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Distinct Glycoprotein Inhibitors of Influenza A Virus in Different Animal Sera

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Normal horse and guinea pig sera contain the glycoprotein inhibitor α_2-macroglobulin, which inhibits the infectivity and hemagglutinating activity of influenza A viruses of the H2 and H3 subtypes. In the current study, the presence of inhibitors of influenza A virus in pig and rabbit sera was investigated. Variants of influenza virus type A/Los Angeles/2/87(H3N2) that were resistant to horse, pig, or rabbit serum were isolated. Analysis of the variant viruses with anti-hemagglutinin (HA) monoclonal antibodies revealed that antigenic changes occurred with the development of serum inhibitor resistance. Characterization of the inhibitors in pig and rabbit sera by using periodate and receptor-destroying enzyme demonstrated that carbohydrate is an important constituent of the active portion of both inhibitor molecules and that sialic acid is involved in the interaction of the inhibitors with influenza virus HA. Nucleotide sequence analysis of the HA molecule revealed that the serum-resistant variants each acquired a different set of amino acid alterations. The multiply resistant variants maintained the original amino acid changes and acquired additional changes. Sequence modifications in the HA involved the conserved amino acids within the receptor binding site (RBS) at position 137 and the second-shell RBS residues at positions 155 and 186. Amino acid changes also occurred within antigenic site A (position 145) and directly behind the receptor binding pocket (position 220). Amino acid alterations resulted in the acquisition of a potential glycosylation site at position 128 and the loss of potential glycosylation sites at positions 246 and 248. The localization of the amino acid changes in HA1 to the region of the RBS supports the concept of serum inhibitors as receptor analogs. The unique set of mutations acquired by the serum inhibitor-resistant variants strongly suggests that horse, pig, and rabbit sera each contain distinct glycoprotein inhibitors of influenza A virus.

Hemagglutinin (HA), the major surface glycoprotein of influenza virus, functions in binding to host cell sialyloligosaccharide receptors. This binding is crucial for the initiation of an influenza virus infection. The HA molecule is composed of a stalk region that includes the HA1/HA2 cleavage site and a globular domain containing the receptor binding site (RBS) (35). The RBS is located at the distal tip of the HA molecule in a surface depression (or pocket) which is lined by a series of conserved amino acid residues and is surrounded by variable antigenic sites. The sialic acid moieties of host cell receptors must become oriented in the RBS in a defined fashion and interact with specific amino acids within the pocket (34). Receptor binding specificity is determined by the ability of the HA molecule to correctly associate with different cell surface sialyloligosaccharides. Comparisons of influenza viruses from various animal species have elucidated differences in their abilities to recognize particular terminal sialic acid sequences (6, 22, 26). The variation in receptor specificities among human, avian, and equine influenza A viruses suggests that the presence of different receptors in their different host animals and the ability of HA to bind to these receptors are factors associated with viral host range and tissue tropism (10, 20, 25).

Naturally occuring inhibitors of HA activity of influenza viruses have been demonstrated in different animal sera (including human sera) and in fluid secretions such as ovomucin, bovine submaxillary mucin, and pleural fluid (for reviews, see references 2, 13, and 19). First discovered in 1947 by Francis (11), the alpha class of inhibitors were found to be heat-stable sialylated glycoproteins that inhibit hemagglutination but do not neutralize viral infectivity. Beta inhibitors, on the other hand, are heat-labile components of normal sera which are thought to be proteins and which possess virus-neutralizing activity (3, 12). The gamma class of inhibitors are heat-stable sialylated glycoproteins, but unlike alpha inhibitors, they can neutralize viral infectivity (5, 8). Inhibitors in the alpha and gamma classes are known to function as receptor analogs by competing with host cell receptors for binding to HA.

