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# Enzyme-linked Immunosorbent Assay for the Activator Protein Specific for the Enzymic Hydrolysis of $G_{M1}$ in Urine

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**We have established a sensitive and specific enzyme-linked immunosorbent assay (ELISA) for the detection of the activator protein which stimulates the enzymic hydrolysis of $G_{M1}$ ($G_{M1}$-activator) in human urine. The level of $G_{M1}$-activator in 19 normal, adult urine samples was estimated to be 370.7 ± 33.2 ng/ml. The amounts of $G_{M1}$-activator excreted in 24 h were estimated to be between 0.28 and 1.1 mg. The coefficient of variation for this method is 4.3% for the intra-assay and 14.4% for the inter-assay. Urine samples, without purification, can be used directly for the ELISA.**

Through the work of many laboratories, it has been well established that in addition to glycosidases, the hydrolysis of sugar chains in glycosphingolipids requires the presence of activator proteins [1]. In 1983, we reported the presence of activator proteins ($G_{M1}$-activator and $G_{M2}$-activator) for the enzymic hydrolysis of $G_{M1}$-ganglioside ($II^3$ NeuAc-GgOse$_4$Cer) ($G_{M1}$) and $G_{M2}$-ganglioside ($II^3$ NeuAc-GgOse$_3$Cer) ($G_{M2}$) respectively, in normal human urine [2]. The assay for activator proteins essential for the catabolism of glycosphingolipids has been limited to the demonstration of the *in vitro* stimulation of the hydrolysis of glycosphingolipid substrates. This *in vitro* assay, however, is not suitable for the detection of activator proteins in biological fluids due to the low content of activators and the presence of interfering substances. We have developed a sensitive and specific enzyme-linked immunosorbent assay (ELISA) for the detection of $G_{M1}$-activator in human urine.

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## Materials and Methods

### *Materials*

Disposable polystyrene Nunc-Immuno® plates II-96F were obtained from Vangard International, U.S.A.. $G_{M1}$-Activator from human liver and rabbit anti-$G_{M1}$-activator antiserum were prepared as described previously [3]. The peroxidase-conjugated goat anti-rabbit immunoglobulin and the color reagent, 2,2' azino-di-(3-ethylbenzthiazoline sulfonic acid), were obtained from Sigma Chemical Company, U.S.A.. All chemicals and reagents used were of the highest quality available commercially.

### *ELISA for $G_{M1}$-Activator*

In each step, the wells of polystyrene plates were filled with 0.2 ml of the designated solution. The wells were first coated with 25 ng/ml of $G_{M1}$-activator in 0.1 M carbonate-bicarbonate buffer, pH 9.6. After leaving the plate at 4°C overnight, the solution was removed and the wells were washed four times with phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-Tween). The coated plates could be stored at 4°C for at least two weeks before use.

Urine samples at three different dilutions (20 ×, 40 × and 80 ×) and a series of standard solutions of $G_{M1}$-activator, ranging from 1.5 to 50 ng/ml, were incubated with rabbit anti $G_{M1}$-activator antiserum (final dilution 8 000 ×) overnight at 4°C. All dilutions were made in PBS-Tween solution containing 1 mg/ml of ovalbumin. Then, 0.2 ml each of the incubated solutions were pipetted into the coated wells in triplicate, and the plate was left standing at room temperature (22°C) for 2 h. The wells were subsequently washed four times with PBS-Tween and again filled with a solution of peroxidase conjugated goat anti-rabbit IgG at 1 000 × dilution. After incubating at room temperature for 2 h, the wells were washed four times with PBS-Tween, and refilled with the color reagent. The color reagent consisted of 100 mg of 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) and 10 µl of 30% hydrogen peroxide in 100 ml of 0.1 M phosphate-citrate buffer, pH 4.0. The color was developed at room temperature for 1 h and the extent of the color was estimated by reading the absorbance at 405 nm using a Bio-Tech spectrophotometer. A standard curve was constructed by plotting the $G_{M1}$-activator concentration on a logarithmic scale against the % absorbance on a linear scale (see Fig. 2). The % absorbance of the sample is the % ratio of the absorbance between the sample and the control which contains only the antiserum without the soluble antigen.

## Results

$G_{M1}$-Activator can be easily coated on a plastic surface and the solid-phase bound $G_{M1}$-activator can bind the specific antibodies. This binding depends on both the amount of coated antigen and the concentration of the antiserum as shown in Fig. 1. A typical standard curve (Fig. 2) demonstrates the ability of the soluble antigen to inhibit the binding of the anti-$G_{M1}$-activator antibodies to the solid-phase bound antigen. As shown in Fig. 2, the working range for this assay is between 1.5 and 25 ng/ml of $G_{M1}$-activator. The extreme values obtained beyond either end of this range do not obey

