Lymphocyte Neuraminidase Activity and Sialic Acid Concentration in Normal Subjects

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SUMMARY An assay method for neuraminidase in human lymphocyte has been developed. The method was based on the determination of enzymatically liberated 4-methylumbelliferyl 4-MU) by a fluorometric procedure.

Homogenates of lymphocyte cells cleaved the 4-MU from the 4-methylumbelliferyl \( \alpha \)-ketoside of N-acetylneuraminic acid. Maximum activity occurred at pH 4.5 in sodium acetate buffer. The Km value was \( 2.35 \times 10^{-4} \)M. The hydrolysis proceeded linearly with the incubation time up to 150 min and with enzyme protein concentration; up to 0.2mg. The enzyme was inhibited by cholic acid, sodium laurylsulfate and also by isotonic concentrations of LiCl, KCl, NaCl, and CaCl2.

The normal values of enzyme activity was somewhat lower in females (2.38±0.90 4-MU/mg protein/h) than in males (3.03±1.30). In contrast, sialic acid concentration of the same lymphocyte was higher in females (87.8±31.5mg/g protein) than in males (58.5±32.5).

Introduction

Neuraminidase(EC 3.2.1.18, sialidase, \( \alpha \)-N-acetylneuraminosyl glycohydrolase) activity has been detected in viruses, bacteria, mycoplasmas and animal tissues. According to the wide distribution and possible involvement in such phenomena as viral and bacterial propagation, mammalian reproduction, blood clot formation, hormone-membrane interaction and neurotransmission, there has been considerable interest in this enzymatic activity\(^1\). The presence of this enzyme in human blood was first reported in 1960 by Warren and Spearing in commercial preparation of human blood glycoprotein\(^2\). Although the possible biological roles of this enzyme in human blood are still unclear, there are...
several reports concerning the presence of this activity in human leucocyte\(^5\)\(^\text{--}\)\(^4\) and platelet\(^5\).

Neuraminidase activity has been usually quantitated by measuring the release of sialic acid by chemical method such as thiobarbituric acid or by enzymatic method\(^6\)\(^\text{--}\)\(^8\). An alternate approach to the assay of neuraminidase activity is to measure the aglycon rather than the liberated sialic acid. For example, 3-methoxyphenol can be measured colorimetrically, after enzymatic hydrolysis of the synthetic substrate, 2-(3′-methoxyphenyl)-N-acetyl-\(\alpha\)-1-neuraminic acid\(^9\)\(^\text{--}\)\(^11\). Recently a fluorogenic substrate, 4-methylumbelliferyl \(\alpha\)-ketoside of N-acetylneuraminic acid (4-MU-NANA), has come to be synthesized independently by several groups of workers\(^4\)\(^\text{--}\)\(^12\)\(^\text{--}\)\(^19\). It was demonstrated that this compound was cleaved by bacterial\(^4\)\(^\text{--}\)\(^12\), by human fibroblast\(^4\)\(^\text{--}\)\(^12\)\(^\text{--}\)\(^19\) and leucocyte\(^4\) neuraminidases. Because of the high sensitivity of the fluorometric measurement of the reaction product, 4-methylumbelliferylone, the assay proved to be much more sensitive than the assay measuring the released free sialic acid\(^12\).

In this paper, we describe the application of this fluorogenic substrate for the assay of human lymphocyte neuraminidase. The assay method could be used for the determination of lymphocyte neuraminidase for the clinical purpose, and the levels of enzyme activities were examined in blood from healthy adults.

**Materials and Methods**

**Isolation of Human Lymphocyte and Preparation of Enzyme**

About 4ml of venous blood was drawn into heparinized tubes, lymphocytes were separated using Separate-L gradient (Muto Chemicals Ltd., SG=1.077 at 20°C) in a conventional method. The freshly prepared pellet cells were suspended in acetate buffer (pH 4.5) and were disrupted by ultrasonication for 60 seconds (Kontes Bio-Sonicator, Kontes Co., Ltd., USA). All studies were conducted using an aqueous suspension of lymphocyte homogenates.

**Assay of Neuraminidase in Cell Homogenate Using 4-MU-NANA**

Unless otherwise stated the assay was performed using 100\(\mu\)l of freshly prepared cell homogenate in a total volume of 200\(\mu\)l which contained 0.125mM 4-MU-NANA [a gift from Dr. Y. C. Lee, Anal. Biochem. 101, 166 (1980)]\(^12\) in 0.025M sodium acetate buffer (pH 4.5). After incubation for 120 min at 37°C, the mixture was diluted with 1.8ml of 0.1M glycine-NaOH, pH 10.4. Fluorescence was read with Eppendorf fluorophotometer (excitation, 366nm, emission 440nm). A standard curve was prepared by dissolving an appropriate amount of 4-MU (Sigma Chemical Company) in 0.1M glycine-NaOH buffer, pH 10.4 and determining the fluorescence as described above. Activity was expressed as nanomoles of 4-MU released per hour per milligram of protein as determined by the method of Lowry et al.\(^13\).