The gamma-type inhibitor present in horse and guinea pig sera, α_2-macroglobulin, is the best-characterized nonspecific inhibitor (14, 23, 28). It is active against human influenza A viruses of the H2 and H3 subtypes. Studies with variant H3 viruses, which are resistant to horse serum, have provided insight into the nature of the binding of the receptor or the inhibitor within the RBS of HA. Human influenza viruses of the H3 subtype recognize *N*-acetylneuraminic acid-α2,6-galactose (NeuAc2,6Gal) linkages of host cell sialic acids and are sensitive to horse serum inhibitor, whereas avian and equine influenza viruses recognize NeuAc2,3Gal linkages and are resistant to horse serum inhibitor (26). A single amino acid change of leucine to glutamine at position 226 of HA1, which lies within the receptor binding pocket, confers horse serum resistance to NeuAc2,6Gal-specific viruses and changes the specificity of the virus to NeuAc2,3Gal (26). Horse and guinea pig α_2-macroglobulin contains the sialic acid 4-*O*-acetyl-*N*-acetylneuraminic acid, which resists hydrolysis by viral neuraminidase (14). The presence of this uncommon sialic acid combined with a spatial arrangement

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of sialic acid groups that allows optimal interaction with HA is thought to be the basis for the high-potency inhibition found with equine and guinea pig sera (14, 23).

In contrast to the inhibitors in horse and guinea pig sera, little is known about the inhibitors in other animal sera. The study of naturally occurring receptor analogs may provide useful information for the development of antiviral agents whose mechanism of action is that of inhibition of viral attachment via HA blockade. We undertook the current study in order to answer the following questions. (i) Do glycoprotein inhibitors distinct from those found in horse and guinea pig sera exist in other animal sera? (ii) If different inhibitors exist, what is the nature of the inhibitory molecules? (iii) How do these inhibitors interact with the HA molecule of influenza A viruses? To address these questions, we examined the sensitivity of a human H3 influenza A virus to inhibitors in horse, pig, and rabbit sera. Serum inhibitor-resistant variants were analyzed with respect to their patterns of sensitivity to the different sera and were examined at the molecular level for changes in the HA molecule acquired during the development of serum inhibitor resistance.

MATERIALS AND METHODS

Viruses and viral RNA. The parent virus, A/Los Angeles/2/87(H3N2) [A/LA/2/87(H3N2)], was cloned by limiting dilution three times in embryonated chicken eggs. Serum inhibitor-resistant variants were isolated at limiting dilution in 11-day-old embryonated chicken eggs after incubation of the virus with an equal volume of the appropriate undiluted animal serum at room temperature for 1 h. The isolation procedure was repeated until the titer of the variant virus grown in the presence of serum was equal to that in the absence of serum. All sera were pooled and obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, Pa.). They were heat inactivated at 56°C for 30 min and stored at −20°C.

RNA was isolated by treatment of purified virus with proteinase K and sodium dodecyl sulfate followed by extraction with phenol-chloroform (1:1) and ethanol precipitation as described previously (4).

Nucleic acid sequencing. Nucleotide sequences of the HA gene were determined by direct RNA sequencing by the dideoxynucleotide-chain termination method (1, 29) using purified viral RNA as the template and reverse transcriptase. Ambiguities found by RNA sequencing were clarified by sequencing the cloned HA gene (15) by using alkali-denatured DNA templates (7). The sequences of the oligonucleotides used as primers will be provided on request.

Serologic tests. Hemagglutination titrations and hemagglutination inhibition (HI) reactions were performed by standard procedures in microtiter plates with 0.5% (vol/vol) chicken erythrocytes. Anti-H3 HA monoclonal antibodies were obtained from J. Katz (16, 33) (St. Jude Children's Research Hospital, Memphis, Tenn.). The animal sera were treated with periodate and *Vibrio cholerae* receptor-destroying enzyme (RDE) as described previously (21).

RESULTS

Inhibitors in horse, pig, and rabbit sera select biologically different variants. To look for inhibitors of influenza A virus different from those known to exist in horse serum, we first examined the sensitivity of A/LA/2/87, the parent virus, to horse, pig, and rabbit sera in HI assays. A/LA/2/87 was sensitive to all three sera (Table 1). Growth of the parent virus was also effectively neutralized by the presence of horse, pig, or rabbit sera with at least a 100,000-fold reduction in the 50% egg-infectious dose (EID_{50}) (Table 2). We then isolated variant viruses individually resistant to neutralization by horse, pig, or rabbit sera. The horse serum-resistant variant, A/LA HS, when examined by the HI test, was found to be resistant to horse serum while reacting to both pig and rabbit sera at a titer fourfold lower than that of the parent virus. A/LA PS (the pig serum-resistant variant) and A/LA RS (the rabbit serum-resistant variant) were resistant to a four- to eightfold-greater concentration of pig and rabbit sera than the parent virus, while remaining sensitive to horse serum. The differential sensitivity of the variants to neutralization and HI by the animal sera suggested that the inhibitors in pig and rabbit sera were different from those in horse serum. Although growth of A/LA PS and A/LA RS was not affected by their homologous sera (Table 2), hemagglutination of these variants was inhibited to some extent by the sera, suggesting the presence of nonneutralizing inhibitors (Table 1).

