The Neuraminidase of Human Parainfluenza 1 Virus (HA2 Virus)

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SUMMARY

Neuraminidase activity could be demonstrated in highly concentrated preparations of human parainfluenza 1 virus, strain C 35 (HA2 virus). Among the substrates used, the most suitable are N-acetyl neuramin lactose and fetuin. Mucin type I and type II were not hydrolysed. The neuraminidase exhibited some characteristics similar to those of the other paramyxoviruses (Sendai, NDV, mumps, human parainfluenza 2 virus): the optimum pH ranged between 5 and 5.4, and the $K_m$ value was $5 \times 10^{-3}$ M when tested with N-acetyl neuramin lactose. Its optimum activity was between 37 and 40 °C and it was thermostable, the enzymic activity being reduced to 50% in 5 min at 45 °C and entirely destroyed at 50 °C in the same period. The thermal inactivation constants of neuraminidase and haemagglutinin and the temperature which inactivated 50% of both these activities were very similar to those already shown for NDV. Haemagglutinin and neuraminidase activities were rapidly destroyed by ionic detergents, but not by non-ionic detergents.

INTRODUCTION

Human parainfluenza 1 virus, strain C 35 (HA2 virus) exhibits particular biological properties: a pathogenicity restricted to the human species, a very narrow host range in vitro and specific envelope antigens except for a very slight degree of cross reactivity between its haemagglutinin and that of Sendai virus. In complement fixation tests, a great similarity could be found between these two viruses, since the Sendai antiserum showed the same antibody titre with the HA2 strain as with the Sendai strain.

The neuraminidase activity of Sendai virus is easy to demonstrate, and its properties as well as its structure have been elucidated (Tozawa, Homma & Ishida, 1967; Maeno et al. 1970; Mountcastle, Compans & Choppin, 1971; Homma & Ohuchi, 1973; Scheid & Choppin, 1974; Famulary & Fleissner, 1976). To extend our knowledge of human parainfluenza 1 we found it of interest to detect and study the neuraminidase activity. Enzymic activity of HA2 strain was detectable in highly concentrated and purified virus preparations and its principal characteristics were determined: substrate specificity, influence of pH and temperature, incubation time, effects of detergents. At the same time, the effects of these various factors were studied on the haemagglutinin in order to establish a correlation between these two activities, both carried by the glycoprotein spikes of the virus envelope (Scheid et al. 1972).
**METHODS**

*Virus.* HA2 virus was derived from the Chanock strain of human parainfluenza I virus, isolated by Chanock *et al.* (1958) under the name of 'haemadsorbing virus'.

**HA2 virus production and purification.** The virus was grown on primary kidney cell cultures from Macacus rhesus in 199 medium without calf serum. Heat-inactivated rabbit anti-SV5 antiserum was added to the medium to prevent contamination by this virus. The cultures were infected with 0.1 to 1 TCD50 of virus per cell. The virus was harvested in the culture medium 5 to 7 days after incubation at 33 °C. Large debris were removed by centrifugation at 3500 rev/min for 30 min at 4 °C.

The virus was pelleted from the supernatant by centrifugation at 100000g for 1 h at 4 °C. The pelleted virus was taken up in 1/50 of the initial volume of 0.01 M-phosphate buffer pH 7.2, and submitted to a second cycle of centrifugation at low and high speed. The virus pellet was then taken up in 1/1500 of the initial volume of 0.01 M-phosphate buffer, pH 7.2. The concentrated virus was used as such or submitted to two cycles of purification on linear sucrose gradients [22 to 60 % (w/w) for 4 h at 120000g at 4 °C]. The purified virus was 1000- to 1500-fold concentrated compared with the initial suspension, and its protein concentration was about 1 mg/ml.

*Neuraminidase and neuraminidase inhibition test (NI).* Neuraminidase (EC. 3.2.1.18) tests were performed according to Aymard-Henry *et al.* (1973) in 0.1 M-citrate–0.2 M-phosphate buffer, pH 5.2.

Four substrates were tested: N-acetyl neuramin lactose (Sigma, 85 % of α 2 → 3 bonds and 15 % of α 2 → 6 bonds), mucin I from bovine submaxillary glands (Sigma, 5 % of sialic acid), mucin II from porcine stomach (Sigma, 1 % of sialic acid), and fetuin (prepared as described by Han & Puck, 1962). N-acetyl neuramin lactose at 1 mg/ml was chosen as substrate for all enzymic studies except for neuraminidase inhibition which was tested using fetuin at 20 mg/ml. Incubation was at 37 °C for 18 h except for a few experiments (Km determination for example).

