SIALIC ACID RESIDUES EXPOSED ON MAMMALIAN CELL SURFACE: THE EFFECT OF ADSORPTION OF DENATURED VIRUS PARTICLES

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Received January 17,1974

SUMMARY: The sialidase preparation from Cl. perfringens was used to determine the number of residues of sialic acid which were exposed on the cellular surface of monkey kidney (MK) cells and mouse L cells. When the denatured particles of influenza virus was adsorbed to MK cells, there was an apparent increase in the number of sialic acid residues. This suggests the rearrangement of glycoproteins and glycolipids on the cellular surface.

It is well known that glycoproteins and glycolipids are located on mammalian cell surface and that some of end group of the saccharide chains are covalently linked to sialic acid residues (1,2). The early events in the mammalian cells infected with certain virus, especially myxo- and paramyxovirus, is the adsorption of the virus particles to the receptors such as the sialic acid residues (3-5). Little is known about the process which follows the adsorption of the virus particles and brings about their penetration through the cellular membrane. However, it is reasonable to assume that some structural change in the cellular membrane or some spatial rearrangement of the membrane components such as glycoproteins and glycolipids may occur during the viral adsorption and penetration. Since saccharide chains or sialic acid residues are linked to these membrane components, specific reagents which selectively react with these residues might be useful in detecting the rearrangement of the membrane components.

In the present communication tritium labeled phytohemagglutinin (PHA) and concanavalin A (Con A) were employed as specific reagents for saccharide chains and the sialidase preparation from Cl. perfringens as a specific rea-

gent for sialic acid residues. With these reagents we tried to detect the structural change in the cellular membrane of MK cells and mouse L cells caused by the adsorption of virus particles. The A/Aichi/2/68 (H3 N2) strain of influenza virus was heated at 60°C for 5 minutes and the denatured virus particles which retained not infectivity nor sialidase activity but only hemagglutination activity were employed in order to observe only the direct effect of viral adsorption on the cellular membrane.

MATERIALS AND METHODS

Virus strains: The A/Aichi/2/68 (H3 N2) strain of influenza virus and the Hemagglutinating Virus of Japan (HVJ) strain of parainfluenza virus were propagated. Virus in the fluids was concentrated by differential centrifugation and purified by 10 to 50% linear sucrose gradient centrifugation (6,7). Titrations of hemagglutination were carried out according to the conventional method.

Cells: Primary monkey kidney (MK) cells were prepared by trypsinization and were grown according to the conventional method. The suspension cultures of mouse L cells were grown as described by Kobayashi and Sano (8) and were harvested at $3X10^5$ to $5X10^5$ cells per ml.

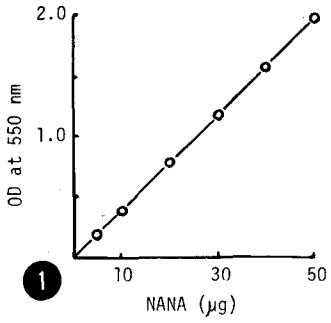
Binding studies of phytohemagglutinins: Bacto-phytohemagglutinin-P (prepared from Phaseolus vulgaris) and concanavalin A (prepared from Canavalis ensiformis) were purchased from Difco Laboratories, U. S. A. and Miles-Yeda Ltd., Israel, respectively. They were acetylated with tritiated acetic anhydride (400 mCi/m mole, purchased from New England Corp., U. S. A.) and purified with Sephadex G-50 chromatography according to the method of Hille et. al. (9). Quantitative binding studies were performed by mixing a fixed number of cells with ^{3}H-PHA and ^{3}H-Con A in centrifuge tubes. $4.7X10^{14}$ molecules of ^{3}H-PHA (409,600 dpm) or $1.2X10^{14}$ molecules of ^{3}H-Con A (385,400 dpm) were incubated with $2X10^7$ MK cells and $3X10^7$ L cells in phosphate buffered saline (PBS) of pH 7.2, respectively. After incubation at 35°C for 1 hour, the cells were collected by centrifugation and washed once with the same buffer. The cells were dissolved in 1.5 ml of NCS solubilizer (Amersham/Searle, U. S. A.) at 37°C for 30 minutes (10), mixed with 8.5 ml of Bray's solution (11) and counted in a liquid scintillation spectrometer. Binding studies were carried out in duplicate.

