
Delivery of Fungal β-Galactosidase to Rat Brain by Means of Liposomes

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TAKADA, G., ONODERA, H. and TADA, K. Delivery of Fungal β-Galactosidase to Rat Brain by Means of Liposomes. Tohoku J. exp. Med., 1982, 136 (2), 219-229 — A significant increase in β-galactosidase activity was observed in the brain of rats 1 hr after an intravenous injection of liposomes containing β-galactosidase purified from Aspergillus oryzae. The increased activity was proved to have features of the fungal enzyme by differentiating it from rat's native β-galactosidase in both heat stability and immunochemical studies. Blood content of rat brain tissue under the experimental conditions employed was estimated as 0.83% (v/w) from an infusion experiment of 131I-labeled human serum albumin. The net uptake of fungal β-galactosidase by rat brain was calculated as equal to 10 µg protein of the fungal enzyme or 0.31% of the injected dose/g tissue, which gave rise to 4.4-fold net increase in enzyme activity above control levels. The experiments clearly demonstrated that liposome-entrapped fungal enzyme was allowed to penetrate the blood-brain barrier and to gain access to rat brain, suggesting liposomes as an effective carrier for exogenous enzyme delivering to the central nervous system of patient with inherited lysosomal storage diseases.

Delivery of missing enzyme to the central nervous system (CNS) of patients with inherited lysosomal storage diseases is one of the major problems for effective enzyme replacement therapy. To date, several human clinical trials of either intravenous or intrathecal injection of enzymes have been fruitless in targeting them to the CNS, suggesting that more sophisticated methodology than simple injection of enzyme itself is needed. Only recently, however, two techniques to deliver administered enzyme to the CNS have been reported. Rattazzi et al. (1980) demonstrated human β-hexosaminidase in the brain of the cats which were given 6×10^6 units of the enzyme per kg body weight under hyperbaric oxygen treatment. Barranger et al. (1980) succeeded to deliver α-mannosidase into rat brains by temporal opening of the blood-brain barrier using either mannitol- or arabinose-induced osmotic alteration. In our previous report (Takada et al. 1981) it was suggested from comparative experiments using liposome-entrapped or unentrapped 131I-fungal β-galactosidase that liposomes would be an effective carrier of exogenous enzymes to the brain. The purpose of this report is to present several

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lines of evidence of neural entry of liposome-entrapped fungal $\beta$-galactosidase following intravenous injection.

**MATERIALS AND METHODS**

**Reagents**

4-Methylumbelliferin (4-MU)-$\beta$-D-galactopyranoside was obtained from Koch-Light Laboratories, Ltd. (Colnbrook Bucks, England). Egg phosphatidylcholine (Lecithin), cholesterol and dicetylphosphate were from Sigma Chemical Co. (St. Louis, USA). DEAE-cellulose was from Whatman, Ltd. (Springfield Mill, England). $^{125}$I-Human serum albumin (0.5 mCi/5 mg protein per 0.5 ml) was from Daiichi Radio Isotope LADS., Ltd. (Tokyo). Freund’s complete and incomplete adjuvants were from Difeo (Detroit, USA). All other reagents including ethylene diamine tetraacetic acid (EDTA) were of analytical grade and obtained from Wako Pure Chemical Industries (Osaka). $\beta$-Galactosidase purified from *Aspergillus oryzae*, which showed a single protein band on 7.5% polyacrylamide gel electrophoresis at pH 9.4, was a kind gift from Tokyo Tanabe Co., Ltd. Specific activity was 94.4 o-nitrophenyl-$\beta$-galactopyranoside (ONPG) units/mg protein. One unit is defined as the enzyme activity liberating 1 $\mu$mole of o-nitophenol per min.

**Preparation of liposomes**

Preparations of liposomes consisting of phosphatidylcholine, cholesterol and dicetylphosphate (7:2:1 molar ratio) were carried out as described elsewhere (Takada et al. 1981). In this experiment, however, liposomes containing enzyme were separated from unentrapped enzyme by centrifugation at 100,000 X g for 60 min and were washed once with five volumes of buffered saline [6.6 mM sodium phosphate buffer (pH 7.2) with 0.16 M NaCl].

**$\beta$-Galactosidase assay**

$\beta$-Galactosidase activity was measured by the method of Suzuki (1977) using 4-MU-$\beta$-D-galactopyranoside as a substrate. In brief, 0.1 ml of diluted enzyme solution was incubated at 37°C for 60 min with 0.2 ml of the substrate dissolved in 0.1 M citrate buffer with 50 mM NaCl, pH 5.0. The reactions was terminated by the addition of 3 ml of 0.2 M glycine-NaOH buffer pH 10.4 and resulting fluorescence was measured in a Hitachi Fluorescence Spectrophotometer-204 with filter setting of maximal excitation at 365 nm and maximal emission at 448 nm.