To establish the difference between inhibitors in horse and pig sera, the neutralizing activity of pig serum against A/LA HS was examined. A/LA HS was sensitive to neutralization by pig serum, with a greater than 1,000-fold reduction in the EID_{50} (Table 2). The fact that A/LA HS was sensitive to neutralization by pig serum supported previous indications that the inhibitors in the two sera were different. A/LA HSPS BB was the serum-resistant variant isolated from

TABLE 1. Selection of different serum inhibitor-resistant variants with normal horse, pig, and rabbit sera

Virus	Virus isolated in the presence of serum	HI titer[a] of serum:		
		Horse	Pig	Rabbit
A/LA/2/87	None	>1,280	>1,280	>1,280
A/LA HS	Horse	40	320	320
A/LA PS	Pig	>1,280	160	320
A/LA RS	Rabbit	1,280	160	320
A/LA HSPS BB[b]	Horse→pig	<10	20	160
A/LA HSPS[c]	Horse and Pig	<10	20	80
A/LA HSPSRS[d]	Horse and pig→rabbit	<10	20	20

[a] HI titers are reciprocals of the highest dilution of serum inhibiting 4 HA units of virus as determined in microtiter plates using 0.5% (vol/vol) chicken erythrocytes.
[b] Isolated from A/LA HS after exposure to pig serum.
[c] Isolated from A/LA/2/87 after simultaneous exposure to horse and pig sera.
[d] Isolated from A/LA HSPS after exposure to rabbit serum.

TABLE 2. Differential neutralization of A/LA/2/87 and serum inhibitor-resistant variants by normal horse, pig, and rabbit sera[a]

Virus	Serum tested	Virus titer (log_{10} EID_{50}/ml)	
		Without serum	With serum
A/LA/2/87	Horse	7.8	1.5
A/LA/2/87	Pig	7.8	2.1
A/LA/2/87	Rabbit	7.8	1.5
A/LA HS	Horse	7.8	≥7.5
A/LA PS	Pig	6.8	7.5
A/LA RS	Rabbit	6.3	5.8
A/LA HS	Pig	>6.0	2.5
A/LA HSPS	Rabbit	8.5	3.5

[a] Viruses were isolated at limiting dilution in 11-day-old embryonated chicken eggs after incubation of the virus with an equal volume of undiluted animal serum at room temperature for 1 h.

TABLE 3. HI of A/LA/2/87 by periodate- and RDE-treated animal sera

Treatment	HI titer[a] of serum		
	Horse	Pig	Rabbit
None	>1,280	>1,280	>1,280
Periodate[b]	<10	<10	40
RDE[c]	80	<10	20

[a] HI titers are reciprocals of the highest dilution of serum inhibiting 4 HA units of virus.
[b] Sera were treated with 0.1 M KIO_4 for 15 min at 20°C. The KIO_4 was inactivated with 1% glycerol saline (21).
[c] Sera were treated with *V. cholerae* RDE for 12 h. The RDE was inactivated with 2.5% sodium citrate (21).

A/LA HS after exposure to pig serum. By the HI assay, A/LA HSPS BB was resistant to both horse and pig sera and had reduced sensitivity to rabbit serum. A/LA HSPS was subsequently isolated by exposure of the parent virus to horse and pig sera simultaneously. This variant had to overcome the inhibitory effects of two sera at the same time in order to grow in eggs. By the HI assay, A/LA HSPS was resistant to both homologous sera and had reduced sensitivity to rabbit serum. This data clearly separated the inhibitors in horse serum from those in pig serum.

