

INTERACTION BETWEEN SENDAI VIRUS AND THE CELL MEMBRANE. I. RESTRICTED NUMBER OF SENDAI VIRUS PARTICLES ENGAGED IN FUSION WITH CHICKEN RED CELL MEMBRANE

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Summary. — The interaction between Sendai virus (HVJ) and the cell membrane was studied from a quantitative point of view, using artificially induced haemolytic virus particles and chicken red blood cells (CRBC). It was shown that one artificially induced haemolytic virus particle can lyse one erythrocyte. A method to accurately enumerate the actual number of virus particles fused to one CRBC was established. The number of virus particles adsorbed on to the CRBC increased with increasing multiplicities of infection (moi). The highest number of virus particles fused with one CRBC was 20 and was obtained from an inoculation of about 100 virus particles per CRBC (moi 100).

Key words: Sendai virus; membrane fusion; chicken blood cell

Introduction

Two kinds of spikes, HANA (Shimizu *et al.*, 1974) and F (Sheid and Choppin, 1974), have been isolated from the Sendai virus (genus *Paramyxovirus*) envelope. The HANA spike possesses both haemagglutinating and neuraminidase activity (Tozawa *et al.*, 1973; Sheid and Choppin, 1974), while the F spike is involved in cell fusion and haemolytic activity (Homa and Ouchi, 1973; Shimizu *et al.*, 1974; Sheid and Choppin, 1974; Hosaka *et al.*, 1974). The HANA spike controls two processes: one is the adsorption of the virus to the host cells (HA function), and the other, the NA function, is the release of the virus particles from the cells (Hirst, 1959; Palese *et al.*, 1974). After the incubation of CRBC and Sendai virus at 4 °C and temperature shift to 37 °C, haemolysis, controlled by the F spikes (Shimizu *et al.*, 1974), is reported to occur before or after the release of an extra amount of adsorbed virus (Hirst, 1959). However, a quantitative analysis of such sequential reactions has not been attempted.

Both cell fusion and haemolysis have long been considered to be the same phenomena (Howe and Morgan, 1969; Apostolov and Almeida, 1972) but

recently they have been reported to be two different reactions (Homma *et al.*, 1976; Shimizu, Y. K. *et al.*, 1976). Sendai virions harvested shortly after the end of the one-step growth cycle in eggs (hereafter to be referred to as "native virion") did not show any haemolytic activity, whereas they possessed complete cell fusion capability and infectivity (Homma *et al.*, 1976). When native virions were frozen and thawed or sonicated, the envelope was partially damaged resulting in a change in permeability allowing these virions to induce haemolysis (Shimizu, Y. K. *et al.*, 1976). The cell fusion activity of the native virions, however, is not affected by either of these treatments. This information led to the suggestion that the haemolytic particles might be increased among the native particle population simply by increasing the number of cycles of freezing and thawing. Using the above observations and conclusions, the present experiments were designed to quantitatively clarify the interaction between Sendai virus and the cell membrane, using artificially induced haemolytic virions.

Materials and Methods

Virus. The Z strain of Sendai virus (HVJ) was used. Native, non-haemolytic virions were harvested from the chorioallantoic fluid of eggs infected for 20 to 24 hr (Homma *et al.*, 1976). To avoid induction of haemolytic virions, care was taken not to freeze the harvested fluid before use. Haemolytic virions were prepared after a single, or repeated cycles of freezing and thawing (Shimizu, Y. K. *et al.*, 1976).

Erythrocytes. Chicken red blood cells (CRBC) were prepared daily. 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, then resuspended and centrifuged at 1,500 rev/min for 10 min. The packed cells were assumed to be 100% CRBC.

Titration of haemagglutinating activity. Serial twofold dilutions of the virus samples were made in 0.5 ml of PBS, and an equal volume of a 0.5% suspension of CRBC was added. The haemagglutinating titre of the viruses was determined as the reciprocal of the highest dilution which showed complete agglutination.

Assay of neuraminidase activity. Fetuin (Spiro method; Grand Island Biological Co., Berkeley, CA, U.S.A.) was used as the substrate. A viral suspension in 1 ml of PBS was added to 0.1 ml of fetuin dissolved in water (10 mg/ml). The mixture was incubated at 37 °C for 10 min and the titre of the N-acetyl neuraminic acid released was measured by the thiobarbituric acid method (Aminoff, 1961).