**Determination of Sialic Acid Concentration of Cell Homogenates**

Sialic acid concentration of lymphocyte homogenate was measured by the fully enzymatic method as described elsewhere\(^8\)\(^\text{--}\)\(^14\).
Fluorometric Assay for Human Lymphocyte Neuraminidase and Its Relationship to the Sialic Acid Content

Results

Properties of Human Lymphocyte Neuraminidase

Kinetic studies—the pH activities curve showed the maximum activity at pH 4.5 (Fig. 1). As the pH of the reaction mixture rose, the enzyme activity was depressed markedly; almost no activity was detected at around pH 6.5. The optimal pH of bacterial enzyme was slightly higher than that of lymphocyte neuraminidase, showing the maximum at around pH 5.0 to 6.0. The rate of hydrolysis of 4-MU-NANA proceeded linearly with the incubation time up to 150 min. A linear relationship was generally observed between protein concentration and 4-MU release (Fig. 2). The projected curve, however, did not pass through the zero point, thus indicating the deviation at low protein concentration. The apparent Km value, calculated from 1/v versus 1/S plot was $2.35 \times 10^{-4}$ M for 4-MU-NANA.

Effect of cations—the effect of several cations (Li⁺, K⁺, Na⁺, Ca++) at concentrations between 0.05—0.2 M was studied with the use of their respective chlorides. As shown in Fig. 3 more than
30 per cent inhibition was observed at $2 \times 10^{-1}$ M concentration of these salts.

Effect of detergent ..... neuraminidase of human lymphocyte appeared to be slightly activated by Triton X-100 and deoxycholic acid (Fig. 4). The optimum concentration of Triton X-100 and deoxycholic acid was 0.1% (v/v), which caused a 30% stimulation; higher concentration of Triton X-100 caused a smaller loss of activity. The activity of human lymphocyte increased with the addition of cholic acid. The optimum concentration of this detergent was 0.025% (v/v), which caused a 15% stimulation. Higher concentration resulted in a gradual loss of this stimulation. At 0.4% of cholic acid, a 20% inhibition of the initial activity was recorded. Sodium laurylsulfate (SDS) was deterious to human lymphocyte neuraminidase. Only about 10% of the initial activity was maintained in the presence of 1.0mM of SDS. Higher concentrations of this detergent failed to cause any further loss of activity.

Effect of buffer concentration on the reaction rate ..... the human lymphocyte neuraminidase appeared to be inhibited by higher concentration of buffer. In Na-acetate buffer (pH 4.5), 0.05mol/l concentration gave a maximal velocity, showing a gradual decrease with increasing concentration of buffer. At a final concentration of 1.0mol/l, 55% inhibition of the enzyme was observed. The effect of phosphate-citrate buffer concentration (pH 4.5) was also studied. This buffer gave a maximal velocity at 0.1mol/l. Higher concentrations resulted in a gradual loss of activity (Fig. 5).

Sialidase and Sialic Acid Content of Normal Human Lymphocyte

The normal level of enzyme activity

Fig. 4 Effect of Triton X-100 (●—●), of deoxycholic acid (x—x), of sodium laurylsulfate (SDS, ●—●) and of cholic acid (○—○) on human lymphocyte neuraminidase. Increasing concentrations of these compounds were added to the assay mixture. Incubation time was 2h.

Fig. 5 Effect of buffer concentration on human lymphocyte neuraminidase. The reaction mixtures were those described under “Materials and Methods” excepting the concentration of the indicated buffers.
in human lymphocyte was determined in samples of venous blood obtained from normal adult subjects. The average neuraminidase activity of freshly prepared lymphocytes, from 14 control male individuals (of 22 to 77 years of age) was 3.03 ±1.30 (SD) nmol of 4-MU released per milligram of protein per hour with a range from 1.39 to 5.80 and that from females (of 21 to 65 years of age) was 2.38±0.90 with a range from 1.32 to 4.19. Neuraminidase activity was somewhat lower in females than in males. In contrast, sialic acid concentration of the same lymphocyte was higher in females (87.8±31.5mg per g protein) than in males (58.5±32.5) (Table 1).

**Discussion**

This paper reports a new fluorometric assay for lymphocyte neuraminidase using 4-MU-NANA (4-methylumbelliferyl-α-D-N-acetyl-neuraminic acid) as a substrate.

