Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 7782–7785, October 1988
Medical Sciences

Hemagglutinin polymorphism as the basis for low- and high-yield phenotypes of swine influenza virus

(nucleotide sequence/point mutation/virulence/pleiotropism/antigenic variation)

EDWIN D. KILBOURNE*†, ALEXANDER H. TAYLOR‡, CHARLES W. WHITAKER*§, RAGHAVENDRA SAHAI*¶, AND ANDREW J. CATON‡

*Mount Sinai School of Medicine of the City University of New York, New York, NY 10029; and ‡The Wistar Institute, 36th and Spruce, Philadelphia, PA 19104

Contributed by Edwin D. Kilbourne, July 7, 1988

ABSTRACT **Single amino acid substitutions at the rim of the receptor binding site of the hemagglutinin molecule of swine influenza virus markedly influence the replicative capacity of the virus in chicken embryos, Madin–Darby canine kidney cells (MDCK), and swine as well as its antigenic phenotype. Mutants of low-yield (L) phenotype replicate poorly in chicken embryos and produce small plaques in MDCK cells but are highly infective for swine. Such mutants have lysine at position 153 and glycine at position 155 of the hemagglutinin (residues 156 and 158 in the H3 model). High-yield (H) mutants have the converse replicative characteristics and can be antigenically distinguished from L mutants (and from each other) based on their differential reactivity with two monoclonal antibodies, 9C8 and Sa-13. H mutants differ from L mutants in that the H mutants express glutamic acid at either position 153 or 155. L and H mutants act in an allelic fashion in effecting predictable one-step adaptation to different hosts. Selection for replication (e.g., high-yielding) phenotype results in concordant pleiotropic change in antigenic phenotype and in genotype. Conversely, immunoselection leads to change in replicative phenotype. Although the mechanism by which these mutations affect viral replication has not yet been defined, they may reflect differences in the affinity of each mutant for different host receptors.**

Single amino acid substitutions at the rim of the receptor binding site of the hemagglutinin (HA) molecule of swine influenza virus markedly influence the replicative capacity of the virus in various hosts, as well as its antigenic phenotype. Mutants of A/NJ/11/76 (H1N1) influenza virus of low-yield (L) phenotype replicate only to low titer in the allantoic sac of the chicken embryo and in Madin–Darby canine kidney (MDCK) cells, but they replicate efficiently in swine, the natural host. High-yield (H) mutants of the virus have the opposite characteristics; they multiply efficiently in laboratory hosts but poorly in swine (1). The mutants are distinguishable with monoclonal antibodies (2), and their pleiotropic phenotypes have been related to transitional mutations resulting in Gly → Glu changes at amino acid 155 of the HA [analogous to amino acid 158 in the H3 HA model (3, 4)]. For the most part, L-phenotype viruses prevail, but both L- and H-phenotype viruses are found in nature (5) and are readily selected in the laboratory by passage with an appropriate monoclonal antibody or on the basis of growth characteristics.

In recent studies of these mutants, we have noted the emergence of the L-phenotype virus in swine inoculated with cloned H-phenotype virus (6). In investigating this remarkable phenomenon, we noted differences in the antibody reactivity pattern of this H isolate from H-phenotype viruses sequenced previously (3). This finding and our interest in the *in vivo* evolution of L-phenotype virus from H-phenotype virus in the course of a single infection prompted the present inquiry into the apparent polymorphism of the HA with respect to its role in viral replication.

MATERIALS AND METHODS

Viruses. All viruses studied represent mutants of the H1N1 swine influenza virus isolated since 1976 or reassortants in which only their HA or their HA and neuraminidase genes have been segregated in context to genes derived from the A/PR/8/34 (PR8) virus (Table 1).

L and H mutants isolated sequentially from the same pig (no. 115) experimentally infected with A/NJ/11/76 (H) phenotype virus (6) were reassorted with PR8 virus (3, 7, 8) by coinfection of chicken embryos to produce the high-yielding viruses 115(L)R and 115(H)R. Hemagglutination inhibition (HI) and neuraminidase inhibition tests demonstrated that both reassortants contained the HA gene derived from the A/NJ/11/76 (swine virus) parent and the neuraminidase gene from the PR/8 virus. All other genes of these viruses were derived from the PR8 virus as was shown by polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled viral proteins from infected MDCK cells. Viral M proteins were identified in ELISA tests (9) with a monoclonal antibody specific for the M1 protein of the PR8 virus (D. Bucher, personal communication).

