

## Regulation of Receptor Binding Affinity of Influenza Virus Hemagglutinin by Its Carbohydrate Moiety

MASANOBU OHUCHI,<sup>†</sup> REIKO OHUCHI,<sup>‡</sup> ANKE FELDMANN, AND HANS-DIETER KLENK\*

*Institut für Virologie, Philipps-Universität Marburg, 35011 Marburg, Germany*

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**The hemagglutinin (HA) of the fowl plague virus (FPV) strain of influenza A virus has two N-linked oligosaccharides attached to Asn123 and Asn149 in the vicinity of the receptor binding site. The effect of these carbohydrate side chains on the binding of HA to neuraminic acid-containing receptors has been analyzed. When the oligosaccharides were deleted by site-specific mutagenesis, HA expressed from a simian virus 40 vector showed enhanced hemadsorbing activity. Binding was so strong under these conditions that erythrocytes were no longer released by viral neuraminidase and that release was significantly reduced when neuraminidase from *Vibrio cholerae* was used. Similarly, when these oligosaccharides were removed selectively from purified viruses by N-glycosidase F, such virions were unable to elute from receptors, although they retained neuraminidase activity. Thus, release of FPV from cell receptors depends on the presence of the HA glycans at Asn123 and Asn149. On the other hand, receptor binding was abolished when these oligosaccharides were sialylated after expression in the absence of neuraminidase (M. Ohuchi, A. Feldmann, R. Ohuchi, and H.-D. Klenk, *Virology* 212:77–83, 1995). These observations indicate that the receptor affinity of FPV HA is controlled by oligosaccharides adjacent to the receptor binding site.**

The carbohydrate moiety of glycoproteins has been shown to have a large variety of different functions, such as protection against proteolysis, stabilization of protein conformation, receptor binding of microorganisms, clearance of proteins from the circulatory system, targeting in intra- and intercellular traffic, and cell-cell recognition (for a review, see reference 28). Besides these signalling and stabilizing functions, modulation of protein activities has also been assigned to oligosaccharides. However, direct evidence for the latter effects has been obtained in only a few instances.

Important contributions to our understanding of the biological significance of glycoprotein glycosylation came from studies of the influenza virus hemagglutinin (HA). HA is a homotrimeric transmembrane protein with an ectodomain composed of a globular head and a stem region (31). Both regions carry N-linked oligosaccharides (10). The oligosaccharides attached to the head region show considerable variations in structure and number among the different influenza A viruses, whereas the stem oligosaccharides are quite conserved. Biosynthesis of HA involves transport through the exocytotic assembly route. HA is the major antigen at the virus surface, and it is the mediator of virus entry. After binding of HA to neuraminic acid, which is the receptor determinant at the cell surface, the virus is internalized through receptor-mediated endocytosis. HA then induces fusion of the viral envelope with the endosomal membrane, thus allowing delivery of the nucleocapsid into the cytoplasm. To show fusion activity, HA has to undergo a biphasic activation process involving proteolytic cleavage by host proteases (11) followed by a conformational change at low pH, resulting in the exposure of a hydrophobic domain on the cleavage fragment HA<sub>2</sub> that presumably inter-

acts with the lipid bilayer of the target membrane (3). Most of the functional properties of HA have been shown to be affected by glycosylation at specific sites. Thus, glycosylation at antigenic epitopes interferes with the access of antibodies (13, 26) and may therefore contribute to the antigenic drift of influenza virus (25). Oligosaccharides near the cleavage site modulate proteolytic activation and, therefore, spread of infection and pathogenicity (5, 14). Oligosaccharides in the stem region maintain HA in the metastable form required for fusion activity (18).

In this study, we focused on the biological role of oligosaccharides adjacent to the receptor binding site. We have previously reported that, in addition to its well-established role as a promoter of virus release from the infected cell, the viral neuraminidase (NA) has another function in virus multiplication. Depending on the virus strain, the NA was found to be necessary for the receptor binding activity of HA (16). This concept was derived from studies in which HA of the fowl plague virus (FPV) strain, when expressed from a simian virus 40 (SV40) vector, was found to be unable to induce hemadsorption, although it was exposed at the cell surface. However, treatment of HA-expressing cells with bacterial NA resulted in extensive hemadsorption. Mutant HA which had lost the complex-type oligosaccharides in the vicinity of the receptor binding site did not require NA treatment to show hemadsorption. NA treatment also enhanced hemadsorption of vector-expressed HA of the WSN strain, which has a complex-type oligosaccharide in the vicinity of the receptor binding site, but had no effect on hemadsorption of Hong Kong-type HA, which has a high-mannose-type oligosaccharide adjacent to the receptor binding site. These results indicated that sialic acid on oligosaccharides near the receptor binding site interferes with hemadsorption. We show now that deletion of the oligosaccharides at the binding site enhances hemadsorbing activity and that binding of erythrocytes is so strong under these conditions that they can no longer be released by NA. These results demonstrate that the receptor affinity of the FPV HA is attenuated by adjacent oligosaccharides in a way that allows both binding to and elution from the receptor.

\* Corresponding author. Mailing address: Institut für Virologie, Philipps-Universität Marburg, Postfach 2360, 35011 Marburg, Germany. Phone: (6421) 28 6253. Fax: (6421) 28 8962.

<sup>†</sup> Present address: Department of Microbiology, Kawasaki Medical School, 577 Matsushima, Kurashiki 701-1, Japan.

<sup>‡</sup> Present address: Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu, Tokyo 183, Japan.