Haemolytic reaction. The virus sample (0.1 ml) was added to 2 ml of 2% CRBC and the mixture was incubated at 37 °C for 2 hr. The haemolytic activity was expressed as optical density reading (O.D.) of the released haemoglobin at 540 nm.

Calculation of multiplicity of input viruses. Two different methods were used for the enumeration of virus particles per erythrocyte. One was by means of HA titration, based on the description of Okada *et al.* (1961) which states that the number of virus particles becomes equivalent to the number of CRBC at the maximum dilution of haemagglutination. The other was by measuring the neuraminidase activity of the viruses. In any assay, a lyophilized standard egg-produced virus containing 100 HA units was measured for NA activity in parallel to calibrate the obtained assay value. This value has been shown to be in direct proportion to the number of virus particles.

Calculation of the number of virus particles adsorbed on to CRBC. The number of adsorbed virus particles was obtained by subtracting the number of unadsorbed virus particles after incubation at 4 °C for 1 hr from the total number of input particles. Both the haemagglutinating and neuraminidase activities of the virus particles were used in the assays of input and unadsorbed virus particles.

Calculation of the number of virus particles remaining on or fused with the CRBC membrane after incubation at 37 °C. Three different methods of calculation were employed. The virus-infected

CRBC were incubated at 37 °C for 2 hr and then centrifuged. 1) The supernatant which should contain both unadsorbed and released viruses, was measured with respect to its haemagglutinating and neuraminidase activities to obtain the number of remaining virus particles adhered to the CRBC indirectly. 2) The CRBC pellet was subjected to analysis of neuraminidase activity. The quantitation may be believed to give the number of virus particles remaining on, or fused with, the CRBC membrane directly (Yasuda *et al.*, unpublished data). 3) The number of virus particles fused to the CRBC was calculated by measuring the degree of haemolysis. Further details of this calculation procedure will be given along with the results.

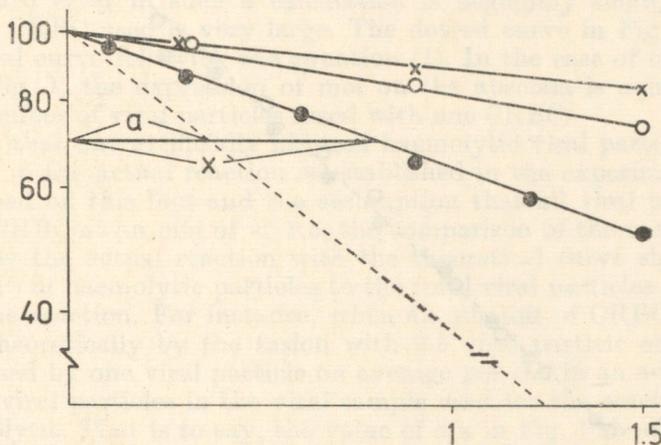


Fig. 1.

Effect of repeated freezing and thawing on production of haemolytic Sendai virus

Abscissa: moi; ordinate: % unlysed CRBC

The early egg harvest was subjected 1 (x), 5 (O) and 18 (●) cycles of freezing and thawing and the kinetics of haemolysis of each sample was examined as described in "Materials and Methods". For further details see text.

Results

Kinetics of haemolysis of CRBC induced by artificially induced haemolytic Sendai virus

The early egg harvest of Sendai virus (native virion) which is known to be non-haemolytic was subjected to freezing and thawing for 1, 5, and 18 cycles, respectively, and the haemolytic activities were compared. An equivalent number of CRBC were physically lysed in distilled water and the O.D. value of the supernatant referred to as 100% haemolysis.

As seen in Fig. 1, although the starting material only contained non-haemolytic particles, the haemolytic population increased with the frequency of freezing and thawing. Also each preparation clearly showed a linear relationship between haemolytic activity and the number of virus particles in the reaction. This fact clearly demonstrates the single hit kinetics between

haemolytic viral particles and CRBC. That is to say, one haemolytic virion is sufficient to lyse one erythrocyte.

When the CRBC were mixed with the virus samples at an moi of < 1.5 , neither neuraminidase activity nor haemagglutinating activity were detected in the supernatant fractions after incubation of the reaction mixture at 37°C for 2 hr.