To distinguish the inhibitors in pig and rabbit sera, the neutralizing activity of rabbit serum against A/LA HSPS was examined and the variant A/LA HSPSRS was isolated. Growth of A/LA HSPS was neutralized by the presence of rabbit serum with a 100,000-fold reduction in EID_{50}. The sensitivity of A/LA HSPS to neutralization by rabbit serum established that the inhibitors in rabbit serum were not identical to those in horse and pig sera. A/LA HSPSRS showed marked resistance to all three sera in the HI test.

The patterns of sensitivity of the viruses to neutralization and HI by horse, pig, and rabbit sera verify the presence of heat-stable, neutralizing inhibitors in all three sera and provide strong evidence that the inhibitors in each type of serum are different.

Preliminary characterization of the inhibitors in pig and rabbit sera. To understand the chemical nature of the inhibitors in pig and rabbit sera and to compare them with the horse serum inhibitors, the sera were treated with either periodate or RDE and then tested for their neutralizing activity against A/LA/2/87. Periodate, which oxidizes *cis*-hydroxyl groups on carbohydrates, removed all neutralizing activity found in horse, pig, and rabbit sera (data not shown). RDE treatment of serum cleaves *N*-acetylneuraminic acid residues from sialyloligosaccharides. As with periodate treatment, RDE treatment of the three sera essentially removed all virus-neutralizing activity (data not shown). The sera were then analyzed by the HI assay for inhibitory activity (Table 3). Periodate removed all inhibitory activity found in horse and pig sera, while residual inhibitory activity was found in periodate-treated rabbit serum. RDE treatment of pig serum destroyed all inhibitory activity, whereas RDE-treated horse and rabbit sera retained some inhibitory activity. The results with periodate and RDE suggest that carbohydrate and sialic acid are important components of the inhibitor molecules in all three sera.

Antigenic variation associated with the development of serum inhibitor resistance. Influenza viruses which differ in their receptor binding specificities and in their sensitivities to horse serum inhibitor are also known to differ in the antigenicity of their HA molecules (9). To assess whether the development of resistance to inhibitors in pig and rabbit sera is associated with antigenic changes in the HA molecule, the different variants were tested in HI reactions using a panel of monoclonal antibodies (Table 4). Resistance to either horse (A/LA HS), pig (A/LA PS), or rabbit (A/LA RS) serum resulted in changes in the antigenic character of the original virus. However, the pattern of reactivity with the monoclonal antibodies was different for each of the three variants. The development of multiple resistance (in A/LA HSPS, A/LA HSPS BB, and A/LA HSPSRS) led to the most noticeable changes in the pattern of reactivity. A/LA HSPS and A/LA HSPS BB lost reactivity with five of the nine antibodies (E12/2, M12/3, M2/1, M2/4, and M2/5). The addition of resistance to a third inhibitor (A/LA HSPSRS) caused further variation in the reactivity to the monoclonal antibodies tested. These results indicate that antigenic changes can be associated with the development of serum inhibitor resistance.

Nucleotide and deduced amino acid sequences of parent and variant viruses. To examine the molecular basis for the resistance to the inhibitors in different sera and the altered reactivity to monoclonal antibodies, we deduced the amino acid sequence of HA1 from the nucleotide sequences of parent and variant viruses (Table 5). The nucleotide sequences of the HAs of all the viruses were derived from genomic RNA, and the ambiguities were verified by sequence analysis of cloned cDNA. In order to facilitate comparison with other H3 strains, and for localization of amino acid residues in the three-dimensional structure, we used the amino acid numbering system of A/Aichi/2/68(H3N2) (32). All of the amino acid sequence changes in the serum inhibitor-resistant variants occurred in the globular domain of HA1 either within or around the RBS (Fig. 1). The

TABLE 4. Antigenic variation associated with the development of resistance to serum inhibitors

Monoclonal antibody to H3 HA	HI titer[a]						
	A/LA/2/87	A/LA HS	A/LA PS	A/LA RS	A/LA HSPS BB	A/LA HSPS	A/LA HSPSRS
E12/2	200	<100	<100	<100	<100	<100	<100
M12/3	>12,800	<100	200	1,600	<100	<100	<100
M2/1	>12,800	200	1,600	1,600	<100	<100	<100
M2/4	>12,800	400	3,200	3,200	<100	<100	<100
M2/5	>12,800	800	<100	1,600	<100	<100	<100
B45/5	6,400	3,200	400	1,600	1,600	800	200
B49/4	>12,800	3,200	100	400	100	100	100
B85/1	800	400	400	400	800	3,200	800
B106/2	>12,800	3,200	100	100	100	200	100

[a] HI titers are reciprocals of the highest dilution inhibiting 4 HA units of virus.