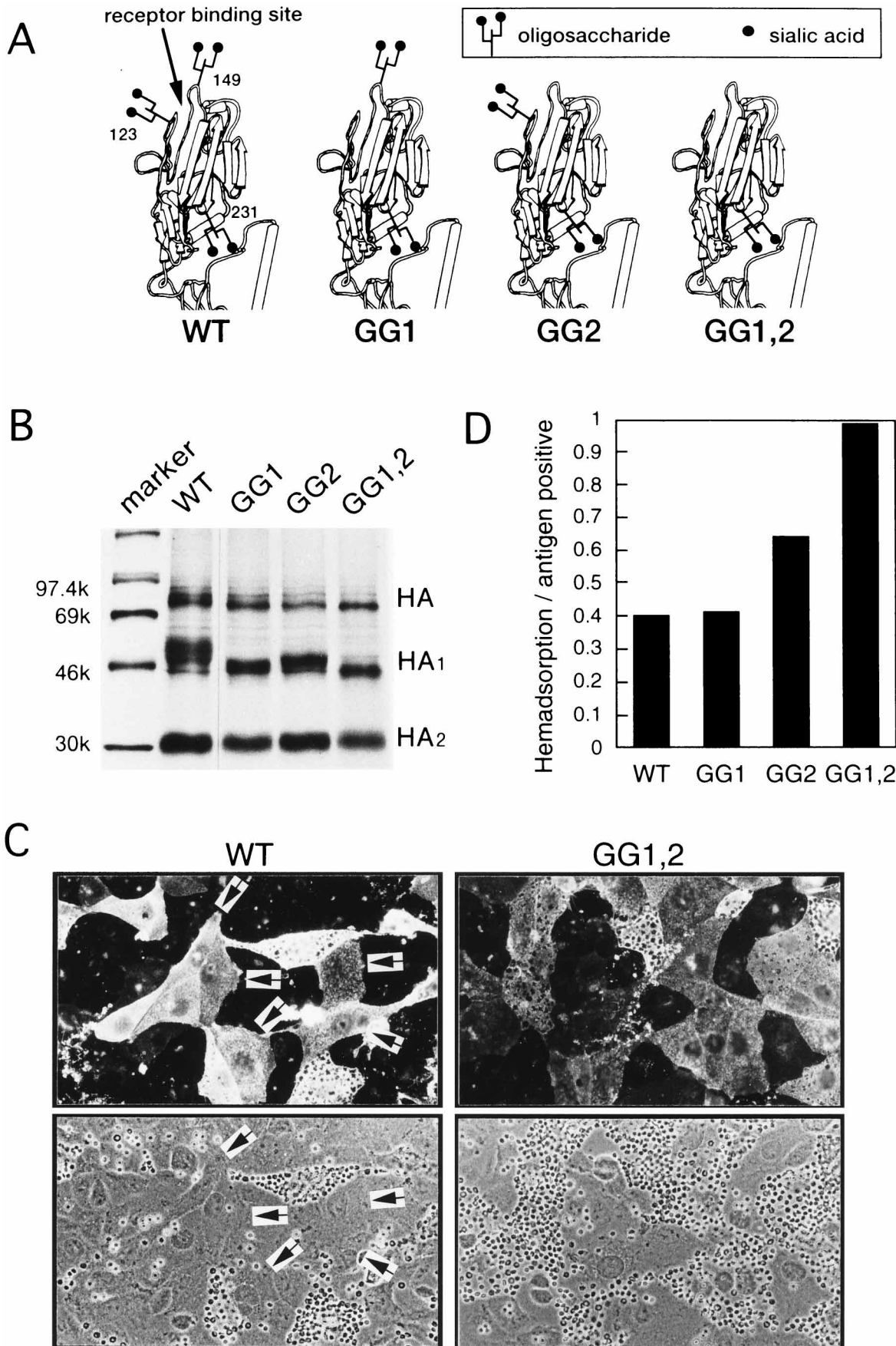


FIG. 1. Effect of oligosaccharide deletion from the vicinity of the receptor binding site on hemadsorption activity. (A) Positions of oligosaccharides in the head region of FPV HA. Mutants GG1 and GG2 lack the glycosylation sites at Asn123 and Asn149, respectively. Both sites are deleted in GG1,2. (B) Two days after infection with HA-SV40 recombinants, CV-1 cells were labeled with [<sup>35</sup>S]methionine for 3 h, and HA was examined by PAGE after immunoprecipitation. Molecular weights (in thousands) are shown on the left. (C) After pretreatment with VCNA, HA-expressing cells were subjected to hemadsorption with freshly prepared guinea pig erythrocytes. Cell monolayers and adsorbed erythrocytes were fixed with 1% paraformaldehyde and then stained with anti-FPV rabbit serum, anti-rabbit IgG biotinylated donkey serum, and streptavidin-fluorescein. The same microscopic fields were analyzed by immunofluorescence to visualize HA antigen on the cell surface (upper panels) and by inspection in visible light to detect hemadsorption-positive cells (lower panels). Cells which were antigen positive but hemadsorption negative are indicated (arrows). (D) Ratios of hemadsorption-positive cells to antigen-positive cells. WT, wild type.

## MATERIALS AND METHODS

**Expression of cloned cDNAs of HA and NA.** cDNAs encoding wild-type and mutant HAs of influenza A/FPV/Rostock/34 (H7N1) virus were subcloned by using the *Bam*HI site in the SV40 expression vector pA11SVL3. The cDNA for NA of A/PR/8/33 (H1N1) was also cloned in the *Eco*RI site of the SV40 vector. Stocks of HA- and NA-SV40 recombinant virus were prepared after transfection of the vector DNA together with helper virus DNA SVdl1055 in CV-1 cells as described previously (17). CV-1 cells were cultivated in Dulbecco's minimum essential medium supplemented with 5% fetal calf serum.

