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Postreassortment Changes in Influenza A Virus Hemagglutinin Restoring HA–NA Functional Match

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An important function of influenza virus neuraminidase (NA) is the removal of sialic acid residues from virion components in order to prevent the aggregation of virus particles. In previous communications we have reported that reassortant viruses containing the NA gene of A/USSR/90/77 (H1N1) virus and HA genes of H3, H4, H10, or H13 subtypes had a tendency to virion aggregation at 4°C and that the virion clusters irreversibly dissociated after the treatment with bacterial neuraminidase. It was concluded that in such reassortants the removal of sialic acid residues is inefficient. Nonaggregating variants of the reassortants were selected in the course of serial passages in embryonated chicken eggs. In the present paper a reassortant virus, R2, having the HA gene of A/Duck/Ukraine/1/63 (H3N8) virus and the other genes of A/USSR/90/77 (H1N1) virus, as well as its non-aggregating passage variants and both parent viruses, have been studied in order to reveal the presence of unremoved sialic acid residues in the virions. An assay of sialic acid content by high-performance liquid chromatography with fluorescent detection has revealed the presence of sialic acid in the purified virus preparations of A/USSR/90/77 (H1N1) virus and the R2 reassortant and its nonaggregating variants, whereas only trace amounts of sialic acid have been detected in the A/Duck/Ukraine/1/63 (H3N8) parent virus. The data obtained with the use of the labeled "indicator" virus suggest that the unremoved sialic acid residues are present at the virion surface. The nonaggregating variants have been shown to possess a lower affinity toward high-molecular-weight sialic acid-containing substrates compared to the initial reassortant R2. Sequencing of HA genes has revealed amino acid changes in the nonaggregating variants compared to the initial reassortant. One substitution, N248D in HA1, is the same in two independently selected nonaggregating variants. The presented data suggest that the complete removal of sialic acid residues by viral NA from the virion components is not obligatory for the absence of virus particle aggregation: the latter may be achieved (in the reassortants and, presumably, in the wild-type virus) through a balance between the degree of HA affinity toward the sialic acid-containing receptors and the extent of the removal of sialic acid residues by NA.

INTRODUCTION

The functions of influenza virus glycoproteins, hemagglutinin (HA), and neuraminidase (NA) are interrelated. HA is the attachment protein involved in the binding of influenza virions to the cells via terminal sialic acid residues in the oligosaccharides exposed on cell-surface glycoproteins or glycolipids, whereas NA cleaves off the sialic acid residues in both cellular and viral components. It was shown that the impairment of NA function by ts mutation (Palese *et al.,* 1974), deletion (Liu and Air, 1993), or inhibition (Palese and Compans, 1976; Gubareva *et al.,* 1996) leads to the attachment of the virions to each other and to the cell surface with the formation of large clusters. It was concluded that the functional role of NA is to remove the sialic receptors from the viral components, thus preventing the formation of the aggregates and adherence of the virion clusters to the cell surface.

In our previous studies (Rudneva *et al.,* 1993, 1996) we observed that influenza A virus reassortants having NA gene of A/USSR/90/77 (H1N1) strain and HA genes originating from the viruses of H3, H4, H10, or H13 subtypes have a strong tendency to virion aggregation at 4°C. The treatment with a bacterial neuraminidase resulted in an irreversible dissociation of the virion clusters. The data suggested that the removal of sialic acid residues from the virion surface was incomplete in such reassortants. It was concluded that NA of A/USSR/90/77 virus strain is not completely functional when combined with an "alien" HA in a reassortant.