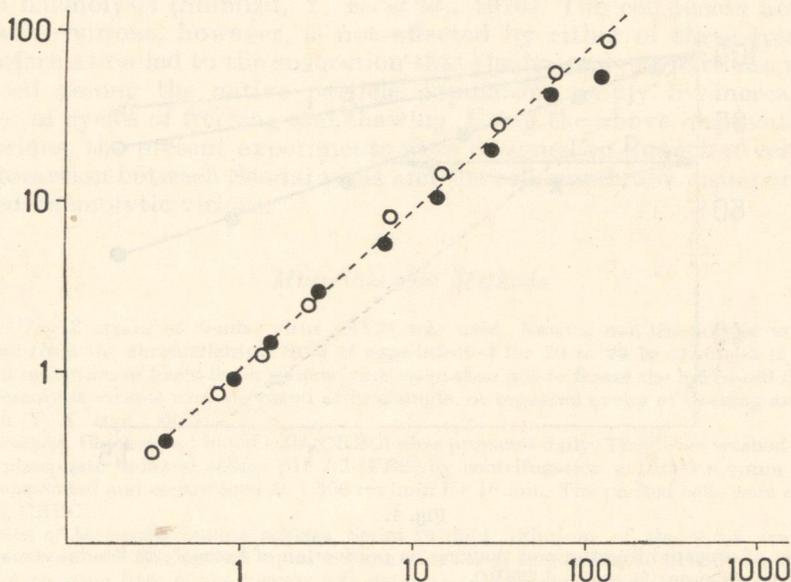


Fig. 2.

Adsorption of Sendai virus to CRBC at 4°C

Abseissa: moi; ordinate: No. of virus particles adsorbed. Half milliliter of 10% CRBC was mixed with 2 ml of native virus particle suspension, kept at 4°C for 1 hr under occasional shaking and centrifuged in the cold at 2,000 rev/min for 5 min. The number of adsorbed viral particles per cell was estimated by subtracting the concentration of the particles left in the supernatant from the original concentration. Both HA (○) and NA (●) activities were measured.

The total input virus suspension used for the reaction surely contained both activities. The observation suggests that almost all of the viral particles fused with CRBC at an moi of below 1.5.

The theoretical curve showing the quantitative relationship between the average number of viral particles fused with one CRBC and haemolysis was obtained under the assumption that 1) all the viral particles in a preparation are haemolytic and 2) one virus particle could lyse one CRBC. On these assumptions, when some percentage (R) of the CRBC was finally lysed by fusing with an average number (m) of viral particles per one CRBC, the probability of the appearance of unlysed CRBC is e^{-m} , according to the

Poisson distribution. The probability of the appearance of lysed CRBC, therefore, is expressed as $1 - e^{-m}$. Then on the basis of the equation of $R/100 = 1 - e^{-m}$, the average number (m) of viral particles fused with one CRBC will be calculated as follows:

$$m = - \frac{\log(1 - R/100)}{\log e} \quad (1)$$

The standard error in such a calculation is negligibly small, because the number of CRBC used is very large. The dotted curve in Fig. 1 represents a theoretical curve following the equation (1). In the case of the theoretical curve in Fig. 1, the expression of m on the abscissa is equivalent to an average number of viral particles fused with one CRBC.

The fact that one artificially induced haemolytic viral particle could lyse one CRBC in the actual reaction is established in the experiment described above. Based on this fact and the assumption that all viral particles could fuse with CRBC at an m of < 1.5 , the comparison of the curves in Fig. 1, obtained by the actual reaction with the theoretical curve should indicate the ratio (P) of haemolytic particles to the total viral particles in the sample used for the reaction. For instance, when an amount of CRBC which could be lysed theoretically by the fusion with 0.5 viral particle on average per cell was lysed by one viral particle on average per cell in an actual reaction, $1/2$ of the viral particles in the viral sample used for the reaction should be non-haemolytic. That is to say, the value of a/x in Fig. 1 should express the value of P . On such a basis, $1/7$ of the particles were found to become haemolytic after a single cycle of freezing and thawing, and $1/2$ after 18 cycles. When the standard suspension of CRBC mixed with a viral sample in which the value of P was apparent were lysed as a result of fusion with an average number m of the haemolytic virions per cell after incubation at 37°C , the average number (N) of fused viral particles per cell should be expressed as

$$N = m/P = - \frac{\log(1 - R/100)}{P \log e} \quad (2)$$

According to equation (2), the average number of viral particles fused with one CRBC (N) should be obtained by knowing the percentage of CRBC in a reaction lysed by incubation with a viral sample in which the value of P was apparent. However, as described above, the experiment was conducted on the assumption that all viral particles could fuse with CRBC at an m of < 1.5 in an actual reaction.