**Deletion of oligosaccharides from FPV HA by site-specific mutagenesis.** N-glycosylation sites at amino acid positions 123 and 149 of FPV HA were abolished by exchanging Thr125 and Ser151 for Ala, respectively, as described previously (16).

**Analysis of expressed HA and NA proteins.** At 2 days postinfection (p.i.) with recombinant virus, HA- or NA-expressing CV-1 cells were labeled in a petri dish (5-cm diameter) for 3 h with 100  $\mu$ l of [<sup>35</sup>S]methionine in 1 ml of methionine-free medium, chased for 1 h, and then solubilized in radioimmunoprecipitation buffer. HA and NA were precipitated with anti-FPV or anti-PR/8 rabbit serum and protein A-Sepharose CL-4B (Sigma, St. Louis, Mo.) and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels.

**Immunofluorescent staining of HA-expressing cells.** CV-1 cells were seeded on a Nunc LAB-TEK chamber slide and infected with HA-SV40 recombinant virus. At 2 days p.i., the hemadsorption test was done as described below. After the cell cultures were washed to remove free erythrocytes, the cells were fixed with 1% paraformaldehyde and stained by indirect methods with anti-FPV hyperimmune rabbit serum, anti-rabbit immunoglobulin G (IgG) biotinylated donkey serum, and streptavidin-fluorescein (Amersham Co., Amersham, United Kingdom).

**Hemadsorption test.** Cells infected with HA-SV40 recombinants were subjected to hemadsorption tests at 2 days p.i. In most cases, HA-expressing cells were treated before the hemadsorption test with *Vibrio cholerae* NA (VCNA) (Test Neuraminidase; Behringwerke AG, Marburg, Germany) at a final concentration of 25 mU/ml for 1 h at 37°C. The VCNA-treated cells were washed intensively with phosphate-buffered saline (PBS) containing calcium and magnesium ions, and then hemadsorption tests were done with a freshly prepared suspension of guinea pig erythrocytes at 4°C for 0.5 h. After three washes with PBS, hemadsorption-positive cells were counted under an inverted microscope at 100-fold magnification. Hemadsorption-positive cells were counted in 10 microscopic fields (area of each field, approximately 1.8 mm<sup>2</sup>).

**NGase digestion of vector-expressed HAs.** HA-expressing cells were labeled in the presence of VCNA (10 mU/ml) with [<sup>35</sup>S]methionine for 3 h as described above and then digested with N-glycosidase F (NGase) (10 U/250  $\mu$ l) for 2 h at 37°C. HA protein was recovered by immunoprecipitation and analyzed by PAGE. In the case of digestion of denatured HA, [<sup>35</sup>S]methionine-labeled undigested HA was recovered by immunoprecipitation and then denatured with 0.1% SDS and 1% 2-mercaptoethanol in 10  $\mu$ l of 10 mM phosphate buffer (pH 7.5) at 95°C for 3 min. After excess SDS was blocked with Triton X-100 at a final concentration of 1%, NGase digestion was done at a concentration of 0.5 U/12.5  $\mu$ l for 4 h at 37°C, and then HA was analyzed by PAGE.

**NGase digestion of purified FPV.** FPV was propagated in 11-day-old embryonated eggs for 2 days, and the propagated virus was recovered from allantoic fluid of the eggs. The virus was concentrated by ultracentrifugation, purified by sucrose density gradient centrifugation, and finally suspended in PBS. A 50- $\mu$ l suspension of purified virus (hemagglutinating titer,  $1.6 \times 10^4$ ) was digested with NGase (Boehringer GmbH, Mannheim, Germany) at a final concentration of 2 U/50  $\mu$ l for 2 h at 37°C. An aliquot of the digested virus was subjected to PAGE in a 10% acrylamide gel. After Western blotting, viral proteins were detected by use of anti-FPV rabbit antiserum, peroxidase-conjugated anti-rabbit IgG donkey serum, and an ECL protein detection kit (Amersham). Other aliquots of the NGase-digested virus suspension were used for hemagglutination and NA assays and for testing adsorption and release in Madin-Darby canine kidney (MDCK) cell cultures.

**Hemagglutination assay.** Virus suspensions were subjected to twofold serial dilutions in a 96-well standard microtiter plate. HA titrations were done with 1% chicken erythrocyte suspensions.

**NA assay.** A 10- $\mu$ l aliquot of virus suspension was mixed with 20  $\mu$ l of a fetuin solution (3 mg of fetuin/ml in PBS), and the mixture was incubated for 0.5 h at 37°C. Released sialic acid was assayed according to Warren's standard method, with some modifications. In brief, the reaction mixture was incubated with 25  $\mu$ l

of 25 mM sodium periodate for 0.5 h at 37°C. After excess periodate was blocked with 20  $\mu$ l of a 2% NaAsO<sub>2</sub> solution, 200  $\mu$ l of a 0.1 M 2-thiobarbituric acid solution was added. The specific pigment was extracted into 500  $\mu$ l of acid butanol, and the optical density at 549 nm was measured by photometry.

**Fixation of MDCK cells.** MDCK cells were grown in a plastic petri dish with a 5-cm diameter, and confluent monolayer cells were fixed with 1% paraformaldehyde for 1 h at 4°C. The fixed monolayers were washed intensively with PBS containing 0.5% bovine serum albumin and then kept at 4°C in PBS with bovine serum albumin until use.

**Assay of virus release from MDCK cell monolayers.** NGase-treated and non-NGase-treated virus suspensions were added to fixed MDCK cell monolayers, and the mixtures were incubated at 0°C for 1 h. After the cells were washed with chilled PBS to remove nonadsorbed virus, the MDCK cell monolayers were incubated at 37°C with 500  $\mu$ l of prewarmed PBS. Aliquots (50  $\mu$ l) of the incubation fluid were removed at appropriate intervals to determine released virus by hemagglutination assay.