Sialidase preparation and determination of sialic acid: The method of purification of sialidase from Cl. perfringens and the assay of its activity were described previously (12-14). One sialidase unit was defined as the amount of enzyme that released 25 μg of N-acetylneuraminic acid (NANA) from excess of bovine sialyllactose (BSL) in 1 hour at 37°C and pH 6.0. The liberated sialic acid was determined by the thiobarbituric acid method as described by Warren (15) except that cyclohexanone was replaced by n-butanol containing 5% of concentrated hydrochloric acid. In our hands the reading at 550 nm was linearly dependent on the amount of synthetic NANA (Fig. 1). During the course of experiments all the residues of sialic acid were assumed as NANA.

RESULTS

Release of sialic acid from the cellular surface by sialidase treatment:

Before using the sialidase preparation as a specific reagent for sialic acid



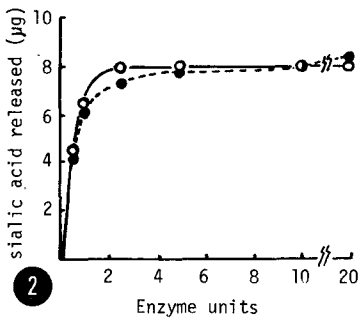


Fig. 1. Determination of NANA by thiobarbituric acid method

Fig. 2. Release of sialic acid by sialidase from the cellular surface. Each tube containing $3x10^7$ cells in PBS of pH 7.2 was incubated with increasing amount of the sialidase (Cl. perfringens). After 1 hour incubation at 37°C, the supernatant was separated by centrifugation and the determination of the liberated sialic acid was carried out as described in the text. The cell pellet was hydrolyzed with N/10 H_2SO_4 for 1 hour at 80°C and determined for sialic acid. The amount of sialic acid in the supernatant and that in the cell pellet was summed up to make the total amount in the whole cells.
O——O MK cell ●-----● L cell

residues, it should be ascertained that the enzyme action is restricted only to the exposed residues on the cellular membrane. As shown in Fig. 2, the amount of released sialic acid from MK cells reached a plateau level which was almost 40% of the total residues, when the increasing amount of the enzyme was used. Based on that data, 3 unit of the enzyme was employed against $3X10^7$ of MK cells during the course of experiments. In the case of L cells, the plateau level was much lower and approximately 18% of the total residues.

Quantitative studies on the binding of ^{3}H-PHA and ^{3}H-Con A to the cells after treatment with sialidase: Table 1 shows the effect of sialidase treatment on the binding of ^{3}H-PHA and ^{3}H-Con A to MK and L cells. The number of molecules of phytohemagglutinins which bound to the cells was not affected by the treatment with sialidase. The presence of sialic acid residues on the terminal of saccharide chains has no effect on the binding of PHA and Con A.

Quantitative studies on the binding of ^{3}H-PHA and ^{3}H-Con A to the cells after

Table 1. Binding of phytohemagglutinins to MK cells and L cells after treatment with sialidase

Cells	Sialidase	Phytohemagglutinins	moles/cell
MK	−	^{3}H-PHA	1.7×10^{6}
	−	^{3}H-Con A	2.2×10^{7}
	+	^{3}H-PHA	1.6×10^{6}
	+	^{3}H-Con A	2.2×10^{7}
L	−	^{3}H-PHA	1.9×10^{6}
	−	^{3}H-Con A	2.2×10^{7}
	+	^{3}H-PHA	1.8×10^{6}
	+	^{3}H-Con A	2.2×10^{7}

After the cells were treated with 3 unit of Cl. perfringens sialidase for 1 hour at 37°C, the radioactive phytohemagglutinins were adsorbed as described in the text.

adsorption of the denatured virus: Denatured virus was prepared by heat treatment. The purified A/Aichi/2/68 (H3 N2) and HVJ strains were suspended in PBS of pH 6.0 and dispensed into test tubes. The tubes containing 0.4 ml of 1,000 HA of virus were heated at 60°C for intervals up to 1 hour and the samples were tested for the hemagglutinin titer and the sialidase activity. As shown in Fig. 3, the sialidase activity of A/Aichi/2/68 (H3 N2) strain was destroyed completely by 4 minutes incubation but hemagglutinin activity remained completely intact for 15 minutes under condition described above. However, we can not discriminate the sialidase activity and the hemagglutinin activity of HVJ strain by heat treatment (50°C).