A/Ty/Ks/4880/80 and A/Ty/Mo/1/81 are H1N1 viruses, isolated from turkeys, that are antigenically indistinguishable from contemporary swine influenza strains (10). The viruses were generously provided by V. S. Hinshaw and R. G. Webster (St. Judes Medical Center, Memphis, TN). L and H phenotypes were selected in the Mount Sinai laboratory (E.D.K.).

All viruses were stored at −70°C as chicken embryo allantoic fluid seeds and were propagated in chicken embryos for extraction of RNA or antigenic and replication studies.

Antibody Preparations and Antigenic Analysis. The nature and derivation of monoclonal antibodies Sa-13 and 9C8 and methods for antigenic analysis have been described (2).

Plaquing of Viruses. Plaquing of viruses was carried out in MDCK cells with trypsin-containing minimum essential medium by methods described previously (2).

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Abbreviations: HA, hemagglutinin; HI, hemagglutination inhibition; PR8, A/PR/8/34 (H1N1); NLNH, non-L, non-H.

†To whom reprint requests should be addressed.
§Present address: Parke Davis, Biological Support, Rochester, MI 48063.
¶Present address: Waksman Institute of Microbiology, Rutgers, The State University of New Jersey, Piscataway, NJ 08854.

Table 1. Characterization of viruses employed

No.	Laboratory designation	Swine influenza virus antecedent	Reassortant*	Surface antigen HA	Surface antigen NA	Other viral protein	Selection	Ref(s).
1	115(L)†	A/NJ/11/76 (H1N1)		SW‡	SW	SW	From 115(H) in swine	6
2	115(H)†	A/NJ/11/76 (H1N1)		SW	SW	SW		6
3	115(H) (9C8-S)	A/NJ/11/76 (H1N1)		SW	SW	SW	Further egg passage of 115(H) with 9C8 antibody	—
4	115(L)R	A/NJ/11/76 (H1N1)	+	SW	P§	P		—
5	115(H)R	A/NJ/11/76 (H1N1)	+	SW	P	P		—
6	115(L)R H^2(NS)	A/NJ/11/76 (H1N1)	+	SW	P	P	L to H^2 phenotype mutant from egg passage	—
7	X-53	A/NJ/11/76 (H1N1)	+	SW	SW	P		1
8	X-53 PR8(L) NLNH	A/NJ/11/76 (H1N1)	+	SW	P	P		—
9	X-53a	A/NJ/11/76 (H1N1)	+	SW	SW	P		1, 8
10	X-53a PR8(H) NLNH	A/NJ/11/76 (H1N1)	+	SW	P	P		1
11	A/Ty/Ks/4880/80(L)	A/Ty/Ks/4880/80¶		SW	SW	SW		10
12	A/Ty/Ks/4880/80(H)	A/Ty/Ks/4880/80¶		SW	SW	SW		10
13	A/Ty/Mo/1/81(L)	A/Ty/Mo/1/81‖		SW	SW	SW		10

NA, neuraminidase.
*From reassortment with PR8.
†Isolated from swine.
‡Derived from A/NJ/11/76 (H1N1) or related swine influenza viruses (viruses 11–13).
§Derived from PR8.
¶Field strains of "swine" (H1N1) viruses isolated from turkeys.
NLNH, non-L, non-H (not reactive with Sa-13 or 9C8 antibody in HI).

Nucleotide Sequence Analysis of Swine Influenza Virus HA Genes. Synthetic deoxyoligonucleotide primers of defined sequence were prepared by phosphoramidite chemistry on the Biosearch SAM-1 automated DNA synthesizer. Nucleotide sequence analysis of viral RNA was performed essentially as described (11).

