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N-Acetylneuraminic Acid (NANA) in Measles Virus (40109)

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The envelope glycoproteins and glycolipids of paramyxoviruses appear to be derived from the host cell plasma membrane during viral release (1, 2). Measles envelope glycoprotein spikes effect attachment of the virion to cell surfaces, thereby initiating either hemagglutination (HA) or infection (3, 4). Most viruses which mature at the cell surface incorporate NANA from the plasma membrane (5–9). Myxoviruses and paramyxoviruses do not do so, because they contain virus-coded neuraminidase (10–12). NANA residues are detectable on purified vesicular stomatitis virus (VSV) (5, 8, 12, 13), avian tumor (7), rabies (9), Sindbis (14, 15) and Semliki Forest viruses (16), as well as on temperature-sensitive mutants of influenza virus type A which lack neuraminidase (17). In this report, we present evidence to show that measles virions likewise possess NANA residues which are involved in the adsorption of virus to the surface of cells.

Materials and methods. VERO cells, obtained from the American Type Culture Collection, were grown in Eagle's minimal essential medium (MEM) in Earle's balanced salt solution (BSS) with 10% heat-inactivated fetal calf serum (FCS) and were maintained in the same medium with 2% FCS. The Edmonston strain of measles virus, grown in VERO cells, was partially purified from infected culture fluids by banding in a 20–40% sucrose gradient in a swinging bucket rotor (SW 27) at 80,000*g* for 3 hr. Parainfluenza virus type 2 (18) and VSV were propagated in VERO cells. Influenza virus was grown in 11-day old embryonated eggs, and mumps virus in primary cultures of chicken embryo fibroblasts.

Plaque titrations were carried out on confluent monolayers of VERO cells in 35 mm plastic dishes (Falcon), using a single overlay of 1% agar in maintenance medium. Plates were stained with neutral red 6 days after infection and the plaques counted two days later. Rhesus erythrocytes,[2] freshly drawn into dextrose-citrate, washed three times and resuspended to 0.5% in phosphate-buffered (pH 7.2) saline (PBS), were used for microhemagglutination and inhibition assays with measles virus. To measure inhibition of influenza virus hemagglutination (19), serial doubling dilutions of measles virus in PBS were mixed with equal volumes of influenza A virus containing 4 HAU, followed by equal volumes of 0.5% chicken erythrocytes. The HI titer was taken as the highest dilution of measles virus which inhibited 4 HAU of influenza virus.

Protein was determined by the method of Lowry (20) with bovine serum albumin as standard. *N*-acetylneuraminic acid (NANA) was determined by the method of Warren (21) using crystalline NANA as standard.

Results. As shown in Table I, measles virus pelleted from infected culture fluids gave slightly higher NANA: protein ratios then washed and gradient-purified virus. The ratios in all viral preparations, however, were higher than those found in normal VERO cells. Measles virus (6×10^6 PFU) and VSV (5×10^7 PFU) were each treated for 90 min at 37° with *Vibrio cholerae* neuraminidase (Behring Diagnostics) at a concentration of 20 U/ml in Tris–HCl buffer, pH 7.0 containing 0.5 μmoles $CaCl_2$. After incubation, the virus–enzyme mixtures were chilled, the virus was pelleted by centrifugation for 90 min at 125,000*g*, washed once and then resuspended in PBS to the original starting volume. Initial virus-free supernatants were saved for analysis. Results are shown in Table II. Supernatants from neuraminidase-treated measles virus were found to contain approximately 80% of the hydrolyzable NANA found in pelleted virions not previously exposed to enzyme. No chromogen was found in unhydrolyzed supernatants of untreated virus and

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no residual chromogen could be detected in acid hydrolyzates of neuraminidase-treated measles virus. Supernatants from neuraminidase-treated virions contained no more protein measurable by the Lowry method than supernatants from untreated virus (Table II), indicating that the neuraminidase preparations used had no contaminating protease activity. Acid hydrolyzates of untreated VSV contained substantial amounts of chromogen. No NANA was found in hydrolyzed influenza A virus. Measles virus and VSV each inhibited influenza HA significantly. Neuraminidase treatment abolished this inhibition as well as the infectivity of both viruses. Measles virus treated with either neuraminidase or trypsin no longer agglutinated rhesus erythrocytes and retained virtually no infectivity.