So the Aichi strain of influenza virus was denatured by 5 minutes incubation at 60°C for further experiments. Table 2 shows the binding of ^{3}H-PHA and ^{3}H-Con A to MK cells after adsorption of the denatured virus. The number of molecules of phytohemagglutinins which bound to MK cells increased only

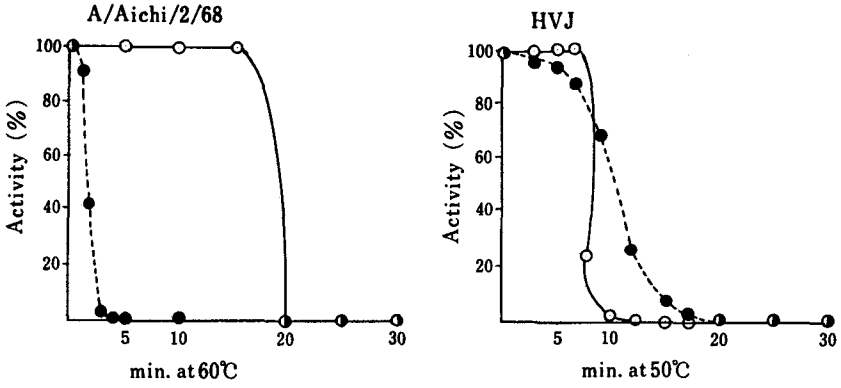


Fig. 3. Activity of hemagglutinin and sialidase of viruses after heat treatment. Each tube containing 1000 HA of virus was heated at 60°C for A/Aichi/2/68 strain and at 50°C for HVJ strain: The other conditions were described in the text. Activity of the hemagglutinin and the sialidase were determined by the standard procedure and expressed as percentage.
O——O hemagglutinin, ●----● sialidase

slightly but the difference was not significant.

Release of sialic acid from MK cell surface by sialidase treatment after the binding of denatured virus: Table 3 shows the number of molecules of sialic acid liberated from MK cells by the sialidase from Cl. perfringens after the adsorption of denatured virus to the cell. $3X10^7$ MK cells were incubated with 1 to 500 HA of the denatured virus in PBS (pH 7.2). After 1 hour incubation at 35°C, the cells were collected by centrifugation and resuspended in PBS (pH 6.0) and the enzyme solution containing 3 enzyme unit was added and the mixture was incubated for 1 hour at 37°C. The liberated sialic acid in the supernatant fraction was determined. There was an apparent and significant increase in the amount of sialic acid liberated by Cl. perfringens sialidase after the adsorption of mroe than 100 HA of denatured virus.

When L cells were used, the sialidase treatment released a rather small amount of sialic acid and the binding of denatured virus did not cause a significant difference in that amount.

Table 2. Binding of phytohemagglutinins to MK cells after treatment with denatured virus

Cell	A/Aichi/2/68 (HA)	Phytohemagglutinins	moles/cell
MK	500	^{3}H-PHA	1.9×10^{6}
	100		1.9×10^{6}
	—		1.8×10^{6}
	500	^{3}H-Con A	2.6×10^{7}
	100		2.6×10^{7}
	—		2.5×10^{7}

After the 2.5×10^{7} MK cells were treated with the denatured virus for 30 minutes at 4°C, the radioactive phytohemagglutinins were adsorbed as described in the text.

Table 3. Effect of the binding of denatured virus on the release of sialic acid from the surface of MK cells by sialidase treatment

A/Aichi/2/68 (HA)	Sialic acid released (μg/3×10^{7} cell ± S.D.)
500	6.98 ± 0.1 (6)
100	6.94 ± 0.08 (6)
30	6.51 ± 0.39 (6)
10	6.38 ± 0.21 (6)
1	6.10 ± 0.27 (6)
0.1	6.02 ± 0.09 (6)
Control	6.02 ± 0.15 (6)

Each figure is the average of six separate experiments together with S.D.
The same lot of cells were employed for the whole experiments.

