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Hemagglutinin Mutants of Swine Influenza Virus Differing in Replication Characteristics in Their Natural Host

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In two mutant clones (L and H) of A/NJ/11/76 (Hsw1N1) influenza viruses which differ slightly antigenically and markedly in replication characteristics in chicken embryos and Madin Darby canine kidney cells, these pleiotropic differences are mediated by mutation in the hemagglutinin gene (E. D. Kilbourne, Proc. Natl. Acad. Sci. U.S.A. **75**:6258-6262, 1978). Experimental infection of swine with either the mutant L and H clones or recombinant viruses differing genetically only with respect to the presence of L or H hemagglutinin demonstrated greater infectivity for the natural host of viruses bearing the L hemagglutinin. Introduction of the L but not the H hemagglutinin gene into the human influenza virus A/PR/8/34 rendered it infective for swine. Both L and H variants were isolated from pigs naturally infected with contemporary swine influenza viruses when selective conditions for the suppression of the more prevalent L mutant were employed. The L and H mutants of swine influenza virus are yet another example of viral dimorphism in nature and probably are not mere artifacts of laboratory selection. In any event, the frequent apparent allelic appearance of the two forms suggests frequent mutation and/or reversion involving a point mutation in the hemagglutinin gene. The present studies demonstrate the importance of a single gene in the pathogenesis of an influenza viral infection in its natural host.

Recently isolated swine influenza viruses have been shown to possess two antigenically distinguishable subpopulations (4, 5) that differ pleiotropically in their replicative characteristics in chicken embryos (eggs) and Madin Darby canine kidney cells. One mutant type, "L," is low yielding in these hosts and reactive with a heterotypic antiserum, and the other "H" variant is high yielding and nonreactive with this antiserum (5). Recombinational reassortment has demonstrated that differences in the two mutants are mediated through mutation in the hemagglutinin gene; i.e., segregation of the L or H hemagglutinin gene in recombinants in which all other genes are derived from the human virus A/PR/8/34 results in preservation of the L and H characteristics manifest in the wild-type mutants (5) (see Table 1).

The significance of the relatively stable association in nature of two mutant forms (viral genetic dimorphism) (2, 5) is unclear. Therefore, we investigated the replication of these well characterized L and H mutants and their recombinants in experimental infections of swine, their natural host. In addition, we have made preliminary investigations of the occurrence of L and H mutants in material from swine suffering natural infection.

MATERIALS AND METHODS

Viruses. The viruses used in this study were described in detail in a previous report (5) and are further characterized with respect to their properties in Table 1. They represent two mutants of A/NJ/11/76 (Hsw1N1) virus ("L" and "H" variants) and recombinants X-53-PR8 and X-53a-PR8 derived from their recombination with A/PR/8/34 virus. The genealogy and ribonucleic acid genotype of the viruses are shown in Fig. 1.

Antiserum. Antisera to A/sw/Cam/39 influenza virus was prepared in ferrets or rabbits as described previously (5). A 40-day bleeding of rabbit serum (R-2008) was obtained after a single intravenous injection of 3,000 hemagglutinating (HA) units of virus and was the serum principally used in the characterization of L and H phenotypes.

Experimental infection of swine. The pigs were young (4 to 10 weeks), purebred Yorkshire pigs ranging in weight from 10 to 40 kg. They were obtained from two different herds known to be free of swine influenza. They were infected by intranasal instillation of 1 ml of virus preparation in each nostril (see Table 2 for the various doses). The virus was suspended in brain heart infusion broth (Difco 0037-01). Blood was collected before infection and tested for influenza virus antibody. Nasal swabs were collected daily for 8 days after infection. The swabs were transported in 25% glycerol-phosphate-buffered saline, pH 7.2, containing penicillin, dihydrostreptomycin, and tylosine. These were

stored frozen at −70°C until they could be tested for the presence of virus. Rectal temperatures were recorded daily for 8 days, and the pigs were observed daily for any signs of disease.

Virus isolation. Virus isolation was carried out by inoculation of 10- or 11-day-old chicken embryos intra-allantoically or monolayers of trypsin-treated Madin Darby canine kidney cells (9), or by both procedures.