TABLE 5. Amino acid differences in HA1 between A/LA/2/87 and serum-resistant variants

Virus	Amino acid at residue[a]:							
	128	137	145	155	186	220	246	248
A/LA/2/87	Asn	Tyr	Asn	Tyr	Ser	Arg	Asn	Thr
A/LA HS	Thr[b]	Tyr	Lys	His	Ser	Arg	Asn	Thr
A/LA PS	Asn	Tyr	Asn	Tyr	Ile	Arg	Asn	Thr
A/LA RS	Asn	Tyr	Asn	Tyr	Ser	Gly	Asp[c]	Thr
A/LA HSPS BB	Thr[b]	Asp	Lys	His	Ile	Arg	Asn	Thr
A/LA HSPS	Asn	Tyr	Asp	Tyr	Ile	Arg	Asn	Thr
A/LA HSPSRS	Asn	Tyr	Asp	Tyr	Ile	Arg	Asn	Ile[c]

[a] Amino acid numbers correspond to the published sequence of A/Aichi/2/68 (31); Underlined amino acids signify changes from the parent amino acid.
[b] Results in the acquisition of a potential glycosylation site (Asn-126-Trp-127-Thr-128).
[c] Results in the loss of the potential glycosylation site Asn-246-Ser-247-Thr-248.

horse serum-resistant variant (A/LA HS) acquired amino acid changes at positions 128 (Asn→Thr), 145 (Asn→Lys), and 155 (Tyr→His). The amino acid change at position 128 resulted in the acquisition of a potential glycosylation site involving amino acids 126 through 128 (Asn-126-X-Thr-128). A/LA PS had a single amino acid change at position 186 (Ser→Ile). A/LA RS acquired amino acid changes at positions 220 (Arg→Gly) and 246 (Asn→Asp). The alteration at