RESULTS

On the basis of their comparative titers in chicken embryo allantoic fluid and plaque size in MDCK cells, the mutant viruses are separable into L and H phenotypes [e.g., compare 115(L) with 115(H), Table 2]. Moreover, the expression of each phenotype correlates with the reactivity pattern each virus displays with the Sa-13 and 9C8 antibodies. Thus, L-phenotype viruses are clearly separable from H-phenotype viruses in their nonreactivity with monoclonal antibody Sa-13 in HI tests. Viruses of H phenotype exhibit either of two different reactivity patterns with Sa-13 and 9C8 antibodies. One type, which we now term H^1, resembles the previously described virus X-53a (3). H^1 mutants are inhibited to higher titer by Sa-13 than by 9C8 antibody. The other type, termed H^2, reacts to high titer with Sa-13 antibody but, unlike H^1 viruses, also reacts with the 9C8 antibody. Accordingly, the L, H^1, and H^2 phenotypes are definable by nonoverlapping HI titer ratios.

Reassortant viruses containing the 115(L) and 115(H) HAs express the same antigenic characteristics as were observed in the swine parental viruses. In addition, virus 115(L)R H^2(NS) [a 115(L)R revertant], L and H mutants of the

Table 2. Antigenic and replication phenotype of swine influenza virus HA mutants in relation to point mutations in the HA

No.	Virus	Reassortant	Log_2 HI* titer mAb Sa-13	Log_2 HI* titer mAb 9C8	HI titer ratio, Sa-13/9C8	Log_2 HA titer	Plaque size, mm	Virus phenotype	Deduced amino acid sequence 153†	154	155	156	157	158	159	160	161	162	163
1	115(L)		1	12	0.08	2.0	2.5	L											‡
2	115(H)		6	12	0.50	6.0	4.0	H^2											‡
3	115(H) (9C8-S)		7	4	1.75	7.0	4.0	H^1	Glu	—	—	—	—	—	—	—	—	—	Arg§
4	115(L)R	+	2	11	0.18	9.0	3.0	L	Lys	Lys	Gly	Asn	Ser	Tyr	Pro	Lys	Ser	Lys	Lys¶
5	115(H)R	+	6	11	0.55	12.0	5.0	H^2	Glu	—	—	—	—	—	—	—	—	—	—¶
6	115(L)R H^2(NS)	+	6	11	0.55	12.0	5.0	H^2	Glu	—	—	—	—	—	—	—	—	—	—§
7	X-53	+	1	5	0.20	9.0	3.0	L	—	—	—	—	—	—	—	—	—	—	—§
8	X-53 PR8(L) NLNH	+	<1	<1		10.0		NLNH	—	Glu	—	—	—	—	—	—	—	—	—§
9	X-53a	+	9	4	2.25	12.0	5.0	H^1	—	—	Glu	—	—	—	—	—	—	—	—¶
10	X-53a PR8(H) NLNH	+	<1	<1		12.0	6.0	NLNH	—	—	Glu	—	—	—	Thr	—	—	—	—§
11	A/Ty/Ks/4880/80(L)		1	5	0.20	7.0	4.0	L	—	—	—	—	—	—	—	—	—	—	—§
12	A/Ty/Ks/4880/80(H)		5	<1	5.00	10.0	5.5	H^1	—	—	Glu	—	—	—	—	—	—	—	—§
13	A/Ty/Mo/1/81(L)		1	5	0.20	3.0	2.0	L	—	—	—	—	—	—	—	—	—	—	—§

mAb, monoclonal; —, indicates that the residue is identical to that in the 115(L) reassortant (virus no. 4)
*Expressed as the reciprocal of dilution at endpoint.
†Corresponds to amino acid 156 in the H3 (X-31) HA sequence.
‡No sequence data obtained, defined by phenotype only.
§Partial sequence.
¶Complete HA RNA nucleotide sequence obtained.

wild-type turkey virus A/Ty/Ks/4880/80, and the previously described X-53 and X-53a reassortants display antigenic phenotypes that permit their classification either as L, H^1, or H^2. Although the reassortant and turkey viruses in each case grow to higher titer and produce larger plaques than the parental 115(L) and 115(H) viruses described above (reflecting a different background of non-HA genes), both turkey and reassortant viruses show the same correlation between growth characteristics and antigenic phenotype as was found in the swine parental viruses of the reassortants.

