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Substituted Sialic Acid Prosthetic Groups as Determinants of Viral Hemagglutination

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Inhibitors of hemagglutination by type A2 influenza virus and a recently isolated strain of type B influenza virus were separated by sucrose density gradient centrifugation and agarose gel filtration from horse serum. Using selected reagents, it was demonstrated that the active substituent on the horse serum inhibitor of A2 influenza virus was 4-*O*-acetyl-*N*-acetylneuraminic acid; however, the active substituent on the inhibitor of the influenza B virus was shown to be *N*-acetylneuraminic acid (NANA). Sodium metaperiodate treatment of a component of horse serum resulted in a 10 to 15-fold enhancement of inhibitory activity against the type B virus, whereas the A2 inhibitor was completely destroyed. Since this enhancement did not occur with influenza B viruses isolated prior to 1965, it was considered that this sensitivity to an oxidized NANA glycoside may have been a reflection of an antigenic change which occurred at that time. The use of different virus strains and selected chemical reagents to define the important sialic acid prosthetic groups active in inhibition was described.

Inhibitors of influenza virus hemagglutination have been discovered in many naturally occurring substances, including various animal sera and secretions. These inhibitors, which are capable of blocking hemagglutination by prior reaction with the virus, provide a system of competitive inhibition which allows the study of the virus-red cell reaction in considerable chemical detail.

Early work in the field of hemagglutination inhibitors was instrumental in establishing the importance of *N*-acetylneuraminic acid (NANA) as the essential prosthetic group responsible for linking the influenza virus to the surface of the red cell (7, 11). All of the well-characterized inhibitors of viral hemagglutination that have been purified so far, such as the urinary mucoprotein isolated by Tamm and Horsfall (22) and ovine submaxillary gland glycoprotein (8), contain NANA as the biologically active grouping. The recognition of neuraminidase-insensitive inhibitors indicates that another receptor grouping, besides NANA, may be implicated in the binding of some of these viruses (5, 20). The neuraminidase-resistant inhibitor of influenza A2 virus, present in horse serum, is well known, but the identity of the inhibitory receptor grouping as a sialic acid other than NANA has only recently been established (16). The isolation of this inhibitor in a sufficiently purified state to enable the investigation of the types of neuraminic acids present has made this work possible (2, 15).

This report describes the use of chemical reagents which make relatively minor alterations of the sialic acids but which result in profound changes in the inhibition of the virus. Most of the work was done using a component of horse serum, equine α-macroglobulin (EαG), which contains the inhibitor of influenza A2. Evidence is presented that horse serum also contains an inhibitor to contemporary influenza B viruses which can be physically separated from the already described A2 inhibitor.

MATERIALS AND METHODS

Viruses. The two strains of influenza virus used in this study were the inhibitor-sensitive variant of A2/Singapore/57 (5) previously used in this department and B/England/13/65, originally received from A. S. Beare. The viruses were grown and purified as already described (14).

Hemagglutination (HA) and hemagglutination-inhibition (HI). One method used to determine the HA and HI titers was the standard "pattern" method using eight agglutination doses of virus in plastic trays (24). A second method, a continuous-flow technique utilizing the Technicon Autoanalyzer, was used to screen column- and density-gradient fractions

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for inhibitory activity. The technique is based on that described by Cohen (4), except that a second channel was added to allow samples to be screened against two viruses simultaneously. In this technique, a single sampler module is used, a sample being taken up through a pair of probes with each probe feeding into a separate channel. The results, expressed as optical density of hemoglobin at 420 nm, are continuously displayed on a recorder. One unit of optical density (Technicon unit) is defined as an excursion from the virus baseline of 3 mm. This system was used mainly to give a semiquantitative indication of the inhibitor distribution in column and gradient samples.

Sera and serum fractions. Whole horse serum, from a single horse, was obtained from Burroughs Wellcome & Co. (Beckenham, England) and was stored at −15 C.

A 5-g sample of EαG, lyophilized and salt-free, was obtained from Calbiochem (Los Angeles, Calif.; grade B, Cohn fraction IV, batch no. 55391) and stored at 4 C.

