

# Molecular Mechanisms of Serum Resistance of Human Influenza H3N2 Virus and Their Involvement in Virus Adaptation in a New Host

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H3N2 human influenza viruses that are resistant to horse, pig, or rabbit serum possess unique amino acid mutations in their hemagglutinin (HA) protein. To determine the molecular mechanisms of this resistance, we characterized the receptor-binding properties of these mutants by measuring their affinity for total serum protein inhibitors and for soluble receptor analogs. Pig serum-resistant variants displayed a markedly decreased affinity for total pig serum sialoglycoproteins (which contain predominantly 2-6 linkage between sialic acid and galactose residues) and for the sialyloligosaccharide 6'-sialyl(*N*-acetylglucosamine). These properties correlated with the substitution 186S→I in HA1. The major inhibitory activity in rabbit serum was found to be a  $\beta$  inhibitor with characteristics of mannose-binding lectins. Rabbit serum-resistant variants exhibited decreased sensitivity to this inhibitor due to the loss of a glycosylation sequon at positions 246 to 248 of the HA. In addition to a somewhat reduced affinity for 6'-sialyl(*N*-acetylglucosamine)-containing receptors, horse serum-resistant variants lost the ability to bind the viral neuraminidase-resistant 4-O-acetylated sialic acid moieties of equine  $\alpha_2$ -macroglobulin because of the mutation 145N→K/D in their HA1. These results indicate that influenza viruses become resistant to serum inhibitors because their affinity for these inhibitors is reduced. To determine whether natural inhibitors play a role in viral evolution during interspecies transmission, we compared the receptor-binding properties of H3N8 avian and equine viruses, including two strains isolated during the 1989 to 1990 equine influenza outbreak, which was caused by an avian virus in China. Avian strains bound 4-O-acetylated sialic acid residues of equine  $\alpha_2$ -macroglobulin, whereas equine strains did not. The earliest avian-like isolate from a horse influenza outbreak bound to this sialic acid with an affinity similar to that of avian viruses; a later isolate, however, displayed binding properties more similar to those of classical equine strains. These data suggest that the neuraminidase-resistant sialoglycoconjugates present in horses exert selective pressure on the receptor-binding properties of avian virus HA after its introduction into this host.

Influenza A viruses possess two envelope glycoproteins: hemagglutinin (HA) and neuraminidase (NA). HA binds to cell surface sialoglycoconjugates and mediates virus attachment to target cells (19, 30). NA cleaves the  $\alpha$ -glycosidic linkage between sialic acid and an adjacent sugar residue, facilitating elution of virus progeny from infected cells and preventing self-aggregation of the virus (1, 13). Natural sialoglycoconjugates are structurally diverse (37, 40), and the preferential recognition of distinct sialyloligosaccharides by HA and NA correlates with the host species from which the viruses are isolated (reviewed in references 19, 30, and 38; see also references 4, 6, 7, 11, and 28).

The receptor-binding activity of influenza viruses can be inhibited by certain molecules present in the sera and fluid secretions of animals (see references 14 and 21 for reviews). These inhibitors are classified as  $\alpha$ ,  $\beta$ , and  $\gamma$  types based on their thermal stability, virus-neutralizing activity, and sensitivity to inactivation by NA and periodate treatments. The  $\beta$  inhibitors are thermolabile mannose-binding lectins that interact with the oligosaccharide moieties on viral glycoproteins.

They neutralize virus by steric hindrance of HA and by activation of the complement-dependent pathway (2, 3). By contrast, the  $\alpha$  and  $\gamma$  inhibitors are heat-stable sialylated glycoproteins that mimic the structure of the cellular receptors of influenza viruses and competitively block the receptor-binding sites of HA. Influenza viruses are neutralized by  $\gamma$  inhibitors but not by  $\alpha$  inhibitors, which are considered to be sensitive to viral NA. However, the distinction between  $\alpha$  and  $\gamma$  inhibitors is strain dependent and rather arbitrary, as described by Gottschalk et al. (14). Although inhibitors in serum or other body fluids are believed to influence the selection of influenza virus receptor variants in natural hosts, no direct experimental support for this hypothesis has been presented.

A potent  $\gamma$  inhibitor of H2 and H3 human influenza viruses, equine  $\alpha_2$ -macroglobulin (EM), contains a Neu4,5Ac<sub>2</sub>-6Gal moiety that is insensitive to viral NA and thus resists inactivation by this enzyme (16, 24, 31). Cultivation of human H3 influenza viruses in the presence of horse serum results in the selection of variants that have a decreased affinity for the Neu5Ac<sub>2</sub>-6Gal-specific receptors due to a single amino acid substitution (226L→Q) in their HA (32, 33). One of these mutants (X31/HS strain) does not bind the Neu4,5Ac<sub>2</sub> (4-O-acetylated sialic acid) species (25). Therefore, there are at least two mechanisms by which a virus can become resistant to the horse serum inhibitor: a change in the recognition of the type

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TABLE 1. Serum-resistant variants of LA/87 influenza virus (34)

Variant	Serum used for selection	Substitution in the HA1 portion of the variant relative to LA/87 at position:							
		128	137	145	155	186	220	246	248
H	Horse	N→T <sup>d</sup>		N→K	Y→H				
H-P	Horse→pig <sup>a</sup>	N→T <sup>d</sup>	Y→D	N→K	Y→H	S→I			
P	Pig					S→I			
R	Rabbit						R→G	N→D <sup>e</sup>	
HP	Horse and pig <sup>b</sup>			N→D		S→I			
HP-R	Horse and pig→rabbit <sup>c</sup>			N→D		S→I			T→I <sup>e</sup>

<sup>a</sup> Isolated from H after its exposure to pig serum.<sup>b</sup> Isolated from LA/87 after its simultaneous exposure to horse and pig sera.<sup>c</sup> Isolated from HP after its exposure to rabbit serum.<sup>d</sup> Substitution results in the acquisition of a potential glycosylation site at position 126 of HA.<sup>e</sup> Substitution results in the loss of a potential glycosylation site at position 246 of HA.

of Sia-Gal linkage, and a change in the recognition of the 4-O-acetylated sialic acid. The relative contributions of these mechanisms to the resistant phenotype are yet to be defined.