Neuraminidase activity was expressed in terms of dilution [the last dilution giving an E549 > 0.5 due to released N-acetyl neuraminic acid (NANA)] or in terms of enzymic activity (nmol NANA/min/mg virus protein).

Anti-neuraminidase antibodies were titrated by the enzyme inhibition test with fetuin as substrate following the procedure used for myxoviruses (Aymard-Henry *et al.* 1973). The serum titre corresponded to the reciprocal of the last serum dilution able to inhibit 50 % of enzymic activity.

*Haemagglutination (HA) and haemagglutination inhibition test (HI).* These tests were performed by a microtitre method. A mixture of the specimen to be tested, 5 × 10–8 M-PO4, 0.15 m-NaCl buffer and 1 % guinea-pig red blood cell suspension (total volume 0.15 ml), was incubated at room temperature for 1 h in Cooke microtitre plates.

HI was measured by adding 4 HAU of virus to doubling dilutions of antiserum. After 1 h the guinea-pig red blood cell suspension was added and the test read after a further hour. The HI titre was expressed as the reciprocal of the highest dilution giving 50 % inhibition.

*Preparation of antisera.* Antisera were prepared in rabbits. The animals received two intramuscular inoculations with 108 TCD50 of purified virus with Freund’s adjuvant at an interval of 1 month. Three weeks after the second inoculation they were bled.

*Protein estimation.* Virus protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.
HA2 neuraminidase

Fig. 1. Effect of pH on HA2 virus neuraminidase activity. Samples were incubated with N-acetyl neuramin lactose (0·25 mg/ml final concentration) and citrate-phosphate buffer for 18 h at 37 °C. Results are expressed as % of the enzyme activity obtained at the optimum pH (50 nmol NANA released).

RESULTS

Neuraminidase study

Neuraminidase activity was found in concentrated preparations but could not be detected in crude virus suspensions. It was necessary to concentrate as much as 1000- to 1500-fold. The specific neuraminidase activity of HA2 virus was about 15 nmol of NANA released/min/mg virus protein, using N-acetyl neuramin lactose as substrate.

Substrate specificity

Four substrates were tested. Optimal activity was obtained with N-acetyl neuramin lactose (1 mg/ml). If this value was considered to be 100%, the neuraminidase activity was only 30% with fetuin (20 mg/ml) and was absent with mucin types I and II. This specificity allowed us to assume that HA2 virus neuraminidase hydrolysed the $\alpha 2 \rightarrow 3$ bonds present in neuramin lactose and fetuin while it did not hydrolyse the $\alpha 2 \rightarrow 6$ bonds present in mucin types I and II.

Optimum pH range

For this study a citrate (0·1 M)-phosphate (0·2 M) buffer with pH values ranging from 2·3 to 7·4 was chosen. The optimum pH for HA2 neuraminidase activity was found to be 5 to 5·4 (Fig. 1). This enzyme was very sensitive to acid pH (less than 10% activity at
Fig. 2 Kinetic study of HA2 virus neuraminidase. Samples were incubated with N-acetyl neuramin lactose (0.25 mg/ml final concentration) and citrate-phosphate buffer, pH 5.2, at 37 °C. Results are expressed as % of the maximum of NANA released (50 nmol at 18 h).

Fig. 3 Effect of incubation temperature on HA2 virus neuraminidase activity. Experiments were run for 6 h with N-acetyl neuramin lactose (0.25 mg/ml final concentration) as substrate in citrate-phosphate buffer, pH 5.2.

pH 4.2). Fifty % neuraminidase activity was observed at pH 4.7 and 6.4. pH 5.2 was chosen for all subsequent enzymic studies.

Kinetic study of enzyme activity

The NANA released from N-acetyl neuramin lactose at various incubation times at 37 °C is shown in Fig. 2. The initial reaction velocity was 140 nmol of released NANA/min/mg virus protein. This velocity was constant for the first 2 h and then decreased to reach 50 nmol/min/mg virus protein at the 6th hour. By this time, the amount of released NANA was 75 % of the total amount which was released after 18 h incubation. This amount remained stable at least until 24 h.

