric mean of four fluids.
‡ EID_{50} titer = $10^{-7.5}$/0.1 ml.
§ Repeated second passage at high dilution without antiserum.

was inoculated together with A/swine/Cambridge/39 virus and both viruses were inoculated singly as controls. Hemagglutination titers of allantoic fluid removed after the initial (recombination) passage revealed evidence of viral autointerference with the X-53 inoculum. Upon further passage of all groups with A/swine/Cambridge/39 antiserum, hemagglutination titers (indicative of total viral yield) increased in the experimental group in which X-53 alone was being propagated. Thus the practical objective of obtaining a higher yielding variant (X-53a) from X-53 had been attained without recombination.

The possibility that mutation of the virus during passage had occurred seemed unlikely because of our ability to repeat the experiment with either partially activated or nonactivated X-53 virus with the same result. It was concluded that X-53 was a mixture of virions of H and intermediate-yield potential and that conditions favorable for the emergence of a H variant had been fortuitously provided. Because the selective factor appeared to be the use of A/swine/Cambridge/39 antiserum, it was suspected that selection was mediated against one of the external viral antigens of A/sw/NJ/11/76 with consequent suppression of the lower yielding X-53 virus. Limit dilution passage of either inactivated or noninactivated X-53 virus did not result in the emergence of X-53a-like virus in the absence the selective effect of the heterotypic A/swine/Cambridge/39 antiserum.

Demonstration of Low-Yield (L) and H Variants in A/NJ/11/76. A search for preexisting variants of L and H potential in the A/NJ/11/76 virus from which X-53 had been derived (1) was made by a similar selective passage of the uncloned virus with A/swine/Cambridge/39 antiserum. The results of this experiment, summarized in Table 2, demonstrate again the selective effect of the antiserum in eliciting the emergence of H virus.

Existence of L and H Variants in Other Swine Influenza Viruses Recovered during 1976. In experiments of similar design to that summarized in Table 2, low passage viruses A/NJ/8/76, A/NJ/9/76, and A/NJ/10/76 were passaged with and without A/swine/Cambridge/39 antiserum. In each case 4- to 8-fold higher hemagglutination titers were observed after a single low dilution passage of virus with antiserum than in its absence. L or H variants were isolated by limit dilution cloning in eggs for further study. When subsequent isolations of virus were made from humans and swine during 1976, the similar existence of two viral populations in these isolates was revealed by this method. These viruses were A/Wisconsin/56/76 (HswN1), isolated from a pig in Wisconsin and A/Wisconsin/263/76 (HswN1) recovered from a human case unrelated to epidemic activity in man.

Recombination of Cloned L and H Variants of A/sw/NJ/11/76 Virus with A/PR/8/34. Having demonstrated that wild-type virus A/NJ/11/76 (used previously in recombination with A/PR/8/34 virus to produce X-53 and X-53a) comprised both L and H particles, I next carried out experiments in which cloned L and H mutants were recombined separately with A/PR/8/34. Cloning was conducted by the limiting dilution technique in the host system (chicken embryo allantoic sac) in which the primary observations have been made. Cloning of the H variant was facilitated by the use of A/sw/Cam/39 antiserum, but cloning of L was dependent upon dilution alone.

Recombination of L and H variants with A/PR/8/34 was carried out in separate experiments by methods described above. Passage with antiserum suppressive to PR8 virus resulted in the isolation of two recombinants of HswN1sw serotype with different hemagglutination titers (Table 3). Recombinant L-2, which resembled X-53 virus in yield, was shown by RNA gel electrophoresis to have derived all but the hemagglutinin and

Table 3. Recombination of cloned L and H variants of A/NJ/11/76 with A/PR/8/34

Parental variant	No.	Recombinants not suppressed by PR8 antiserum: Phenotype* Antigens	Titer	Genotype†
L‡	L-1	$HswN1_{sw}$	64§	PPPSSSPP
	L-2	$HswN1_{sw}$	512	PPPSPSPP
H¶	H-1	$HswN1_{sw}$	4096	PPPSPSPP
	H-2	$HswN1_{pr8}$	8192	PPPSPPPP