We have previously shown that horse, pig, and rabbit sera all contain distinct heat-resistant inhibitors of the H3N2 human influenza virus A/Los Angeles/2/87 (LA/87), because variants resistant to these sera possess unique mutations in their HA receptor-binding regions (34). The major inhibitor in pig serum was later identified as  $\alpha_2$ -macroglobulin that contains predominantly 2-6 linkage between sialic acid and galactose (35). Gimsa et al. (12) recently showed that pig serum-resistant human and swine strains exhibit decreased affinity for human erythrocytes that had been modified to contain terminal Neu5Ac2-6Gal residues. However, the nature of the rabbit serum inhibitor and the mechanisms of influenza virus resistance to each serum inhibitor remain unknown.

To understand the molecular mechanisms by which influenza viruses become resistant to horse, pig, and rabbit serum inhibitors, we compared the receptor-binding characteristics of LA/87 and its serum-resistant variants and analyzed these data in relation to the known amino acid substitutions in the HA of the mutants. We then analyzed the receptor-binding properties of viruses isolated during an equine influenza outbreak that was caused by an avian virus, in order to evaluate the influence of natural inhibitors on the evolution of virus in a new host.

#### MATERIALS AND METHODS

**Viruses.** Variants were isolated from the parent virus LA/87 by growth in embryonated chicken eggs in the presence of horse, pig, or rabbit serum, or their mixtures, and characterized as previously described (24) (Table 1). Avian and equine influenza A virus strains were from the virus repository of St. Jude Children's Research Hospital; their isolation and characteristics have been described elsewhere (5, 15). All viruses were grown in 9- to 10-day-old chicken eggs. The allantoic fluids were clarified by low-speed centrifugation; the viruses were pelleted by high-speed centrifugation, resuspended in 0.1 M Tris buffer (pH 7.2) containing 50% glycerol, and then stored at  $-20^{\circ}\text{C}$ .

**Soluble receptor analogs and serum inhibitors.** Sera were obtained from Pel-Freez Biologicals (Rogers, Ark.) and stored at  $-20^{\circ}\text{C}$  in aliquots. Free *N*-acetylneuraminic acid (Neu5Ac), 3' sialyllactose (3' SL; Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4Glc), and all neutral monosaccharides used were purchased from Sigma, St. Louis, Mo. 6'-Sialyl(*N*-acetylglucosamine) (6' SLN; Neu5Ac $\alpha$ 1-6Gal $\beta$ 1-4GlcNAc) was a gift from V. E. Piskarev, Nesmeyanov Institute of Organoelement Compounds, Moscow, Russia. Sialylglycopolymers 3' SL-PAA and 6' SLN-PAA, which contained 20 mol% 3' SL and 6' SLN, respectively, attached to a soluble polyacrylamide carrier were described previously (11). They were kindly provided by N. V. Bovin and A. B. Tuzikov, Shemyakin Institute of Bio-Organic Chemistry, Moscow, Russia.

To study heat-stable (sialylglycoprotein) serum inhibitors, sera were heat inactivated for 30 min at  $56^{\circ}\text{C}$ . To further discriminate between NA-sensitive and NA-resistant sialylglycoprotein components, the sera were either treated with LA/87 virus NA or mock treated. To 60  $\mu\text{l}$  of heat-inactivated sera, 540  $\mu\text{l}$  of Ca-Tris-buffered saline (Ca-TBS) buffer (6.8 mM  $\text{CaCl}_2$ , 0.02 mM Tris [pH 7.3], 0.9% NaCl) and 15  $\mu\text{l}$  of purified LA/87 virus (HA titer of 16,000) were added. The mixture was then incubated for 4 h at  $37^{\circ}\text{C}$ . Viral HA and NA were inactivated by heating the mixtures for 1 h at  $56^{\circ}\text{C}$ . This treatment was deter-

mined, through the control experiments, to be sufficient to eliminate any contribution of the virus to the subsequent assay of serum inhibitors in the preparations. The mock-treated sera were handled similarly except that Ca-TBS was used instead of virus.

To study the binding of EM by avian and equine influenza viruses (Table 2), partially purified EM was prepared from the heat-inactivated horse serum by using gel chromatography as previously described (29). A fraction of this preparation (50  $\mu\text{l}$ ) in Ca-TBS buffer was incubated with 2.5 mU of *Vibrio cholerae* NA (type III; Sigma) at  $37^{\circ}\text{C}$  for 4 h and then at  $56^{\circ}\text{C}$  for 1 h to inactivate the NA. The control EM was treated in the same way but without the NA incubation.

**Rabbit serum  $\beta$  inhibitor.** Freshly prepared samples of rabbit serum  $\beta$  inhibitor were used each day to avoid deterioration of the preparations during storage. The serum was thawed, incubated at  $56^{\circ}\text{C}$  for 30 min, and then chilled on ice. To inactivate sialic acid containing  $\alpha$  and  $\gamma$  inhibitors, 3 ml of 0.011 M  $\text{NaIO}_4$  was added to 1 ml of serum. After a 15-min incubation at room temperature, excessive periodate was inactivated by the addition of 0.2 ml of 10% glycerol solution in 1 M Tris buffer (pH 7.3).

