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INTERACTION BETWEEN SENDAI VIRUS AND THE CELL MEMBRANE. II. THE ROLE OF SENDAI VIRUS NEURAMINIDASE IN HAEMOLYSIS

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Summary. — When chicken erythrocytes (CRBC) were pretreated with non-haemolytic Sendai virions or isolated HANA spikes, they acquired a resistance to the haemolytic action of "second challenge" viruses. This resistance was dependent on the quantity of N-acetylneuraminic acid liberated from the surface of the CRBC by the initial virus, and not on the use of different viral sources. When exposed to Sendai virus, CRBC were more difficult to be lysed and easier liberated N-acetylneuraminic acid than human 0 erythrocytes. The restricted number of virions able to fuse CRBC was explained by such neuraminidase function of the HANA spike of the virion.

Key words: *Sendai virus*; *neuraminidase*; *haemolysis*: *cell fusion*; *chicken blood cell*

Introduction

Previously (Yasuda *et al.*, 1980), we reported that the number of Sendai virus particles that finally fused with the membrane of CRBC was restricted. As the cause of this restriction, loss of receptor sites for additional virions to reveal their F spike activity was proposed. To clarify the correlation of the activities of the two viral spikes on the cell membrane, we examined the role of the HANA spike neuraminidase in haemolysis, which is mediated through the function of the F spike.

The biological function of neuraminidase is to detach the virions from the cells by destroing the cell surface receptors (Hirst, 1959; Palese *et al.*, 1974) which consist of N-acetylneuraminic acid (Hoyle, 1968; Haywood, 1974). However, the mechanism of participation of the F spikes in membrane or cell-fusion, in particular in connection with the function of HANA spikes, is not clear. Yamamoto *et al.* (1974) demonstrated that horse erythrocytes containing no receptors for Sendai virus and thus being normally insusceptible to agglutination or lysis by the virus, acquired these properties when they were coated with Concanavalin A. This observation suggests that the haemagglutinating activity could be essential for the exhibition of the function of the F spikes.

Materials and Methods

Virus. The Z strain of Sendai virus (HVJ) was used. The egg-grown non-haemolytic (native virion or early harvest) was defined previously (Yasuda *et al.*, 1980). MK-Sendai virus was also used as non-haemolytic (and also non-infectious) virion. This was grown and harvested from 72 hr culture fluid of LLC-MK_2 cells infected with egg-grown Sendai virus. HANA spikes isolated from egg-grown HVJ by Ampholine isoelectric focusing electrophoresis were kindly provided by Dr. T. Kohama.

Erythrocytes. Chicken red blood cells (CRBC) and human O type red blood cells (HRBC) were used. They were washed twice with 0.01 M phosphate buffered saline, pH 7.2 (PBS) by centrifugation at 2,000 rev/min for 5 min. The packed cells were resuspended in PBS and centrifuged at 1,500 rev/min for 10 min. The sediment was considered to be 100% erythrocytes.

Quantitation of N-acetylneuraminic acid (further on briefly neuraminic acid) was carried out by the thiobarbituric acid method (Aminoff, 1961).

Complete liberation of N-acetylneuraminic acid from erythrocytes by Sendai virus. Both CRBC and HRBC were incubated with Sendai virus at a concentration of 50 virions per cell at 37 °C for 2 hr. This treatment was regarded as causing the release of the maximum amount of neuraminic acid into the reaction mixture.

Haemolytic reaction and calculation of the number of viral particles per erythrocyte were performed as described previously (Yasuda *et al.*, 1980).

Results

Haemolysis of CRBC pretreated with various types of Sendai virus

Two milliliters of a 5% suspension of CRBC were pretreated with 200 haemagglutinating units (HAU) of non-haemolytic egg-grown Sendai virions or with an amount of MK-Sendai virus or isolated HANA spikes which had the same neuraminidase activity as that of 200 HAU of egg-grown Sendai virus in the reaction mixture containing fetuin as substrate. After predetermined incubation periods, the amount of neuraminic acid released from CRBC into the reaction medium was measured. Samples in which a suitable amount of neuraminic acid was detected in the reaction medium were employed for the following experiment.