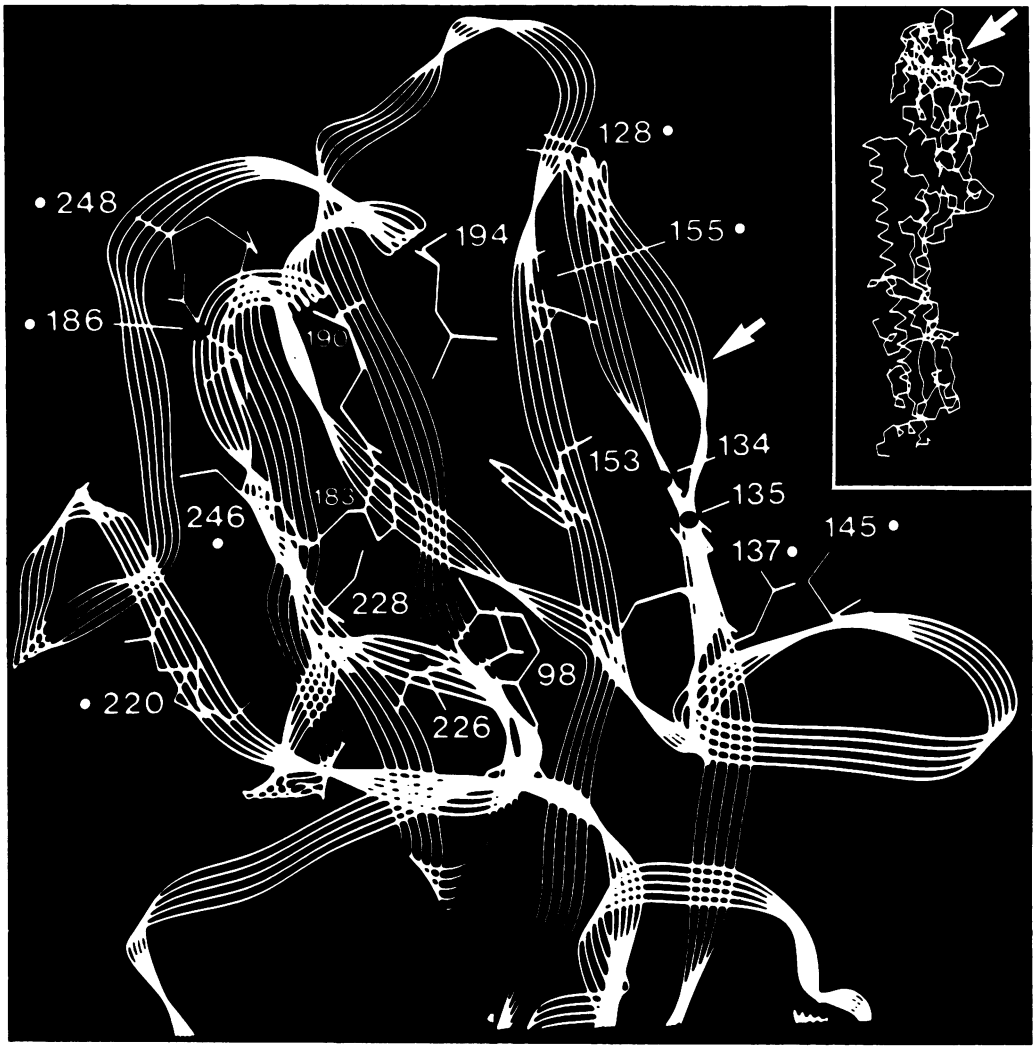

FIG. 1. Schematic diagram of the globular head of the H3 HA molecule, including the RBS, demonstrating the location of the amino acid changes found in the serum-resistant variants. Labeled amino acids include conserved residues in the RBS and altered residues (white dots) found in the variants. The insert shows a diagram of the whole HA monomer. Arrows indicate the RBS.

position 246 resulted in the loss of the potential glycosylation site involving amino acid residues 246 through 248 (Asn-246-X-Thr-248).

A/LA HSPS BB, the variant isolated from A/LA HS after exposure to pig serum, retained all of the amino acid changes seen with A/LA HS. In addition, A/LA HSPS BB had a change at position 186 (Ser→Ile) and an alteration at position 137 (Tyr→Asp) not seen in either A/LA HS or A/LA PS. A/LA HSPS, the variant isolated after simultaneous exposure of the parent virus to horse and pig sera, had an amino acid change at position 145 (Asn→Asp) and the same amino acid change at position 186 (Ser→Ile) seen in A/LA PS. A/LA HSPSRS, derived from A/LA HSPS upon exposure to rabbit serum, differed from A/LA HSPS only at position 248 (Thr→Ile), which resulted in the loss of the potential glycosylation site (Asn-246-X-Thr-248).

Three amino acid changes in the variants occured either directly within the RBS (137) or at residues corresponding to the second shell of RBS residues (155 and 186). The other amino acid changes occured in a loop of amino acids adjacent to the RBS (145), behind the RBS (220), and at potential glycosylation sites (128, 246, and 248) (Fig. 1). The location of the amino acid changes in the variants in or near the RBS strongly supports the concept of the serum inhibitors as substances binding to HA via the RBS, thereby inhibiting the binding of the virus to a host cell receptor. These findings also indicate that different mutations in HA are required to overcome the inhibitory effect of the glycoprotein inhibitors in horse, pig, and rabbit sera, strongly suggesting that the inhibitors in these animal sera are structurally distinct.