In order to determine the effect of enzymes or periodate treatment on susceptibility to infection, monolayers of VERO cells in 35 mm plates were treated at 37° with *V. cholerae* neuraminidase (Worthington) (20 U/ml), trypsin (1 mg/ml), pronase (1 mg/ml) or metaperiodate (0.011 *M*) for different periods of time up to 60 min. After treatment, which in each case caused only minimal rounding and no detachment of cells, the monolayers were washed three times with 5 ml PBS, infected with serial tenfold dilutions of measles virus, overlaid with agar and incubated. Staining and estimation of plaques were then carried out as described.

As shown in Fig. 1, treatment of cells with either trypsin or pronase before infection reduced by 60–70% the number of plaques which should have appeared in comparison to untreated controls. After 10 min of periodate treatment, the plaque count was reduced by about 90% as compared with untreated controls; and no plaques appeared in cells which had been treated for 60 min. In contrast, pretreatment up to 30 min with neuraminidase appeared to enhance slightly the susceptibility of cells to infection.

Erythrocytes were treated with the same reagents in order to determine the effect on HA receptors. Cells were suspended in 0.011 *M* sodium metaperiodate ($NaIO_4$) and allowed to stand at room temperature for 30 min, after which an equal volume of 40% glucose was added to stop the reaction. Erythrocytes in 10% suspension were also treated with *V. cholerae* neuraminidase (Behring), 40 U/ml, for 1 hr at 37°, or with pancreatic trypsin (1 mg/ml, Difco 1:250) for 30 min at room temperature. Treated cells were washed three times with PBS and resuspended to 0.5% in PBS, and then tested for agglutinability by virus. The results, not otherwise given in detail, showed that desialylation of rhesus erythrocytes enhanced measles virus HA titers 8- to 16-fold. Influenza A virus, which agglutinates normal rhesus erythrocytes weakly, did not agglutinate neuraminidase-treated rhesus erythrocytes. Pretreatment of rhesus erythrocytes with either trypsin or periodate completely eliminated their agglutinability by measles virus. Human erythrocytes remained inagglutinable by measles virus after treatment with either neuraminidase or trypsin.

Discussion. We have shown that measles virus, which lacks neuraminidase activity and matures by budding from the cell surface (22, 23), contains a chromogen which has an adsorption maximum identical to that of NANA (Table I) and which is released from concentrated purified measles virions by the action of exogenous neuraminidase (Table II). Although most of this NANA undoubtedly stems from measles virus particles, the possibility cannot be excluded that some portion of the NANA detected in gradient purified virus may have been derived from frag-

TABLE I. NANA CONTENT OF MEASLES VIRUS

	Micrograms NANA[a]	Milligrams protein[b]	Ratio NANA/protein × 10^3
VERO cells[c]	40	18	2.2
Measles virus[d] pelleted from medium containing			
2% FCS	4.8	1.4	3.4
5% FCS	5.6	1.6	3.5
2% FCS and washed 2× in PBS	4.3	1.4	3.1
2% FCS, washed 2×, banded in sucrose gradient (20–40%), repelleted	4.0	1.3	3.1

[a] Determined by thiobarbituric acid procedure (28) after hydrolysis 30 min 80°C in 0.1 *N* H_2SO_4 referred to crystalline NANA.

[b] Lowry method referred to bovine serum albumin (20).

[c] 1×10^6 cells.

[d] 6×10^5 PFU used for each analysis.

TABLE II.

Virus	Concentration of NANA (μg/ml)	Concentration of Protein (mg/ml)	Influenza viral HI titer[a]	HA titer	Infectivity (PFU treated/PFU untreated)
Measles					
Neuraminidase treated[b]					
Pellet	0[c]	1.1	0	0	0.05
Supernatant	28	0.1			
Trypsin treated (Pellet)				0	0.003
Control					
Pellet	37[c]	1.2	16–32	32	
Supernatant	0	0.1			
VSV					
Neuraminidase treated	nd	nd	0	nd	0.002
Control	78[c]	1.6	128		

[a] Mumps virus gave no inhibition.

[b] PFU treated virus/PFU untreated virus, titered simultaneously. In four experiments with neuraminidase, mean titer of treated virus was $4.2 \times 10^4 \pm 2.2$, untreated virus $4.6 \times 10^6 \pm 2.1$, $P = <0.01$.