The pretreated CRBC samples obtained as described above were washed in cold PBS and diluted with it to 2 % suspensions. Thereafter the egg-grown haemolytic viruses were added at an input multiplicity (moi) of 5 and incubated at 37 °C for 2 hr to test for resistance against haemolysis. The results are shown in Fig. 1, in which an optical density of 0.3 for liberated neuraminic acid was equivalent to about 50 % of the total neuraminic acid releasable in 2 ml of 5% CRBC suspension by Sendai viruses. It is evident that the respective pretreatments rendered the CRBC resistant to the haemolytic action of challenge virus. Furthermore, the greater the amount of neuraminic acid liberated by the pretreatment, no matter what kind of virus was used for the pretreatment, the higher the resistance demonstrated on second challenge.

To explain the resistance of the CRBC to haemolysis observed in the experiment, we first examined the adsorption of second challenge virus to CRBC which had been pretreated with non-haemolytic egg-grown virus. After pretreatment, the CRBC were suspended in PBS to make a 10% suspension and 400 or 1,600 HAU of virions were added, which corresponded to an moi of 5 or 20 respectively. As shown in Table 1, almost all virions were adsorbed on to the pretreated CRBC. When 50% of total neuraminic acid was

liberated from CRBC by the pretreatment, unadsorbed virus represented only 0.5% of the input virus after incubation at 4 °C for 1 hr, and even when 86% of neuraminic acid was liberated, unasorbed virus still repressented only 2%. This result seems to exclude the possibility that the resistance of the CRBC to haemolysis observed might be due to decrease ability to be adsorbed of the second challenge virus; it rather supports the possibility that the resistance is due to a restricted ability of the second challenge virus to lyse CRBC.

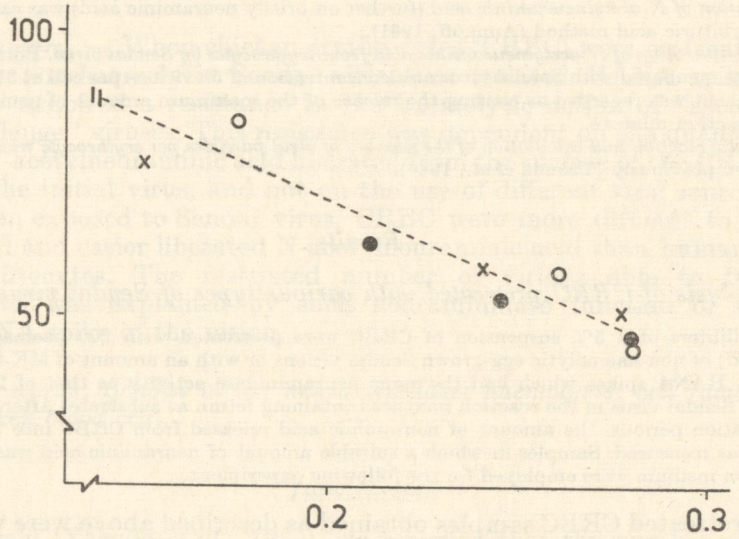

Fig. 1.

The acquisition of resistance to haemolysis of CRBC pretreated with various types of Sendai virus

Percentage of CRBC lysed by the second challenge virus (ordinate) was plotted against the amount of neuraminic acid liberated by pretreatment with egg-grown Sendai virus (×), MK-Sendai virus (○), or isolated HANA spikes (●) (abscissa; OD_{549})

Table 1. Adsorption of Sendai virus on to CRBC pretreated with non-haemolytic Sendai virus

moi of second challenge virus	% neuraminic acid liberated*				
	0	8.6	25	46	86
5	< 2	< 2	< 2	2	8
20	< 2	< 2	2	4	32

*By pretreatment at 37 °C for 2 hr with non-haemolytic egg-grown Sendai virus. The values indicate haemagglutinating units in supernatant after incubation of pretreated CRBC with second challenge Sendai virus at 4 °C for 1 hr.