More detailed studies to be presented in this communication suggest that, more likely, NA of A/USSR/90/77 strain is intrinsically unable to remove efficiently sialic acid residues from the virion surface, both in the reassortants and in the parent virus. Moreover, this feature of NA may be functionally compensated by the mutations in

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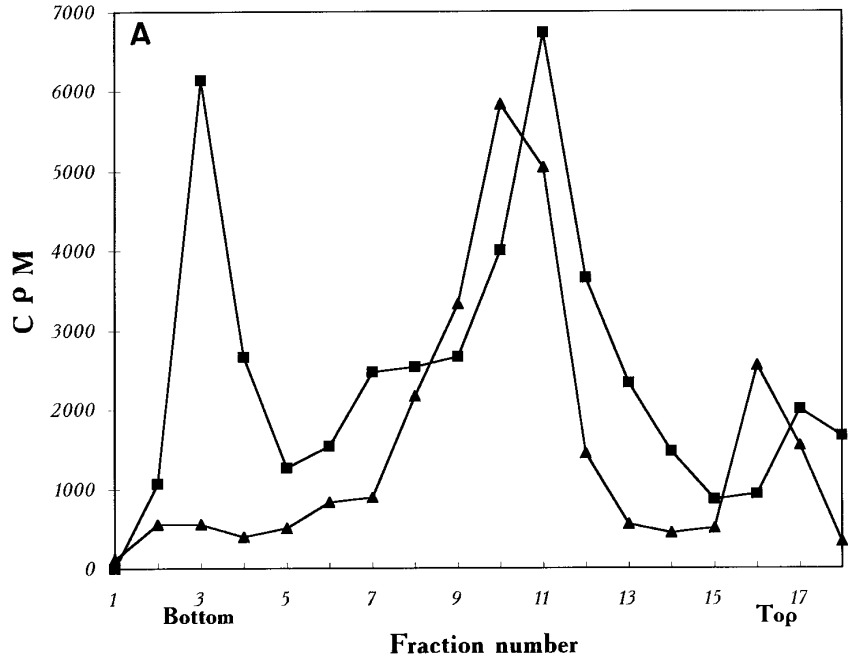

FIG. 1. Formation of mixed virion clusters as a test for the presence of sialic acid residues on the virion surface. Virus preparations were heated at 37°C for 30 min, mixed with [^{3}H]-uridine-labeled purified "indicator" influenza A/FPV/Rostock virus, and incubated overnight at 4°C. Each sample was layered on top of 15–30% sucrose gradients with a 2-ml 60% sucrose cushion at the bottom and centrifuged at 23,000 rpm for 60 min in a SW27-1 rotor. Fraction volume, 1 ml. (A) (■) A/USSR/90/77, (▲) A/Duck Ukraine/1/63; (B) (○) R2, (■) R2-XXI, (●) R2 treated with *Cl. perfingens* neuraminidase (0.15 U/ml, 30 min, 37°C).

the HA gene associated with a decrease in the affinity of HA to high-molecular sialic acid-containing substrates.

RESULTS

Formation of mixed virion aggregates

In our previous studies we observed that reassortant viruses containing the NA gene of A/USSR/90/77 (H1N1) virus and the HA gene of H3, H4, H10, or H13 subtypes form virion aggregates at 4°C (Rudneva *et al.,* 1993). The virion clusters partially dissociated when incubated at 37°C and reaggregated again at 4°C. The treatment with a bacterial neuraminidase resulted in an irreversible dissociation of the aggregates. In further studies (Rudneva *et al.,* 1996) a reassortant virus, R2, containing the HA gene of A/Duck/Ukraine/1/63 virus and all the other genes of A/USSR/90/77 virus was used for a selection of a nonaggregating variant, R2-XXI.

In an attempt to assess the aggregation in a more precise way, we tried to use the purified [^{3}H]uridine-labeled influenza A/FPV/Rostock/34 virus as an "indicator" virus. The virus preparations were incubated at 37°C for 30 min, an equivalent amount (as measured by HA activity determination) of ^{3}H-labeled virus was added, and the mixtures were held at 4°C overnight to allow the formation of mixed virion aggregates. Each sample was layered on the top of a sucrose gradient and analyzed by velocity sedimentation. The results (Fig. 1) reveal that the labeled virus is detected in clusters (at the upper surface of a 60% sucrose cushion or in the lower part of a 15–30% sucrose gradient) not only in the samples containing the aggregate-forming reassortant R2, but also in the samples containing the nonaggregating passage variant R2-XXI (Fig. 1B) or the parent virus A/USSR/90/77 (Fig. 1A). On the other hand, the labeled "indicator" virus is present in the position of the peak of single virions (fractions 10 and 11) in the sample containing A/Duck/Ukraine/1/63 virus (Fig. 1A). The treatment of R2 virus with *Cl. perfringens* neuraminidase (0.25 U/ml, 30 min at 37°C) results in the abolition of the formation of clusters containing the ^{3}H-labeled virus (Fig. 1B). The results suggest that both the nonaggregating passage variant R2-XXI and the parent virus A/USSR/90/77 contain sialic acid residues on the virion surface, although in our earlier studies (Rudneva *et al.,* 1996) both viruses were shown not to form virion clusters when incubated at 4°C per se (that is, without the addition of any alien virus, such as the "indicator" virus in the present studies).