Partially purified EαG (PP5) was prepared by pooling the inhibitory fraction from six agarose gel-filtration runs, using as starting material EαG. The pooled fractions were concentrated by ultrafiltration and rerun on the same column to remove aggregates. The active fractions were pooled and run on a sucrose density gradient. The dialyzed and concentrated inhibitor material was examined in the analytical ultracentrifuge and showed one sedimenting peak, which was calculated to be 18*S*.

A number of randomly collected human serum samples were pooled to form a single 30-ml batch. This was stored untreated at −15 C.

Sucrose density gradients. A sample was layered onto a 19-ml linear sucrose density gradient (45 to 9% sucrose in borate-buffered saline, *p*H 7.6) and run in the no. 30 swing-out rotor of the MSE Superspeed 50 ultracentrifuge at 75,000 × *g* for 16 to 18 hr. Fractions (40 drops/ml) were collected by piercing the bottom of the tube and collecting drops in successive tubes by hand.

Molecular exclusion chromatography. A column of agarose beads (4% gel, 120 mesh, 1.16 by 123 cm) was prepared and calibrated (14). The sample was layered on the column and eluted at 3.0 ml/hr with 0.15 M NaCl containing 0.02% NaN_3.

Periodate treatment of inhibitor. The conditions for oxidation of glycoproteins by $NaIO_4$ were those recommended for the specific oxidation of straight-chain cis-hydroxyl groups and which reduce non-Malapradian oxidations to a minimum (17, 19). The concentration of sodium metaperiodate was adjusted to give a ratio of moles of periodate not reduced to moles of periodate consumed of approximately 2 on the basis of the available oxidizable groups. In the case of whole serum, this consisted of three volumes of 0.011 M $NaIO_4$ to one volume of undiluted serum. The reaction was carried out in the dark, at *p*H 4.5 and at 4 C for 15 min; at that time, the reaction was stopped by the addition of one volume of 10% glucose. After an additional 15 min at 4 C, the treated serum or inhibitor was ready for testing. The reagents were made up in 0.15 M NaCl immediately before use. Under these conditions, it is unlikely that extensive oxidation of pyranose rings occurs.

Neuraminidase treatment. Purified *Vibrio cholerae* neuraminidase (Seravac Laboratories, Maidenhead, England; 500 units/ml) was incubated with serum or inhibitor overnight at 37 C. A concentration of 50 to 100 units of neuraminidase to 30 mg of protein was found to be completely effective in destroying inhibitor activity. All dilutions were made in calcium acetate buffer at *p*H 6.2.

Alkali treatment of EαG. A 12-mg sample of EαG was mixed with 1.0 ml of 0.1 N NaOH and, after 20 min at room temperature, the *p*H was adjusted to neutrality with 1.0 ml of 0.1 N HCl and 0.2 ml of 1 M PO_4 buffer. The neutralized mixture was then titrated for inhibitory activity. In a number of experiments, alkali treatment was followed by exposure to bacterial neuraminidase or periodate. When alkaline hydrolysis was followed by addition of neuraminidase, the *p*H was first adjusted to 6.2 with calcium acetate buffer, and the reaction was allowed to proceed for 20 hr at 37 C in the presence of 50 units of enzyme.

Complement fixation. A microtechnique was used to determine the presence of antibody to the type-specific V antigen in the human serum pool (1).

RESULTS

Effect of neuraminidase and periodate on horse and human serum inhibitors. It has been shown previously that the inhibitory effect of horse serum against A2 influenza viruses is readily abolished by treatment with sodium metaperiodate, whereas neuraminidase is ineffective in destroying the inhibitor activity (5). In contrast to horse serum, the human serum inhibitor to A2 influenza viruses is destroyed by both methods of treatment.

In view of these results, it was of interest to determine the effect of these inhibitors and these treatments on the hemagglutinating activity of a newly isolated strain of type B influenza virus, B/England/65. As can be seen in Table 1, the type B virus was relatively insensitive to untreated horse and human serum. However, when the serum was treated with $NaIO_4$, the inhibitory activity to A2 was lost but the activity against B/Eng/65 was enhanced 8- to 15-fold. Conversely, neuraminidase is effective in removal of the B inhibitor in both sera. This phenomena has been observed recently in human serum with other contemporary type B strains (13). Complement fixation tests, using the V antigen of both the A2 and the B virus, confirmed the absence of antibody in horse serum and demonstrated only a low level present in the pooled human serum which is responsible for the residual inhibitory activity after neuraminidase treatment. Also tested were two preparations of horse serum inhibitor, EαG and a highly purified component

EαG-PP5. It was confirmed from these experiments that the semipurified EαG fraction behaved identically to whole horse serum and the highly purified material with respect to the treatments used; due to its substantially higher inhibitory activity, this material was used in all the subsequent experiments.