**Preparation of plasma membranes from CAMs cells.** Chorioallantoic membranes (CAMs) from 12-day-old embryonated chicken eggs were processed in a Dounce homogenizer with a 0.5-mm clearance to detach epithelial cells. The cells were pelleted at  $2,000 \times g$  and resuspended in 50% Percoll solution in TSE buffer (10 mM Tris, 0.15 M NaCl, 0.5 mM EDTA [pH 7.2]). After centrifugation for 5 min at  $10,000 \times g$ , the layer of epithelial cells at the top of the Percoll solution was removed, and the bottom fractions containing erythrocytes were discarded. This procedure was repeated until no admixtures of erythrocytes were visible. The CAM cells were then washed from the Percoll in TSE, suspended in an ice-cold lysis buffer (0.01 M Tris [pH 7.2], 1 mM phenylmethylsulfonyl fluoride), incubated for 10 min on ice, and disrupted in a standard Dounce homogenizer. Nuclei and cellular debris were removed by centrifugation for 1 min at  $1,000 \times g$ . The cell membranes were then pelleted at  $40,000 \times g$  for 1.5 h, resuspended in TSE containing 1 mM phenylmethylsulfonyl fluoride, sonicated for 2 min in an ice bath, and then stored as aliquots at  $-20^{\circ}\text{C}$ .

**Virus receptor-binding affinity.** The virus-binding affinity for serum sialylglycoprotein inhibitors, sialosides, and sialylglycopolymers was assessed by using the solid-phase fetuin-binding inhibition assay as previously described (10, 26). This assay is based on the competition for binding sites on the viral particle between nonlabeled sialic acid-containing compound and enzyme-labeled sialylglycoprotein fetuin. In brief, purified viruses diluted with phosphate-buffered saline (PBS) to a hemagglutination titer of 1:20 to 1:100 were adsorbed to the wells of fetuin-coated polyvinyl chloride enzyme immunoassay microplates at  $4^{\circ}\text{C}$  overnight. After unbound virus was washed off with 0.01% Tween 80 in PBS (PBS-T), 0.05 ml of solutions containing a fixed amount of fetuin labeled with horseradish peroxidase (HRP) and a variable amount of nonlabeled inhibitor were added to the plate, which was then incubated for 1 h at  $4^{\circ}\text{C}$ . The solutions were prepared in PBS supplemented with 0.02% bovine serum albumin, 0.02% Tween 80, and 1  $\mu\text{M}$  sialidase inhibitor 2,3-didehydro-2,4-dideoxy-4-guanidino-*N*-acetyl-*D*-neuraminic acid (GG167), kindly provided by R. Bethel, Glaxo Wellcome Co. After this incubation, the plates were washed with PBS-T, and the amount of labeled fetuin bound was determined by using the standard *o*-phenylenediamine chromogenic substrate.

The association constants of the virus complexes with serum sialylglycoproteins, sialosides, and sialylglycopolymers were calculated for each concentrations of the compound used in the competitive reaction, and the results were averaged. The association constants for the serum inhibitors were calculated by assigning the concentration of the inhibitors in each undiluted serum sample a value of 1 U. Standard deviations of the mean values of the constants obtained in the same experiment varied from 10 to 40%. The variation among the mean values of the constants determined in replicate experiments on different days was higher; however, the overall patterns of binding affinities of different virus strains for different receptor molecules were reproducible.

The effect of bivalent ions on the interaction of the virus with the rabbit  $\beta$

inhibitor was studied by using the same assay, with the following modifications. The virus was incubated separately with the inhibitor and with the labeled fetuin. First, the solid-phase immobilized virus was allowed to interact with dilutions of the heat-inactivated and periodate-treated serum in various buffers, at which time the  $\beta$  inhibitor attaches to the virions. After washing of unbound material, fetuin-HRP conjugate in Ca-TBS-bovine serum albumin buffer, supplemented with 0.05% Tween 80 and 1  $\mu$ M GG167, was added for 30 min at 4°C. This step was followed by standard detection of the bound conjugate (10). The percent inhibition of conjugate binding to the virus as a result of prebound  $\beta$  inhibitor was calculated as  $100 \times (K^+ - A_i)/(K^+ - K^-)$ , where  $A_i$  is the absorbancy in the experimental wells,  $K^-$  is the absorbancy in the control wells without the virus (background conjugate binding), and  $K^+$  is the absorbancy in the control wells without inhibitor (100% conjugate binding).

**Binding of LA/87 and its serum-resistant variants to CAM cell membranes pretreated with NA.** CAM cell membranes, suspended in PBS to a final concentration of about 10  $\mu$ g/ml of total protein, were incubated in 96-well polyvinyl chloride microplates for 4 h at 4°C. Nonadsorbed material was then washed off with PBS. Wells of the same microplates that lacked coating were used as background controls. Adsorbed membranes were treated with decreasing concentrations of *V. cholerae* NA (Sigma type III) for 2 h at 37°C or mock treated (no NA). The viruses were allowed to bind to the membranes, and the amounts of the viruses bound were estimated by overlaying them with fetuin-HRP conjugate as previously described (27). The results were expressed as percent binding to the NA-treated membranes relative to that to the mock-treated preparation.

**Nucleic acid sequencing.** Viral RNA was isolated from virus-containing allantoic fluid and sequenced as previously described (20). In brief, cDNA was synthesized with reverse transcriptase and a random hexamer. The HA genes were amplified by PCR with the cDNA, H3 HA-specific oligonucleotide primers, and *Pfu* polymerase (Stratagene). PCR products were cloned into a plasmid and sequenced with an Autosequencer (Applied Biosystems Inc., Foster City, Calif.) according to the protocol recommended by the company. Three independent cDNA clones were sequenced and found to be identical to each other.

## RESULTS AND DISCUSSION

**H3N2 viruses become resistant to horse serum due to their reduced affinity for 4-O-acetylated sialylglycoconjugates.** The serum-resistant variants of LA/87 that can grow in embryonated hen eggs in the presence of heat-inactivated horse, pig, or rabbit serum differ from their parent virus and from each other in that they possess distinct HA mutations (34) (Table 1). The goal of this study is to understand the molecular basis for viral resistance to each serum. To determine whether those viruses became resistant to serum because of a reduced binding to NA-sensitive ( $\alpha$  inhibitors) and/or NA-resistant ( $\gamma$  inhibitors) sialylglycoconjugates, we compared the binding affinities of the viruses for serum inhibitors that were either untreated or treated with the NA of the parent LA/87 virus (Fig. 1). We found that pig and rabbit sera contained low to undetectable levels of sialylglycoconjugates resistant to the LA/87 NA, because the binding of sialylglycoproteins in these sera to LA/87 decreased 30- and 45-fold, respectively, after NA treatment (Fig. 1C). Given that we did not optimize the treatment conditions, the residual inhibitory activity in the sera may have been the result of incomplete treatment rather than the presence of NA-resistant components.

