Retrograde axonal transport of an exogenous enzyme covalently linked to B-II_b fragment of tetanus toxin

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Attempts to replace enzymes in a number of fatal lysosomal storage diseases involving the central nervous system have as yet been unsuccessful owing to the impermeability of the blood/brain barrier to macromolecules. In order to treat storage diseases due to enzyme deficiencies, we investigated the feasibility of transporting an enzyme into the central nervous system without crossing the blood/brain barrier. Using the B-II_b fragment of tetanus toxin (because it is involved in recognition by the nerve-cell endings), retrograde axonal transport toward the spinal cord and trans-synaptic movement, and glucose oxidase as a marker, we demonstrated that a non-toxic enzyme–vector conjugate was taken up by axon terminals. After injection into the gastrocnemius muscle, the B-II_b–glucose oxidase conjugate was detected, both histologically and electrochemically, distally to a ligature on the sciatic nerve. Thus the B-II_b fragment could serve as a vector for glucose oxidase transport into the central nervous system. It was also verified that the transported enzyme retained its activity. Transport of this 150 kDa molecule by fragment B-II_b of tetanus toxin suggests that other enzymes of a lesser molecular mass may also be transported.

INTRODUCTION

Many lysosomal storage diseases have been reported [1]. Among them, several lipid storage disorders (Tay–Sachs or Gaucher's disease, for example) involve the central nervous system. The clinical consequence of lysosomal accumulation of undegraded metabolites in neurons (gangliosides in particular) is progressive neurological deterioration, resulting ultimately in the patient's death, usually at an early age. Potential therapies for inherited metabolic disorders include replacement of the missing enzyme activity, organ transplantation, bone-marrow transplantation and gene therapy [2]. None of these strategies has worked as yet. In particular, all the attempts to replace enzymes in a number of these conditions have failed until now. These failures can likely be ascribed to the impermeability of the blood/brain barrier to macromolecules. Only water and selected ions, as well as those molecules that are soluble in both water and lipids, are able to cross this barrier [3,4]. The only example of a provoked transfer across the brain capillary wall has been observed after retraction of the endothelial cells that form the blood/brain barrier, as caused, for instance, by the action of hyperosmolar solutions of mannitol or arabinose [5].

The therapeutic delivery of macromolecules to the central nervous system may occur along a pathway by-passing the bloodbrain barrier. Such a pathway, involving uptake of the material by nerve endings, internalization and its subsequent retrograde axonal transport, is used by pathogenic agents such as neurotropic viruses and tetanus toxin, the latter being also able to bring about the transport of another macromolecule. Habig *et al.* [6] demonstrated that the complex formed between antitoxin antibodies and tetanus toxin was transported along the sciatic nerve. Horseradish peroxidase (45 kDa) was chemically linked to tetanus toxin in order to follow its distribution after uptake by adrenergic nerve terminals [7].

Like other toxins, tetanus toxin consists of several domains.

One of these is involved in binding and transport. Mild treatment with papain, or exposure to endogenous proteinases, generates a 45 kDa fragment designated as fragment C [8] or B-II_b [9], which is derived from the C-terminal moiety of the heavy chain of the toxin. This non-toxic fragment binds to brain gangliosides *in vitro* [10], is transported retrogradely [11] and moves trans-synaptically, as does intact tetanus toxin. Evinger & Erichsen [12] have confirmed these findings. The use of fragment B-II_b as a specific carrier for therapeutic agents into the central nervous system was first proposed by Bizzini *et al.* [13].

Glucose oxidase was selected as a model enzyme [14] because its activity is resistant to chemical modification. It can be detected histoenzymically, since the enzyme is absent in mammals and can be easily quantified in tissues [15]. If the 150 kDa glucose oxidase molecule is found to be transported by fragment B-II_b of tetanus toxin, then other enzymes of lesser molecular mass should also be transported. Consequently this work was aimed at demonstrating that a non-toxic enzyme–vector conjugate, the fragment B-II_b–glucose oxidase conjugate, was taken up by axon terminals and conveyed retrogradely to the spinal-cord motoneurons.

MATERIALS AND METHODS

Materials

Purified tetanus toxin-derived B-II_b fragment was prepared essentially as described elsewhere [16]. Purified tetanus toxin assaying 3.1×10^7 MLDs/mg of protein was digested with papain coupled to CNBr-activated Sepharose 4B. Digestion was carried out at 37 °C for 4 h in 100 mM-citrate buffer, pH 7.3, containing 100 mM-sodium hyposulphite and 10 mM-EDTA. At the time of digestion cysteine was added to 5 mM final concentration. The ratio of papain to toxin was taken as equal to 1.67 units of immobilized papain/mg of toxin. At the end of the digestion time, papain was immediately removed by centrifugation for

Abbreviations used: MLD, minimal lethal dose; MMB, methyl 4-mercaptobutyrimate ester hydrochloride.

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5 min at 10000 *g* in a refrigerated centrifuge at 4 °C. The supernatant was filtered through a Sephadex G-100 column (2.5 cm × 80 cm) equilibrated with 100 mM-Tris/HCl buffer, pH 8, containing 1 M-NaCl. The effluent was continuously monitored at 280 nm and collected in 2 ml fractions. The fractions corresponding to the peak with a mean molecular mass of 45 kDa were combined. The volume of the pool of fractions was reduced to 2 ml before being refiltered on a column of Ultrogel AcA44 (2.5 cm × 80 cm) using the same buffer. The fractions representing the main peak were combined, and the volume of the resulting solution was reduced to 3 ml by dialysing it against a 50 % (w/v) poly(ethylene glycol) (20 kDa) solution. Undigested toxin or I_{bc}, which could contaminate this fraction, was adsorbed on to a column of CNBr-activated Sepharose to which the IgG fraction of an anti-I_{bc} serum had been covalently coupled. The unadsorbed fraction contained in the filtrate contains the B-II_b fraction. The B-II_b fragment was freed in this way of any residual toxicity.