Fractionation of EαG and human serum. Treatment of the macroglobulin fraction with neuraminidase destroyed the periodate enhancement of inhibition of the B virus (Table 2), indicating that this effect was dependent on a molecule carrying a substituted neuraminic acid prosthetic group. As the inhibitory effect against A2 virus is not destroyed by neuraminidase, it is conceivable that two distinct molecular species of inhibitor are present in horse serum, one active against A2 and the other active against B/Eng/65. Further evidence to support this hypothesis is the finding that the highly purified horse serum inhibitor (EαG-PP5) showed a lower inhibition of the type B virus relative to whole serum and EαG (Table 1). Since this fraction has been isolated on the basis of A2 virus inhibition, this is also suggestive of multiple inhibitor populations. In contrast, human serum would be expected to contain only one inhibitor active against the two viruses, since treatment with neuraminidase results in equal destruction of inhibitory activity against both viruses.

In an attempt to separate the inhibitory activity present in horse and human serum, sucrose density gradient centrifugation and agarose gel filtration were done. The separation of the A2 and B virus inhibitor peaks was achieved in EαG, substantiating the presence of at least two molecular species responsible for inhibition (Fig. 1, 2). The inhibitory activity of human serum was not resolvable into more than one peak, indicating that most likely only one inhibitor is responsible for the activity of human serum against the two viruses (Fig. 3, 4).

TABLE 1. *Effect of neuraminidase and $NaIO_4$ on the hemagglutination inhibition potency of horse and human serum and derived fractions*

Serum	Treatment	HI titers	
		A2/Sing/57	B/Eng/65
Horse	None	1,280	20
	$NaIO_4$	<5	160
	Neuraminidase	1,280	<5
Human	None	320	480
	$NaIO_4$	60	3,840
	Neuraminidase	80	60
EαG (60 mg/ml)	None	60,000	600
	$NaIO_4$	<10	10,000
	Neuraminidase	60,000	<10
EαG-PP5 (6.1 mg/ml)	None	30,000	8
	$NaIO_4$	<10	24
	Neuraminidase	30,000	<10

TABLE 2. *Effects of multiple treatment with alkali, neuraminidase, and periodate on the hemagglutination inhibition activity of EαG*

Treatment		HI titer	
1	2	A2/Sing/57	B/Eng/65
Untreated		<60,000	600
Neuraminidase		60,000	<10
$NaIO_4$		<10	10,000
0.1 N NaOH		2,560	1,280
Neuraminidase	$NaIO_4$	<25	<25
0.1 N NaOH	Neuraminidase	60	<20
0.1 N-NaOH	$NaIO_4$	<36	18,400
Neuraminidase	0.1 N NaOH	2,560	960

Identification of the inhibitory prosthetic groups

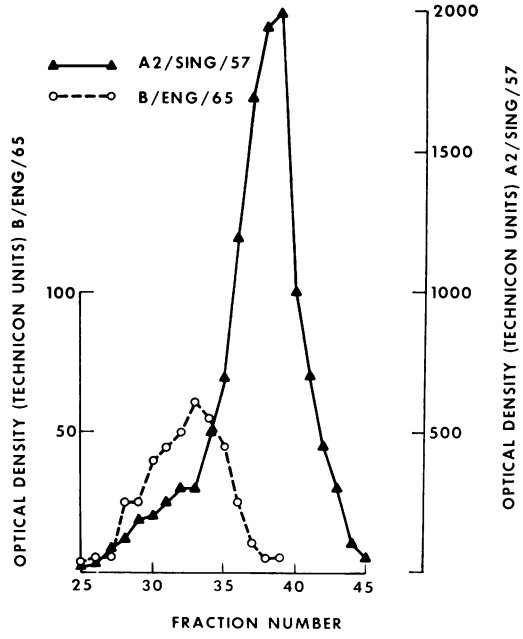

FIG. 1. *Comparison of the distribution of inhibitory activity against A2/Sing/57 and B/Eng/65 in fractions derived from agarose column separation of untreated EαG. A 0.4-ml (24 mg) sample of EαG was layered onto an agarose gel column and eluted at a rate of 3.0 ml/hr with 0.15 M NaCl. Fractions of 1.5 ml were collected, and the HI activity was determined by use of the Technicon autoanalyzer.*

by chemical treatment. The differences in the biological effects of the EαG inhibitors can most likely be ascribed to variations in the relevant inhibitory prosthetic groups present on the respec-