In marked contrast to pig and rabbit sera, horse serum retained up to 30% inhibitory activity against the LA/87 virus after NA treatment, indicating that substantial amounts of NA-resistant inhibitors are present in horse, but not in pig or rabbit, serum. Previous studies have shown that  $\alpha_2$ -macroglobulin is the principal inhibitor in horse serum for H2N2 and H3N2 viruses and that 4-O-acetylated sialic acid, which represents about 30% of the total sialic acids in EM (16), confers this inhibitor's resistance to bacterial and viral NA (16, 24, 25, 31). Our results are consistent with these previous findings in that LA/87 NA is unable to cleave 4-O-acetylated sialic acid residues from  $\alpha_2$ -macroglobulin and in that LA/87 HA does bind this type of sialic acid.

To understand the molecular basis of horse serum resistance, we analyzed the receptor-binding properties of serum-

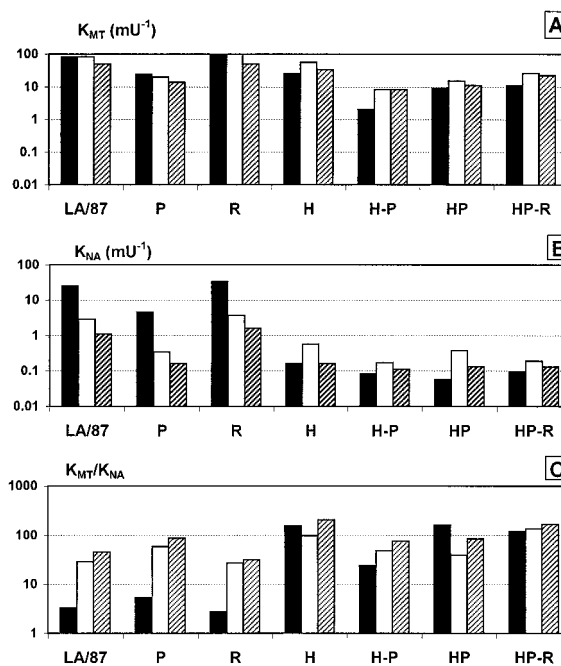


FIG. 1. Binding of inhibitors from animal sera by the LA/87 strain and its serum-resistant variants. Horse (closed bars), pig (open bars), and rabbit (dashed bars) sera were heat inactivated and either treated with LA/87 NA or mock treated as described in Materials and Methods. Association constants of the virus complexes with inhibitors of mock-treated (A) or NA-treated (B) sera ( $K_{MT}$  and  $K_{NA}$ , respectively) were determined in a competitive solid-phase assay; 5 mM EDTA was present in the assay buffer to inactivate possible residual  $\beta$ -inhibitor activity in the sera (references 2 and 3 and this paper). The ratio  $K_{MT}/K_{NA}$  (C) reflects a decrease in the affinity of the serum inhibitors for the virus after NA treatment. The lower this ratio is, the more resistant is the inhibitor to inactivation by NA.

resistant variants of LA/87 (Fig. 1). The serum-resistant variants could be separated into two distinct groups based on their recognition of the NA-treated EM (i.e., their ability to bind NA-resistant 4-O-acetylated sialic acid). Variants LA/87 P and LA/87 R, which were selected from LA/87 with pig or rabbit serum, respectively, displayed substantial affinity for viral NA-treated horse serum (Fig. 1B), indicating their ability to bind 4-O-acetylated sialyloligosaccharides. On the other hand, all of the horse serum-resistant variants lost this ability, as shown by their dramatic decrease in affinity for the NA-treated horse serum inhibitors (Fig. 1C;  $K_{MT}/K_{NA}$  [see legend to Fig. 1 for definition] = 24 to 160). Similar results were obtained with *V. cholerae* NA-treated horse serum, thereby confirming these data (data not shown).

Influenza viruses that become resistant to horse serum inhibitors are thought to do so because of a reduced affinity for the Sia2-6Gal moiety of EM (31–33). We therefore analyzed the receptor-binding specificity of serum-resistant viruses by using a panel of receptor analogs (Fig. 2). We found that the LA/87 H virus, a horse serum-resistant variant without any additional passages with other sera, displayed about threefold-weaker binding compared to the parent LA/87 virus with respect to the Sia2-6Gal-containing receptor analogs 6'SLN and 6'SLN-based synthetic glycopolymer 6'SLN-PAA (Fig. 2). These results suggest that the LA/87 virus acquires resistance to the horse serum inhibitor through a combination of two mechanisms: reduced binding to NA-resistant 4-O-acetylated sialic acid species present in the inhibitor and reduced recognition of 2-6-linked sialyloligosaccharide determinants. How-



