Association of Sendai virus with esterase and leucine aminopeptidase activity; its probable relationship to “haemolysin”

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(Z. Naturforsch. 19 b, 810—814 [1964]; eingegangen am 27. April 1964)


It has been suggested¹ that the release of lysosomal enzymes may explain some of the biochemical changes found in virus-infected cells and may contribute to the cytopathic effects of some viruses. Since concentrated lysosomal preparations hydrolyse tissue fractions², and the lysosomal enzymes β-galactosidase, β-glucuronidase and β-glucosaminidase were reported to cause antigenic changes of erythrocyte surface antigens or even haemolysis,³,⁴ it might be possible that the haemolytic (cytolytic) activity of some viruses results from their association with a lysosomal enzyme (lysosomal enzymes). Recently data suggesting the identity of haemolysin with an enzyme splitting diisopropylfluorophosphate (DFP) were published⁵.

In the present paper the release of lysosomal enzymes occurring in chorioallantoic membranes of chick embryos infected with Sendai virus and the effect of administration of agents, known to influence the stability of lysosomes, on the haemolytic activity of the virus progeny was studied. Furthermore experiments were carried out to find whether the enzymes under investigation become associated with the virus particles. A brief account of the results presented below was already⁶ published.

Materials and methods

Ten days old chick embryos were infected with approximatively 10⁶ EID₅₀ of Sendai virus and incubated at 35 °C. To account for the changes of the level of lysosomal hydrolases occurring during the course of embryonic development⁷, uninfected control embryos were included in the experiment. The incubation of groups consisting each of 5 embryos was interrupted 16, 20, 24, 29 and 37 and 48 hours, respectively, after infection, and after chilling for approximately 8 hours at 4 °C, the allantoic fluids (AF) and the chorioallantoic membranes (CAM), corresponding to each group, were harvested separately. The AF were then clarified (3,000 x g, 20 mins.) and centrifuged twice at 58,000 x g for 60 mins. The sediments (= virus + normal “sedimentable” component), resuspended in 1/15 of the original volume of AF with phosphate buffered saline (0.14 M NaCl, 0.007 M phosphate, pH 7.2) (BS) and the supernatant fluids after high speed centrifugation (SAF) were stored for the assay of enzymatic activities. The CAM, washed with BS, were cut with scissors into small pieces, mixed with 7.5 ml. of 0.25 molar sucrose, homogenized with quartz sand and centrifuged at 15,000 x g for 20 mins. The sediments were discarded and the supernatant fluids (SH) stored for further analysis.

For the estimation of acid phosphatase, β-glucuronidase and β-galactosidase in the individual samples of SH, suitable aliquots (0.05 — 0.30 ml.) were withdrawn, mixed with 0.50 ml. of a solution of the appropriate substrate (0.50 mg/ml.) (phenylphosphate, Lachema; phenolphtalein-β-glucuronide sodium salt, Light; and o-nitrophenyl-β-D-galactopyranoside, Sigma, respectively) and the volume adjusted to 1.5 ml. by addition of 0.1 molar citrate-HCl buffer pH 4.9. After 2 hours incubation at 37 °C, 0.5 ml. of a 5% solution of trichloroacetic acid was added to the samples and the precipitated proteins were sedimented (3,000 x g, 5 mins.). In the supernatant fluids the amount of liberated phenol (addition of 0.1 ml. of phenol-H₂O, 0.1 ml. of a 3% solution of 4-aminoantipyrine and of 0.1 ml. of a 10% potassium ferricyanide solution; optical density (O.D.) read at 530 mµ) phenolphtalein

5. A. R. Neurath, Acta virol. 8, in press.
of 1.5 ml. of 0.1 molar glycine-NaOH buffer pH 10; and L-leucyl-p-naphtylamide hydrochloride (B grade, Calbiochem) as substrates. Suitable aliquots of samples (0.05 – 0.35 ml.) were mixed with 1 ml. of substrate solutions (freshly prepared by mixing 1 part of a 1% stock solution in acetone with 50 parts of distilled water) and the volume was adjusted to 2 ml. by addition of 0.1 molar phosphate buffer pH 7.2. The assay of esterase and amidase activities was performed at the absence or the presence of DFP (final concentration 2 x 10^-5 molar solution of DFP to 2 ml. of the reaction mixture). After 1 hour's incubation at 37 °C the reaction was stopped by addition of 0.5 ml. of a 2 molar citrate-HCl buffer pH 4.2 to the samples. The amount of enzymes was determined spectrophotometrically at 545 (525) mμ 40 minutes after further addition to the samples of 0.5 ml. of a solution of o-aminoazotoluene diazonium chloride (C grade, Calbiochem; 3 mg/ml. in 10% Tween 20). The O.D. was read against controls containing the substrates and reagents only.