RESULTS

Experimental infection of swine with A/NJ/11/76 hemagglutinin mutants. After inoculation of the L phenotype mutant in graded dosage, virus was recovered from all 15 pigs inoculated on one or more days of the 8-day observation period (Table 2). In all but one animal inoculated with the smallest amount of virus (10^2 50% egg infective dose [EID_{50}]), virus was isolated on three or more successive days after inoculation. Significant signs of disease or elevation of temperature was not observed with these or other animals inoculated in the present experiments.

In the case of animals inoculated with equivalent EID_{50} of the H hemagglutinin mutant, fewer than 50% of swine were infected. Only one of five animals excreted virus in the group given 10^2 EID_{50} and then only on the terminal day of observation. Virus was not reisolated from this animal (see below).

Virus was reisolated from 40 of 47 specimens from which reisolation was attempted in eggs and from 35 specimens concomitantly inoculated in Madin Darby canine kidney cells monolayers, in which plaque formation was demonstrated. All reisolates were characterized on the basis of yield in eggs as L (HA titer 1:4 to 1:16) or H (HA titer 1:32 to 1:128) and in each instance proved indistinguishable from the virus inoculated. Serological testing of all H reisolates confirmed their identity as such; those L reisolates tested had also maintained their serological characteristic during swine passage.

TABLE 1. *Summary of characteristics of wild-type and recombinant viruses containing L or H hemagglutinin*

Virus	Inhibited with A/sw/Cam/39 antisera[a]	Plaque size in MDCK[b] cells (mm)	Virus yield in eggs
A/NJ/11/76 (L) ($Hsw1N1_{sw}$)	+	1.0–2.5	16[c]
X-53-PR8 (L) ($Hsw1N1_{PR8}$)	+	1.0–3.0	2,048
A/NJ/11/76 (H) ($Hsw1N1_{sw}$)	0	2.0–4.0	256
X-53a-PR8 (H) ($Hsw1N1_{PR8}$)	0	2.0–5.0	8,192

[a] In HA-inhibition and neutralization tests (for details see reference 5).
[b] MDCK, Madin Darby canine kidney.
[c] HA titer.

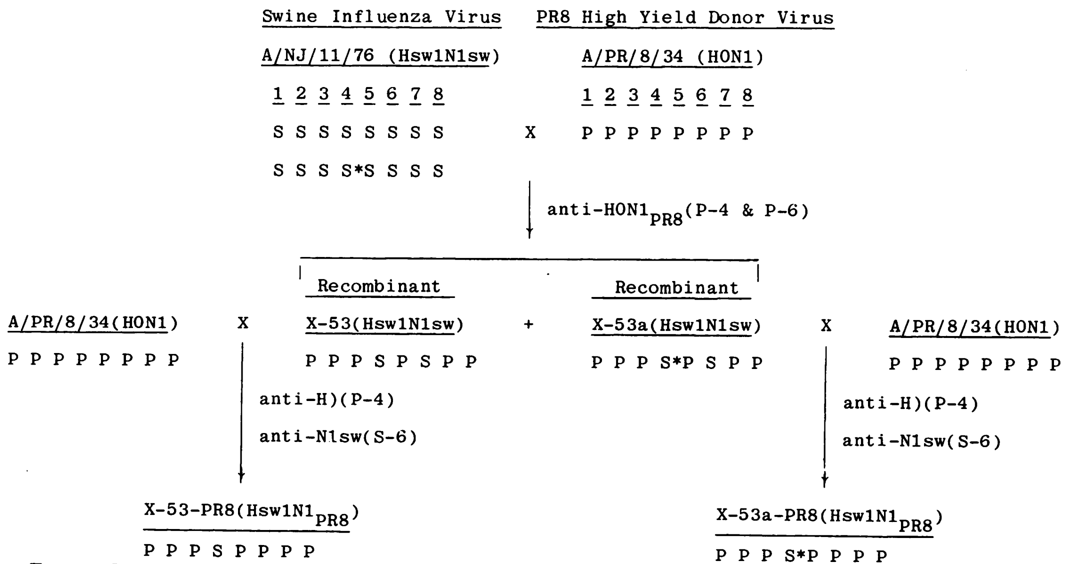

FIG. 1. *Genealogy and genotype of swine PR8 (high-yield donor), and their recombinant progeny influenza viruses. Swine influenza virus (strain A/NJ/11/76) is represented as a mixture containing antigenically distinguishable hemagglutinin mutants S-4 and S*-4 (L and H, respectively). Ribonucleic acid genes are numbered in decreasing order of molecular size as determined by ribonucleic acid gel electrophoresis by Peter Palese. Genes 4 and 6 code for hemagglutinin and neuraminidase antigens corresponding with serotype designation given parenthetically (Hsw1 = S-4 etc.). See also footnote to Table 2. Data from reference 5.*