* Defined by hemagglutinin in inhibition and neuraminidase inhibition tests with hemagglutinin- and neuraminidase-specific antigens produced by injection of rabbits with antigenically hybrid recombinant viruses.
† Defined by electrophoresis on polyacrylamide gels of isolated viral RNA (1). Genes are numbered in decreasing order of RNA size (left to right). RNAs 4 and 6 code for hemagglutinin and neuraminidase respectively. P-RNA, migration pattern characteristic of RNA of A/PR/8/34 virus. S-RNA, characteristic of A/NJ/11/76 virus.
‡ Characterized by low (<1024) hemagglutination titer and suppression on passage with A/sw/Cam/39 antiserum.
§ Reciprocal of dilution at titration end point. Geometric mean of hemagglutination titrations of four allantoic fluids after inoculation at 10^8 EID_{50} and 40–44 hr of incubation at 37°C.
¶ Characterized by high (≥4096) hemagglutination titer and nonsuppression upon passage with A/sw/Cam/39 antiserum.

neuraminidase genes from A/PR/8/34 (as had X-53) (1). Recombinant L-1, a low yielding virus also of X-53 serotype, was found by RNA gel analysis to lack the fifth PR8 gene coding for the viral nucleoprotein; this gene is probably requisite for "high yielding" viruses derived from A/PR/8/34 (9).

Recombinants H-1 and H-2 derived from the H variant were high yielding and hence "X-53-like." H-1 was antigenically identical to X-53a but H-2 derived only the hemagglutinin gene from the A/NJ/11/76 (H) donor. These experiments reinforced our concept that the yield limitation of X-53 (and X-53′) was imposed by one of the swine influenza virus external antigens—probably the hemagglutinin.

Segregation of Hemagglutinins of L and H Viruses by Additional Recombination of X-53 and X-53a Recombinants. X-53 and X-53a viruses were cloned by the limiting dilution technique just prior to recombination with A/PR/8/34 with the purpose of segregating the hemagglutinin genes of the recombinants from the only other non-PR8 gene that they contained—i.e., the A/NJ/11/76 neuraminidase gene. In these experiments, selection was accomplished by passage with antiserum to recombinant PR8-HK, selective against the PR8 hemagglutinin, and antiserum to recombinant X-54 (Heq1N1sw) selective against the neuraminidase (N1sw) of X-53. (Fig. 1) In the case of X-53 virus, the desired $Hsw1N1_{pr8}$ recombinant (X-53–PR8) was provisionally identified by its higher yield (hemagglutinin titer 1:4096), prior to definitive identification by neuraminidase inhibition testing with NWS-PR8 antiserum specific for the PR8 neuraminidase in this system. Recombinant X-53a–PR8 also emerged as slightly higher yielding in hemagglutinin than was parental X-53a in comparable passage.

Antigenic Characterization of L and H Variants and Their Recombinants. Preliminary analysis of the A/NJ/11/76 L and H variants and their respective X-53 and X-53a recombinants by HI testing with L- and H-specific antisera showed them to be closely related and distinguishable only with the heterotypic antisera to A/sw/Cam/39 virus used in their selection (Table 4). The selective inhibition of L variants was effected principally with sera from rabbits that had received a single injection of antigen or from ferrets convalescent from infection with A/sw/Cam/39 virus. Sera from rabbits in which a secondary response had been stimulated by additional injections of virus were broadly crossreactive and did not distinguish between L and H variants. However, absorption of such antisera with X-53a–PR8 (H) virus removed antibody reactive with H variants but not antibody reactive with L variants.

The availability of recombinants in which the swine virus L and H hemagglutinins had been segregated permitted their comparative antigenic analysis uninfluenced by possible differences in their associated neuraminidase antigens. Hemagglutination inhibition testing of X-53–PR8 and X-53a–PR8 viruses with antisera to four swine influenza virus antigenic variants including A/sw/Wisc/61, A/NJ/11/76(H), and A/sw/Iowa/15/30 viruses and the related PR8 hemagglutinin revealed no significant differences in reaction patterns for the two viruses other than their differing reaction with A/sw/Cam/39 antiserum. X-53–PR8, the hemagglutinin of which was derived from the L variant, was inhibited at the same titer observed with A/NJ/11/76 (L) and its parental X-53 virus, whereas X-53a–PR8 virus, containing the H hemagglutinin from X-53a, was not inhibited. Similarly, in plaque neutralization tests, X-53–PR8 and X-53a–PR8 as well as the original L and H wild-type variants were differentially inhibited with rabbit or ferret antisera to A/sw/Cam virus.