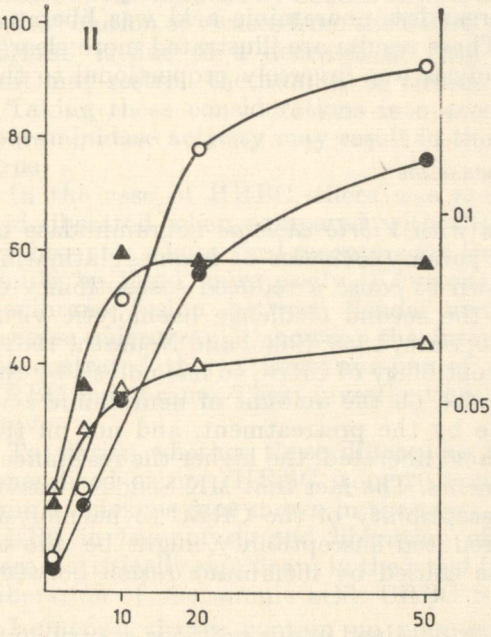

Fig. 2.

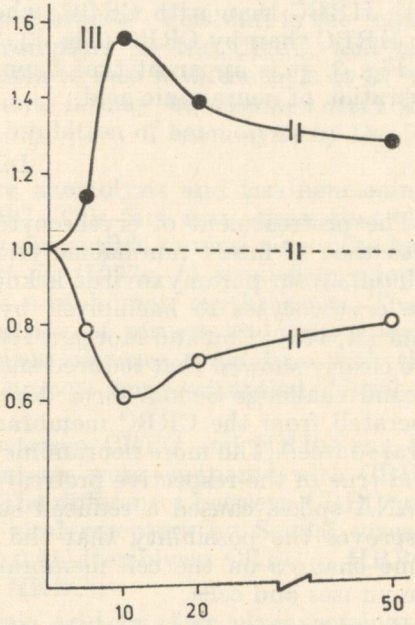

Fig. 3.

Fig. 2. The difference between CRBC and HRBC in haemolysis and liberation of neuraminic acid by Sendai virus. Percentage of lysed CRBC (●—●) or HRBC (○—○) (left ordinate) and the amount of neuraminic acid liberated from CRBC (▲—▲) or HRBC (△—△) (right ordinate; OD_{549}) by Sendai virus were plotted against the moi (abscissa).

Fig. 3. The relationship between haemolysis and liberation of neuraminic acid. Data from Fig. 2 were redrawn by plotting the value of haemolysis (●—●) of, and liberation of neuraminic acid (○—○) from HRBC (ordinate) against each moi (abscissa), assuming the value for CRBC to be 1.

Difference in susceptibility of viral receptors on CRBC and HRBC to viral neuraminidase

To correlate the liberation of neuraminic acid and the restriction of the haemolytic activity of Sendai virus, HRBC were compared with CRBC, as preliminary experiments revealed that it was more difficult to liberate neuraminic acid from HRBC than from CRBC by viral neuraminidase.

Haemolytic Sendai virus was added into 5% suspensions of both CRBC and HRBC at moi of 1, 5, 10, 20, and 50, respectively. The mixtures were kept at 4 °C for 30 min and then at 37 °C for 1 hr. The amonts of neuraminic acid in the supernatants of the incubation mixtures were measured and the haemolysis of both RBCs was examined using 2% erythrocyte suspensions under standard conditions (Fig. 2). For the control of haemolysis, the same amounts of both CRBC and HRBC were physically lysed in distilled water

and referred to as 100% haemolysis. Haemolysis was more pronounced with HRBC than with CRBC, whereas less neuraminic acid was liberated by HRBC than by CRBC (Fig. 2). These results are illustrated more clearly in Fig. 3. It is apparent that haemolysis was inversely proportional to the liberation of neuraminic acid.