Influence of temperature

Neuraminidase activity was tested after 6 h incubation in a water bath at temperatures ranging from 18 to 55 °C, from samples diluted to release a maximum amount of 50 nmol NANA. The optimum temperature for the reaction ranged from 37 to 40 °C. Fifty % of the neuraminidase activity was observed at 24 and 44 °C (Fig. 3).

Thermal inactivation of neuraminidase

Samples diluted to release a maximum of 50 nmol NANA after 18 h incubation were heated at temperatures ranging from 37 to 50 °C for 0 to 20 min and then chilled to 4 °C before being incorporated into the reaction. The residual enzymic activity of the samples in relation to the thermal inactivation time at a given temperature is shown in Fig. 4(a).

The data collected during periods of exponential decline in activity are presented in terms of the rate constant defined in the following equation for a first order inactivation:
Fig. 4. Heat inactivation of (a) neuraminidase and (b) haemagglutinin activities. Virus samples were diluted to give sufficient activity to release 50 nmol NANA from N-acetyl neuramin lactose. Samples were incubated at the temperatures indicated and immediately placed in an ice-bath prior to assay. Results are expressed as log₁₀ residual activity. Numbers in brackets are rate constants.

\[ 2.3 \log_{10} \left( \frac{N}{N_0} \right) = -kt, \]

where \( N_0 \) represents the original activity and \( N \) the activity at some time, \( t \), after heating; \( k \) is expressed as min⁻¹ (Fig. 4(a)). The temperature which inactivated 50% of the virus-bound enzyme after 5 min exposure was 45 °C. The activity totally disappeared after 5 min exposure at 50 °C.

**Variation of reaction velocity with substrate concentration**

Determination of the Michaelis constant (\( K_m \)) of the reaction, after 2 h incubation so as to be in the initial velocity phase, using concentrations of N-acetyl neuramin lactose ranging between 0.1 and 2 mg/ml, is shown in Fig. 5. The \( K_m \) value of HA2-bound neuraminidase for neuramin lactose, obtained by Lineweaver-Burk plots, was \( 5 \times 10^{-8} \) M and the maximal velocity of reaction was 345 nmol of NANA released/min/mg virus protein.

**Effect of ethylenediaminetetra-acetate (EDTA)**

A chelator such as EDTA inhibited enzymic activity. Fifty % inhibition was obtained with EDTA at a concentration of 6.25 mM (Fig. 6).

**Effect of detergents**

The effects of ionic detergents such as sodium deoxycholate (DOC) and sodium sarcosyl sulphate (SSS) and non-ionic detergents such as Triton X-100 and Nonidet P-40 were investigated. The ionic detergents, even at a very low concentration (0.1% and over), inhibited at least 75% of the enzymic activity while the non-ionic detergents did not affect it.
Antigenic characterization of HA2 virus neuraminidase

The low mol. wt. of N-acetyl neuramin lactose does not allow the study of virus neuraminidase inhibition by specific antibodies (Drzeniek, 1972) so this substrate was replaced by fetuin which enabled us to detect about 30% of the activity obtained using N-acetyl neuramin lactose. Several sera were tested: a hyperimmune anti-HA2 horse serum (CDC Atlanta, HI = 640), a rabbit antiserum prepared against purified HA2 virus (HI = 1280) and a number of human sera (HI = 640). None of the sera inhibited the neuraminidase activity except the rabbit antiserum diluted fivefold in buffer. We observed that the HA/NA activity ratio of HA2 virus was about $2 \times 10^2$ but the HA/NA activity ratio of rabbit anti-HA2 antiserum was $2.5 \times 10^3$. The feeble anti-neuraminidase antibody production could be attributed to the low specific activity of the enzyme (15 nmol NANA/min/mg virus protein) and not necessarily to a low immunogenic capacity. Because of the lack of anti-neuraminidase antibody, the specificity of HA2 virus neuraminidase was assayed by mixing HA2 virus and anti-Sendai antiserum on the one hand, and Sendai virus and anti-Sendai antiserum on the other hand. If the Sendai anti-Sendai reaction was considered as the 100% control, the cross-reaction between HA2 virus and anti-Sendai antiserum was only 5%. The neuraminidase of the two strains of parainfluenza type I virus seem to be different.