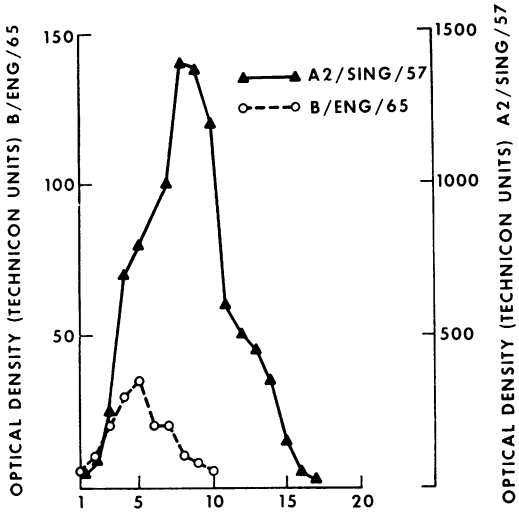

FIG. 2. *Comparison of the distribution of inhibitory activity against A2/Sing/57 and B/Eng/65 in fractions collected from sucrose density gradient centrifugation of EαG. A 0.5-ml (30 mg) sample of EαG was layered onto a 19-ml linear sucrose gradient and run for 18 hr at 75,000 × g. The HI activity was determined by use of the Technicon autoanalyzer.*

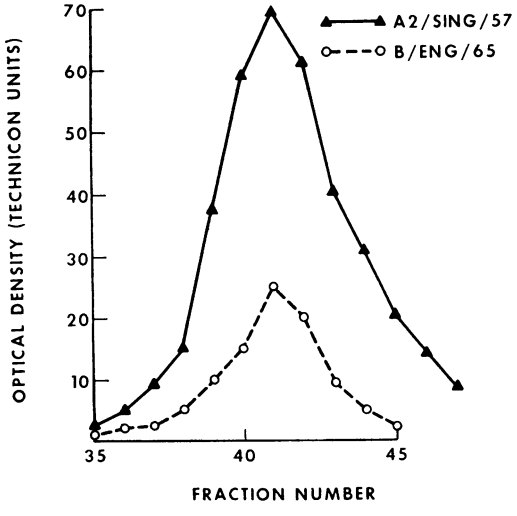

FIG. 3. *Comparison of the distribution of inhibitory activity against A2/Sing/57 and B/Eng/65 in fractions derived from agarose column separation of pooled human serum. A 0.5-ml sample of human serum was layered onto the agarose gel column and eluted with 0.15 M NaCl. Fractions of 1.5 ml were collected, and the HI activity was determined by use of the Technicon autoanalyzer.*

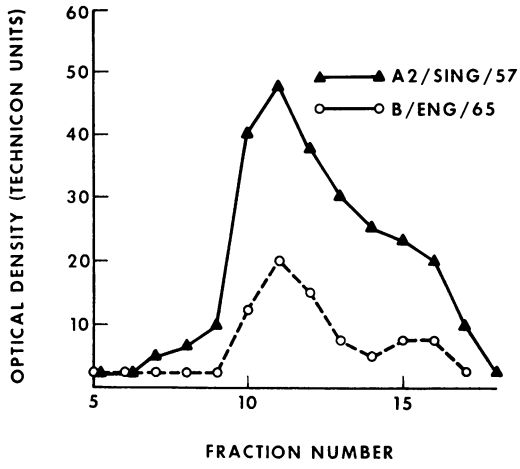

FIG. 4. *Comparison of the distribution of inhibitory activity against A2/Sing/57 and B/Eng/65 in fractions collected from sucrose density gradient centrifugation of pooled human serum. The 0.5-ml sample was layered onto a 19-ml linear sucrose gradient and run for 18 hr at 75,000 × g. Fractions of 1.0 ml were collected, and the HI activity was determined by use of the Technicon autoanalyzer.*

tive inhibitory molecules. Therefore, attempts were made to further characterize these inhibitors by chemical means.