Sialic acid content in virus proteins

Highly purified virus preparations essentially free from contaminating cellular proteins have been used for the measurement of sialic acid content by means of a liquid chromatography technique with fluorescence detection (Anumula, 1995). The results reveal that the content of sialic acid in the preparations of A/Duck/Ukraine/1/63

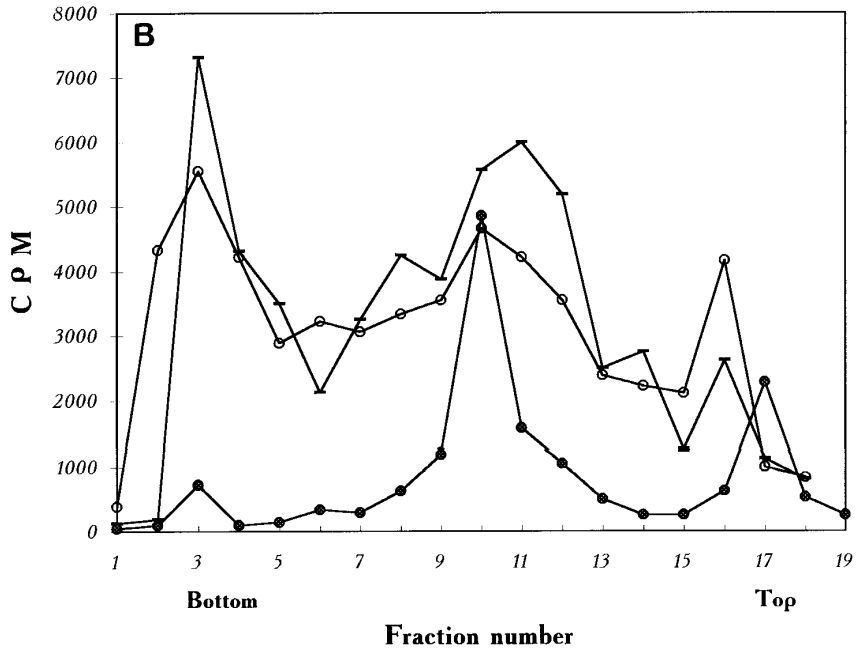

FIG. 1—*Continued*

parent virus is low, whereas in the viruses containing N1 neuraminidase (A/USSR/90/77, the initial reassortant R2, both nonaggregating passage variants) it is several times higher (Table 1). The difference between R2 and the passage variants R-XXI and R2-XIIIa is not statistically significant.

Affinity of the reassortants to natural and synthetic sialic acid-containing substrates

The passage variants were shown earlier to have lost the tendency to virion aggregation at 4°C (Rudneva *et al.,* 1996), whereas both the reaction with "indicator" virus (Fig. 1) and the direct measurement of sialic acid content (Table 1) revealed the presence of unremoved sialic acid residues in the virions. A plausible explanation for the loss of aggregation may be a decrease in the affinity of HA to sialic receptors. In order to verify this suggestion a quantitative assay of the virus binding to sialic acid-containing substrates has been performed. In a direct binding test with horseradish peroxidase-conjugated fetuin a drastic decrease in the binding of the passage variants compared to the initial R2 reassortant was registered (Table 2). In the competitive binding assay no difference between the initial reassortant and the passage variants was observed in the affinity to low-molecular-weight substrates (sialic acid and 3′-sialyllactose), whereas with respect to a high-molecular-weight substance, 3′-sialyllactose attached to a polyacrylic acid carrier (Gambaryan *et al.,* 1997), a decrease in the affinity of the passage variants compared to R2 is as evident as in the direct fetuin binding assay (Table 2).