## RESULTS

**HA mutants lacking oligosaccharides adjacent to the receptor binding site have increased hemadsorbing activity.** FPV HA has seven oligosaccharide side chains, three of which are located in the head region. The head oligosaccharides attached to Asn residues 123 and 149 are adjacent to the receptor

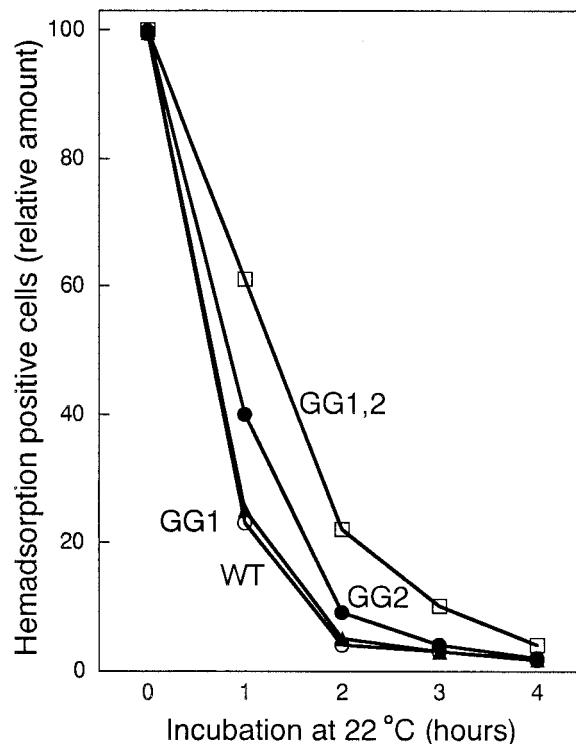


FIG. 2. Kinetics of erythrocyte release from HA-expressing cells by VCNA treatment. After hemadsorption, monolayers were incubated at 22°C with 2 mU of VCNA in 2 ml of PBS containing Ca and Mg ions. Hemadsorption-positive cells were counted after 1, 2, 3, and 4 h. The percentage of hemadsorption-positive cells after VCNA treatment was calculated relative to the number of hemadsorption-positive cells before VCNA treatment.



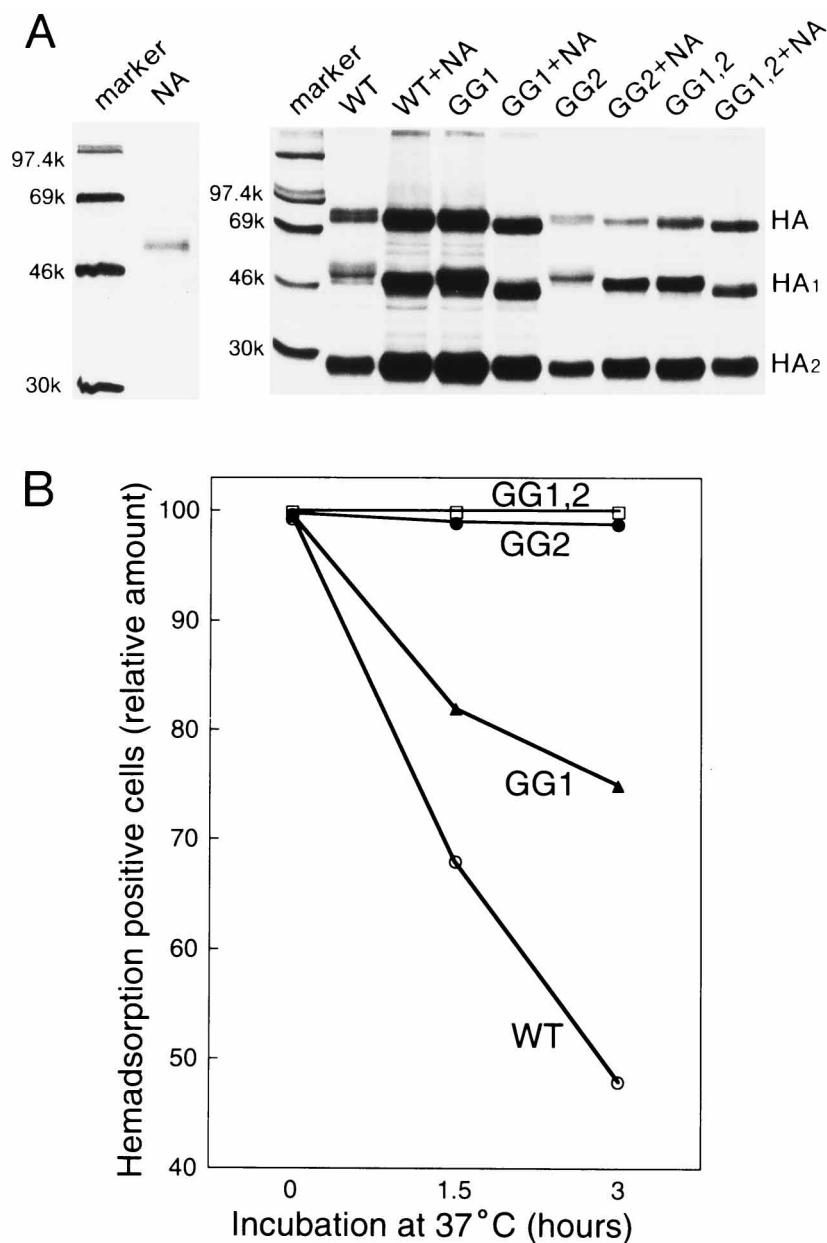


FIG. 3. Kinetics of erythrocyte release from HA-expressing cells by coexpressed NA. NA of influenza virus A/PR/8/33 (H1N1) was expressed alone (A) or coexpressed with HA (B). Although an NA band was not visible after expression with HA, NA expression is indicated by the increased electrophoretic mobility of HA and HA<sub>1</sub>, reflecting the removal of sialic acid. Release of erythrocytes from cells coexpressing HA and NA was examined as described in the legend to Fig. 2, but the incubation was done for 1.5 to 3 h at 37°C. WT, wild type. Molecular weights (in thousands) are indicated to the left of the gels.