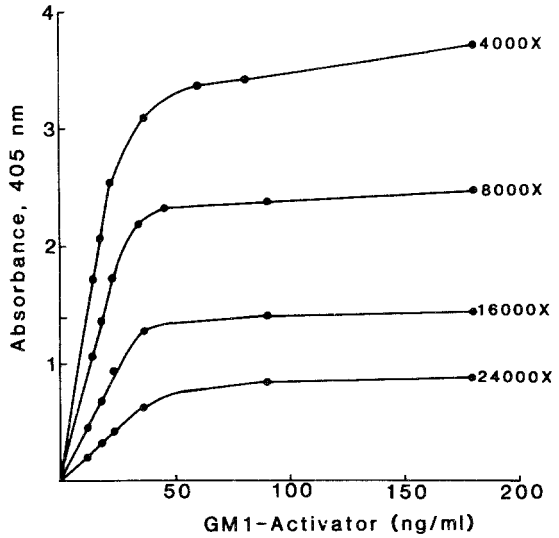


**Figure 1.** Interaction between various dilutions of rabbit anti-$G_{M1}$-activator antibodies and various amounts of solid phase-bound $G_{M1}$-activator protein. The amounts of $G_{M1}$-activator used for coating the wells are indicated on the abscissa.

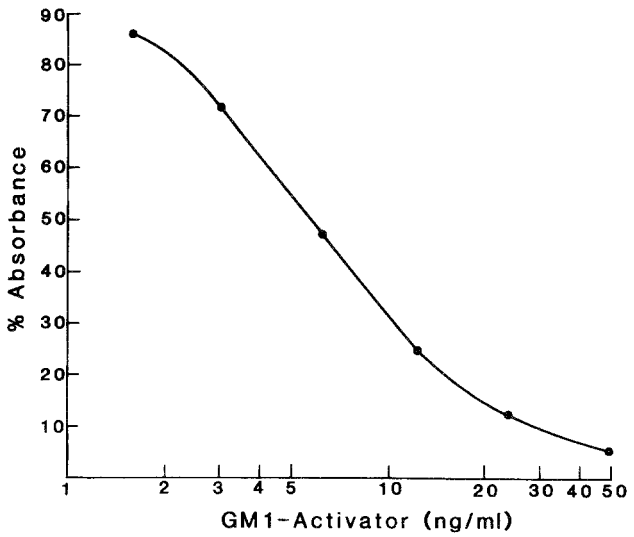


**Figure 2.** Standard curve for the quantitation of $G_{M1}$-activator by ELISA. This curve illustrates the inhibition of the interaction between antibody and the solid-phase bound $G_{M1}$-activator by the soluble antigen. Rabbit anti-$G_{M1}$-activator antiserum was diluted 8 000 times. Detailed assay conditions are described in "Materials and Methods".

**Table 1.** The level of $G_{M1}$-activator in 24 h urine samples of normal human adults.

| Subject & (Sex) | Urine No. | ml/day | Prot.[a] μg/ml | Creat.[b] mg/ml | $G_{M1}$-Activator ng/ml | μg/24h | μg/mg prot. | μg/mg creat. |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2400 | 12.4 | 0.58 | 272 | 653 | 21.9 | 0.47 |
| | 2 | 1680 | 24.4 | 0.78 | 441 | 741 | 18.1 | 0.57 |
| | 3 | 1549 | 27.2 | 1.53 | 443 | 686 | 16.3 | 0.29 |
| I (M) | 4 | 2375 | 15.2 | 0.51 | 225 | 534 | 14.8 | 0.43 |
| | 5 | 2727 | 23.9 | 0.73 | 335 | 914 | 14.0 | 0.46 |
| | 6 | 2253 | 17.6 | 0.59 | 220 | 496 | 12.5 | 0.37 |
| | 7 | 2050 | 18.4 | 0.75 | 292 | 599 | 15.9 | 0.39 |
| II (M) | 8 | 1098 | 22.6 | 1.65 | 535 | 587 | 23.7 | 0.32 |
| | 9 | 1240 | 20.8 | 1.32 | 275 | 341 | 16.4 | 0.21 |
| III (M) | 10 | 1000 | 30.5 | 1.20 | 497 | 497 | 16.3 | 0.41 |
| | 11 | 2400 | 19.7 | 0.75 | 357 | 857 | 18.1 | 0.48 |
| IV (M) | 12 | 1760 | 17.7 | 1.25 | 585 | 1030 | 33.1 | 0.47 |
| | 13 | 1600 | 23.1 | 1.82 | 707 | 1131 | 30.6 | 0.39 |
| | | | | Mean: | 398.7 | 697.4 | 19.4 | 0.40 |
| | | | | S.D.: | ±41.8 | ±63.6 | ±1.7 | ±0.02 |
| V (F) | 14 | 1100 | 15.2 | 0.81 | 367 | 404 | 24.1 | 0.45 |
| | 15 | 1020 | 23.9 | 0.98 | 502 | 512 | 21.0 | 0.51 |
| VI (F) | 16 | 3190 | 13.8 | 0.47 | 165 | 526 | 11.9 | 0.35 |
| | 17 | 2315 | 17.9 | 0.59 | 360 | 810 | 19.5 | 0.59 |
| VII (F) | 18 | 1210 | 9.9 | 0.49 | 230 | 278 | 23.2 | 0.47 |
| | 19 | 1755 | 10.0 | 0.59 | 245 | 430 | 24.5 | 0.42 |
| | | | | Mean: | 309.8 | 493.3 | 20.7 | 0.46 |
| | | | | S.D.: | ±49.4 | ±73.0 | ±1.9 | ±0.03 |
| | | | Overall | Mean: | 370.7 | 632.9 | 19.8 | 0.42 |
| | | | Overall | S.D.: | ±33.2 | ±53.0 | ±1.3 | ±0.02 |