TABLE 1

The Content of Sialic Acid in Purified Virus Protein

Virus	Subtype	Sialic acid content (pM per 1 μg of virus protein)[a]
A/Duck/Ukraine/1/63	H3N8	0.42 ± 0.12
A/USSR/90/77	H1N1	3.52 ± 0.28
R2	H3N1	3.55 ± 0.40
R2-XXI	H3N1	2.65 ± 0.39
R2-XIIIa	H3N1	2.43 ± 0.25

[a] Mean of four determinations ± standard error. A separate virus preparation was used for each determination.

Amino acid changes associated with the loss of the virion aggregation

The results presented above suggested that the changes leading to the loss of the virion aggregation feature in the passage variants occurred in HA rather than in NA: first, the incomplete removal of sialic acid residues seemed to be unaltered or only slightly affected in the nonaggregating variants; second, the binding to sialic acid-containing substrates decreased in the passaged variant. Therefore, HA genes of the reassortant R2 and its nonaggregating variants were sequenced. Since the sequencing of the HA gene of R2 revealed several differences compared to the published sequence of A/Duck/Ukraine/1/63 virus HA (Fang *et al.,* 1981), we have also sequenced the HA gene of the parent A/Duck/Ukraine/1/63 virus used in these studies.

The results (Table 3) revealed that the strain we used has two amino acid changes compared to the published

TABLE 2

Changes in the Affinity of HA toward Sialic Acid-Containing Substrates Associated with the Loss of Virion Aggregation

Virus	Substrate			
	Fetuin–HRP conjugate	3′-Sialyllactose attached to a polyacrylic acid carrier	α-*N*-Acetyl neuraminic acid	3′-Sialyllactose
R2	0.16 ± 0.02[a]	0.97 ± 0.14	312 ± 43	463 ± 60
R2-XXI	1.17 ± 0.12	19.2 ± 2.2	420 ± 51	770 ± 98
R2-XIIIa	0.95 ± 0.08	14.6 ± 3.0	513 ± 33	460 ± 92

[a] K_{aff} (expressed in μM sialic acid) determined for fetuin conjugate by direct solid-phase enzyme-linked assay (Gambaryan and Matrosovich, 1992) and for SA-polyacrylate, Neu5Ac, and 3′-SL by competition assay (Gambaryan *et al.,* 1997). The data represent means ± standard error. For each substrate four preparations of each virus were assayed.

sequence (S91T and G146D). There are also two silent nucleotide changes: C versus T in position 827 and A versus C in position 929. The former is important, since it changes AAC triplet (coding for Asn-250) into AAT, and this is a prerequisite for N250S substitution in a nonaggregating variant (AAT versus AGT). The HA sequence of R2 is identical to the sequence of the A/Duck/Ukraine/1/63 strain used as the parent virus in our experiments.

The sequencing of the HA genes of R2-XXI and R2-XIIIa variants revealed several amino acid changes compared to R2: four substitutions in R2-XXI and two substitutions in R2-XIIIa. Only one substitution (N248D) is the same in both passage variants (Table 3).

DISCUSSION

Influenza virus neuraminidase has one well-defined function as an enzyme, but physiologically it is multifunctional. Although it seems to have no obvious role in viral entry, replication, assembly, and budding (Liu *et al.,* 1995), it was reported to influence the proteolytic activation of HA (Schulman and Palese, 1977) and to be necessary for the acquisition of receptor binding activity by HA of some virus strains (Ohuchi *et al.,* 1995). However, the most obvious and well-documented function of NA is to prevent the binding of the virions to each other and to the surface of the virus-producing cell. The function is performed by cleaving off the terminal sialic acid residues in oligosaccharides, thus removing the HA binding receptors from cell and virion surfaces. Among the first data indicating that the role of NA is to remove sialic acid residues from the virion components were the results demonstrating the absence of sialic acid in the virions (Klenk *et al.,* 1970). Since then a vast amount of data has accumulated demonstrating that if the function of NA is impaired or blocked with an inhibitor, ts mutation, or deletion (Palese and Schulman, 1976; Palese *et al.,* 1974; Gubareva *et al.,* 1996; Liu and Air, 1993), virus particles form clusters and adher to the cell surface. Virus particle aggregation was also observed when influenza virions were artificially sialylated with sialyltransferase (Lakshmi and Schulze, 1978). On this basis it is generally accepted that in wild-type viruses under normal conditions sialic acid residues are removed by viral NA from the virus components; if the removal is blocked, the virions aggregate, and the clusters adhere to the cell surface.