Discussion

The pretreatment of erythrocytes with *Vibrio cholerae* neuraminidase or with that of intact non-haemolytic paramyxoviruses or haemagglutinating subunits from paramyxovirus is known to cause a reduced susceptibility of the erythrocytes to haemolysis by the second challenge haemolytic virus (Burnet, 1949; Chu and Morgan, 1950; Sato, 1958; Sokol and Neurath, 1962). We clearly showed that reduced susceptibility of CRBC to haemolysis by the second challenge Sendai virus depended on the amount of neuraminic acid liberated from the CRBC membrane by the pretreatment, and not on the viral source. „The more neuraminic acid liberated, the higher the resistance" held true in the respective pretreatments. The fact that MK-Sendai virus or HANA spikes caused a reduced susceptibility of the CRBC to haemolysis disproves the possibility that the reduced susceptibility might be due to some changes on the cell membrane caused by membrane fusion between the viruses and cells.

Based on this fact and on the results obtained in our previous experiments showing that the maximum number of virus particles responsible for fusion was restricted on CRBC (Yasuda *et al.*, 1980), it may be reasonable to consider the interaction of the virus and RBC on the premise described as follows; 1) There are two possible fates of virus adsorbed on to the CRBC; one, infection of the cell by envelope-membrane fusion, and the other, release from the cell. These alternative pathways should be controlled by the haemagglutinating and neuraminidase activities of the HANA spikes and the fusion activity of the F spikes, in relation to receptor sites on the membrane. 2) The adsorption of the virus to the cell must be derived from the multivalency between the HANA spikes of the virion and the receptors on the cell surface.

In the case of CRBC, the number of neuraminic acid residues susceptible to Sendai virus neuraminidase is about 10^7 per cell (data not shown). When the total surface area of CRBC is divided by the assumed virus axial area, there should be about 700—800 neuraminic acid residues on a CRBC membrane in the area occupied by one viral particle. This is the situation when CRBC were not pretreated. Even when 80% of the receptors are removed and therefore only 140—160 residues per viral particle remain on the CRBC membrane, these receptors can still be useful for the adsorption of virus. This is the situation of CRBC after pretreatment. An "equilibrium" may exist among haemagglutinating activity, neuraminidase activity and the fusion activity of the F spike of Sendai virus in the interaction between the virus and cell membranes. The reduced susceptibility of the pretreated cells

to haemolysis induced by Sendai virus is caused by a shift of the "equilibrium" to the "elution of viruses from the CRBC membranes". This shift in the "equilibrium" is due to a decrease of viral receptors on the CRBC, and the shift may restrict the number of viruses able to fuse with the cells at 37 °C.

Taking these considerations into account, it may be assumed that the neuraminidase activity may result in the inhibition of haemolysis by Sendai virus.

In the case of HRBC, there was more haemolysis and less neuraminic acid liberated when compared with CRBC. This fact may mean that the erythrocytes whose viral receptors are less susceptible to viral neuraminidase should be lysed more easily. Knutton *et al.* (1977*a*, *b*) studied envelope-membrane fusion between Sendai virus and human erythrocytes. Their electron micrographs showing the interaction of viruses and cells clearly demonstrated that a large number of virus particles could fuse with the HRBC membrane. Their investigations support our assumption described above.

To clarify whether these differences between CRBC and HRBC can be generalized or not, HRBC from 20 volunteers were compared with CRBC from 4 chickens (not shown in the text). The differences between CRBC and HRBC in haemolysis and liberation of viral receptors by Sendai viruses were statistically significant in the t-test (p 0.01) Hemolysis: CRBC < HRBC. Liberation of neuraminic acid: CRBC > HRBC).

Influenza viruses contain no neuraminic acid in their glycoproteins or glycolipids since they possess neuraminidase (Klenk *et al.*, 1970a, b); Klenk and Choppin, 1970). Palese *et al.* (1974) investigated the neuraminidase-less mutants of influenza virus and suggested that the biological significance of the neuraminidase of influenza virus is to remove neuraminic acid from its own envelope. Sendai viruses have no neuraminic acid in their envelope proteins (Kohama, 1978, personal comunication) and the same explanation as for influenza virus will hold true. However, another more important role of neuraminidase of Sendai virus might be to regulate the infective dose of virus to each cell and thus to give an infectivity of high efficiency.

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