The following experiments were done to confirm the validity of equation (2) and to quantitatively clarify the interaction between viral particles and CRBC at higher m .

Quantitation of the amount of virus per CRBC before and after incubation at 37°C

The number of adsorbed virus particles was first calculated by increasing the m beyond 1.5 up to 500. Fig. 2 clearly shows a linear increase of adsorbed

virus particles per cell in relation to an increase of input virus (moi). The two different methods of quantitation of the virus in the supernatant (HA and NA titration) gave closely comparable values at each point. This experimental series also showed that the number of unadsorbed viruses could be detected in the supernatant when the moi was > 20 .

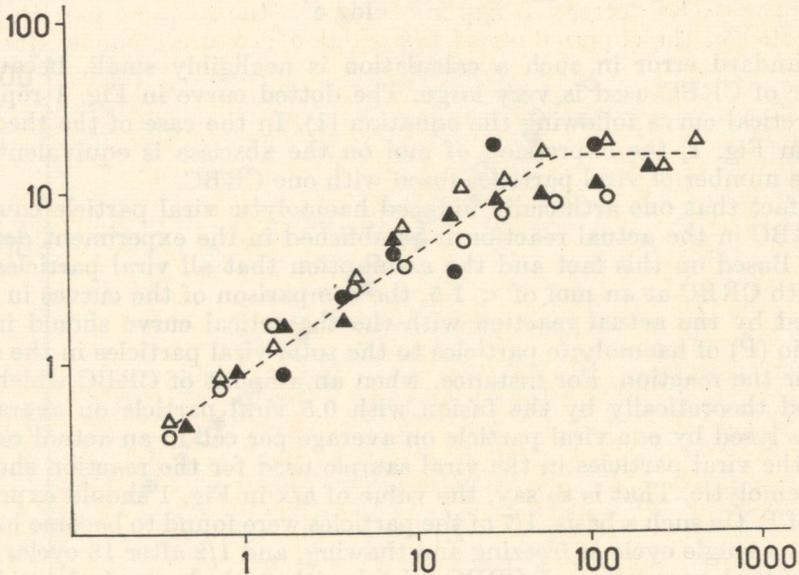


Fig. 3.

Number of virus particles fused with or remaining on the CRBC membrane after incubation at 37 °C

Abscissa: moi; ordinate: No. of virus particles fused CRBC with virus adsorbed as described in Fig. 2 were washed once, resuspended in PBS and incubated at 37 °C for 2 hr. The number of viral particles fused with or remaining on the CRBC membrane after incubation was estimated by haemolytic activity (Δ), haemagglutinating activity (\circ), neuraminidase activity in the supernatant (\bullet) and neuraminidase activity on CRBC membrane (\blacktriangle).

To confirm the validity of equation (2), the procedures described in Materials and Methods were employed to calculate the number of viral particles remaining on or fused with one CRBC membrane after incubation of the reaction mixture of CRBC and a viral sample at 37 °C for 2 hr (Fig. 3). As a result, three different procedures for virus quantitation gave almost equivalent values. The quantitation also clarified the fact that the number of the viruses which stayed on the cell membrane after incubation of the reaction mixture at 37 °C for 2 hr was limited and that the number of virus particles per cell never exceed 20, despite increases in virus input. Released virus particles from CRBC membrane after incubation at 37 °C could be detected when the moi was > 2 .

The overall quantitation in the interaction between Sendai virus and CRBC membrane is illustrated in Fig. 4. Although the number of adsorbed particles at 4 °C increased with increasing moi, unadsorbed virus appeared when the moi was over 20. When the temperature was raised to 37 °C, almost adsorbed particles were fused with the CRBC membrane within an moi or 2, but beyond this moi the number of fusing particles among the adsorbed particles decreased. Beyond moi of 2, the released particles could be detected in the supernatant of the reaction mixture incubated at 37 °C.