[c] After hydrolysis in 0.1 N H_2SO_4, 80°C, 30 min.

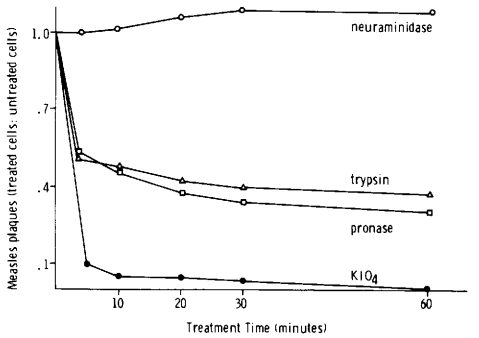

FIG. 1. Effect of pretreatment of VERO cells with neuraminidase, trypsin, pronase or periodate on susceptibility to infection with measles virus.

ments of infected host cell membrane containing predominantly virus-coded antigen as well as from defective, noninfectious particles (24) which might have cosedimented with complete virions. The latter two sources of NANA are still of viral origin. The amount of NANA absorbed to virions from calf serum in the medium was negligible.

Viruses which contain sialoglycoproteins have been reported to form aggregates (17) and to exhibit receptor activity for influenza virus, demonstrable by inhibition of hemagglutination (19). On this basis, our results provide further evidence for the presence of sialic acid on the measles virion, in contrast to mumps virus, which, because it bears intrinsic neuraminidase, lacks NANA and therefore fails to interact with influenza virus HA. Trypsin reduced both infectivity and HA to a greater extent than did neuraminidase, suggesting that the NANA required for these activities is attached to glycoprotein rather than to glycolipid.

Studies on VSV (25, 26) and those reported to date on measles virus (4) indicate that envelope glycoproteins mediate adsorption of viral particles to cells. NANA may play a central role in this process, which initiates infection in Vero cells and effects hemagglutination. Accordingly, infectivity was reduced and HA was eliminated by treatment with neuraminidase. That other as yet unidentified components of the viral envelope may be essential to attachment is suggested by the finding that trypsin treatment completely abolished infectivity, whereas some infectivity remained after neuraminidase treatment. This is consonant with the finding that trypsinization of desialylated VSV was reported to result in a 100-fold decrease in infectivity (26), suggesting that portions of the sialopeptide proximal to terminally linked NANA had some part in attachment of virions to cell receptors. The role of NANA in VSV infectivity is still not clear, however, since other workers have found that three different strains of VSV, after being stripped of NANA, remained fully infective for BHK and mouse cells, but lost HA activity (27). NANA appears not to be involved in the attachment of other enveloped RNA viruses to cells. Both Sindbis (25) and Semliki Forest viruses (16) were reported to retain biological function after treatment with neuraminidase. Sindbis virus grown in *Aedes albopictus* cells, which lack NANA, was biologically equiva-

lent to virus produced in either duck or hamster cells (14). It is apparent that no generalizations are possible regarding the role of viral NANA in the attachment of virions to cells susceptible to infection.

In contrast to its essential role in the measles virion, the presence of NANA in the glycoprotein receptor site appears not to be essential for viral attachment. Accordingly, treatment of VERO cells with neuraminidase resulted in moderate increase in their capacity to adsorb virus; and neuraminidase treatment of rhesus erythrocytes markedly enhanced agglutinability by virus. It is possible that removal of NANA residues may unmask receptors, and, by reducing the negative surface charge, promote attachment of measles virions. Trypsin, pronase or periodate each greatly reduced the susceptibility of Vero cells to infection, and abolished the reactivity of rhesus erythrocytes with measles HA. Oxidation of terminal sugar residues, including NANA, may account for the more pronounced effects of periodate as compared with proteolytic enzymes in reducing the capacity of VERO cells to absorb virus and in eliminating hemagglutination. These findings indicate that the receptor groupings required for viral attachment, while not exclusively in terminal linkage, are still distal to the site of tryptic cleavage.

Lastly, the residual bioactivity seen after treatment of cells with proteolytic enzymes (Fig. 1) may be a reflection of the experimental conditions or it may indicate that the virus is able to utilize active sites on the cell surface which are not affected by the enzymes tested.

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