DISCUSSION

In the current study, we have demonstrated that nonspecific glycoprotein inhibitors of influenza A virus exist not only in horse serum but also in pig and rabbit sera. Inhibitors of influenza virus have been observed previously in rabbit serum; however, the serum was not assessed for neutralizing activity (31). To our knowledge there are no published data dealing with the nonspecific inhibitors in swine sera. Our data establish the presence of glycoprotein inhibitors in pig and rabbit sera which inhibit HA activity, neutralize virus infectivity, and are thermostable. The inhibitors in the animal sera could be distinguished by differential virus neutralization. The nucleotide sequences of the HAs of the serum-resistant variants demonstrated that horse, pig, and rabbit sera each generate unique modifications in HA. Thus, these data affirmed at the molecular level that different mutations in the HA molecule are required to overcome the inhibitory activity of each of the sera. These results support the hypothesis that there are distinct glycoprotein inhibitors present in the sera of different animal species, each of which interacts with the HA of influenza A virus in a specific manner.

Characterization of the serum inhibitors by chemical modification with periodate and RDE provided evidence that carbohydrate and sialic acid are important elements of the active portions of the inhibitors in pig and rabbit sera. The importance of these two constituents has already been established for the glycoprotein inhibitor α-2-macroglobulin in horse serum (14, 23). Isolation, purification, and detailed chemical analysis of the pig and rabbit serum inhibitors are in progress as a means of thoroughly understanding the nature of the inhibitor molecules and how they interact with HA as receptor analogs.

Molecular analysis of the HAs of the variants revealed that distinct amino acid changes were required in each variant to overcome the inhibitory effect of horse, pig, or rabbit serum (Fig. 1). A/LA HS acquired an amino acid alteration at position 145, which is part of a loop of amino acids lying immediately adjacent to the RBS and constituting part of antigenic site A (35). A change in this region of the HA molecule could conceivably alter the orientation of sialic acid on the inhibitor within the RBS. A/LA HS also acquired a change at position 155, and A/LA PS acquired a change at position 186. These amino acids are within the receptor binding pocket but do not interact directly with the sialic acid of the receptor via hydrogen bonds or van der Waals contacts (34). Instead, they can be considered part of a second shell of residues which stabilize the RBS and interact secondarily with sialic acid through other residues in the binding pocket. On the basis of the proposed interactions of sialic acid with RBS residues set forth by Weis et al. (34), amino acid residue 155 interacts with amino acid 194, which binds to *N*-acetylneuraminic acid via the hydroxyl group of C7. Amino acid residue 186 interacts secondarily with the hydroxyl group of C9 via amino acid residues 190 and 228. This suggests that the inhibitors in horse and pig sera may be modified at these areas of the sialic acid moieties (e.g., by acetylation to 7-*O*-acetyl or 9-*O*-acetyl neuraminic acid) and thereby induce the alterations at positions 155 and 186. A/LA RS acquired an amino acid alteration at position 220, which lies posterior to the RBS. This amino acid is near amino acids 224 through 228, forming one wall of the pocket. The change from arginine to glycine at this position results in a considerable change in the size and charge of the amino acid residue, which may change the conformation of the HA in such a way as to subsequently affect receptor binding with residues 224 through 228. A/LA HS and A/LA RS both had alterations at amino acid residues which could affect the glycosylation of the HA molecule. These amino acid residues (128 and 246) are located at points distant from the RBS. However, the loss or acquisition of glycosylation sites could indirectly influence binding of the inhibitor by interfering with the proper orientation of the inhibitor in the pocket or by altering the access of the inhibitor to certain critical residues within the pocket.

A/LA HSPS BB maintained all the amino acid alterations seen in its precursor A/LA HS and in addition acquired the alteration seen in A/LA PS. The new alteration in A/LA HSPS BB at amino acid residue 137 (an alteration not seen in A/LA HS or A/LA PS) lies within the RBS. This residue is involved in the direct linkage of HA with sialic acid molecules. It can be speculated that this mutation is required for complete resistance upon the background of the other amino acid alterations. A/LA HSPS, although exposed to both horse and pig sera simultaneously, appears to have selected for pig serum resistance first since it acquired the alteration seen in A/LA PS at position 186 without acquiring the changes seen with the development of horse serum resistance. To then become resistant to horse serum required only one additional change at position 145. Selection for horse serum resistance first would have resulted in specific amino acid alterations at positions 128, 145, and 155, alterations which were not found in this variant (see below). An alteration at position 186 appears to be important for the acquisition of resistance to pig serum inhibitors, because the same amino acid change occured in each of the pig serum-resistant variants. A/LA HSPSRS differed at only one amino acid residue (248) from its precursor A/LA HSPS (before exposure to rabbit serum). This change at position 248,

which results in the loss of the potential glycosylation site (Asn-246-X-Thr-248), suggests that loss of carbohydrate at this position is important for acquisition of resistance to rabbit serum inhibitors, since A/LA RS also lost this potential glycosylation site.