Neuraminidase-haemagglutinin relationships

The haemagglutinin of HA2 virus could be demonstrated in crude virus suspensions by using a 1% suspension of guinea-pig red blood cells in buffer ($5 \times 10^{-3} \text{M-PO}_4-0.15 \text{M-NaCl, pH 7}$). The average values obtained were 15 HAU/50 µl virus suspension. After concentration and purification, the specific activity was about $5 \times 10^4$ HAU/mg protein.
Fig. 7. Effect of concentration of several detergents on (a) neuraminidase and (b) haemagglutinin activities of HA2 virus. Samples were incubated with various concentrations of ionic and non-ionic detergents and incubated for 18 h at 37 °C with N-acetyl neuramin lactose (0.25 mg/ml final concentration) in citrate-phosphate buffer, pH 5.2. Percent activities are shown. Ionic detergents: DOC (■—■) and SSS (▼—▼); non-ionic detergents: Triton X-100 (□—□) and Nonidet P-40 (▽—▽).

Thermal inactivation of haemagglutinin

Samples of concentrated virus were diluted to 100 HAU/50 μl and were treated as previously described for neuraminidase. Residual haemagglutinating activity in relation to the thermal inactivation time at various temperatures is shown in Fig. 4(b). Haemagglutinin inactivation constants were calculated and it can be seen that they are very close to those of the neuraminidase (Fig. 4b).

Effect of detergents on haemagglutinin

Ionic and non-ionic detergents were used at concentrations ranging from 0.1 to 5% DOC and SSS concentrations of 0.1 to 0.2% destroyed 50% of the haemagglutinin, which completely disappeared with concentrations of 1% and over. In contrast, the non-ionic detergents did not alter the biological activity of HA2 haemagglutinin (Fig. 7b).

Antigenic characterization

HA2 virus haemagglutinin was inhibited by homologous antisera: hyperimmune anti-HA2 horse antiserum and anti-HA2 rabbit antiserum were raised using crude and purified viruses respectively. The rabbits immunized with purified HA2 virus produced an antiserum containing 1000 to 2000 units/50 μl of HI antibody. This haemagglutinin was specific for the HA2 strain except for a slight cross reactivity with Sendai virus (2%).

DISCUSSION

In this study, we have reported the first determination and characterization of the neuraminidase of human parainfluenza 1 virus (HA2 virus). Neuraminidase activity could only be detected in highly concentrated virus preparations, and the ratio of the titres of
neuraminidase to haemagglutinin was only 0.005. This value was much lower than any of those given for other paramyxoviruses: 0.03 for NDV (Hertz strain), 0.44 for Yucaipa and 2.28 for Bangor viruses (Alexander, 1974), and 0.05 for Sendai virus grown in eggs or cells (unpublished data).

The HA2 neuraminidase showed optimal activity using N-acetyl neuramin lactose which possesses 85% of α 2→3 bonds as indicated by the manufacturer. A minor activity was observed with fetuin; the α 2→3 bonds were also present in this substrate (Spiro, 1964) but the relative abundance of NANA [3 units of carbohydrate per molecule of fetuin (Spiro, 1970)] was much lower than in N-acetyl neuramin lactose. In contrast, no activity could be detected using mucin types I and II containing α 2→6 bonds (Nisizawa & Hashimoto, 1970). HA2 virus neuraminidase attacked specifically the α 2→3 bonds; indeed with the indications provided by the manufacturer (75% purity, 85% α 2→3 bonds and 15% α 2→6 bonds) we obtained too% α 2→3 bonds hydrolysed. Only the α 2→3 bonds were hydrolysed because α 2→6-linked N-acetyl neuramin lactose is not attacked by neuraminidases (Nisizawa & Hashimoto, 1970). This affinity for the alpha ketose α→3 linkage seems to be a characteristic of the different paramyxoviruses studied so far: NDV (Drzeniek, 1973), NDV, Yucaipa and Bangor (Alexander, 1974) and a number of orthomyxoviruses (Drzeniek, 1973).

With regard to the pH optimum of the neuraminidase, paramyxoviruses can be placed in one of two categories. The first one contains HA2 virus, Sendai (Tozawa et al. 1967; Brostrom, Bruening & Bankowski, 1971), NDV (Brostrom et al. 1971; Alexander, 1974), mumps (Bromstrom et al. 1971) and human parainfluenza 2 (Darrell & Howe, 1964); the optimum activity is at pH 5 to 5.4. The second one includes viruses whose optimum activity is in the much lower pH range of 4 to 4.5: bovine parainfluenza 3 (Drzeniek, Bogel & Rott, 1967), SV5 (Scheid et al. 1972), and Yucaipa and Bangor viruses (Alexander, 1974).