From these experiments, I conclude that the differing serologic activity of the L and H variants is determined by the viral hemagglutinin and that the hemagglutinin has not undergone

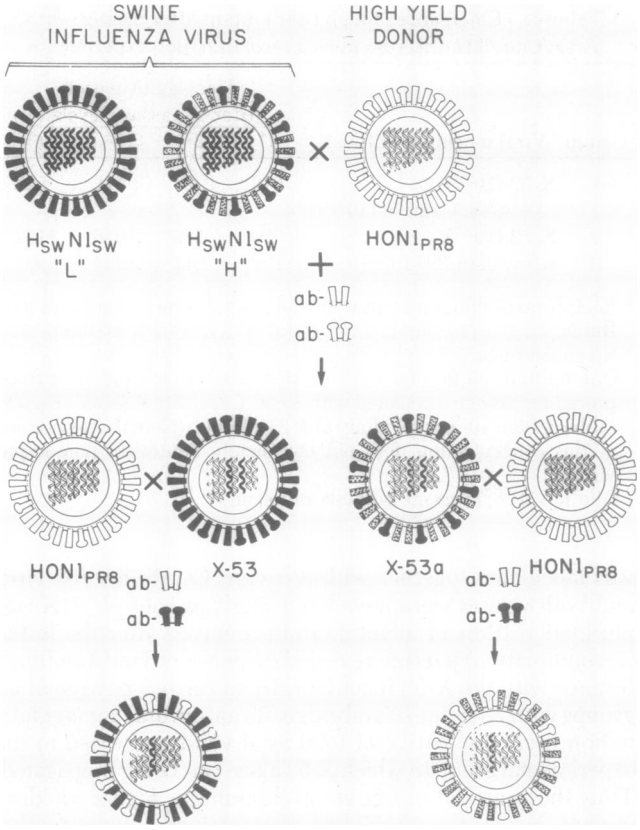

FIG. 1. Schematic representation of the results of recombination of the dimorphic hemagglutinin mutants L and H of A/NJ/11/76 (HswN1) with A/PR/8/34 (H0N1) influenza virus. Selection with antibody (ab) against hemagglutinin (polygonal spikes) and neuraminidase (mushroom-like spikes) of $H0N1_{PR8}$ after mixed infection yielded X-53 with hemagglutinin derived from the L mutant and X-53a deriving its hemagglutinin from the H mutant in the mixed wild-type population of swine influenza virus particles. Genotype determination by RNA gel electrophoresis demonstrated that X-53 and X-53a had derived only hemagglutinin and neuraminidase genes from swine influenza virus and all other genes (internal helices) from $H0N1_{PR8}$. Further recombination of cloned X-53 and X-53a recombinants with $H0N1_{PR8}$ virus produced X-53–PR8 and X-53a–PR8 deriving respectively only the L or H hemagglutinin gene from swine influenza virus. Selection of these recombinants was facilitated by the use of antibody to swine virus neuraminidase.

detectable mutation during its segregation by recombination.

Differences in Biologic Activity Related to L and H Variation. L and H variants of A/NJ/11/76 virus were originally recognized on the basis of their different yields in the allantoic sac of the chicken embryo. Yields of hemagglutinating virus in MDCK cells after inoculation of X-53 virus were one-eighth of that with X-53a virus. Mean plaque size of the L-containing viruses in MDCK cells was less (1.0–3.0 mm) than that of H-containing viruses and L plaques were clear and sharply defined compared to those of the H variant and its recombinants. As summarized in Table 5, these differences and the antigenic differences previously noted appear to be determined by mutation in the A/NJ/11/76 virus hemagglutinin gene.