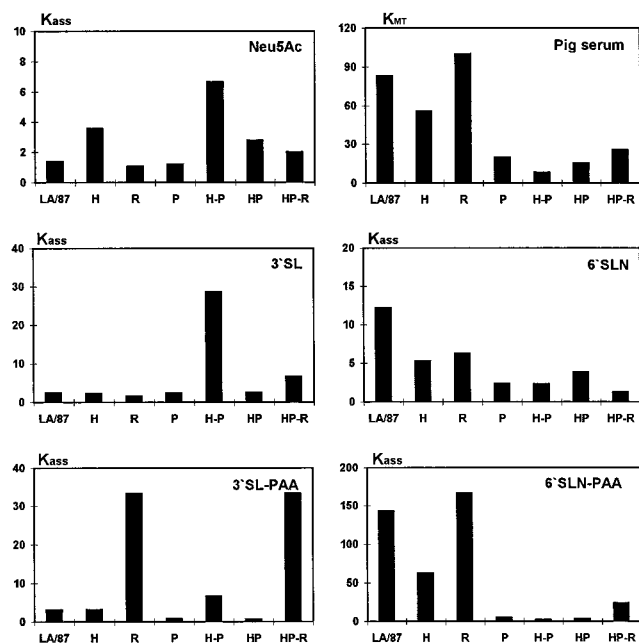


FIG. 2. Binding of receptor analogs by LA/87 and its serum-resistant variants. The association constants of the virus complexes with free Neu5Ac, 3'SL, 6'SLN, and sialylglycopolymers of 3'SL and 6'SLN (3'SL-PAA and 6'SLN-PAA) were determined in a competitive solid-phase assay as described in Materials and Methods. The constants are expressed in  $\text{mM}^{-1}$  for the monovalent sialosides and in  $\mu\text{M}^{-1}$  sialic acid for the sialylglycopolymers. Our data on the binding of total heat-inactivated pig serum from Fig. 1 ( $K_{MT}$ ,  $\text{mU}^{-1}$ ) are also represented (Pig serum) to facilitate the comparison of binding patterns.

ever, the first mechanism plays a more important role in horse serum resistance, because the LA/87 P variant, which has an even lower affinity for 6'SLN and 6'SLN-PAA than does LA/87 H, binds 4-O-acetylated sialic acid and is horse serum sensitive. This conclusion highlights the contribution made by viral NA to virus resistance to neutralization by receptor analogs. This contribution should be taken into account when developing inhibitors of virus receptor binding (see references 23 and 29 and references therein).

Comparison of the HA amino acid sequences (Table 1) revealed that substitutions at position 145 of HA1 (either  $\text{N} \rightarrow \text{K}$  or  $\text{N} \rightarrow \text{D}$ ) are the only changes common among all four horse serum-resistant variants and separate them from other sensitive strains. Therefore, these substitutions should be primarily responsible for the resistance. The three-dimensional structure of X31 (H3N2) virus HA complexed with the  $\alpha$ -methyl glycoside of 4-O-acetyl-5N-acetylneuraminic acid (36) shows that the 4-O-acetyl group of sialic acid contacts the side chain of the amino acid at position 145 (Fig. 3). Clearly, changes in the size ( $\text{N} \rightarrow \text{K}$ ) or charge ( $\text{N} \rightarrow \text{K}$  or  $\text{N} \rightarrow \text{D}$ ) of the amino acid at this position could directly interfere with the fit of the 4-O-acetyl moiety to the receptor binding site.

**Pig serum-resistant variants have a decreased affinity for Sia2-6Gal-containing sialylglycoconjugates.** Unlike horse serum, pig serum did not contain substantial amounts of NA-resistant inhibitors of the LA/87 virus; the inhibitory activity of the serum decreased 30-fold after NA treatment (Fig. 1). We found that four pig serum-resistant variants (LA/87 P, LA/87 H-P, LA/87 HP, and LA/87 HP-R) clearly differ from the parent virus and from other non-pig serum-adapted variants (LA/87 H and LA/87 R) in that the former have a lower affinity for the total inhibitors of pig serum (Fig. 1A and 2, pig serum).

The main neutralizing inhibitor of LA/87 in pig serum is  $\alpha_2$ -macroglobulin (35). Lectin-binding analysis has shown that 2-6 linkages are predominant between the sialic acid and the penultimate sugar residue in the sialyloligosaccharide chains of  $\alpha_2$ -macroglobulin (35). To determine how influenza viruses become resistant to this pig serum inhibitor, we analyzed the receptor-binding specificity of these viruses by using a panel of receptor analogs (Fig. 2). All of our pig serum-resistant variants had a lower binding affinity for 6'SLN and 6'SLN-PAA (Fig. 2). This finding is consistent with the data of Gimsa et al. (12), who showed that pig serum-resistant viruses bind poorly to Sia2-6Gal-containing erythrocytes, and suggests that resistant variants escape neutralization by pig serum inhibitor due to their decreased affinity for Sia2-6Gal sialyloligosaccharide determinants.

Because all pig serum-resistant variants contain a common amino acid substitution in their HA, 186S $\rightarrow$ I (Table 1), this mutation must be responsible for the decreased binding of Sia2-6Gal and, therefore, for the resistance. In X31 HA, the amino acid at position 186 does not directly contact the sialic acid moiety (Fig. 3), nor does it directly interact with the 2-3- and 2-6-linked asialic parts of sialyloligosaccharides (9, 36). The side chain of this amino acid does, however, contact amino acid residues at positions 190 and 228, which form hydrogen bonds with the terminal hydroxyl group of the polyhydroxyl tail of sialic acid (36, 41) (Fig. 3). Homology modeling suggests that the substitution of Ser by the bulkier Ile would move the side chains of amino acids 190 and 228 inside the pocket toward the C9-OH group of Neu5Ac (24a). It is not clear at present how this change could decrease the binding of Neu5Ac2-6Gal-terminated receptor analogs without substantially decreasing the binding of free Neu5Ac and Neu5Ac2-3Gal-terminated receptors (Fig. 2).

**The inhibitor in rabbit serum is a mannose-binding lectin.** Neither rabbit serum-resistant variant LA/87 R nor LA/87 HP-R showed any decrease in its affinity for the total sialylglycoprotein inhibitors in rabbit serum compared with its parent virus, LA/87 or LA/87 HP, respectively (Fig. 1A; compare rabbit serum  $K_{MT}$  values for LA/87 and LA/87 HP to those for LA/87 R and LA/87 HP-R). In addition, the affinity of LA/87 R and LA/87 HP-R for NA-treated rabbit serum was unchanged (Fig. 1B; compare rabbit serum  $K_{NA}$  values for LA/87 and LA/87 HP to those for LA/87 R and LA/87 HP-R). Because rabbit serum-resistant variants showed no change in affinity for the  $\alpha$  and  $\gamma$  inhibitors, it seems likely that these inhibitors were not responsible for the selection of these variants. We therefore hypothesized that rabbit serum contains a thermostable  $\beta$  inhibitor, which was not completely inactivated during the standard heat treatment procedure (30 min, 56°C) that we used (34). Two lines of evidence indirectly supported this suggestion. First, both rabbit serum-resistant strains lost a potential glycosylation site at Asn<sub>246</sub> (Table 1; Fig. 3). This is a typical feature of resistant mutants selected in the presence of  $\beta$  inhibitors (17, 18). Second, one of the known serum  $\beta$  inhibitors, bovine conglutinin, is relatively thermostable (17). To examine the presence of heat-stable  $\beta$ -inhibitory activity, the sera were incubated at 56°C for 30 min and then treated with sodium periodate to destroy sialic acid-containing inhibitors (2, 21). The hemagglutination inhibition (HAI) titers of horse, pig, and rabbit sera treated in this way were 16, 16, and 256. Therefore, a substantial nonsialoside-mediated inhibitory activity was present in the heat-inactivated rabbit serum.