DISCUSSION

The number of binding sites for PHA and Con A on the cellular membrane of MK and L cells substantially coincides with the data obtained by Stein et. al. (10). Table 1 furthermore demonstrates that the exposed sialic acid residues is not essential for the binding of these phytohemagglutinins to the saccharide chains of glycoproteins and glycolipids. Table 2 shows that the denatured virus which has only HA activity does not compete with PHA and Con A and that it binds to the cellular membrane only through the sialic acid residues. The RDE has been employed to demonstrate certain biochemical alteration of host cells which accompanied with virus infections (3,4,16,17).

The sialidase from Cl. perfringens releases sialic acid from glycoproteins and glycolipids more effectively than from BSL and is shown to be useful tool to detect any change in the cellular membrane. It is quite reasonable to assume that the adsorption of a bulky substance such as the denatured virus particles to the cellular membrane causes the rearrangement of glycoproteins and glycolipids within membranes. In normal cells, some of the sialic acid residues are exposed to the outside but others are located intra-membrane and are protected from the action of the hydrolyzing enzyme. This hypothesis is supported by a previous study, showing that a large proportion of virus receptors on chick embryo cells are refractory or relatively resistant to enzyme action (4). If any change occurs in the cellular membrane and causes the rearrangement of glycoproteins and glycolipids, these inner residues of sialic acid are made accessible to the action of sialidase. Actually, if more than 100 HA of the denatured virus was adsorbed to $3X10^7$ MK cells, there was an apparent increase in the number of released sialic acid residues (Table 3). In the above condition each cell can be considered to adsorb 100 or more of the viral particles. It seems essential to use a large amount of the denatured virus in order to amplify the change which occur as the initial event in the whole process of infection. In case of L cells it can not be concluded whether there was really no change in the amount of

hydrolyzable residues or the difference was too small to detect.

In the present communication the denatured virus was adsorbed to the cells and so the change we observed in the cellular membrane can be considered as the direct result of the adsorption of virus particles. Further investigation is in progress to determine whether any change in the structure or level of various enzyme activities in the cellular membrane following virus adsorption is essential for penetration.

REFERENCES

1. Gottschalk, A., in The chemistry and biology of sialic acid and related substances, Cambridge Univ. Press, London (1960), p. 75.
2. Roseman, S., Chem. Phys. Lipids 5, 270 (1970).
3. Ackermann, W. W., Ishida, N. and Maassab, H. F., J. Exptl. Med. 102, 545 (1955).
4. Haff, R. F. and Stewart, R. C., J. Immunol. 94, 842 (1965).
5. Lipkind, M. A. and Tsvetkova, I. V., Arch. Ges. Virusforsch. 29, 370 (1970).
6. Noll, H., Aoyagi, T. and Orlando, J., Virology 18, 154 (1962).
7. Nerome, K., Kumagai, M. and Aoyagi, T., Arch. Ges. Virusforsch. 39, 353 (1972).
8. Kobayashi, S. and Sano, E., Proc. Japan. Virol. Soc. 20, 1035 (1972).
9. Hille, M. B., Barrett, A. J., Dingle, J. T. and Fell, H. B., Exptl. Cell Res. 61, 470 (1972).
10. Stein, M. D. and Sage, H. J., Arch. Biochem. Biophys. 150, 412 (1972).
11. Bray, G. A., Anal. Biochem. 1, 279 (1960).
12. Cassidy, J. A., Jourdian, G. W. and Roseman, S., J. Biol. Chem. 240, 3501 (1965).
13. Noll, H., Aoyagi, T. and Orlando, J., Virology 14, 141 (1961).
14. Aoyagi, T., Yagisawa, M., Kumagai, M., Hamada, M., Okami, Y., Takeuchi, T. and Umezawa, H., J. Antibiotics 24, 860 (1971).
15. Warren, L., J. Biol. Chem., 234, 1971 (1959).
16. Fisher, T. N. and Ginsberg, H. S., Virology 2, 637 (1956).
17. Ginsberg, H. S. and Blockman, J. R., Virology 2, 618 (1956).