Difference in L and H Hemagglutinins. L and H hemagglutinins extracted from X-53-PR8 and X-53a-PR8 viruses respectively did not differ with respect to their state of proteolytic cleavage, a possible factor influencing influenza virus replication (10, 11). Hemagglutinins from sodium dodecyl sulfate-disrupted L and H viruses migrated at equivalent rates on polyacrylamide gels, and under reducing conditions HA1 and

Table 4. Antigenic comparison by reciprocal hemagglutination inhibition tests of L and H variants and their recombinants

Antiserum*	Virus (L)	X-53	(H)	X-53a	A/sw/Cam/39
(L) 2186‡	20‡	20	20	40	40
(L) 2187†	40	40	20	80	40
(H) 2188§	320	1280	1280	640	320
(H) 2189§	320	640	1280	640	80
A/sw/Cam/39 R-2008¶	20	20	<10	<10	320

Homologous titers are underlined.
* Serum obtained from rabbits 47 days after initial immunization; second injection at 40 days.
† Immunized with A/NJ/11/76(L) virus.
‡ Reciprocal of serum dilution at titration endpoint.
§ Immunized with A/NJ/11/76(H) virus.
¶ Bleeding (40 day) from "unboosted" rabbit—i.e., no second injection.

HA2 (heavy and light chains) were distinguishable with both viruses. L and H hemagglutinins did not differ in susceptibility to proteolysis with trypsin or to thermal degradation at 56°C. L and H viruses grown in MDCK cells retained their characteristic antigenic phenotypes, lessening the possibility that these differences reflect host determined differences in the glycosylation of their hemagglutinins.

Mechanisms by Which the Two Variant Viral Populations are Maintained in a Dimorphic State. Although only six strains were studied, in each instance genetic dimorphism of the viral populations was easily demonstrated by passage of virus with selective (A/sw/Cam/39) antiserum. When initially characterized, all strains were of the L phenotype, but from each virus the H phenotype emerged after a single passage. Reexamination of the A/NJ/11/76 seed used in recombination to produce X-53, demonstrated a marked preponderance of L phenotype particles with less than 10^3 EID_{50} of H phenotype detectable. Furthermore, recombination of A/PR8/34 virus with each of four A/NJ/76 uncloned strains resulted in the isolation of only X-53-like (i.e., L-like) recombinants under nonselective conditions.

The question arises, then, how H virions arise and by what mechanism are they maintained in association with the numerically predominant L particles. In the chicken embryo host in which H virions are by definition high yielding, their eventual emergence on passage would seem likely. Yet in early passages after isolation from man, L virions predominate. When plaque-cloned A/NJ/11/76 (L) and A/NJ/11/76 (H) variants were inoculated together in equal amounts ($10^{4.7}$ EID_{50}) into chicken embryos and the effects on mutual replication were studied, little mutual interference as measured by reduction of infected virus was demonstrable. However, in another experiment in which higher concentrations of virus were used for inoculation, L virus reduced the final (40 hr) yield of egg infective virus by 2.7 logs. When the ratio of the L recombinant, X-53, to the H recombinant, X-53a, in the inoculum was 10^4, reduction of total H viral yield (measured by hemagglutination) of 1.4 logs was observed. L and H variants did not differ in susceptibility to canine interferon in MDCK cells as measured by plaque inhibition, nor did they differ in replication at 35°C and 39°C as ascertained by plaque-forming unit titration in MDCK cells. Therefore I conclude that L variants can interfere with replication of H variants by mechanisms undefined but probably not related to a greater susceptibility of H to temperature or to interferon. Interference with L replication by H was not possible to measure precisely in the chicken embryo because of overlap of plaque size of L and H variants in virus recovered from the yield of mixed infection.

Table 5. Linkage of three phenotypes to L-H hemagglutinin mutation

Virus	RNA gel "genotype"	Viral yield*	Inhib. by A/sw/Cam/39 antiserum†	Plaque size (mm) in MDCK cells
A/NJ/11/76(L)	SSSSSSSS‡	16	20	1–2.5 (clear)
X-53	PPPSPSPP§	512	20	1–3
X-53-PR8	PPPSPPPP	1024	20	1–3
A/NJ/11/76(H)	SSSS¶SSSS	128	<10	2–4 (turbid)
X-53a	PPPS¶PSPP	4096	<10	2–5
X-53a-PR8	PPPS¶PPPP	8192	<10	2–5

* Hemagglutination titer.
† Reciprocal of serum dilution at end point.
‡ S = migration of RNA on polyacylamide gel characteristic of A/NJ/11/76 virus (1).
§ P = migration of RNA on polyacrylamide gel characteristic of A/PR/8/34 virus (1).
¶ Mutation of hemagglutinin gene not detected by RNA gel migration but only by phenotype.