Studies by Anders and colleagues (2, 3, 17, 18) identified  $\beta$  inhibitors from bovine, guinea pig, and mouse sera as calcium-dependent mannose-binding lectins. We therefore examined whether the rabbit serum  $\beta$  inhibitor might also be a mannose-

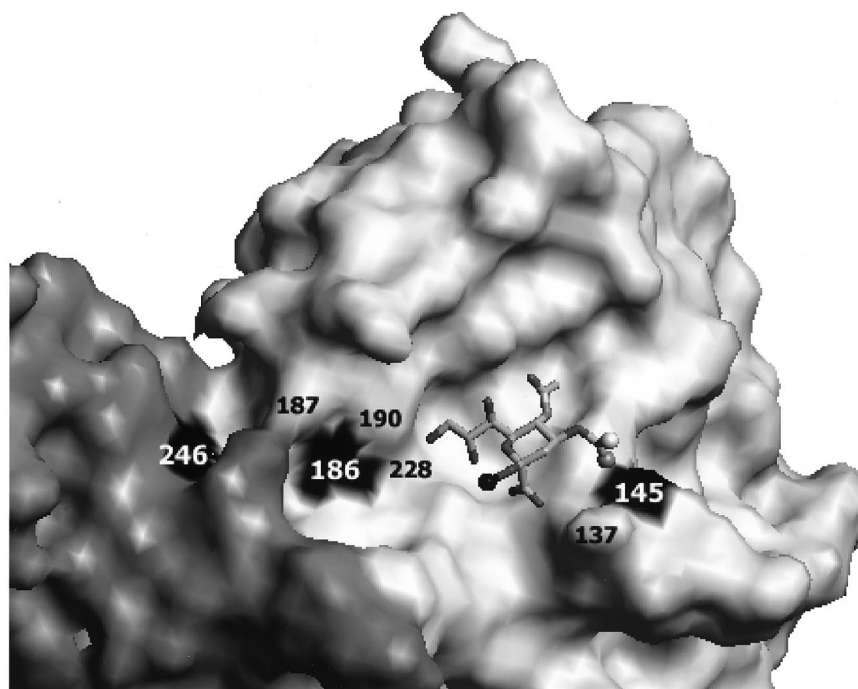


FIG. 3. Key mutations (black) in the HA of serum-resistant variants of LA/87 shown on a model depicting the complex between X31 virus HA and the  $\alpha$ -methyl glycoside of 4-*O*-acetyl-*N*-acetylneuraminic acid (1HG1 structure; Brookhaven Protein Databank [36]). The solvent-accessible surface of the receptor-binding site of the HA monomer A (white) and the adjacent part of the second monomer C (gray) are represented. The molecule of Neu4,5Ac<sub>2</sub>Me is shown as a stick model (heavy atoms only); the carbon and oxygen atoms of the 4-*O*-acetyl group and the carbon atom of the 2-*O*-methyl group are shown as white, gray, and black balls, respectively. Black numbers indicate the positions of the amino acids discussed in the text. The figure was generated using the Preview version of WebLab ViewerPro 3.0, Molecular Simulations, Inc., San Diego, Calif.

binding lectin. As shown in Fig. 4A, the inhibitory activity of the rabbit preparation was abrogated in the presence of EDTA and restored by the addition of Ca<sup>2+</sup> but not Mg<sup>2+</sup> ions. The HAI activity of the rabbit serum  $\beta$  inhibitor was blocked in the presence of the monosaccharides D-mannose, L-fucose, and *N*-acetyl-D-glucosamine but unaffected by D-galactose and *N*-acetyl-D-galactosamine (Fig. 4B). These features indicate that the residual  $\beta$ -inhibitory activity of heat-treated rabbit serum was mediated by a mannose-binding calcium-dependent lectin. Finally, we examined the activity of the rabbit serum  $\beta$  inhibitor against viruses in the HAI test. The variants LA/87 R and LA/87 HP-R selected in the presence of this serum were substantially less sensitive to the rabbit serum  $\beta$  inhibitor than were LA/87 and the other variants (Fig. 4C). This finding supports our contention that the  $\beta$  inhibitor is responsible for the selection of these serum-resistant variants. Both variants have lost a glycosylation site at the tip of their HA, which is consistent with the known mechanism of influenza virus resistance to  $\beta$  inhibitors (17, 18).

**Virus-binding affinity to CAM cells.** The data presented above indicate that all serum-resistant variants differ from the parent virus by their decreased affinity for serum inhibitors (either  $\alpha$  [pig serum],  $\gamma$  [horse serum], or  $\beta$  [rabbit serum]). Although this effect alone could account for the resistance, we wanted to know whether an increased affinity of the mutants for target cells might additionally help the virus to escape serum inhibitors. To this end, the relative affinity of the viruses for CAM cells was estimated by the method of Yewdell et al. (42). Plasma membranes prepared from CAM cells were adsorbed to wells of microtiter plates and treated with increasing concentrations of *V. cholerae* NA to gradually decrease the density of sialyloligosaccharide receptors. Virus binding to

these cell membranes was then assayed. This assay relies on the facts that the higher the NA concentration used, the lower the receptor density left on the cell surface, and that the viruses that bind to the membranes with lower receptor density have a higher affinity for the receptor. We found that all of the variants demonstrated similar or higher affinities for CAM cell membranes than did the parent virus (Fig. 5); LA/87 R, LA/87 HP-R, and LA/87 H-P variants had the highest affinities. The enhancement of virus affinity for cells is thought to promote virus escape from neutralization by antibodies (8, 22, 39, 42). Therefore, the higher affinity of LA/87 H-P, LA/87 R, and LA/87 HP-R for CAM cells may contribute to their serum-resistant phenotypes in addition to reduced virus binding to the serum inhibitors. However, the possibility remains that the enhanced binding of these serum-resistant variants to CAM cells is merely a consequence of the selection for reduced virus binding to serum inhibitors.

