Haemolysis by Sendai Virus: Lack of Requirement for Neuraminidase

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Since both neuraminidase and the haemolytic activity of Sendai virus were inhibited by Cu²⁺ ions, it was suggested that neuraminidase was essential for haemolysis (Howe & Morgan, 1969). Haemolysis by Newcastle disease virus (NDV) appeared to be intimately associated with the capacity of the virus to become irreversibly attached to the cell (Burnet, 1950), indicating that neuraminidase, responsible for elution of the virus from red blood cells (RBC), was perhaps not required for, or may even have hindered haemolysis.

The results of this study, aimed at the elucidation of the role of neuraminidase in Sendai virus-induced haemolysis, show that: (1) treatment of the virus by disodium ethylenediaminetetraacetate (EDTA) or hydroxyethylethlenediaminetriacetate (HEDTA) causes a simultaneous decrease in neuraminidase activity and an increase in haemolytic activity of the virus. (2) 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DDANA), a reversible inhibitor of neuraminidase (Meindl & Tuppy, 1969; Meindl et al. 1971), does not interfere with, but augments haemolysis.

It has been determined previously that incubation of different influenza viruses in the absence of Ca²⁺ ions (Dimmock, 1971) or in solutions of EDTA (Boschman & Jacobs, 1965) gradually inactivated the neuraminidase. We observed a similar effect with Sendai and B/MASS/66 influenza virus.

Equal volumes of pre-warmed (37 °) solutions of 0.2 M-EDTA, pH 7.0, and of either Sendai (1024 H.A.U./ml.) or B/MASS/66 influenza (128 H.A.U./ml.) viruses, which had been partly purified from allantoic fluid by centrifuging at high speed (Spinco rotor 21, 21,000 rev./min. for 90 min.) and resuspended in 0.14 M-NaCl, 0.001 M-CaCl₂, 0.01 M-tris, pH 7.0 (TB), were mixed and incubated at 37 °. Periodically, 0.2 ml. samples were withdrawn, mixed with 0.1 ml. of the substrate solution (1 mg./ml. of N-acetylneuraminic acid lactose (NANLA) in TB) and incubated at 37 °. The amount of released N-acetylneuraminic acid (NANA) was determined by the method of Aminoff (1961), except that 0.1 ml. of 1 M-MgCl₂ and 0.2 ml. of 1 N-H₂SO₄ were added to the samples before periodate was added. As shown in Fig. 1, the percentage of residual neuraminidase activity (calculated from calibration curves relating the amount of released NANA to the concentration of intact viruses) decreased during incubation of the viruses in 0.2 M-EDTA.

The Sendai and B/MASS/66 viruses treated for 30 min. with EDTA were sedimented by centrifuging, resuspended in 0.075 M-phosphate, 0.072 M-NaCl, pH 7.2 (PBS) to a concentration of 1000 to 6000 H.A.U./ml., mixed with an equal volume of 10 to 20 % chick RBC at 0 ° and kept at this temperature for 30 min. Only about 5 % of the adsorbed viruses was eluted from the RBC within 12 hr of subsequent incubation at 37 °. Untreated viruses were completely eluted within 1 hr. The same treatment enhanced the haemolytic activity of Sendai virus about 2.5-fold.

HEDTA-treated virus was prepared as follows: 2.5 to 10 × 10⁶ H.A.U. of pelleted Sendai virus were resuspended in a 10 % solution of HEDTA, incubated for 50 min. at 37 ° and then either dialysed against PBS or pelleted by centrifuging at high speed and resuspended in PBS. The HEDTA solution was freshly prepared from a 70 % stock solution (Chas. Pfizer & Co.,
Fig. 1. Kinetics of inactivation of Sendai (○—○) and B/Mass/66 influenza (●—●) virus neuraminidase by EDTA.

Fig. 2. Comparison of the kinetics of haemolysis and virus elution between untreated and HEDTA-treated Sendai virus. ●—●, percentage haemolysis; ○—○, percentage of eluted virus.

Inc., New York) by proper dilution and adjustment of the pH value to 7.0 by adding concentrated phosphoric acid. When NANLA or ovomucoid were used as substrates, the neuraminidase activity of the treated virus was 20 and 13 % respectively, of the activity of untreated virus. The residual neuraminidase was associated with the virus particles, as established by rate zonal centrifugation of the treated virus. The kinetics of neuraminidase inactivation were not studied because of the interference of HEDTA in the determination of N-acetylneuraminic acid (NANA).