binding site (Fig. 1A). To analyze the effects of these oligosaccharides on receptor binding, wild-type HA and mutants lacking the glycosylation sites at either Asn123 (GG1) or Asn149 (GG2) or at both positions (GG1,2) were expressed in CV-1 cells by an SV40 vector. Expression was done in the presence of 10 mM ammonium chloride to protect FPV HA from acid denaturation during exocytotic transport (15, 27). PAGE of radiolabeled HA revealed that the mutants were cleaved into the subunits HA<sub>1</sub> and HA<sub>2</sub> as well as the wild type, indicating that oligosaccharide deletion had no effect on intracellular transport and processing. Also, there were no significant differences in expression levels. The different migration rates of

the HA and HA<sub>1</sub> bands reflected the loss of side chains in the mutants (Fig. 1B).

After pretreatment of HA-expressing cells with VCNA at a concentration of 25 mU/ml at 37°C for 1 h (16), hemadsorption was analyzed with freshly prepared guinea pig erythrocyte suspensions at 4°C for 30 min. After the cells were washed with PBS, hemadsorbing cells were counted under a microscope. Specific hemadsorbing activity was determined by comparing in the same culture dish the number of hemadsorbing cells with the number of cells expressing HA antigen at their surface. This was done by fixing cells with 1% paraformaldehyde after hemadsorption and then subjecting them to immunofluores-

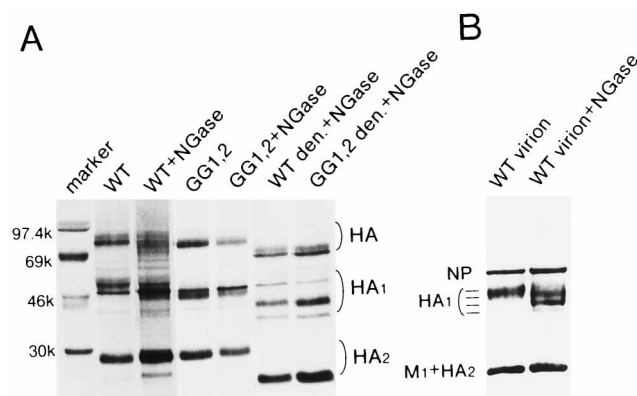


FIG. 4. Controlled removal of oligosaccharides from vector-expressed HA and virion HA by NGase. (A) Wild-type (WT) and GG1,2 HA-expressing cells were labeled with [<sup>35</sup>S]methionine for 3 h in the presence of VCNA (10 mU/ml) and then digested with NGase (10 U/250  $\mu$ l) for 2 h at 37°C. Digested (lanes WT+NGase and GG1,2+NGase) and nondigested (lanes WT and GG1,2) HA was recovered by immunoprecipitation with anti-FPV antiserum and analyzed by PAGE with a 10% acrylamide gel. HA was also digested with NGase (0.5 U/12.5  $\mu$ l) for 4 h at 37°C after immunoprecipitation and denaturation by boiling with SDS and 2-mercaptoethanol (lanes WT den.+NGase and GG1,2 den.+NGase). Molecular weights (in thousands) are indicated on the left. (B) Purified FPV was digested with NGase (2 U/50  $\mu$ l) for 2 h at 37°C, and an aliquot was subjected to PAGE and Western blotting. Virus proteins were visualized with an anti-FPV rabbit serum, anti-rabbit IgG peroxidase-conjugated donkey serum, and ECL reagents (Amersham). NP, nucleocapsid protein; M<sub>1</sub>, matrix protein.

cence staining with an FPV-specific antiserum without permeabilization. The cultures were then inspected under a microscope with both visible and UV light. Figure 1C demonstrates that after expression of wild-type HA, a fair number of immunofluorescent cells did not show hemadsorption. Only highly stained cells showed hemadsorption, indicating that accumulation of a large amount of HA at the cell surface is required for hemadsorption. On the other hand, after expression of mutant GG1,2, almost all immunofluorescent cells were also hemadsorption positive. Comparison of the hemadsorption/immunofluorescence ratios indicates that receptor binding is efficient with GG1,2, low with the wild type and GG1, and intermediate with GG2 (Fig. 1D). These results indicate that the oligosaccharides in the vicinity of the receptor binding site interfere with the activity of HA binding to the erythrocyte receptor.

**Deletion of oligosaccharides adjacent to the receptor binding site of HA reduces elution of erythrocytes by NA.** To examine the kinetics of dissociation of HA from erythrocyte receptors, CV-1 cells expressing HA were incubated with VCNA at a concentration of 1 mU/ml at room temperature after hemadsorption. After different incubation intervals of up to 4 h, released erythrocytes were removed by a washing with PBS. The remaining hemadsorption-positive CV-1 cells were counted, and hemadsorption rates were calculated relative to hemadsorption before VCNA treatment. As shown in Fig. 2, erythrocytes were released most rapidly from cells expressing wild-type and mutant GG1 HAs (half-life, 45 min), followed by mutant GG2 and, finally, by mutant GG1,2 (half-life, 90 min), which again proved to be the strongest binder. Essentially the same results were obtained when erythrocyte release was evaluated by measuring the optical density of hemoglobin after lysis of residual erythrocytes. The data show that release of receptor binding by VCNA is down-regulated when the oligosaccharides near the receptor binding site are lost.