Molecular Basis for L and H Phenotypes. Without exception, L-phenotype viruses have lysine at position 153 and glycine at 155. These include X-53 (3), the 115(L) reassortant, and the two swine wild-type influenza viruses isolated from turkeys (10). In contrast, viruses of the H phenotype have acquired a glutamic acid residue either at position 153 or 155. Accordingly, the appearance of a glutamic acid residue at one of two positions that are in close proximity to each other on the rim of the receptor binding site results in the altered growth phenotype of these viruses.

The dissection of the H-phenotype viruses into H^1 and H^2 results from the different epitopes that each antibody recognizes. The binding of the 9C8 antibody [which was generated by immunization with an L-type virus (2)] is abolished by the Gly → Glu change at position 155 but not by the Lys → Glu change at position 153 [compare X-53a with 115(H)R, Table 2]. The reduced binding of this antibody to virus 115(H) (9C8-S) is most readily interpreted as resulting from the immunoselection by this antibody of the Lys → Arg change at position 163. The failure to bind other viruses cannot be accurately accounted for since interpretation is based on partial sequence analysis; however, the Lys → Glu change at position 154 might account for the failure to bind X-53 PR8(L) NLNH. X-53 may carry an undetected mutation that reduces the binding affinity of 9C8 antibody, which results in a lower HI titer.

The Sa-13 antibody (which was generated by immunization with PR8) binds to all H-type viruses but does not bind to L-type viruses. Table 3 shows a comparison of the relevant amino acid sequence of PR8 HA with sequences of the mutant swine viruses; in addition, positions at which amino acid substitutions were previously shown to abolish binding of this antibody to PR8 or not to affect binding are indicated. Relative to PR8, all of the swine viruses display substitutions at positions 156, 161, and 163. Viruses 115(H)R and X-53a also display individual differences at positions 153 and 155, but these differences do not prevent binding. However, when both these substitutions are present [as in virus 115(L)], antibody binding is abolished, indicating that it is the additive effect of these mutations that prevents binding. The failure of Sa-13 to bind X-53a PR8(H) NLNH can be accounted for by the Pro → Thr change at position 159 [based on previous characterization of PR8 mutant viruses (11)]. Finally, the Lys → Glu interchange at position 154 apparently blocks binding of both 9C8 and Sa-13 antibodies to X-53 PR8(L) NLNH.

DISCUSSION

The L and H HA mutants of swine influenza virus are intrinsically interesting because they appear to act allelically in effecting predictable one-step adaptation of the virus to different hosts. In the present studies, we have confirmed the importance of amino acid substitutions at or in the immediate vicinity of HA position 155 (position 158 in the H3 model) in markedly altering the replicative capacity of the virus and its reactivity with two HA-specific monoclonal antibodies. We have shown polymorphism with regard to virus of the H phenotype with the demonstration that glutamic acid substitution not only at position 155 but alternatively at position 153 can diminish the replication of virus in swine (6) and enhance replication in chicken embryos and MDCK cells.

It is important to stress that both the Glu-153 and Glu-155 H mutants manifest their biologic phenotype not only in laboratory hosts but also in swine, their natural host, in which infectivity is diminished 50- to 100-fold (5, 6). A previous study has shown that an H^1 reassortant of PR8 and A/NJ/11/76 (not discussed here) is incapable of infecting swine, whereas an L reassortant with identical gene composition can (5). Data presented elsewhere show that the H^2 mutant not only is poorly infective for swine but is rapidly replaced by the L variant during the course of H^2 virus infection (6).

The construction of high-yielding reassortants in which the swine influenza virus HA gene has been segregated from other genes of the virus not only has facilitated isolation of viral RNA for sequence determination but also reinforces evidence that the virus phenotype is determined by the HA gene (1, 3) and shows clearly that HA modulation of virus replication occurs in the context of either swine or PR8 viral genes. Furthermore, selection for replication (high-yielding and large plaque) phenotype results in a concordant pleiotropic change in antigenic phenotype. Conversely, escape mutants from immunoselection with monoclonal antibodies or spontaneous revertants (3) are demonstrably changed in biologic phenotype.