[a] Urinary protein was determined by the method of Lowry *et al* [5] after being precipitated by 10% trichloroacetic acid.

[b] Creatinine was determined according to the method of Hawks *et al* [6].

the straight line relationship, therefore a repeat experiment must be performed with suitable adjustment of the sample concentration.

Table 1 summarizes the levels of $G_{M1}$-activator in 19 different 24 h urine samples collected from seven (4 males and 3 females) normal adult human subjects. On subject I, seven different samples were analyzed and on each of the six others, analyses were made on two separate samples. The levels of $G_{M1}$-activator in these 19 urine samples were found to be between 165 and 707 ng/ml with a mean of 370 ±33.2 ng/ml. The amounts of $G_{M1}$-activator excreted in the 24 h urine were calculated to be between 280 and 1130 μg with a mean of 632 ±53 μg. When expressed as μg/mg protein or μg/mg creatinine, the levels of $G_{M1}$-activator in these urine samples were found to be rather constant. The results in Table 1 also indicate that there are no significant differences in the amount of $G_{M1}$-activator excreted in the urine of the two sexes.

This method for the determination of $G_{M1}$-activator appeared to be specific, since the urine samples from dog, cat, rabbit and rat gave the same values as that found in the

control wells. Furthermore, if the wells were coated with $G_{M2}$-activator [4] instead of $G_{M1}$-activator, only the background values were obtained for all the samples. When a known amount of $G_{M1}$-activator was added to the urine samples, the recovery of the activator was found to be 100% by this method. The coefficient for intra-assay variation between the triplicated wells was estimated to be 4.3% and that for inter-assay, calculated from the variation of the 20 repeated assays on each of three urine samples was estimated to be 14.4%.

## Discussion

The current assay method for $G_{M1}$-activator is to determine the stimulation of $G_{M1}$ hydrolysis carried out by human $\beta$-galactosidase. This method is often limited by the availability of $G_{M1}$ and a highly purified $\beta$-galactosidase from human tissues. In addition, because of the low level of $G_{M1}$-activator and the existence of interfering substances in tissues or biological fluids, the *in vitro* assay cannot be directly applied to crude tissue extracts or biological fluids. The ELISA for $G_{M1}$-activator is very simple, sensitive and specific. As little as 0.1 ml of urine would be sufficient for a duplicate experiment. The ELISA for $G_{M1}$-activator appears to be very specific, since no urine samples from dog, cat, rabbit and rat showed any positive reaction. However, the urine samples from the higher primates, such as baboon, green monkey and rhesus monkey, who are immunologically closely related to man, showed various degrees of cross reactivity.

So far, no false negative result has been observed, since 100% recovery was obtained when $G_{M1}$-activator was externally added to a urine sample. Among several brands of microtiter plates tested, $G_{M1}$-activator could not be adsorbed on the surface of Dynatech Immunolon 1 and Limbro E.I.A. plates. Dynatech Immunolon 2, Dynatech Micro ELISA and Nunc-Immuno plates were all satisfactory for binding $G_{M1}$-activator.

The ELISA for $G_{M1}$-activator has been reasonably reproducible judged from the variation coefficient from the intra-assays (4.3%) and inter-assays (14.4%). This method is also economical and can be carried out routinely once the dilution factor for the antibody is established. As ELISA is one of the established and accepted micromethods for the quantitation of various compounds, this method has been successfully adapted for the assay of $G_{M1}$-activator.

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## References

1 Li Y-T, Li S-C (1983) in The Enzymes, Vol XVI, ed. Boyer P, Academic Press, New York, p 427-45.
2 Li Y-T, Muhiudeen IA, DeGasperi R, Hirabayashi Y, Li S-C (1983) Am J Hum Genet 35:629-34.

3 Li S-C, Li Y-T (1976) J Biol Chem 251:1159-63.
4 Li S-C, Hirabayashi Y, Li Y-T (1981) J Biol Chem 256:6234-40.
5 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) J Biol Chem 193:265-75.
6 Hawk PB, Oser BL, Summerson WH (1950) in Practical Physiological Chemistry, 13th edition, eds. Hawk PB, Oser BL, Summerson WH, McGraw-Hill, New York, p 899-901.