To analyze the effect of influenza virus NA on hemadsorption, HA was coexpressed with NA. Since we failed to express

FPV NA from an SV40 vector, NA of the PR8 strain, which, like FPV NA, belongs to the N1 subtype, was used. Coexpression of the two proteins was monitored by radioimmunoprecipitation followed by PAGE. The HA and HA<sub>1</sub> bands showed increased electrophoretic mobilities under these conditions, indicating that neuraminic acid had been removed by enzymatically active NA (Fig. 3A). Hemadsorption was done at 4°C without VCNA pretreatment, which was not required because of coexpression of NA. After hemadsorption, the cultures were incubated at 37°C for up to 3 h, and hemadsorption-positive cells were counted following washing of the monolayers with PBS. The proportions of hemadsorbing cells before and after the shift to 37°C were determined as described above. As shown in Fig. 3B, erythrocytes were released from cells expressing wild-type HA and, with lower efficiency, from cells expressing GG1. There was no release, however, from cells expressing GG2 and GG1,2, indicating that binding was so tight that NA was unable to remove the receptor after attachment of HA. These observations again demonstrate that the oligosaccharides near the receptor binding site reduce the receptor affinity and that the effect depends on the number and position of these oligosaccharides.

**Controlled NGase treatment of HA abolishes elution of virus particles from cell receptors.** The exposure of the oligosaccharides attached to Asn123 and Asn149 at the tip of the HA spike suggested that these oligosaccharides are susceptible to selective removal by controlled glycosidase treatment. To test the feasibility of this approach, we first carried out a comparative analysis in which native and denatured wild-type HA and GG1,2 HA were incubated with NGase (Fig. 4A). Both HAs were expressed from SV40 vectors and labeled with [<sup>35</sup>S]methionine for 3 h in the presence of VCNA and then digested with NGase at a concentration of 10 U/250  $\mu$ l for 2 h at 37°C without a denaturing agent. After being washed with PBS, the cells were lysed, and HA was analyzed by immunoprecipitation and PAGE. As a result of NGase digestion, the major part of the HA<sub>1</sub> subunit of the wild type had a reduced molecular weight which was similar to that of GG1,2 HA<sub>1</sub>, whereas most of HA<sub>2</sub> was unchanged (Fig. 4A, lanes WT, WT+NGase, and GG1,2). There were also minor bands representing undigested HA<sub>1</sub> which still persisted when higher NGase concentrations and longer incubation periods were used. Modification of incubation conditions also had little effect on the appearance of a minor band representing HA<sub>2</sub> lacking one of its two oligosaccharide side chains. Without VCNA treatment, the efficiency of NGase digestion was generally low. No mobility changes were observed with GG1,2 after NGase treatment (Fig. 4A, lanes GG1,2 and GG1,2+NGase). These observations support the view that the oligosaccharides at Asn123 and Asn149 are susceptible to

TABLE 1. Biological activities of NGase-digested virus

Virus condition	HA titer	NA activity <sup>a</sup>
Purified virus		
Before digestion	16 × 10 <sup>3</sup>	21.6
After NGase digestion	8 × 10 <sup>3</sup>	16.2
Virus suspension <sup>b</sup>		
Nontreated (WT) <sup>c</sup>	512	0.71
NGase treated (WT <sub>NGase</sub> ) <sup>c</sup>	512	1.05

<sup>a</sup> Measured as optical density at 549 nm.

<sup>b</sup> Virus suspension prepared for the adsorption and release test in MDCK cell culture.

<sup>c</sup> See Fig. 5B.