This is not to say that yield or plaque phenotypes are necessarily monocistronically determined only by HA—e.g., we expect that not all large plaque L to H revertants will necessarily be H antigenically but that some may reflect mutation in other viral genes that affect plaque size. However, taken together, yield and plaque size markers thus far have been consistent predictors of antigenic phenotype (Table 2).

We do not yet know the mechanism by which single amino acid changes at positions 153 and 155 so markedly influence host-determined differences in replication of swine influenza virus. Mutation at position 155 (Gly → Glu change) (position 158 in the H3 HA model) has been shown to alter the affinity of virus for receptors containing the sialic acid α-galactose (2, 6) linkage (12). Also, with H3N2 virus, Katz *et al.* (13) found Lys → Glu changes at the site analogous to position 153

Table 3. Influence of amino acid substitutions on binding of antibody Sa-13 to mutant viruses

Virus	Sa-13 binding	Deduced amino acid sequence										
		153*	154	155*	156*	157†	158	159†	160†	161	162*	163*
PR8		Glu	Lys	Glu	Gly	Ser	Tyr	Pro	Lys	Leu	Lys	Asn
115(H)R	+	—	—	Gly	Asn	—	—	—	—	Ser	—	Lys
X-53a	+	Lys	—	—	Asn	—	—	—	—	Ser	—	Lys
115(L)R	−	Lys	—	Gly	Asn	—	—	—	—	Ser	—	Lys

Virus sequences in the region of HA in which substitutions were found are shown. They are numbered according to the sequence of WSN (H1N1) virsus HA. —, indicates that the residue is identical to that in the PR8 virus.

*Positions at which substitutions were found not to prevent binding (when present as single mutations in PR8 mutant viruses).

†Amino acids in the PR8 sequence at which substitution has previously been seen to prevent binding of Sa-13 to PR8 mutant viruses.

(position 156 in the H3 HA model) in association with adaptation to laboratory hosts and antigenic variation. Furthermore, variation in receptor binding affinity has been found in natural (i.e., nonselected) strains of virus, and their epidemiological implications have been pointed out (reviewed by Wiley and Skehel in ref. 14). Gly → Glu changes at the position (residue 158) equivalent to swine influenza virus residue 155 have been found in natural antigenic variants of H3N2 viruses (15). In the present case, the correlation between the appearance of glutamic acid at either position and the L phenotype in swine may reflect a decreased affinity for the swine cellular receptor induced by the introduction of a negatively charged amino acid residue at particular positions on the rim of the receptor binding site. Conversely, the same substitution apparently increases the affinity of the virus for the cellular receptor that is present in the chicken embryo and on MDCK cells.

Regardless of the mechanism, the present studies show that influenza virus genetic polymorphism exists not only with respect to the general evolution of influenza A virus genes (16) and to the dimorphic coexistence of dissimilar phenotypes within a viral population (1, 17) but also with respect to determination of a single biologic phenotype. Thus, enhanced replication in laboratory hosts and diminished infectivity for swine can be accomplished either by a Gly → Glu change at position 155 or by a Lys → Glu change at position 153. If selection pressure by monoclonal antibody 9C8 is put on the latter (H^2) mutant, it becomes indistinguishable from H^1 in replication and antigenic phenotype through another single nucleotide change substituting arginine for lysine residue 163. Thus, phenotype H^1 comprises at least two genotypes.

The swine influenza virus L and H phenotypes are not mere laboratory curiosities of antibody selection, but they represent yet another survival strategem of influenza viruses. In the course of a single infection of swine with the H phenotype, emergence and eventual predominance of the L phenotype rapidly and predictably occurs (6), just as L is supplanted by the H phenotype virus during passage of virus in laboratory hosts.

The excellent technical work of Ms. B. A. Pokorny is gratefully acknowledged. This work was supported by Public Health Service Grant AI 09304.

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