In the present study, we did not attempt to obtain multiple isolates resistant to the same serum, except with horse serum. The nucleotide sequence of a second (independently isolated) horse serum-resistant variant was found to be identical to that of the first (data not shown). However, it is possible that resistant strains containing different amino acid substitutions could be selected by the same serum.

Antigenic comparison using anti-HA monoclonal antibodies revealed changes in the HAs of the serum-resistant variants. The development of resistance to both horse and pig sera (A/LA HSPS and A/LA HSPS BB) or to all three sera (A/LA HSPSRS) resulted in a greater degree of antigenic variation than one would expect if the alterations were simply additive. This suggests that additional changes in the HA may lead to alterations in the conformation of the HA at antigenic sites, around the RBS, or between HA subunits, thus decreasing the ability of antibodies to bind.

Host cell selection of antigenic variants has been shown to occur with influenza A and B viruses (10, 16, 24, 30). The HA gene of recent human H3N2 viruses from the same individual grown either in eggs or in MDCK cells differs antigenically and at one or more amino acid residues in HA1 (18). Since the nucleotide sequence of the HA gene of MDCK cell-grown virus is identical to that of virus obtained in an individual throat wash sample (17), amino acid changes found in egg-grown viruses (e.g., at residues 111, 126, 138, 145, 156, 173, 186, 193, 199, 229, 246, and 248) may be important for the adaptation of H3 viruses to growth in eggs (16, 17, 18, 33). Many of these amino acid residues are also altered in our serum-resistant variants, supporting the concept that these amino acid residues are important for the interaction of HA with host cell receptor molecules.

In the case of the early human H3N2 virus X-31, horse serum resistance has been shown previously to result in a single amino acid change at position 226, which is part of a group of conserved amino acids forming one lateral wall of the RBS of the HA molecule (27). However, the horse serum-resistant variant derived from A/LA/2/87 did not have an amino acid change at position 226. It is not known why the development of horse serum resistance with A/LA/2/87 did not result in a change at amino acid 226. It is possible that variations in HA as a result of genetic drift have occurred over time such that an amino acid change at position 226 no longer confers resistance to horse serum. Within the same context, the neutralization of A/LA/2/87 observed with the animal sera in our studies may be due to inhibitors previously characterized as nonneutralizing with earlier H3 viruses. The fact that RDE-treated horse serum did not inhibit hemagglutination of A/LA/2/87, in contrast to what has been shown with other H3 viruses, provides support for variations in the H3 hemagglutinin (23).

The presence of nonneutralizing inhibitors in normal pig and rabbit sera could account for the residual activity observed with A/LA PS and A/LA RS, respectively, in the HI assay (Table 1). This type of inhibitor would not have interfered with isolation of the variant in eggs but would remain active in the HI test. The removal of all neutralizing inhibitory activity in the sera with periodate and RDE without complete removal of the HI activity corroborates the presence of nonneutralizing inhibitors.

Immunoprophylaxis of influenza virus infection through the use of inactivated vaccines has been suboptimal because of antigenic drift among the viruses and the rapidity with which immunity declines after vaccination. Amantadine and rimantadine, the antiviral agents currently available for chemoprophylaxis and therapy of influenza A virus infections, are limited in their effectiveness by the emergence of resistant virus strains. The prevention and/or treatment of influenza would be possible if synthetic receptor analogs that bound to viral HA, thereby inhibiting virus attachment to host cells, could be manufactured. A thorough understanding of the exact molecular mechanism of the interaction of HA with host cell receptors is necessary for achieving such a goal. The present study established that different amino acid mutations are required to overcome the activities of the inhibitors in different animal sera. Thus, the structural features required for each inhibitor to be active might be incorporated into a single multivalent receptor analog to alleviate the problem of drug resistance encountered with currently available agents.

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