Mutation of L Variant to H Form. Although the maintenance together of L and H forms might occur as the result of the higher yielding capacity of H and the interfering effect of L, it is not credible that this genetic dimorphism could be maintained in nature without the interconversion of the two forms by mutation.

When A/NJ/11/76 L virus cloned by four successive plaque-to-plaque passages was subjected to five serial passages at low dilution (10^0–10^{-1}) in eggs, the viral "L" phenotype remained unchanged. At the sixth passage, at the 10^{-3} (but not at the 10^{-1}) dilution, virus of high hemagglutination titer was detected in one of eight eggs. This virus after a cloning passage in eggs was shown to be of H phenotype; low titer virus from another egg in the same group was verified to be of L phenotype with respect to antigenic and plaque characteristics.

This experiment provides evidence for the derivation of an H mutant from an L viral population cloned by the exacting criterion of sequential plaque-to-plaque passage.

The search for an L mutant of A/NJ/11/76 (H) virus was conducted by inoculation of 24 MDCK cell culture dishes with approximately 75 plaque-forming units per dish followed by isolation of small plaques. Only 3 of 1800 plaques were less than 3 mm in diameter. Virus from these was of H phenotype. This experiment is obviously inconclusive in answering the question of H to L mutation. In the absence of a selective system against H, the question is answerable only by examination of 10^4–10^6 plaques (probable mutation frequency of 10^{-4}–10^{-6}) derived from H virus.

DISCUSSION

It is well known that animal viruses comprise genetically heterogeneous populations (12–15), and recent evidence from oligonucleotide mapping suggests a high degree of mutability of RNA viruses even under standard conditions of laboratory passage (16). The mutation of influenza virus glycoprotein antigens is commonly observed in nature and is central to the problem of influenza epidemiology and control. In the laboratory, minor antigenic variants are readily isolated after sequential selective passage with homotypic or heterotypic antiserum suppressive to wild-type virus. However, although nonantigenic variation has been remarked with individual viral isolates and strains (17) including differences in morphology (18) and inhibitor susceptibility (19), the existence of mixed

populations of antigenically distinguishable viral particles has been described only recently for swine influenza viruses (20) and has been ascribed to the cocirculation in swine of two types of virus, one (analogous to "H" variants) prevalent between 1957 and 1968, and the other (analogous to "L" variants) emerging about 1971 to comprise the majority of recent isolates from swine.

Supplementing these independent and concurrent epidemiologic observations, the present study has demonstrated (*i*) the predominance of virus of L antigenic serotype under standard conditions of isolation and passage in the chicken embryo; (*ii*) the predictable emergence of the H antigenic serotype related to specific suppression of L particles with heterotypic A/swine/Cambridge/39 antiserum; (*iii*) the segregation of L and H phenotypes by genetic reassortment involving transfer of the hemagglutinin gene; (*iv*) pleiotropism with respect to L and H serologic variation affecting viral growth characteristics and plaque formation; (*v*) mutation of plaque-cloned L genotype to H genotype in the absence of antiserum selection [not observed by Kendal *et al.* (20)].

The circumstances of viral isolation, the ease of demonstrating the two phenotypes, and the evidence for mutation of one phenotype to the other suggests that this is another example of viral genetic dimorphism (19), that is, a relatively stable association of two allelic mutants under conditions of replication and serial passage. Dimorphism with respect to neuraminidase content per particle, viral morphology, and inhibitor susceptibility have been noted (summarized in ref. 17).

The present observations are of special interest in that they demonstrate either (*i*) that change in viral replicative capacity or virulence (plaque size) may fortuitously be associated with antigenic change related to a single mutation or (*ii*) that antigenic variation may be selected for as a concomitant of selection for other properties of survival value (e.g., replication rate, virulence, stability, etc.). Therefore, the biologic properties of an antigenic variant (virulence, transmissibility, etc.) do not necessarily reflect only changed (lesser) modulation by host antibody.