The data presented in this paper suggest that this is not so or, at least, that this rule has exceptions. Both the data of direct assessment of sialic acid content in purified virus preparations (Table 1) and the data on the interaction of virions with "indicator" virus (Fig. 1) suggest that the viruses containing the NA gene of the A/USSR/90/77 (H1N1) virus possess unremoved sialic acid residues on the virion surface, irrespective of whether they have the intrinsic property of virion aggregation (without

TABLE 3

Amino Acid Changes in H3 Hemagglutinin Associated with the Loss of Virion Aggregation

Virus	HA1						HA2
	91	145	146	155	248	250	111
A/Duck/Ukraine/1/63[a]	S	N	G	T	N	N	A
A/Duck/Ukraine/1/63	T	N	D	T	N	N	A
R2	T	N	D	T	N	N	A
R2-XXI	T	N	D	S	D	S	T
R2-XIIIa	T	D	D	T	D	N	A

[a] Published sequence (Fang *et al.,* 1981).

the addition of "indicator" virus). The loss of the aggregation in the passage variants correlates with a decrease in their affinity toward high-molecular-weight sialic acid-containing substrates. Altogether the data presented may be regarded as a possible indication that the absence of virion aggregation is not necessarily the result of a complete removal of sialic acid residues from the virion components; it may result from a balance between the extent of sialic acid removal by NA and the binding capacity of HA toward sialic receptors.

Mutations in the HA gene selected under conditions of impairment of NA function by a specific inhibitor were recently described (McKimm-Breschkin *et al.,* 1996; Gubareva *et al.,* 1996). The mutations were situated in the receptor binding pocket of HA, or close to it (McKimm-Breschkin *et al.,* 1996), or in HA2 (Gubareva *et al.,* 1996) in the vicinity of the second binding site identified by crystallographic analysis (Sauter *et al.,* 1992). In our studies the sequencing of HA genes of the R2 reassortant and its nonaggregating passage variants (Table 3) revealed several amino acid changes acquired in the course of the selection of the variants, four in R2-XXI and two in R2-XIIIa. One substitution, N248D, is identical in both passage variants. This position is situated at the top of the globule close to the edge of the receptor binding pocket (Wilson *et al.,* 1981). All reported avian H3 sequences have Asn in this position, and some human H3 strains have Thr (Bean *et al.,* 1992). To our knowledge, a change to Asp has never been reported. The other changes differ in R2-XXI and R2-XIIIa: among them position 145 is situated in close proximity to the receptor binding pocket, and 155 right inside the pocket. A change in position 155 was reported for a variant resistant to a neuraminidase inhibitor (McKimm-Breschkin *et al.,* 1996).

The decrease in the affinity of the nonaggregating variant to sialic acid-containing substrates (Table 2), although registered both in direct assay with a natural substrate and in a competitive assay with a synthetic substrate, was not observed with the low-molecular-weight competitors *N*-acetylneuraminic acid and 3′-sialyllactose. A similar situation has been observed for changes associated with egg adaptation of influenza virus isolates (Gambaryan *et al.,* 1997). The effect may be attributed to a weakening of electrostatic interactions with a negatively charged high-molecular-weight substrate in the passage variant due to a negative shift produced by the N248D mutation.