The haemagglutination (HA) titers and the haemolytic activity of Sendai virus were determined using the methods already published 8, 9.

The conditions of density gradient centrifugation were identical with those published recently 10, except that fractions of 0.30 ml. each were collected.

The effect of agents known to influence the stability of lysosomes and to act on the surface of cells and subcellular particles, on the haemolytic activity of Sendai virus, obtained by high speed centrifugation of lysosomes and to act on the surface of cells and subcellular particles, on the haemolytic activity of Sendai virus, was studied as follows: 0.1 ml. of suspensions (solutions) of hydrocortisone acetate (Spofa Prague, 10 mg/m.l.), A vitamin acetate (Spofa, 50,000 m.u./m.l.), A vitamin (Hudson Vitamin Products, Inc. N.Y., derived from fish liver oil), histamine (Organofarma Prague, 2.5 mg/m.l.); sodium deoxycholate (50 μg/ml.) and BS (= control) were administered intraaallantoically to groups each consisting of 5 embryos, 16 hours after infection with the virus. The HA titre of the purified virus suspensions, prepared separately from each embryo, was adjusted to the same level by appropriate dilution with BS and then tested for haemolytic activity.

The inactivation of Sendai virus by heat (50 °C, 20 mins.), formaldehyde (at pH 9.2, 12 mins. at 37 °C) and phenylisothiocyanate was performed as described in detail elsewhere 11. The conditions of treatment of Sendai virus by ether were identical with those already published 12. The water layer together with the interphase precipitate were used for the assay of haemolytic, esterase and leucine aminopeptidase activities.

To test whether esterase and leucine aminopeptidase activities were adsorbed onto red blood cells (RBC) together with viral haemolysin, 0.4 ml. aliquots of a suspension of Sendai virus (16,000 HA/ml.) were mixed with 6 ml. of RBC suspensions (5, 2.5 and 1.25%, respectively) at 4 °C. After standing for 30 mins. at the same temperature, the suspensions were centrifuged at 1,000 x g for 5 mins. and the virus (+ host components) not adsorbed onto RBC from the supernatant fluid was concentrated by high speed centrifugation (80,000 x g, 60 mins.), and then the haemolytic and enzymatic activities of the sediments were determined. Thus the proportion of activities adsorbed onto RBC could be calculated.

**Results**

The administration to the infected chick embryo of A vitamin or sodium deoxycholate, which are known to enhance the release of lysosomal hydrolyses 13-15 and to act on cellular membrane systems 16-20, and of histamine results in an increased haemolytic activity of the virus progeny (Fig. 1). On the other hand, administration of hydrocortisone, which increases the in vitro and in vivo stability of lysosomes 21-24 and suppresses in

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8 A. R. NEURATH and F. SOKOL, Acta virol. 6, 66 [1962].
9 A. R. NEURATH, Acta virol. 6, 412 [1962].
11 A. R. NEURATH, Acta virol. 8, 143 [1964].
12 F. SOKOL and A. R. NEURATH, Acta virol. 6, 122 [1962].
19 H. A. BLOUGH, Virology 19, 349 [1963].
20 H. A. BLOUGH, Virology 19, 112 [1963].
flammatory reactions in chick embryos infected with influenza viruses, reduces the haemolytic activity. This result is compatible with previous suggestions that haemolytic activity is not an invariable property of "haemolytic" myxoviruses and expresses changes which have occurred in host cells as a consequence of viral infection, and supports the assumption that the formation of "haemolytic" virus particles might be in some relation to the release of lysosomal hydrolases.

In the course of infection by Sendai virus of chick embryos, an increased release of the lysosomal enzymes: acid phosphatase, \( \beta \)-glucuronidase and \( \beta \)-galactosidase, as well as of esterase could be demonstrated in homogenates of CAM (Fig. 2). The release of lysosomal enzymes occurring in vivo in infected cells as compared with non infected controls may be even more pronounced, as the method of homogenization possibly involves an artificial release of enzymes caused by mechanical trauma of lysosomes.

As no \( \beta \)-glucuronidase or \( \beta \)-galactosidase activities, which hypothetically might be involved in the destruction of RBC, could be demonstrated in the resuspended sediments obtained after high speed centrifugation of infectious or control allantoic fluids, further experiments were confined to the estimation of esterase and leucine aminopeptidase activities, which probably take part in the process of cell destruction. It is evident from Fig. 3, that in the course of infection an increase of esterase and leucine aminopeptidase activities, concomitant with a raise of haemolytic/H.A activity, could be demonstrated in both sediments and supernatant fluids obtained after high speed centrifugation of allantoic fluids. The higher ratio of aminopeptidase/esterase, and esterase resistant to DFP/esterase inhibitable by DFP activities of the sediments in comparison with those of the supernatant fluids, the esterase activity of the latter being completely inhibited in the presence of \( 10^{-6} \) molar DFP, indicates that we are dealing more than with a single enzyme splitting \( \beta \)-naphthylacetate. No acetylcholine splitting activity could be demonstrated in the sediments.