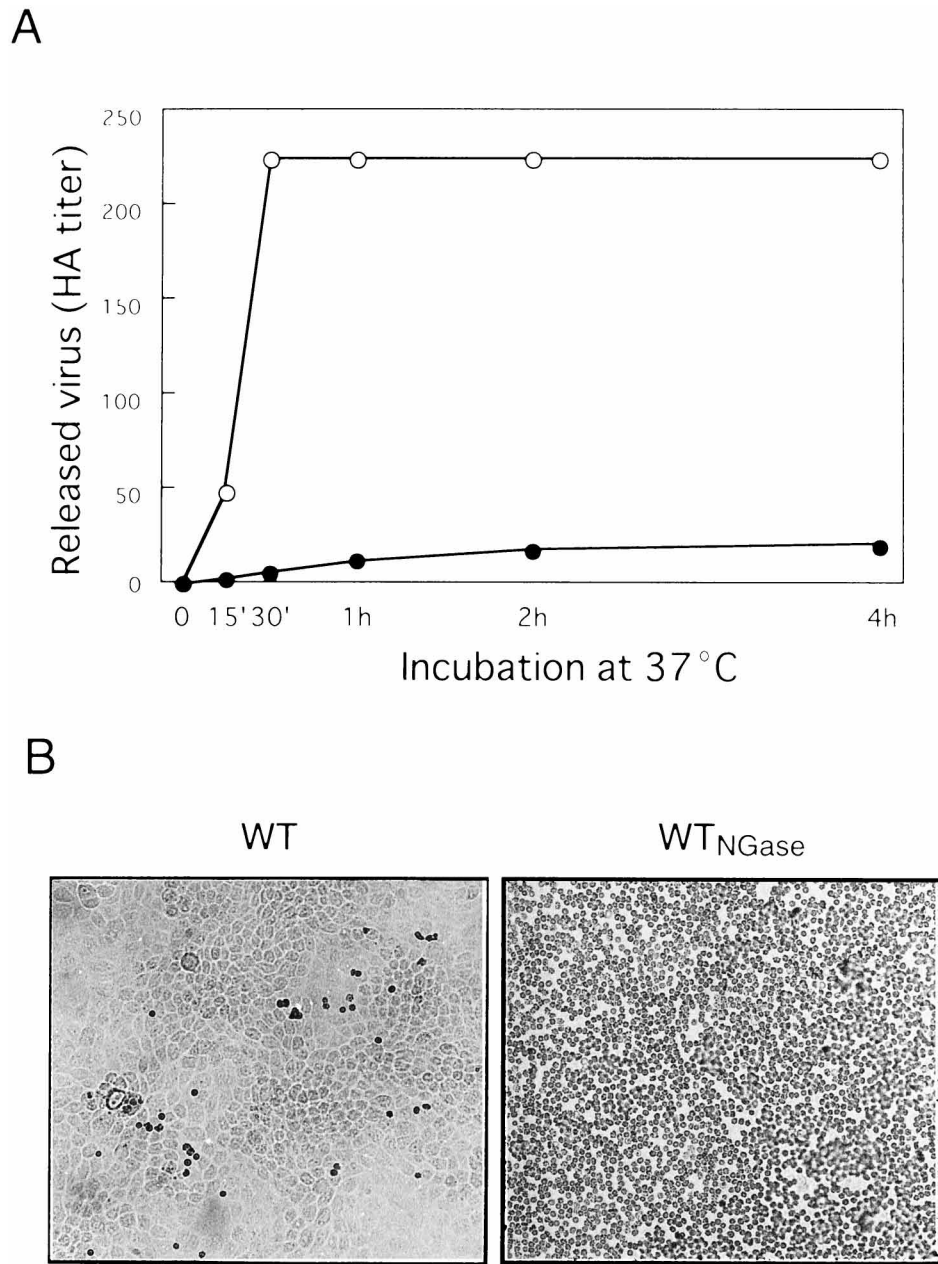


FIG. 5. Release of virus from MDCK monolayer cells. (A) After adsorption of nontreated or NGase-treated FPV to fixed MDCK cell monolayers at 0°C for 1 h, virus was eluted at 37°C. Aliquots of 50  $\mu$ l were removed from the culture fluid periodically, and HA titers were determined. Results for nondigested and NGase-digested virus (empty and solid circles, respectively) are shown. (B) After virus elution, MDCK cell monolayers were analyzed for residual virus by hemadsorption. On the left, MDCK cells after adsorption and release of nontreated virus are shown; there are almost no erythrocytes left on the monolayer. On the right, a monolayer exposed to NGase-treated virus is shown; the cells are densely packed with erythrocytes.

NGase digestion, whereas the other oligosaccharides of HA are resistant when the native glycoprotein is used as a substrate. On the other hand, when NGase digestion was performed on HA that had been denatured by boiling with SDS and mercaptoethanol, most of the oligosaccharides were removed from both HA<sub>1</sub> and HA<sub>2</sub>, and the differences in enzyme susceptibility between the wild type and GG1,2 were no longer observed (Fig. 4A, lanes WT den.+NGase and GG1,2 den.+NGase). Thus, we have shown that the carbohydrate side chains adjacent to the receptor binding site can be cleaved from intact spikes with relatively high selectivity.

It was then of interest to see if these oligosaccharides could also be removed from HA incorporated into virions. Purified FPV particles were digested with NGase at a concentration of 2 U/50  $\mu$ l under the nondenaturing conditions described above but without VCNA incubation, since mature influenza virus virions do not contain neuraminic acid. When this virus was analyzed by PAGE and Western blotting, the single HA<sub>1</sub> band observed in untreated virus was replaced by four bands, representing fully glycosylated HA<sub>1</sub> and HA<sub>1</sub> lacking one, two, and three oligosaccharides, among which the species lacking two oligosaccharides prevailed (Fig. 4B). Thus, partial removal

of oligosaccharides was also accomplished with intact virions, and in light of the experiments whose results are shown in Fig. 4A, it can be concluded that most of the carbohydrate side chains have been stripped from Asn123 and Asn149.

These virus particles were then analyzed for their receptor binding activity. As the NGase digestion caused a slight decrease in hemagglutinating and NA activities, virus concentrations were adjusted to the same HA titer ( $2^9$ ) in treated and nontreated samples (Table 1). Virus was adsorbed for 1 h at 0°C to monolayers of MDCK cells that had been fixed before with 1% paraformaldehyde to prevent endocytosis of input virus. The monolayers were washed three times with cold PBS to remove unadsorbed virus and also to remove remaining NGase. The hemagglutination titer of unadsorbed virus was  $2^8$ , indicating that about 50% of input virus was adsorbed to the cells. Virus elution was done at 37°C with prewarmed PBS, and released virus was assayed by hemagglutination titration. As shown in Fig. 5A, nontreated virus was rapidly released, whereas NGase-treated virus was released inefficiently. After elution, the monolayers were examined by hemadsorption assay for residual virus attached to MDCK cells. As shown in Fig. 5B, there was no hemadsorption after elution when nontreated virus was used. In contrast, the MDCK cells were heavily packed with erythrocytes when NGase-treated virus was used, indicating that such virions did not detach from their receptors even after a 4-h incubation with VCNA at 37°C. Unspecific hemadsorption not involving neuraminic acid as a receptor was ruled out by the observation that erythrocytes were completely released when the cultures were incubated after erythrocyte attachment with VCNA at a concentration of 25 mU/ml for 1 h at 37°C. Similarly, erythrocytes that had lost their receptors by pretreatment with VCNA did not attach to NGase-treated virions adsorbed to MDCK cells, again indicating that binding of such virus particles was mediated by neuraminic acid. Taken together, these results demonstrate that release of FPV virions by the viral NA depends on the presence of the oligosaccharides adjacent to the receptor binding site of HA.