To determine the effect of HEDTA on the haemolytic activity of Sendai virus, equal volumes (10 ml.) of a 10 % suspension of chicken RBC and of either untreated or HEDTA-treated virus (1280 H.A.U./ml.) in PBS were mixed at 0°, kept at the same temperature for 25 min. and mixed occasionally. The agglutinated RBC, which had adsorbed more than
95% of the virus, were pelleted, resuspended in 20 ml. of PBS pre-warmed to 37° and then incubated at the same temperature. Periodically, 1 ml. samples were removed and centrifuged for 3 min. at 1500 rev./min. The percentages of lysed RBC and of eluted hemagglutinin (HA) were determined (Neurath & Sokol, 1962). The results shown in Fig. 2 indicate that treatment of the virus by HEDTA enhanced its haemolytic activity and impaired its elution from RBC.

The relationship between the percentage of haemolysis and the concentration of untreated and HEDTA-treated virus, respectively, was investigated. Equal volumes of a suspension of 10% chicken RBC and of serial 1·5-fold dilutions in PBS of the same amounts of untreated or HEDTA-treated Sendai virus were mixed and treated further as described above. Haemolysis was determined after incubation for 2 hr at 37°. The percentage of non-haemolysed RBC was about the same when determined either by comparison of the concentrations of haemoglobin released from virus-treated RBC and from RBC completely lysed in 1% sodium deoxycholate or by direct counting of intact RBC under the microscope.

The following conclusions were drawn from the results presented in Fig. 3.

(1) A linear correlation exists between the log of the concentration of RBC which have not lysed and the concentration of treated virus. This correlation conforms with the Poisson distribution for n = 0. This result suggests, but does not prove, that a single virus particle possessing haemolytic activity is sufficient to lyse one RBC. Since the virus particles display considerable heterogeneity with respect to haemolytic activity (Hosaka, Kitano & Ikeguchi, 1966), direct experimental verification that haemolysis is a 'single-hit' process is not possible at present.

(2) The same correlation for untreated virus is not linear, and the difference between the levels of haemolysis caused by treated and untreated virus increases with increasing virus concentration. This may be ascribed to the faster elution of untreated virus particles from RBC, which may result in either a decreased probability of damage to the RBC membrane or an increased interference by non-haemolytic virus particles in haemolysis (Neurath, 1963).

The attachment of most of the HEDTA-treated virus to RBC membranes appears to be irreversible, since treating the RBC ghosts (obtained by haemolysing 1 ml. of 10% chick

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**Fig. 3.** Dependence of extent of haemolysis on the concentration of Sendai virus.

○---○, untreated virus; ●---●, treated virus.
RBC with 640 H.A.U. of virus) with 100 units of *Vibrio cholerae* neuraminidase for 40 min. at 37° failed to release more than 10 % of the adsorbed virus. However, HA is recovered in the water phase when the ghosts are extracted with diethyl ether in the presence of Tween 80 (1 mg./ml.). The irreversible attachment of the virus to RBC ghosts was confirmed by electron microscopy using the technique described by Howe & Morgan (1969). About the same number of virus particles appeared to be attached to intact RBC, to which virus was adsorbed at 0° and to ghosts obtained by incubating the RBC for 2 hr at 37°. Most of the particles remain intact during their irreversible reaction with RBC membranes. However, some, especially the larger ones, are disrupted, and occasionally virus nucleoprotein strands are seen inside lysed RBC. It is possible that only the disrupted virus particles are actually involved in haemolysis, in agreement with observations of K. Apostolov (personal communication).

The irreversible adsorption of B/MASS/66 influenza virus to RBC does not lead to haemolysis.

The above results do not show whether or not the residual neuraminidase associated with the treated virus may be required for haemolysis. To clarify this question, the effect of DDANA on the neuraminidase and haemolytic activities of Sendai virus was investigated. Two-tenths of a ml. of solutions containing various amounts of DDANA were mixed with 0.1 ml. of the substrate solution (1 mg./ml. of NANLA in TB) and with either 0.05 ml. of a virus suspension (4000 H.A.U./ml. in TB) or with 0.05 ml. of TB (= blanks). The amount of NANA released during 30 min. incubation of the samples was determined by the method of Aminoff (1961) and the percentage of residual neuraminidase was calculated. The neuraminidase activity corresponding to 200 H.A.U. of Sendai virus was decreased by 50 % in $4 \times 10^{-5}$ M-DDANA. About 100 times higher levels of DDANA were needed for complete inhibition of the enzyme (Fig. 4). Similar results were obtained with HEDTA-treated virus or with ovomucoid as substrate instead of NANLA. The haemolytic activity of HEDTA-treated virus (200 H.A.U.) was further enhanced by about 10 % in the presence of DDANA ($4 \times 10^{-3}$ M); that of untreated virus was also increased by DDANA ($> 10^{-3}$ M) and the correlation between the log of the concentration of unlysed RBC and the amount of untreated

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**Fig. 4.** Inhibition of Sendai virus neuraminidase by DDANA.
virus was linear. These results suggest that neuraminidase is not essential for Sendai virus-induced haemolysis.

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