The following points indicate that at least a fraction of the virus particles becomes associated with esterase and leucine aminopeptidase activities:

25 E. D. Kilbourne, J. Immunology 74, 57 [1955].
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Fig. 3. Haemolytic, esterase and leucine aminopeptidase activities in sediments and supernatant fluids after high speed centrifugation of allantoic fluids. Empty columns = controls; shaded columns = infected embryos. 1. Haemolytic activity of sediments; 2. Esterase activity of sediments; black parts = activity not inhibited in the presence of DFP (10^-6 molar). 0.15 ml. aliquots were used for the assay of enzymatic activity. 3. Aminopeptidase activity of sediments (0.15 ml. aliquots). 4. Esterase activity of supernatant fluids. Black parts = leucine aminopeptidase activity. (0.10 ml. aliquots.)

1. A part of both enzymatic activities is adsorbed onto RBC together with the virus (Table 1). Moreover, qualitative evidence was found for the elution of both esterase and aminopeptidase from RBC.

2. Density gradient centrifugation experiments revealed that a part of enzymatic activities sedimented together with the virus (Fig. 4), i.e. at a higher sedimentation rate in comparison with that of the normal "sedimentable" component of allantoic fluid or with that of a virus preparation treated with sodium deoxycholate.29

Table 1. Adsorption of haemolytic, esterase and leucine aminopeptidase activities onto RBC.

<table>
<thead>
<tr>
<th>Final concentration of RBC [%]</th>
<th>Percentage of activities adsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Haemolysin</td>
</tr>
<tr>
<td>4.7</td>
<td>89</td>
</tr>
<tr>
<td>2.3</td>
<td>86</td>
</tr>
<tr>
<td>1.2</td>
<td>81</td>
</tr>
</tbody>
</table>

Fig. 4. Distribution of leucine aminopeptidase (--- ● ---), HA (--- ○ ---) and haemolytic (--- ■ ---) activities in fractions obtained after density gradient centrifugation of sediments from high speed centrifugation of infected (3), and control (2) allantoic fluids, and of a preparation of Sendai virus treated by sodium deoxycholate and filtered through a column of Sephadex G 2529 (1). Fraction 1 — bottom; fraction 15 — top. Analogous results were obtained for the distribution of esterase activities.

Treatments known to cause a loss of haemolytic activity of Sendai virus, impaired both esterase and leucine aminopeptidase activities (Table 2).

Loss of activities in percents (original activity = 100%)

<table>
<thead>
<tr>
<th>Treatment with</th>
<th>Haemolysin</th>
<th>Esterase</th>
<th>Leucine aminopeptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat</td>
<td>99</td>
<td>56</td>
<td>22</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>99</td>
<td>41</td>
<td>39</td>
</tr>
<tr>
<td>Phenylisothiocyan.</td>
<td>100</td>
<td>51</td>
<td>35</td>
</tr>
<tr>
<td>Ether</td>
<td>100</td>
<td>51</td>
<td>36</td>
</tr>
</tbody>
</table>

Table 2. Comparison of the rate of inactivation of haemolytic, esterase and leucine aminopeptidase activities by various treatments.

Discussion

Previous investigations revealed that the component responsible for the haemolytic activity of some myxoviruses is of proteinaceous nature, probably an enzyme splitting DFP. The specific association of this component with the virus particle seems to be a condition for the manifestation of haemolytic activity. The association of Sendai virus particles with esterase and leucine aminopeptidase activities, experimentally evidenced in the present paper, offers an attractive explanation for the haemolytic activity of the virus particles, since an aminopeptidase with esterolytic properties, which is not inhibited by DFP, seems to be involved in cytolysis. Increased aminopeptidase and esterase activity could be demonstrated histochemically in wounded, inflammatory, necrotic and neoplastic tissues and a relationship between tumor invasion and aminopeptidase activity was suggested. In this paper evidence is given for the increase of both enzymatic activities resulting from the infection of CAM with Sendai virus. It seems to be probable that virus particles become associated with these enzymes during the process of emergence from host cells.

For a further study of the haemolytic (cytolytic) activity of some myxoviruses, the biochemical changes occurring with cell membranes and preceding lysis have to be studied. Experiments are now in progress to study the substrate specificity of the enzymes under investigation and to characterize them by means of starch gel electrophoresis.

Acknowledgements: The author is much indebted to Prof. De Bernardi from the University of Trieste, Italy, for the gift of phenolphtalein-β-glucuronide and o-nitrophenyl-β-D-galactopyranoside.

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