It is known from previous studies, performed in this laboratory by Pepper (16), that the sialic acids present in purified horse serum inhibitor are NANA, *N*-glycolylneuraminic acid (NGNA), and the *O*-acetylated derivatives of these acids. The latter constitute over 50% of the sialic acids present in horse serum and are resistant to the action of bacterial neuraminidase. The sialic acid in horse serum, active against A2 virus, is most likely the *O*-acetyl-*N*-acetylneuraminic acid (*O*-acetyl NANA) since NGNA is known to have little inhibitory activity (F. Biddle, *personal communication*) and the glycosidic linkages of NANA are susceptible to the action of neuraminidase. In addition, dilute alkali, under certain conditions, has been recently shown by Schauer and Faillard (18) and Pepper (16) to remove *O*-acetyl groups from substituted neuraminic acids. Thus, when EαG was exposed to dilute NaOH, the inhibitory activity to A2 virus was reduced to 3% of its former level, and the remaining activity was then susceptible to neuraminidase (16). This is presumably due to the removal of the *O*-acetyl group, resulting in NANA which has a low degree of inhibitory activity against A2 virus. Therefore, it seemed useful to examine the effects of mild alkali treatment in combination with enzymatic hy-

drolysis and periodation on the biological properties of the horse serum inhibitors.

In this experiment, a sample of EαG was treated with dilute NaOH and followed by exposure to neuraminidase or periodate. Table 2 shows the inhibitory titer of EαG to the two viruses after the various treatments. As can be seen, treatment with alkali, which removes the *O*-acetyl group, increases the inhibition of the B virus, presumably due to the increased amount of NANA. As would be expected, subsequent $NaIO_4$ treatment resulted in a dramatic rise in the inhibitory titer. Treatment with neuraminidase, after exposure to alkali, removes the nonacetylated neuraminic acids, thereby removing all inhibitory activity to the B virus. It is clear that any treatment which results in an increase in available NANA heightens the HI titer to the B virus, and the production of an oxidized NANA glycoside increases the inhibitory titer approximately 10-fold more. Conversely, the A2 virus is most sensitive to neuraminidase-resistant, acetylated neuraminic acids, and any treatment which results in removal of the acetyl group—i.e., NaOH—or oxidizes the molecule, substantially reduces or destroys the activity.

DISCUSSION

The experiments using treated horse serum inhibitor lead to the conclusion that horse serum contains two inhibitor populations which can be physically separated and which possess at least two different biologically active sialic acids. The probable composition of the active, terminal sialic acid groups can be deduced from their reaction with the various reagents used in this study. To more easily comprehend the sites of attack of these reagents, Fig. 5 shows the points at which the various chemicals are active under the described conditions.

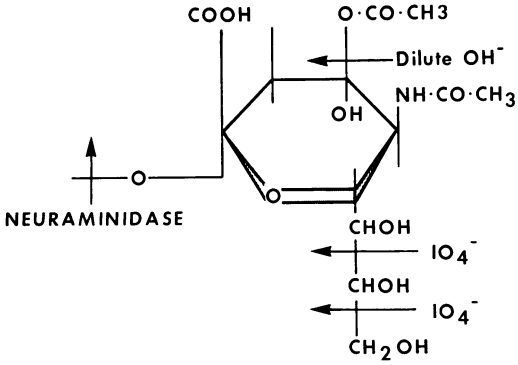

FIG. 5. *Sites of action of $NaIO_4$, neuraminidase, and dilute alkali on the 4-O-acetyl-N-acetylneuraminic acid.*

The susceptibility of the B-virus horse serum inhibitor to neuraminidase, whereby both the inhibition and the periodate enhancement effect are abolished, points to the fact that a simple NANA must be the principle receptor involved in this reaction. It is also clear that the inhibition is at a relatively low level until the oxidation of a portion of the molecule by periodate. Using the described condition for periodate oxidation, the straight-chain cis-hydroxyl groups are more rapidly oxidized than those occurring within the pyranose ring (19). Assuming that the sialic acid group is terminal and is linked at C2 to a hexosamine, the only straight-chain cis-hydroxyl groups occur at C7, C8, and C9, the oxidation of which would result in an aldehyde group at C7. This new prosthetic group is a much more effective inhibitor of hemagglutination than the unoxidized molecule, presumably due to a better fit with the receptor on the virus surface.