## DISCUSSION

The data presented here demonstrate that elimination of carbohydrate side chains adjacent to the receptor binding site increases the affinity of the influenza virus HA for its receptor to such an extent that release by receptor-destroying enzymes is severely impeded or completely inhibited. On the other hand, we have shown previously that HA no longer binds to its receptor when these oligosaccharides are sialylated in the absence of NA expression (16). Taken together, these findings indicate that the oligosaccharides attached to Asn123 and Asn149 regulate the receptor affinity in a manner that enables HA to both bind to and dissociate from its receptor (Fig. 6). The data shown in Fig. 1D, 2, and 3B indicate that the oligosaccharide at Asn149 is the dominant regulator of receptor binding, whereas the oligosaccharide at Asn123 is less effective. GG1,2 was able to induce pH-dependent fusion. It was interesting to see, however, that similar mutants obtained from other HA subtypes had lost fusion activity (unpublished results). Thus, it appears that fusion is abolished when binding is too tight. The experiments employing recombinant HA as well as those using virus particles showed that binding of unglycosylated HA was highly resistant to influenza virus NA, whereas it was partially sensitive to VCNA. The reason for the different releasing potentials of the two enzymes is not clear, but it is reasonable to assume that soluble VCNA, unlike particle-bound viral NA, may have access to the neuraminic acid receptor molecule even when it is enclosed in the binding pocket

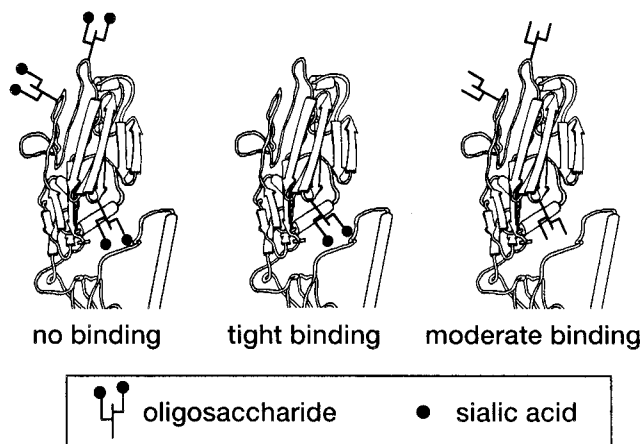


FIG. 6. Regulatory effect of oligosaccharides on the receptor binding affinity. When HA has neuraminic acid-containing oligosaccharides, it is unable to bind to the receptor (left). When it completely lacks the oligosaccharides adjacent to the receptor binding site, it is no longer released after binding (middle). Thus, only HA carrying neuraminic acid-free oligosaccharides is able to both bind to and elute from receptors (right).

of HA. From the observation that receptor binding of unglycosylated HA is not released by NA, it can also be concluded that elution of virus from the cell surface involves two steps: first HA dissociates from the neuraminic acid receptor, and then the receptor is cleaved by NA. Thus, our data clearly demonstrate that there is a close cooperation of HA and NA in virus exit as well as in virus entry, in which attachment of HA to the receptor requires removal of the terminal neuraminic acid residues from oligosaccharides adjacent to the receptor binding site.

Like the FPV HA, the HAs of most other influenza A and B viruses are glycosylated at the tip of the spike, and many of these oligosaccharides are located in the vicinity of the receptor binding site of the same monomer or of an adjacent monomer. Several studies have shown that glycosylation often changes at these sites, depending on the host system in which the virus is propagated. Thus, it has been observed that such glycosylation sites are frequently lost when virus present in clinical specimens is adapted to eggs, whereas they are preserved during passage in MDCK cells (9, 20, 22–24, 29). Other studies have presented evidence that a change in glycosylation at the receptor binding site may alter the receptor specificity of HA. Thus, an unglycosylated variant of an H1 HA reacted with  $\alpha$ -2,6-bound neuraminic acid, whereas the glycosylated form did not (6). Similar results were obtained with another pair of H1 variants, where acquisition of a carbohydrate side chain at almost the same site caused a shift in receptor specificity from binding to  $\alpha$ -2,6-linked neuraminic acid to binding to  $\alpha$ -2,3-linked neuraminic acid. In the latter case, the altered glycosylation pattern was also accompanied by a change in host range (1). In light of these observations, our study supports the concept that by modulating receptor specificity and receptor avidity, glycosylation near the receptor binding site may be an important determinant for cell tropism and host range.

Attenuation of biological activity of protein by its carbohydrate moiety has been reported also for several glycoproteins of nonviral origin. The removal of N-linked oligosaccharides from human granulocyte-macrophage colony-stimulating factor (hGM-CSF) increased the biological specific activity about 20-fold (12), and the increase in activity correlated with the increased receptor affinity of the nonglycosylated form (4).



Okamoto et al. reported that highly glycosylated hGM-CSF showed less *in vitro* specific activity than the nonglycosylated form, but in terms of clearance from blood circulation in the rat, the glycosylated form showed a half-life five times longer than that of the nonglycosylated form (19). The same tendency was observed with human tissue-type plasminogen activator (t-PA), although the effect of deglycosylation was not so drastic as observed for hGM-CSF (2, 7, 21, 30, 32, 33). The specific activity became higher when t-PA was treated with NA or when it was synthesized in the presence of deoxymannojirimycin, indicating that the interfering effect of oligosaccharide on the activity of t-PA is greater when the oligosaccharide contains sialic acid (8, 30). This phenomenon is similar to that observed for FPV HA (16), although desialylation of FPV HA increases the hemagglutinating activity approximately 100-fold, compared to the two- to threefold increase in the case of t-PA.

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