Viral polymorphism provides the virus with obvious advantages for survival beyond those offered by its intrinsic mutational capacity. Although the L variant appears to be at an ultimate disadvantage in the chicken embryo or in MDCK cell culture, it replicates far more efficiently than the H variant in swine, its natural host, under conditions of experimental infection (R. Easterday and E. D. Kilbourne, unpublished results). Therefore, a virus dependent upon more than one host species for its survival could adapt readily to a change in host through selection of a preexisting phenotype or through repetition of the same high frequency mutation.

Specific mutations affecting the hemagglutinin of influenza viruses have been reported to emerge predictably and repeatedly upon selection of antigenic variants with monoclonal antibody (21). Minor antigenic dimorphism of current swine influenza viruses does not appear to explain the transient adaptation of swine virus to man at Ft. Dix, New Jersey in 1976. As reported here and by Kendal *et al.* (20) both variants (L and H in my terminology and subpopulations 1 and 2 in Kendal's) have been demonstrated in chicken embryos after recovery of virus from both human and swine hosts. However, given the demonstrated mutability of one form to the other, it remains unclear whether or not one form alone replicates selectively in one or the other host under natural conditions. An H variant (in the absence of demonstrable L) has been recovered directly from swine in high titer in the absence of selective antiserum, which suggests that the H variant is not merely a mutant selected for during passage in the chicken embryo (unpublished results).

The precise nature of the structural differences of the dimorphic L and H variants should be instructive in determining how a single mutation can influence both antigenicity and viral biologic activity.

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1. Palese, P., Ritchey, M. B., Schulman, J. L. & Kilbourne, E. D. (1976) *Science* **194**, 334–335.
2. Kilbourne, E. D. (1969) *Bull. WHO* **41**, 643–645.
3. Kilbourne, E. D., Schulman, J. L., Schild, G. C., Schloer, G., Swanson, J. & Bucher, D. (1971) *J. Infect. Dis.* **124**, 449–462.
4. Palmer, D. F., Coleman, M. T., Dowdle, W. R. & Schild, G. C. (1975) *Advanced Laboratory Techniques for Influenza Diagnosis*, Immunology Series No. 6 (U.S. Department of Health, Education and Welfare/U.S. Public Health Service, Washington, DC).
5. Sever, J. L. (1962) *J. Immunol.* **88**, 320–329.
6. Wong, S. C. & Kilbourne, E. D. (1961) *J. Exp. Med.* **113**, 95–110.
7. Aymard-Henry, M., Coleman, M. T., Dowdle, W. R., Laver, W. G., Schild, G. C. & Webster, R. G. (1973) *Bull. WHO* **48**, 199–202.
8. Palese, P. & Schulman, J. L. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2142–2146.
9. Schulman, J. L. & Palese, P. (1978) in *Negative Strand Viruses and the Host Cell*, eds. Barry, B. D. & Mahy, B. W. J. (Academic, London), in press.
10. Lazarowtiz, S. G. & Choppin, P. W. (1975) *Virology* **68**, 440–454.
11. Klenk, H. D., Rott, O. M. & Blodorn, J. (1975) *Virology* **68**, 426–439.
12. Walen, K. H. (1963) *Virology* **20**, 230–234.
13. Fenner, F. & Sambrook, J. F. (1964) *Annu. Rev. Microbiol.* **18**, 47–94.
14. Cooper, P. D. (1967) *Br. Med. Bull.* **23**, 155–160.
15. McCormick, K. J. & Murphy, W. H. (1969) *Nature (London)* **221**, 286.
16. Clewley, J. P., Bishop, D. H. L., Kang, C.-Y., Coffin, J., Schnitzlein, W. M., Reichmann, M. E. & Shope, R. E. (1977) *J. Virol.* **23**, 152–166.
17. Mowshowitz, S. & Kilbourne, E. D. (1975) in *Negative Strand Viruses*, eds. Barry, R. D. & Mahy, B. W. J. (Academic, London), Vol. 2, pp. 765–775.
18. Kilbourne, E. D. & Murphy, J. S. (1960) *J. Exp. Med.* **111**, 387–406.
19. Choppin, P. W. & Tamm, I. (1960) *J. Exp. Med.* **112**, 895–920.
20. Kendal, A. P., Noble, E. R. & Dowdle, W. R. (1977) *Virology* **82**, 111–121.
21. Gerhard, W. & Webster, R. G. (1978) *J. Exp. Med.* **148**, 383–392.