**Protein assay**

Protein was measured by the method of Lowry et al. (1951) using crystallin bovine serum albumin as standard.

**Animal experiment**

Littermate rats of both sexes (Sprague-Dawley strain) weighing 40–50 g were employed in all experiments. The rats which had been fasted overnight were divided into two groups; “experimentals” and “controls”, balancing both groups in sex and body weight. Rats of each group were injected from tail veins with either the purified fungal $\beta$-galactosidase-loaded liposomes or buffer-loaded (i.e. empty) liposomes. Injected dose was about 290 ONPG units or 3 mg protein of the fungal enzyme to each of experimentals and an equivalent amount of empty liposomes to controls. They were killed at 60 min postinjection by bleeding. Various organs were immediately removed, washed extensively with ice-cold 0.9\% saline, blotted in filter paper, weighed and homogenized in 9 volumes of 0.25 M sucrose solution containing 1 mM EDTA using Potter-Elvehjem homogenizer with teflon pestle. Four subcellular fractions (i.e. cell debris-nuclear, mitochondrial-lysosomal, microsomal and soluble) were obtained from the brain and liver by differential centrifugation as described by Gregoriadis and Sourkes (1967). $\beta$-Galactosidase activity was assayed in the blood, homogenized tissues and four subcellular fractions, which were preincubated
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with 1% Triton X-100 at 37°C for 30 min and were diluted to optimal concentrations with 0.25 M sucrose containing 1 mM EDTA.

**Differential thermostability of the fungal vs. rat native β-galactosidase**

The thermostability test to differentiate the fungal β-galactosidase from that of rat brain was performed as follows: 0.1 ml of diluted brain homogenate and the same amount of fungal enzyme which was previously mixed with heat-inactivated (at 80°C for 10 min) brain homogenate were incubated for 2 min at various temperatures, respectively. They were transferred to an ice-cold bath and assayed for the enzyme activity.

**Immunization and preparation of antibody against the fungal enzyme**

Another approach to differentiate the two enzymes from different sources was made by immunochemical procedures. Two white rabbits were immunized by subcutaneous injections of fungal β-galactosidase (2 mg protein in one ml of buffer) emulsified with an equal volume of Freund’s complete adjuvant. Four weeks later, they were boosted by similar procedures using Freund’s incomplete adjuvant. High titers of antibody was found in the sera of the sensitized rabbits around 10 days following the second injection. The sera were combined and γ-globulin fraction was isolated as described by Levy and Sober (1960), except that final precipitate was dissolved in 20 mM in sodium phosphate buffer pH 8.0. Control γ-globulin fraction was prepared by the identical procedures from two control rabbit sera.

**Estimation of blood content of rat brain**

The following experiment was carried out to estimate the amount of blood which is contaminated to brain tissue preparations under the present experimental conditions and thus to calculate the extent of contribution of blood-derived fungal enzyme to the observed β-galactosidase activity of brain tissue from experimentals. Another set of litter-mate rats was injected intravenously with 131I-labeled human serum albumin (each rat received 30 μCi in a volume of 0.5 ml of 0.9% saline). Four rats were killed at 30 min and the remaining four at 60 min postinjection. Removed blood and brains which were washed drastically with 0.9% saline and weighed were counted for their radioactivity directly in a Intertechnique γ-counter (PG-4000). The amount of blood contained in thus obtained brain tissues was simply calculated according to the formula:

\[
\text{cpm/g wet weight of brain} \times 100 \% = \frac{\text{cpm/ml of blood}}{\text{cpm/ml of blood}}
\]

**Results**

**Blood content of brain**

Blood content of rat brain is known to be 3.2% wet weight (Everett et al. 1956). Since brain tissues were extensively washed in the present experiment, the mean of blood content of brain was calculated as 0.79% and 0.83% at 30 min and 60 min postinjection, respectively (Table 1).

**Tissue distribution of β-galactosidase activity**

As shown in Fig. 1a, increments in β-galactosidase activity above control levels were observed in all organs examined in the experimentals. The spleen and liver were the two most effective organs for uptake. When uptake was expressed as activity in whole organ, the liver became the most effective one (Fig. 1b), as observed by others (Gregoriadis and Ryman 1972). Relatively high
uptake by kidneys was also observed in this experiment. Since the free enzyme was found to be taken up effectively by kidneys in our previous experiment (Takada et al. 1981), this finding could be explained by that liposomal preparation contained a relatively large amount of free enzyme because of inefficient washing. Of particular importance is that approximately 6-fold of increased activity above control levels was found in the brain of experimentals (Table 3). Since the enzyme activity in the