The data on the direct measurement of sialic acid content in the purified virus preparations should be evaluated with some caution, since the determination of small amounts of any substance in a purified virus preparation is always open to criticism regarding the possible presence of contaminating cellular proteins, even though they were not detected by the analysis of polypeptides in PAGE. However, in conjunction with the other phenotypic characteristics of the reassortant viruses and their variants presented in these studies and previous communications (Rudneva *et al.,* 1993, 1996), it seems likely that at least a fraction of the sialic acid residues registered in the purified virus preparations is present on the virion surface. If one assumes that the sialic acid residues revealed by the direct assay of purified virus preparations are really present in the virion components, one may attempt to evaluate the number of sialic acid residues per virion. If the molecular mass of one virion is 2×10^8 daltons (Choppin and Compans, 1975), the number of sialic acid residues per virion can be estimated as 75 for A/Duck/Ukraine/1/63 (H3N8) virus and as 500 to 700 for A/USSR/90/77 (H1N1) virus and all the reassortant variants. Our data provide no estimate for the number of sialic acid residues exposed at the virion surface and accessible to HA of other virus particles.

The selection of the high-yield nonaggregating variants in our experiments with different N1-containing reassortants was achieved after 3 to 4 passages for H3N1 reassortant R2 used in the experiments presented in this paper, 4 to 7 passages for a H4N1 reassortant, and 19 passages for a H13N1 reassortant (Rudneva *et al.,* 1996). It seems that the establishment of a HA–NA functional match in a reassortant was achieved for some HA subtypes (or for HA of some strains) more easily than for the others. However, eventually it was achieved in all the strains tested. The absence of aggregation in the parent A/USSR/90/77 virus may be regarded as a result of an adjustment of its HA to the incomplete NA function in the course of virus circulation; however, some sublines of A/USSR/90/77 virus exhibit a tendency to virion aggregation (E. Nobusawa, personal communication). The aggregation in the reassortants, most likely, is a result of the substitution of H1 HA with an "alien" HA, more avid toward the unremoved sialic acid residues, in much the same way as it occurs in the course of the interaction with the "indicator" virus.

Although the production of the variants with mutations in HA compensating for the inhibited NA had been described (McKimm-Breschkin *et al.,* 1996; Gubareva *et al.,* 1996), our data are the first, to our knowledge, in which such mutations are shown to overcome the intrinsic inefficiency of NA of a natural isolate (that is, A/USSR/90/77 strain). This observation may be of importance with respect to the problem of HA–NA gene interactions under natural conditions. The results may be regarded as an indication that the adjustment of HA to the peculiarities of the function of a particular NA after reassortment, and the differences in the capability of HA genes to achieve the functional match, may be important as a factor limiting or favoring the appearance of specific HA–NA combinations in the circulating influenza A virus strains.

MATERIALS AND METHODS

Viruses

Influenza viruses A/Duck/Ukraine/1/63 (H3N8), A/USSR/90/77 (H1N1), and A/FPV/Rostock/34 (H7N1) were obtained from the virus repository of the D. I. Ivanovsky Institute of Virology, Moscow. The viruses A/Duck/Ukraine/1/63 and A/USSR/90/77 were the same as used in our previous studies for the production of reassortants (Rudneva *et al.,* 1993). The other viruses used in these studies were: reassortant virus R2 containing the HA gene of A/Duck/Ukraine/1/63 virus and all the other genes of the A/USSR/90/77 virus (Rudneva *et al.,* 1993), and its high-yield variants R2-XXI (Rudneva *et al.,* 1996) and R2-XIIIa, obtained, respectively, by 21 and 13 serial passages in chick embryos. All the viruses were propagated in 10-day-old embryonated chicken eggs. The allantoic fluid was collected and stored at 4°C.

Virus purification

The virus-containing allantoic fluids were clarified by low-speed centrifugation, layered on top of 15–60% discontinuous sucrose gradients prepared in STE buffer (0.1 M NaCl, 0.01 M Tris–HCl, pH 7.4, 0.001 M EDTA), and centrifuged in SW27-1 rotor at 23000 rpm for 1 h at 4°C. The virus was collected from the 20–60% sucrose interphase, diluted in STE buffer, and pelleted under the same conditions of centrifugation. The pellets were resuspended in an appropriate buffer: STE buffer for RNA extraction, ST buffer (0.15 M NaCl, 0.01 M Tris–HCl, pH 7.4) for the assay of virus binding to sialic acid-containing substrates and for the studies on virion clustering, or in STM buffer (0.15 M NaCl, 0.01 Tris–HCl, pH 7.4, 0.0015 M $MgCl_2$) for the assay of sialic acid content. The virus suspension was clarified by low-speed centrifugation, and aliquots were taken for the measurement of protein content and for the analysis of proteins in polyacrylamide gel electrophoresis.