Kinetic properties of human lymphocyte determined with this substrate were almost similar to those of the leucocyte neuraminidase reported by other groups of workers using neuramine-lactose, or 4-MU-NANA as substrates. Apparent Km value determined with this substrate (2.35 x10⁻⁴M) was similar to those determined with neuramine-lactose (5.62 x10⁻⁴M) or with the same substrate from different methodology (2.2 x10⁻⁴M). It was reported that there were two different neuraminidases in mammalian organ, soluble and lysosome-bound neuraminidases. Soluble neuraminidase, which shows maximum activity at pH 5.5 to 5.8, was first detected in human plasma. In contrast to this enzyme, lysosome-bound neuraminidase was reported to have pH optimum at around 4.0 to 4.4. The pH activity curve of human lymphocyte neuraminidase showed a sharp maximum at 4.5 (Fig. 1). Similar results were reported with human leucocyte neuraminidase. When lymphocyte suspension was subjected to such treatment as homogenization or sonication, only about 9% of the activity could be detected in the supernatant fraction (data not shown). These results suggest that most of the human lymphocyte neuraminidase might be strongly bound to the particulate fraction of the cell. Further work is required to elucidate the existence of neuraminidase activity in cytosol fraction of lymphocyte.

<table>
<thead>
<tr>
<th>SEX</th>
<th>No. of samples</th>
<th>Neuraminidase activity (mean±SD) (range)*</th>
<th>Sialic acid concentration (mean±SD) (range)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>14</td>
<td>3.03±1.30* (1.39 to 5.80)</td>
<td>58.5±32.5* (29.0 to 147.6)</td>
</tr>
<tr>
<td>Females</td>
<td>11</td>
<td>2.38±0.90** (1.32 to 4.19)</td>
<td>87.8±31.5** (41.2 to 145.9)</td>
</tr>
<tr>
<td>Males and Females</td>
<td>25</td>
<td>2.75±1.17*** (1.32 to 5.80)</td>
<td>71.4±34.8*** (29.0 to 147.6)</td>
</tr>
</tbody>
</table>

*nano moles of 4-MU released/mg protein/h  **mg sialic acid/g protein

* r = -0.3425  P<0.001.  ** r = -0.6273  P<0.001.  *** r = -0.4961  P<0.001
The release of 4-MU from the substrate was in proportion to the concentration of added cellular protein (Fig. 2). However, the projected curve did not pass through the zero point, thus indicating nonlinearity at lower levels of the enzyme. A similar phenomenon has been reported with human fibroblast homogenate neuraminidase.

The presence of two neuraminidases which differ in behavior towards cations was reported in rat liver; one occurs in soluble form in the cytosol and is inhibited by divalent cations, and the other occurs firmly bound to the lysosomal particles and is inhibited by monovalent cations. As shown in Fig. 3, human lymphocyte neuraminidase was inhibited by both monovalent and divalent cations. It appears that human lymphocyte neuraminidase is a bound form enzyme that differs from that of rat liver. Increasing the buffer concentration from 0.1M to 1.0M also resulted in a decrease in enzyme activity (Fig. 5). Low concentration of Triton X-100 and deoxycholic acid caused a slight activation of the enzyme (Fig. 4). This behavior is similar to those of human brain and human liver lysosomal neuraminidase, and differs from that of human leucocyte neuraminidase which underwent 54% inhibition in the presence of 0.05% (v/v) of Triton X-100. This might be attributed to the difference from substrate and/or incubation protein concentration.

In lymphocyte samples of normal males and females, neuraminidase activity and sialic acid concentration were negatively correlated, r (correlation coefficient) = -0.4961, and the significance level of correlation coefficient (p) was p<0.001 (Table 1). The correlation were more significant in females than in males. This negative correlation would be in agreement with the report presented by Thomas et al. that the deficiency of intracellular neuraminidase in I-cell disease (mucolipidosis II) associated with the increased sialic acid levels in the I-cell fibroblast.

Myers et al. have pointed out that 4-MU-NANA was a very good substrate for neuraminidase, and shown to provide the basis for a fluorometric assay that offers sensitivity, specificity and convenience. The use of 4-MU-NANA is convenient to detect the low levels of neuraminidase in human tissues, particularly to detect the clinical disorders resulting from inherited deficiency of neuraminidase.

In the present paper, we have demonstrated the existence of neuraminidase activity in normal lymphocyte cells and have developed a method to detect the activity by using a fluorogenic substrate, 4-MU-NANA. Neuraminidase activity in lymphocyte cells is expected to change in the case of disease. We are now trying to measure the neuraminidase activity in lymphocyte cells of various types of disease, and pursuing a correlation between neuraminidase activity and disease. The results will be reported elsewhere.

References

Fluorometric Assay for Human Lymphocyte Neuraminidase and Its Relationship to the Sialic Acid Content