We recently showed that CAM cells possess predominantly Sia2-3Gal-terminated oligosaccharides on their surface (20). What is the molecular basis for the significantly enhanced affinity these three virus variants have for those receptors? Variant LA/87 H-P displays increased affinity for free Neu5Ac and 3'SL (Fig. 2). It may be concluded, therefore, that the higher affinity of this variant for CAM cells is provided by enhanced interactions with the sialyloligosaccharide part of the receptors. This enhancement must be due to mutations 137Y→D and 186S→I, given that these are the only amino acid differences between LA/87 H and LA/87 H-P (Table 1). Two rabbit serum-resistant variants, LA/87 R and LA/87 HP-R, differ from the other viruses in that they lack a carbohydrate chain at position 246 of the HA close to the receptor-binding site of the adjacent HA monomer (Fig. 3). Although

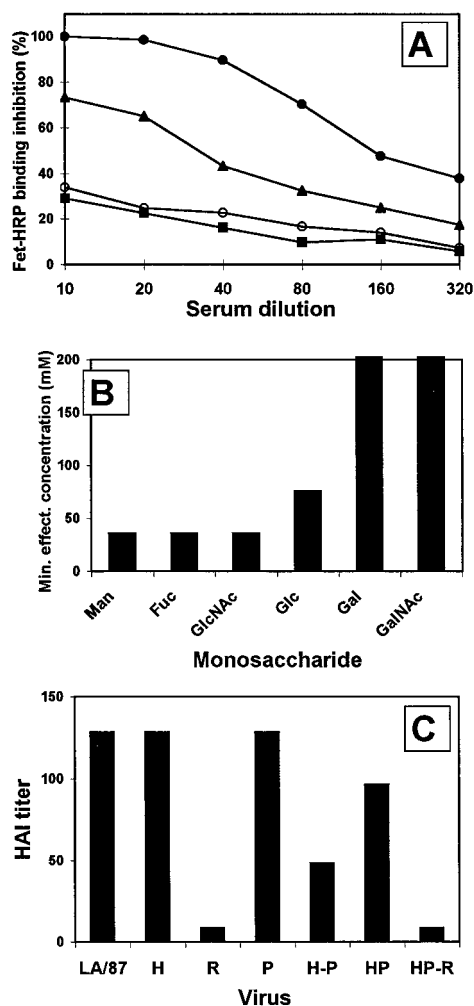


FIG. 4. Properties of the  $\beta$  inhibitor present in rabbit serum after heat inactivation for 30 min at 56°C and periodate treatment. (A) Effect of bivalent ions on the ability of the preparation to inhibit the binding of the fetuin (fet)-HRP conjugate to LA/87 virus (see Materials and Methods for assay details). Serum was diluted in 0.05 M TBS (pH 7.3) (closed circles), TBS supplemented with 5 mM EDTA (TBS-E) (open circles), or TBS-E containing either 25 mM CaCl<sub>2</sub> (triangles) or 25 mM MgCl<sub>2</sub> (solid squares). (B) Sensitivity of the HAI activity of the  $\beta$  inhibitor to competitive blocking by monosaccharides. The ordinates show the minimal concentrations of the sugars D-mannose (Man), L-fucose (Fuc), N-acetyl-D-glucosamine (GlcNAc), D-glucose (Glc), D-galactose (Gal), and N-acetyl-D-galactosamine (GalNAc) that are required to completely inhibit the HAI activity of 4 HAI units of the  $\beta$  inhibitor. The assay was performed in microplates on 4 HAI units of LA/87 virus and 0.5% chicken erythrocytes as described previously (17). No inhibition was observed at the highest concentration (200 mM) of Gal or GalNAc used. (C) HAI activity of the  $\beta$  inhibitor against LA/87 and its variants. Four HAI units of the viruses and 0.5% chicken erythrocytes were used for the assay.

neither variant exhibited any substantial change in affinity for sialic acid or monovalent sialyloligosaccharides compared to the parent virus, both variants demonstrated dramatic increase in their affinity for polymeric 3'SL (Fig. 2, 3'SL-PAA). A similar binding pattern has been reported for the egg-adapted variants of influenza B virus (11), where the loss of a glycosylation site at Asn<sub>187</sub>, which is associated with egg adaptation of the virus, increases the virus's affinity for Sia2-3Gal-containing receptors without significantly changing its ability to bind small sialosides. In this case, the carbohydrate chain at position 187 is thought to sterically interfere with the macromolecular part of Neu5Ac2-3Gal-terminated macromolecular receptors (11).

Because the carbohydrate moiety at Asn<sub>246</sub> must be relatively close to the carbohydrate moiety at Asn<sub>187</sub> of the adjacent HA monomer (Fig. 3), this mechanism could also operate in the case of LA/87 HA variants.