TABLE 2. *Excretion of virus from pigs inoculated with hemagglutinin mutants L and H and their recombinants*

Inoculum											No. pigs infected	No. pigs excreting virus on day:								Summary: virus excretion[c]
Virus	Clone[a]	Viral genotype[b]								Dose (EID_{50})		1	2	3	4	5	6	7	8	
		1	2	3	4	5	6	7	8											
A/NJ/11/76	L	S	S	S	S	S	S	S	S	$10^{6.3}$	6/6	5	6	6	6	6	6	1	0	36/48
										$10^{5.0}$	4/4	0	1	3	4	4	4	3	0	19/32
										$10^{2.0}$	5/5	0	0	1	3	3	4	4	4	18/40
A/NJ/11/76	H	S	S	S	S[d]	S	S	S	S	$10^{5.0}$	2/5	0	2	1	0	0	1	2	1	7/40
										$10^{2.0}$	1/5	0	0	0	0	0	0	0	1	1/40
X-53-PR8	L	P	P	P	S	P	P	P	P	$10^{5.7}$	5/5	1	1	1	1	2	5	1	1	14/40
X-53a-PR8	H	P	P	P	S[d]	P	P	P	P	$10^{5.6}$	0/5	0	0	0	0	0	0	0	0	0/40
A/PR/8/34	—	P	P	P	P	P	P	P	P	$10^{6.3}$	0/5	0	0	0	0	0	0	0	0	0/40

[a] L or H hemagglutinin phenotype (see text).

[b] Viral genotype determined by gel electrophoresis of virion ribonucleic acid by Peter Palese. 1, 2, and 3, Genes for P (polymerase) proteins; 4, hemagglutinin gene; 5, nucleoprotein gene; 6, neuraminidase gene; 7, M protein gene; and 8, gene for nonstructural protein (5).

[c] Number of days on which virus was excreted/number pig days of observation.

[d] Mutation in hemagglutinin gene conferring H phenotype.

In pigs inoculated with the L mutant, a dose response was evident; virus excretion was detectable on the average of 1.2 days after $10^{6.3}$ EID_{50}, 3.0 days after 10^5 EID_{50}, and 5.6 days after the smallest inoculum, 10^2 EID_{50}. Even in these previously thawed specimens, virus concentrations of 10^3 to 10^4 EID_{50} or plaque-forming units per 0.1 ml were demonstrated. In two animals—one given 10^2 EID_{50} and the other given 10^5 EID_{50}—virus was recovered on days 3 to 7 postinfection with peak titers on day 5, providing clear evidence of virus replication. Antibody response was not measured.

Only 3 of 10 animals were infected with the H mutant, two with the larger dose of virus. From only one pig was virus reisolated and in that animal the maximum virus concentration measured was $10^{2.7}$ EID_{50} on day 7 postinfection.

Experimental infection with recombinant viruses in which the swine hemagglutinin gene has been segregated. The different infectiousness for swine of the cloned wild-type mutants could be ascribable to the demonstrated differences in their HA phenotype. On the other hand, mutation in any of the eight viral genes might be expected to influence replication. Therefore, infection was carried out with two recombinant viruses, X-53-PR8 and X-53a-PR8, in which only the mutant L or H HA genes, respectively, had been derived from swine influenza virus. All other genes were equivalent, having been derived from A/PR/8/34 virus (5).

Only the recombinant with the L hemagglutinin (X-53-PR8) infected pigs (Table 2). All of five animals were infected with the dose employed, and the excretion "score" exceeded that recorded for the wild-type H mutant, but excretion was more sporadic than with the L mutant at equivalent dosage. Virus was not recovered from any animal inoculated with the H-containing recombinant X-53a-PR8 or the parental A/PR/8/34 virus.