### Table 1. Estimation of blood content in rat brain

<table>
<thead>
<tr>
<th>Time after injection (min)</th>
<th>Rat No.</th>
<th>Radioactivity in blood (cpm/ml)</th>
<th>Radioactivity recovered in brain (cpm/gww)</th>
<th>Brain/Blood ×100</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>1</td>
<td>6,908,840</td>
<td>53,433</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7,041,100</td>
<td>54,779</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8,156,766</td>
<td>68,050</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6,758,766</td>
<td>52,342</td>
<td>0.77</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>0.79</td>
</tr>
<tr>
<td>60</td>
<td>1</td>
<td>6,822,433</td>
<td>42,721</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7,989,100</td>
<td>78,831</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5,849,500</td>
<td>50,490</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5,716,860</td>
<td>47,646</td>
<td>0.83</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>0.83</td>
</tr>
</tbody>
</table>

Rats were injected with 30 μCi of 131I-human serum albumin from tail veins and killed at either 30 min or 60 min after injection. Removed bloods and brains which were washed extensively with 0.9% saline and weighed were counted for their radioactivity.

![Fig. 1. Tissue distribution of β-galactosidase activity. β-Galactosidase activity was assayed in various tissues of rats 2 hr following i.v. injection of liposomes containing either fungal β-galactosidase or buffer itself. □, controls (n=4); □, experimentals (n=2). Vertical bars represent the range.](image-url)

uptake by kidneys was also observed in this experiment. Since the free enzyme was found to be taken up effectively by kidneys in our previous experiment (Takada et al. 1981), this finding could be explained by that liposomal preparation contained a relatively large amount of free enzyme because of inefficient washing. Of particular importance is that approximately 6-fold of increased activity above control levels was found in the brain of experimentals (Table 3). Since the enzyme activity in the
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Blood from experimentals was 656 µmoles/hr per ml (Table 2) and the amount of blood in brain tissue at 1 hr is 0.83%. Total intravascular activity in the brain would be about 5.4 µmoles/hr per g brain. The subtraction of the control level activity (12.2 in Table 3) and the activity attributable to blood (5.4) from the activity observed in the experimentals (71.8 in Table 3) gives the net increment in activity (54.2 µmoles/hr per g brain or 4.4-fold above control levels) attributable to the fungal enzyme which penetrated the blood-brain barrier and gained access to the CNS. It was calculated as equivalent to approximately 10 µg protein of the fungal enzyme or 0.31% of the injected dose.

**Table 2. β-Galactosidase activity in the blood**

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Control Activity</th>
<th>Experimental Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23.5</td>
<td>623</td>
</tr>
<tr>
<td>2</td>
<td>20.0</td>
<td>689</td>
</tr>
<tr>
<td>3</td>
<td>19.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>22.5</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>21.4</td>
<td>656</td>
</tr>
</tbody>
</table>

β-Galactosidase activities in sera from controls and experimentals drawn at 1 hr after injection were assayed and corrected for their hematocrits. Enzyme activity was expressed as µmoles of 4 MU/ml per 60 min.

**Table 3. Heat inactivation of β-galactosidase in brain homogenates**

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Activity Before inactivation</th>
<th>Activity After inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12.3</td>
<td>7.2</td>
</tr>
<tr>
<td>2</td>
<td>12.1</td>
<td>7.9</td>
</tr>
<tr>
<td>3</td>
<td>11.9</td>
<td>7.4</td>
</tr>
<tr>
<td>4</td>
<td>12.4</td>
<td>8.0</td>
</tr>
<tr>
<td>Mean</td>
<td>12.2</td>
<td>7.6</td>
</tr>
<tr>
<td>Experimental</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>75.3</td>
<td>8.7</td>
</tr>
<tr>
<td>2</td>
<td>68.2</td>
<td>8.2</td>
</tr>
<tr>
<td>Mean</td>
<td>71.8</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Enzyme activity was expressed as µmoles of 4 MU/g wet weight per 60 min. An aliquot of each fraction from control or experimental rat brains was incubated in a test tube at 62.5°C for 2 min, assayed for the enzyme activity and compared with that of without heat treatment. Each number represents the average of duplicate assays.

**Subcellular distribution of β-galactosidase activity**

Fig. 2 shows the result of differential centrifugation of the brain and liver homogenates. Most of the activity was recovered in the soluble fraction from both
tissues. Uptake by the mitochondrial-lysosomal fraction from the brain was also observed. Relatively high activity was associated with the cell debris-nuclear fraction from the brain, suggesting fusion of liposomes with plasma membrane of the brain.

Differential thermostability study

As shown in Fig. 3, a significant dissociation in thermostability was observed in enzymes from two different sources. By incubation at 62.5°C for 2 min, fungal β-galactosidase lost its all activity, while β-galactosidase of rat brain retained about 50% of its activity.