**The receptor-binding phenotype of avian influenza virus changes when the virus is naturally introduced into horses.** We have shown that for influenza viruses to grow in eggs in the presence of horse serum, they must avoid binding to the NA-resistant 4-O-acetylated sialic acid moieties of EM. To determine whether the same mechanism operates in nature, we compared the affinities for NA-treated EM of H3N8 avian influenza viruses, currently circulating H3N8 equine viruses (so-called equine type 2 viruses), and virus strains isolated from horses during the 1989 to 1990 influenza outbreak in China that was caused by the introduction of an influenza virus from birds (15) (Table 2). The type 2 equine viruses differed from the avian viruses in that they bound weakly to NA-treated EM in terms of both absolute binding affinity ( $K_{NA}$ ) and relative affinity compared to the mock-treated EM ( $K_{MT}/K_{NA}$ ). Of interest, the earliest isolate from the equine influenza outbreak, A/equine/Jilin/89, exhibited a binding phenotype similar to that of avian viruses, whereas the phenotype of a strain isolated a year later (A/equine/Heilongjiang/90) was more like that of type 2 equine viruses. These findings indicate that there is a pressure in horses to select viruses that do not bind 4-O-acetylated sialic acid.

Because in the LA/87 virus an amino acid at position 145 contributes to the recognition of the 4-O-acetyl substituent of sialic acid, we compared the H3 HA sequences of avian and equine viruses (5). All avian viruses possess 145S, whereas type 2 equine viruses have 145D, which is also found in LA/87 variants adapted to grow in the presence of horse serum. It can be suggested, therefore, that the same molecular mechanism operated during the adaptation of the avian virus HA in horses. In accord with this notion, the HA of A/equine/Jilin/89 virus, which displays an avian-like binding phenotype, bears 145S (15). To determine the amino acid substitutions responsible for the change in receptor-binding phenotype of A/equine/Heilongjiang/90, we sequenced the HA of this isolate. Five amino acid substitutions (at positions 48, 91, 190, 216, and 261) distinguish the HA1 sequence of A/equine/Heilongjiang/90 from that of A/equine/Jilin/89. Of these substitutions, the mu-

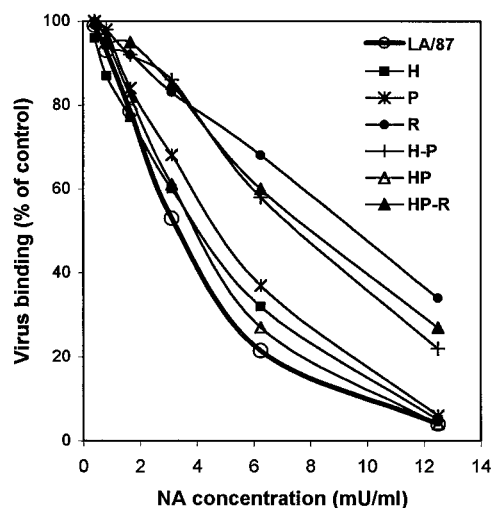


FIG. 5. Effect of *V. cholerae* NA treatment of plasma membranes of chicken embryo CAM cells on the binding of LA/87 and its variants.



TABLE 2. Binding of mock-treated and NA-treated EM by avian and equine H3N8 influenza A viruses<sup>a</sup>

Virus	Association constant (mU <sup>-1</sup> )		Relative affinity (K <sub>MT</sub> /K <sub>NA</sub> )
	K <sub>MT</sub>	K <sub>NA</sub>	
Duck/Memphis/928/74	0.5	0.18	2.8
Duck/Hokkaido/33/80	1.1	0.4	2.8
Duck/Hokkaido/7/82	0.5	0.2	2.5
Duck/Hokkaido/10/85	1.0	0.35	2.9
Equine/Miami/63	0.45	0.01	45
Equine/Algiers/72	0.5	0.02	25
Equine/Fontainebleau/76	0.3	0.015	20
Equine/Santiago/1/85	0.2	0.004	50
Equine/Tennessee/5/86	0.2	0.012	17
Equine/Kentucky/2/86	0.6	0.013	45
Equine/Jilin/1/89 <sup>b</sup>	0.5	0.13	3.8
Equine/Heilongjiang/15/90 <sup>b</sup>	0.8	0.07	11

<sup>a</sup> Partially purified EM was treated with *V. cholerae* neuraminidase (NA) or mock treated (MT), and the binding of both preparations to the viruses was tested in a fetuin-binding inhibition assay as described in Materials and Methods. To calculate the constants, the concentration of the stock EM preparation was arbitrarily assigned a value of 1 U. The ratio K<sub>MT</sub>/K<sub>NA</sub> reflects a decrease in the affinity of the viruses for the EM after NA treatment.

<sup>b</sup> Representatives of the H3N8 avian-like equine influenza virus lineage that emerged in China in 1989 (15).

tation 190E→K in the HA of A/equine/Heilongjiang/90 appears to be primarily involved in the alteration of the receptor-binding properties of the virus. The side chain of 190E interacts with the glycerol tail of the sialic acid moiety in the receptor-binding site (36, 41), and 190E is strictly conserved among all avian influenza viruses (28). The substitution E→K at position 190 probably moves the sialic acid residue toward the "right" edge of the receptor-binding site, leading to steric interference between the 4-O-acetyl group and side chain of 145S (Fig. 3).

Because A/equine/Jilin/89 virus, which binds 4-O-acetylated sialic acid as efficiently as avian viruses, replicated and caused significant disease in horses, the presence of this sialic acid species does not appear to pose an impenetrable barrier for transmission of the avian virus to horses. Rather, this sialic acid species appears to exert a selective pressure that leads to distinct changes in the receptor-binding phenotype of the HA of the virus during its further adaptation in this host. It may be that α<sub>2</sub>-macroglobulin or some other glycoproteins containing 4-O-acetylated sialyloligosaccharides present in horse respiratory secretions are responsible for this selection. It is also possible that 4-O-acetylated sialic acids are present on susceptible cells in horses and that selection is due to the inability of viral NA to remove these sialic acid residues from oligosaccharides and to provide the release of the virus from these cells or to prevent virus self-aggregation. In either event, selection stems from the presence of NA-resistant 4-O-acetylated sialylglycoconjugates in horses. Although we do not know if natural receptor analog inhibitors have played a role in other events of interspecies transmission of influenza virus, the present study demonstrates, for the first time, that a species difference in sialic acid contributes to the evolution of influenza viruses.

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