The optimum temperature for HA2 virus neuraminidase activity ranged from 37 to 40 °C. We have not found any information concerning the other paramyxoviruses in the literature.

The temperature which inactivated 50% of the enzyme after 5 min exposure was 45 °C. The other paramyxovirus strains are more resistant with values of 48 °C for Sendai (Brostrom et al. 1971), 51 to 53 °C for NDV (Brostrom et al. 1971; Alexander, 1974), 55 °C for mumps (Brostrom et al. 1971) and between 50 and 75 °C for human parainfluenza 2 (Darrell & Howe, 1964). The thermosensitivity of the HA2 virus neuraminidase was shown by the value of the enzyme inactivation constants. These were much higher for the HA2 strain (k = 0.78 min⁻¹ at 50 °C) than for other paramyxoviruses: k = 0.2 min⁻¹ at 56 °C for NDV (Pierce & Haywood, 1973); k = 0.25 min⁻¹ at 56 °C for various strains of mumps virus (Leprat, Aymard & Bardeletti, 1975). The thermolabilities of the neuraminidase and the haemagglutinin are identical. Pierce & Haywood (1973) published similar observations for NDV and suggested that the same glycoprotein was responsible for both activities.

The kinetics of enzyme activity were also studied and gave us the appropriate incubation time for determination of the $K_m$ value and other characteristics of the neuraminidase. The $K_m$ value obtained with N-acetyl neuramin lactose was near that given by Alexander (1974) for Bangor virus (3⋅16 × 10⁻³ M) and higher than those given by Alexander (1974) for NDV (1⋅02 × 10⁻³ M) and for Yucaipa (1⋅97 × 10⁻³ M) and by Drzeniek, Seto & Rott (1966) for NDV (1 × 10⁻³ M). Therefore, HA2 virus enzyme has a lower affinity for N-acetyl neuramin lactose than other paramyxoviruses previously described.

HA2 virus neuraminidase and haemagglutinin are very sensitive to ionic detergents. Concerning neuraminidase, these data are in agreement with those of Iinuma et al. (1971)
HA2 neuraminidase

for NDV, though they did not observe any destruction of haemagglutinin using DOC concentrations similar to ours (0.1 to 1% w/v). The non-ionic detergents, Triton X-100 and Nonidet P-40, do not alter the haemagglutinin and neuraminidase activities of HA2 virus. The same observation was made by Brostrom et al. (1971) with Sendai virus. The soluble fraction obtained after treatment by Triton X-100 or Nonidet P-40 still agglutinated guinea-pig red blood cells, in contrast to the results reported by Scheid et al. (1972) for SV5, Scheid & Choppin (1973) for NDV and by Jensik & Silver (1976) for mumps virus. The separation of glycoproteins carrying the different biological activities would thus be difficult.

There is a common antigen which reacts with complement fixing (CF) antibodies in anti-Sendai serum tested with both HA2 and Sendai viruses but the anti-HA2, CF antibodies are strictly specific in contrast to the results obtained by Lief et al. (1975). Sendai and HA2 have a common antigenic determinant which represents only about 2% of cross reactivity in HI test. Because of the lack of anti-neuraminidase antibodies induced by the HA2 strain we could only try to inhibit HA2 neuraminidase with anti-Sendai antiserum. HA2 neuraminidase appears to be specific.

In conclusion, neuraminidase activity is present in the human parainfluenza I virus, strain C 35 (HA2 virus). The specific activity is low, but it was possible to compare its biological properties with those of Sendai virus to which it is related. The enzyme is active in a narrow range of pH, and the optimum pH activity allows us to place this parainfluenza virus in the group of paramyxoviruses showing a maximum neuraminidase activity around pH 5, distinct from the group of paramyxoviruses having an optimum activity around pH 4.

The neuraminidase hydrolyses α 2→3 bonds and shows a low avidity for N-acetyl neuraminylcose. It also shows a remarkable thermolability. These data might be related to the finding that human parainfluenza I virus grows poorly in cell cultures, to the fact that it induces chronic infections in vitro (Ishida et al. 1964) and in vivo (Phillips & Christian, 1970) and shows limited spread among children and usually causes mild disease.

Both neuraminidase and haemagglutinin are solubilized by non-ionic detergents which do not destroy their biological properties, and they both have the same thermal inactivation constants which suggests that these activities are situated on the same glycoprotein component. This hypothesis is now under investigation.

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REFERENCES


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