Measurement of sialic acid content

Purified virus preparations were treated with 0.5% v/v Triton X-100 for 30 min at 16°C and transferred to glass tubes. Five volumes of acetone was added, the preparations were held overnight at −20°C, and the precipitates were pelleted by centrifugation for 20 min at 11,000 rpm in a Sorvall RC2B centrifuge. The pellets were used for the quantitative determination of sialic acid content by high-performance liquid chromatography with fluorescence detection (Anumula, 1995). Briefly, the pellets were dissolved in 0.5 M $NaHSO_4$ at 80°C for mild acid hydrolysis of glycoproteins and derivatized with orthophenylenediamine (OPD). The sialic acid derivatives were separated on a C-18 reversed-phase Ultrasphere-ODS (0.22 × 25 cm, Beckman) column and eluted isocritically using 6.8% 1-propanol, 3.5% acetonitrile, 0.01% trifluoroacetic acid. All the separations were carried out at room temperature using a flow rate of 0.2 ml/min. The column and injector were washed with 90% acetonitrile for 5–10 min and equilibrated for 10–15 min under the initial conditions to ensure reproducibility from run to run. The OPD derivatives of sialic acids were detected with a fluorescence detector (Model 121Gilson). *N*-Propionylneuraminic acid was used as a standard.

Assay of virus binding to sialic acid-containing substrates

The binding of the viruses to fetuin was measured in a direct solid-phase assay with the immobilized virus and horseradish peroxidase-conjugated fetuin (Gambaryan and Matrosovich, 1992). The affinity toward sialic acid, sialyllactose, and 3′-sialylglycopolymer obtained by conjugation of a 1-*N*-glycyl derivative of 3′-sialyllactose with poly(4-nitrophenylacrylate) (Gambaryan *et al.,* 1997) was performed in a competitive assay based on the inhibition of binding of the peroxidase-labeled fetuin (Matrosovich *et al.,* 1993). The following modifications were introduced: the concentration of neuraminidase inhibitor Neu5Ac2en in the reaction mixture was increased to 0.1 mM, and the virus preparations before the immobilization were treated with *Cl. perfringens* neuraminidase (0.1 U/ml, 1 h at 37°C).

Preparation of [^{3}H]uridine-labeled virus

Primary chicken embryo cell cultures were grown in 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution, infected with influenza A/FPV/Rostock/34 virus at m.o.i. 20 to 40 PFU/cell, overlayed with Eagle's MEM containing 0.2% bovine plasma albumin, and incubated at 37°C. [^{3}H]Uridine at a final concentration 500 μCi/ml was added 1.5 h postinfection. The virus-containing culture fluid was collected 16 h postinfection, and the virus purification was performed as described above for the allantoic virus.

Velocity sedimentation analysis

To measure the extent of virion aggregation the virus preparations mixed with the ^{3}H-labeled "indicator" virus were layered on top of linear 15–30% sucrose gradients 16 ml in volume with a 2-ml 60% sucrose cushion at the bottom of the tube and centrifuged at 23,000 rpm in SW27-1 rotor for 60 min at 4°C. The gradient fractions were precipitated with 5% trichloroacetic acid, washed on nitrocellulose filters, and counted in a scintillation counter.

Sequencing of HA genes

The RT–PCR procedure was used to amplify the HA gene. All PCR products were cloned in JM109 cells using the Promega pGEMr-T vector system (Promega Protocol)

and sequenced by the dideoxy chain-termination technique using a set of oligonucleotide primers complementary to several regions of the A/Duck/Ukraine/1/63 HA gene. The primers were kindly provided by Dr. R. G. Webster (St.-Jude Children's Research Hospital, Memphis, Tennessee).

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