We conclude from this experiment (i) that the more efficient replication of the L mutant in swine is determined in large part by the nature of its hemagglutinin and (ii) that optimal replication of virus is dependent as well on other swine influenza virus genes. It is also apparent that the substitution of the L HA gene in PR8 virus is all that is required to convert this virus to one infective for swine.

Characterization of influenza viruses recovered from swine after natural infection. In an effort to determine the relative importance of L and H variants in natural infection of swine, 32 nasal swabbings from which virus had been isolated previously were examined for the presence of either or both forms of virus. Specimens were injected undiluted into eggs with or without A/sw/Cam/39 antiserum at a 1:10 dilution as selective against L antigenic variants. In this concentration, the antiserum neutralized 10^3 EID_{50} of a cloned L mutant. No selective system against H variants was available, but past experience had suggested that such variants were in the minority on initial egg passage.

Viruses were reisolated from 21 swine (Table 3). Only virus of L serotype was isolated from 10 animals. In four others, virus of very low titer (therefore presumably of L phenotype) was detected but could not be serotyped because of insufficient virus titer. In four animals only H virus was demonstrable. However, concomitant inoculation of at least 10^2 EID_{50} of L virus to 1 EID_{50} of H virus is required for significant reduction in H yields (E. D. Kilbourne, unpub-

TABLE 3. *Recovery and characterization of influenza viruses from swine after natural infection*

Virus	Swine no.	Isolation		Viral serotype[a]	Comment
		Date	Place		
1	11187759	11/77	Wisconsin	L	L → H on passage in eggs
2	1112877182	11/77	Ohio	H	8/8 eggs positive for virus
3	12157774	12/77	Illinois	L + H	
4	11477232	1/77	Wisconsin	L	
5	52077295	5/77	Wisconsin	L	
6	112877103	11/77	Ohio (Greenfield)	H	1/8 eggs positive for virus
7	112877136	11/77	Ohio (Greenfield)	L + H[b]	L and H in equal proportions
8	112877164	11/77	Ohio (Xenia)	L + H	
9	112877199	11/77	Ohio (Xenia)	(?)[c]	
10	112877251	11/77	Ohio (Xenia)	L	
11	112877283	11/77	Ohio (Xenia)	(?)	
12	112877298	11/77	Ohio (Xenia)	L	2/4 eggs positive for virus
13	112877302	11/77	Ohio (Xenia)	L	
14	121477328	12/77	Ohio (Xenia)	L	
15	121477328	12/77	Ohio (Xenia)	(?)	
16	121477381	12/77	Ohio (Xenia)	H	8/8 eggs positive for virus
17	121477391	12/77	Ohio (Xenia)	(?)	
18	121477429	12/77	Ohio (Xenia)	L	
19	121477430	12/77	Ohio (Xenia)	H	8/8 eggs positive for virus
20	121477442	12/77	Ohio (Xenia)	L	
21	777716	7/77	Wisconsin	L	

[a] L, Virus inhibited in HA-inhibition test with A/sw/Cam/39 antiserum; H, virus not inhibited in HA-inhibition test with A/sw/Cam/39 antiserum.

[b] In passage with A/sw/Cam/39 antiserum, 1/4 eggs were positive for virus typed as H; in passage without A/sw/Cam/39 antiserum, 1/4 eggs were positive for virus typed as L.

[c] Presumably L, on the basis of 1:4 HA titer. Virus insufficient for serological identification.

lished data), so that the presence of L virus in virus serologically identified as H remains a possibility. Indeed, in the case of virus 2 (Table 3) (serotyped as H), titration of infective virus with and without selective antiserum demonstrated $10^{4.7}$ EID_{50} of virus in the absence of antiserum and $10^{3.7}$ EID_{50} in its presence. Therefore, by inference, 90% of infective virus (i.e., that subject to neutralization) was of the L type. However, in the case of virus 7, in which less than 1 EID_{50} was detected, L and H forms appear to have been present in equal proportions (see footnote to Table 3).

No systematic geographic sampling was conducted in this preliminary study, but only in specimens from Ohio pigs was H virus demonstrated.