The A2 virus inhibition is clearly not due to NANA because of its insensitivity to neuraminidase. Most of the evidence indicates that the inhibition is due to another substituted neuraminic acid, most likely the *O*-acetyl NANA. Here the most significant fact is the demonstration that mild-alkali treatment of the horse serum inhibitor, which removes *O*-acetyl groups, renders the A2 inhibitory component fully susceptible to destruction by bacterial neuraminidase and the residual activity is almost equally active against both viruses. Moreover, it is apparent that alkali treatment converts the inhibitor into one that is also susceptible to periodate enhancement, i.e., NANA. These facts together mean that the anti-A2 component also inhibits by binding through a substituted neuraminic acid group and that both viruses are sensitive to NANA, but optimal inhibition is achieved by slight modifications of the molecule.

It is possible to localize the position of the second acetyl group of the *O*-acetyl NANA to the C4 position by the use of the B virus and appropriate chemical reagents. Acetylation at C7 is not possible, as this substitution would prevent the formation of a chromogen in the Warren thiobarbituric acid assay of sialic acids (23). Since the total substituted neuraminic acid content of EαG and purified equine inhibitor is identical by both the thiobarbituric acid and Svennerholm resorcinol test (21), an acetyl group at C7 is excluded. Acetylation at C8 is not compatible with the data, as this form would not be oxidizable by periodate at all and acetylation at C9 would result, after periodation, in an aldehyde which would be indistinguishable from oxidized NANA glycoside. The combined use of neuraminidase and periodate, which resulted in no effective in-

hibition of the B virus, demonstrated that this virus is insensitive to an oxidized *O*-acetylated NANA. The only remaining possibility is acetylation at C4. On this basis, the essential prosthetic group on the A2 inhibitor is a 4-*O*-*N*-diacetylneuraminic acid. This would agree with the work of Blix and Lindberg (3) and Martensson et al. (12), which showed that the predominate sialic acid present in both horse serum and submaxillary mucins is the 4-*O*-acetyl NANA.

In the case of whole human serum, only NANA is responsible for the inhibition of both viruses. Thus neuraminidase is completely effective in removal of all activity. As would be expected, sodium metaperiodate oxidizes NANA to a molecule which shows a high affinity for the type B virus while destroying all the anti-A2 activity.

The identification of the biologically active sialic acids is interesting since if they are to act as effective competitive inhibitors in the virus-red cell interaction, they must be complementary to the virus surface and reflect its basic configuration. Evidence supporting this hypothesis is that the appearance of the periodate enhancement effect, not seen in influenza B viruses isolated before 1965, is associated with a known antigenic change (13).

In addition to being complementary to the virus surface, the structure of the inhibitor should be analogous to the structure of the cell surface. An interesting question raised by changes in the inhibitor spectrum of a virus is the continued affinity of the virus for the red-cell receptor, which for any particular species remains essentially the same. Kathan and Winzler (9) have characterized the receptor substance previously isolated from human red-cell stroma (10). The sialic acid present is probably NANA, all of which can be removed by neuraminidase. It may be possible that small amounts of other substituted neuraminic acid groups are present on human and chick red blood cells. However, more likely, the specificities of the receptor groups are not absolute; for instance, the two inhibitor components in horse serum clearly cross-react to some degree between the two strains. It may be that slight changes in the virus surface occur which merely modify the binding efficiency of the virus with any given receptor without necessarily destroying it completely. Conceivably, such modification may have profound effects on the general biological effectiveness of the virus.

In a recent article, Gottschalk (6) discussed the criteria that glycoproteins must fulfill to be effective inhibitors of influenza hemagglutination. Work with trypsinized ovine submaxillary gland glycoprotein and the soluble receptor from human red cells has led to the conclusion that the effectiveness of an inhibitory substance is a function of molecular size, the number of terminal sialic acids, and of the three-dimensional structure complementary to an area on the virus surface. It appears, from the work reported here, that a fourth criterion must be considered: the structure of the neuraminic acid.

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