Table 3 shows the result of heat treatment at 62.5°C for 2 min on brain homogenates from both controls and experimentals. After the treatment, almost all of the increased activity in the brain of experimentals was lost specifically and the retained activity was essentially of the same level as controls. This result confirms that the increased β-galactosidase activity observed in the brain from rats injected with liposome-entrapped fungal β-galactosidase was attributable to the fungal enzyme and not to the endogenous enzyme of brain.

Immunohistochemical study

In the Ouchterlony double diffusion analysis, antibody produced a single
precipitin line with the purified fungal enzyme, but not with the rat brain extract, which was prepared from the mitochondrial-lysosomal fraction obtained by differential centrifugation and treated with 1% Triton X-100 at 37°C for 30 min (Fig. 4). Control γ-globulin gave no precipitin line with either of these two enzymes (results not shown).
Fig. 5. Immunochemical titration of fungal and rat brain β-galactosidase with antifungal enzyme antibody. The immunochemical titration study was carried out with a fixed amount of the fungal enzyme (13 µg) in 0.1 ml of 50 mM sodium phosphate buffer, pH 7.0 or of the brain extract (0.1 ml) prepared as described in the text and increasing amounts of antibody. The reaction mixtures in a final volume of 0.5 ml were incubated at 37°C for 30 min and then at 4°C for overnight. β-Galactosidase activity in the supernatant obtained by centrifugation at 1200 × g for 20 min was assayed. A mixture of 13 µg of the fungal enzyme and 600 µg of control γ-globulin was incubated and assayed for the enzyme activity in a parallel fashion. Each point represents the average of four identical assays.

Fig. 6. β-Galactosidase activities in four subcellular fractions from control and experimental rat brains after incubation with either control γ-globulin or anti-fungal enzyme antibody. A mixture of an aliquot of each fraction treated with 1% Triton X-100 at 37°C for 30 min and an excess amount of antibody or control γ-globulin in a final volume of 0.5 ml was incubated and assayed for enzyme activity just like the way described in Fig. 5. □, fractions from controls + control γ-globulin; □, fractions from controls + antibody; □, fractions from experimental + control γ-globulin; □, fractions from experimental + antibody. Vertical bars represent the range.
Immunochemical titration study was carried out with a fixed amount of the fungal enzyme or brain extract and increasing amounts of antibody. As shown in Fig. 5, the amount of fungal activity removed by precipitation was proportional to the amount of antibody added. Again, no cross-reaction between rat brain extract and antibody added was observed. No precipitation reaction was observed between control β-globulin and the fungal enzyme in this system.

Fig. 6 shows the result of treatment of four fractions from both control and experimental brain with either control γ-globulin or antibody. All the increased activity observed in experimentals were specifically decreased and returned to almost the same level with that of controls, while enzyme activity from controls was not affected by the same treatment. Control γ-globulin had no effect on the activities either from controls or experimentals. These results further confirm that the increased activity observed in experimentals derived from the fungal enzyme.

**Latency of β-galactosidase activity in the brain**

The enzyme activities in the four fractions from brains of both controls and experimentals were compared with or without Triton X-100 treatment at 37°C for 30 min. Only the four fractions from experimentals showed latency, that is, full activity was demonstrated solely after the treatment that disrupted liposomal
membranes (Fig. 7). This finding not only supports the above described conclusion, that is, neural entry of exogenous enzyme, but also suggests that most of the fungal enzyme taken up by the brain still remained encapsulated in liposomes.

**DISCUSSION**

The presence of a significant amount of active fungal $\beta$-galactosidase was demonstrated in rat CNS 1 hr following an intravenous injection of the enzyme which was entrapped in liposomes. The property of the fungal enzyme, that is, difference in thermostability as well as immunogenicity from that of rat native $\beta$-galactosidase made it possible to obtain direct evidence of neural entry of exogenous enzyme. By estimating the amount of blood contained in brain tissue using $^{131}$I-human serum albumin, it was ascertained that the increased activity in the CNS was derived from the enzyme which actually penetrated the blood-brain barrier and gained access to the CNS, and not solely derived from the enzyme present in blood. From the present experiments, entrapment of enzymes in liposomes was shown to be effective means of delivering exogenous enzyme to the CNS, in addition to its known effect on elongation of the intracellular half life of administered activity (Weissmann and Finkelstein 1980).

Although the amount of the enzyme allowed to enter the CNS was small in terms of the injected dose, 4.4-fold net increment above control activity would be sufficient for correction of derangement in brain metabolism as far as the amount of enzyme is concerned.

Furthermore, subcellular fractionation of brain revealed the activity was incorporated into the mitochondrial-lysosomal fraction, although most of the enzyme was present in the supernatant fraction.

The present studies suggest liposomes as an effective carrier of exogenous enzyme to deliver into the central nervous system for the purpose of treatment of inherited lysosomal storage diseases, although much work in experimental animals has to be done before clinical application.

**Acknowledgments**

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**References**