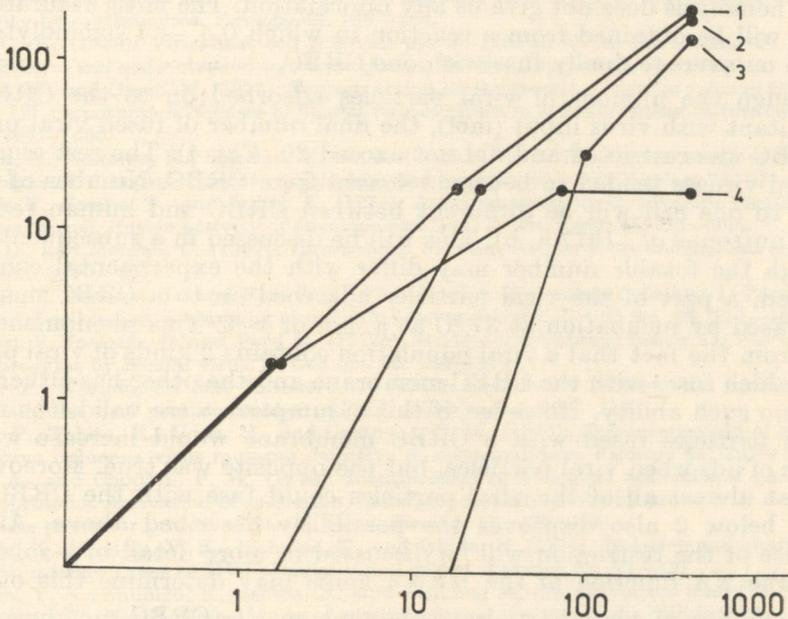


Fig. 4.

Quantitation of the interaction between Sendai virus and CRBC membrane. Number of adsorbed virus particles at 4 °C (curve 1), unadsorbed particles at 4 °C (curve 3), fused particles at 37 °C (curve 4), and released particles at 37 °C (curve 2) (ordinate) plotted against moi (abscissa).

Discussion

Clavell and Bratt (1972a, b), in a study on haemolysis, suggested that a single virus particle is capable of causing haemolysis. Our observations with artificially induced haemolytic virus particles are in good agreement with theirs (Fig. 1). This fact also indicates that artificially induced haemolytic virus particles could react with the cell membrane in the same manner as native virions.

Based on this fact, a method of enumerating the absolute number of virus particles fused with one CRBC was developed (equation 2). The accuracy of the method was repeatedly shown by equivalent calculation methods (Fig. 3), and this immediately indicates the validity of the assumption that all the viral particles could fuse with the CRBC at an moi of < 1.5 .

To enumerate the absolute number of viral particles fused with one CRBC membrane by this method, the CRBC must not be lysed perfectly by the virus sample to be tested. That is to say, the virus sample in which the number of haemolytic viral particles is large enough to induce the CRBC into perfect hemolysis does not give us any information. The most accurate information will be obtained from a reaction in which 0.5 — 1 haemolytic viral particle on average finally fuse with one CRBC.

Although the number of viral particles adsorbed on to the CRBC was concomitant with virus input (moi), the final number of fused viral particles per CRBC was restricted and did not exceed 20 (Fig. 4). The rest amount of adsorbed viruses tended to become released from CRBC. Number of viruses fusible to one cell will be different between CRBC and human red blood cells (Knutton *et al.*, 1977 a, b). This will be discussed in a subsequent paper. Although the fusible number may differ with the experimental conditions employed, a part of the viral particles adsorbed on to a CRBC membrane are released by incubation at 37 °C at a moi of > 2 . This phenomenon may result from the fact that a viral population contains 2 kinds of viral particles one of which fuses with the CRBC membrane and the other has either a very low or no such ability. However, if this assumption were valid, the number of viral particles fused with a CRBC membrane would increase with the number of adsorbed viral particles, but the opposite was true. Moreover, the fact that almost all of the viral particles could fuse with the CRBC at an moi of below 2 also disproves the possibility described above. Although the cause of the restriction will be discussed in more detail in a subsequent paper, the NA function of the HANA spike may determine this outcome.

The number of viral particles remaining on the CRBC membrane after incubation at 37 °C, was enumerated by measuring the neuraminidase activity associated with the CRBC (Fig. 3). The number closely compared with the number of viral particles left on the cell calculated by subtracting the amount of released HA and NA from adsorbed HA and NA, and with the number of fused particles on CRBC membrane estimated by the extent of haemolysis of CRBC (Fig. 3). The former result may implicate that the neuraminidase activity associated with the original number of viral particles fused with the CRBC was shown to be translocated on the CRBC membrane with no reduction in biological activity. Bächli *et al.* (1973), using an immuno-freeze-etching method, and Y. K. Shimizu *et al.* (1976), using the ferritin-labelled antibody technique, demonstrated that the viral envelope antigen of Sendai virus was dispersed on to the membrane of human erythrocytes when the virus fused with the erythrocytes. The results of our experiments not only biologically support these observations but also indicate that the transfer of HANA spikes is proved not only by HA assay but also by NA assay.

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