DISCUSSION

The genetic heterogeneity of animal viruses needs no further confirmation so that the identification of mutants within contemporary swine influenza viruses has not been surprising. Furthermore, the relatively stable association on laboratory passage of apparent allelic forms has been described previously for influenza viruses (3, 5, 8) and has been noted as well with other ribonucleic acid viruses (2, 10). The significance of such allelic mutants and their relation to the pathogenesis of infection has not been established.

The demonstration that minor antigenic (L and H) hemagglutinin variants of recent swine influenza viruses differ significantly in replication and plaque-forming characteristics in laboratory hosts prompted our present study of their effects in swine—the natural and definitive host for the virus. We have shown that less of the L variant (which grows to lower titers in eggs and Madin Darby canine kidney cells) is required to infect pigs than is the case with the H variant, defined as "high yielding" in those laboratory hosts. Furthermore, viral excretion was sporadic in pigs infected with the H mutant.

That differences in in vivo replication were mediated by difference in the viral hemagglutinin gene is strongly suggested by the results of challenge experiments employing recombinant viruses differing only in the presence of L or H hemagglutinin genes. Recombinant X-53-PR8 (L phenotype) infected five of five swine studied for viral excretion, whereas none of five animals given an equivalent inoculum of H phenotype (X-53a-PR8) showed evidence of infection.

Thus, mutation in a single of the eight genes of the influenza virus can significantly alter the infectivity of the virus for its natural host, and incorporation of the L hemagglutinin gene is all that is required for the conversion of the human influenza virus A/PR/8/34 to one infective for swine. Almond has described determination of

host range restriction by a single gene in fowl plague virus in an in vitro plaquing system (1).

Although Kendal and associates have stressed the prevalence of L serotype swine influenza viruses (SG-I in his nomenclature) since 1971 (4), they, as well as we, have had no difficulty in selecting H mutants from early egg passage isolates of recent origin. Therefore, either true viral dimorphism (5) exists with respect to L and H forms or mutation from L to H forms must be a common event. If such mutation is a common event, what advantage does it confer? At least in the past, virus of H (Kendal's SG-II) type fluorished, and clearly the mutation is not lethal in swine as we have now shown by recovery of H mutants after experimental infections with cloned H virus. The mutation, therefore, appears unlikely to be one of trivial significance in the natural host and only fortuitously selected for by chicken embryo passage.

As one approach to answering the question, we have characterized swine influenza virus isolates from naturally infected pigs as they emerge on initial passage in eggs with respect to their L or H phenotype. Although L strains predominated, H strains were detected on initial passage both in the absence and the presence of detectable L mutants. Definitive proof of the occurrence in swine of H virus alone will depend on development of a selective system against H mutants.

In any event, clones of both mutants are capable of replication in swine under experimental conditions, although H mutants appear to be less infective when measured as EID_{50}. Mutation of one form to the other during such infection has not been demonstrated in the limited number of animals studied. Both L and H mutants are also able to infect man (A. S. Beare et al., personal communication), in whom differences in virulence was not noted.

It is unlikely that the hemagglutinin mutation distinguishing the L and H viruses involves differences in the state of cleavage of their hemagglutinins, a factor known to influence influenza viral replication (7). L and H hemagglutinins of chicken embryo-grown virus do not differ with respect to their state of proteolytic cleavage (5). We have not observed significant differences in in vitro replication of L and H mutants in swine testicle and swine kidney cell lines (Kilbourne, unpublished data). However, the possibility remains that replication of the two mutants in intact animals might be differentially influenced by endogenous proteases of the respiratory tract.

If, in fact, L and H mutants are dimorphic variants, then their relative contribution to survival of the virus will be important to assess. Polymorphism implies a balance of functions that justifies survival of both variants. Further studies on replication of the mutants in their natural host will be necessary to ascertain particularly the importance of the H mutant. Although less infective, it might be more persistent, less readily neutralized, or conversely, more virulent and therefore conducive to virus expulsion into the environment. Because only small amounts of virus are required for transmission of infection, infection in nature may often be a cloning event. If so, then the maintenance of allelic hemagglutinin forms must depend on a relatively frequent interconversion of L to H and H to L, presumably by point mutation in the hemagglutinin gene. Influenza viral hemagglutinin antigenic variants selected with monoclonal antibody may differ by only a single amino acid (6).

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