Glucose oxidase (E.C. 1.1.3.4) from *Aspergillus niger* and horseradish peroxidase (E.C. 1.11.1.7), both grade I, were purchased from Böhlinger. Catalase (EC 1.11.1.6) from bovine liver (C-10) was obtained from Sigma. All other reactants were of analytical grade.

Preparation of conjugates by coupling glucose oxidase to B-II_b fragment

Since B-II_b fragment does not contain any readily available thiol groups, these were introduced in the protein by amidination using methyl 4-mercaptobutyrimidate ester hydrochloride (MMB) as described in [18]. Briefly, a B-II_b fragment solution was filtered on a Sephadex G-25 column (1 cm × 10 cm) equilibrated in 25 mM-borate buffer, pH 9. A 50 µl portion of *t*-butanol was added to 2.5 ml of the freshly filtered solution containing 4.84 mg of B-II_b. The solution was cooled in an ice bath and mixed, while being stirred with 160 µl of a methanolic solution containing 4.8 mg of MMB in four portions of 40 µl each at 30 s intervals. The cooled solution was stirred for an additional 30 min and filtered under N₂ on a Sephadex G-25 column (1 cm × 15 cm) equilibrated with 100 mM-sodium phosphate buffer, pH 6.9, containing 1 mM-Na₂EDTA. The content of thiol groups introduced into the protein was determined by using 4,4'-dithiodipyridine as a reagent. A disulphide bond was formed between glucose oxidase and the B-II_b fragment of tetanus toxin by allowing the 2-pyridyl disulphide derivative of glucose oxidase, obtained through the use of *N*-succinimidyl 3-(2-pyridyldithio)-propionate [19], to react with the thiolated B-II_b fragment. The thiol-disulphide exchange reaction was monitored by measuring the absorbance variation at 343 nm as a function of time. The reaction was complete within 30 min. The reaction product was separated on an FPLC 300 SW (1–300 kDa) column (Pharmacia), and elution was monitored by absorbance at 280 nm. Strong evidence for positive co-operativity has been obtained for the uptake and subsequent transport of the B-II_b fragment of tetanus toxin [20]; therefore we did not further purify the conjugate.

Animal model

For retrograde-axonal-transport studies, the technique described by Price *et al.* [21] in rats was adapted to mice [22]. Mice (Swiss, 20–37 g body wt. each) were anaesthetized by intraperitoneal injection of 150 mg of ketamin (Imalgène 500; IFFA-Mérieux, Lyon, France)/kg. After shaving and disinfection of the hind limb, an incision was performed dorsally. A 1 cm length of the sciatic nerve was exposed and two ligatures were performed with silk surgical suture halfway between the gastrocnemius muscle and the spinal cord. After suturing the incision, the enzyme or the conjugate B-II_b-glucose oxidase was

injected into the gastrocnemius muscle as a single dose of 20 µl. Owing to the toxicity of glucose oxidase [23], 16 µl of the conjugate with a glucose oxidase activity of 8.64 µmol·min⁻¹·l⁻¹ were co-injected with 4 µl of catalase (19 µg/µl). The use of a ligature allowed collection of migrating material at a single site, so that small amounts of catalytic activity could be detected. After 24 h the mice were decapitated and exsanguinated, and proximal and distal parts of the nerve were dissected. The controlateral sciatic nerve served as a reference. Nerves were kept at 4 °C in saline (0.9 % NaCl) until tested.

Histological detection of enzyme activity

Glucose oxidase was detected histologically by a technique using 3,3'-diaminobenzidine and horseradish peroxidase [14]. The nerve was excised after exsanguination of the mouse and submerged in Trypan Blue-coloured Tissue Teck (Miles). The samples were frozen at -20 °C and cut into 10 µm slices. The slices were fixed for 90 s in a 2.5 % (v/v) solution of glutaraldehyde in acetone, exhaustively rinsed with 154 mM-NaCl solution and then immersed for either 2.5 or 6 h in a reaction mixture composed of 50 mg of 3,3'-diaminobenzidine, 7.5 mg of horseradish peroxidase and 670 mg of glucose in 100 ml of 50 mM-phosphate buffer, pH 7.4. No other strain was applied. After this treatment, the slices were rinsed with 154 mM-NaCl, dehydrated, and mounted in Eukitt (Prolabo, Paris, France). Control sections were prepared from the controlateral nerve of the injected mouse and from nerves obtained from mice which did not receive an enzyme injection.

Quantification of enzyme activity

Glucose oxidase activity was measured in solution or in crushed tissue samples using an electrochemical technique [15]. Briefly, the natural co-substrate of glucose oxidase, namely O₂, is replaced by an artificial electron acceptor, benzoquinone, which is enzymically reduced to hydroquinone. Detection of this hydroquinone is performed by controlled-potential amperometry using an amperometric unit (PRG-DEL; Tacussel, Neuilly-Plaisance, France) and an anaerobic electrochemical cell including a classical three-electrode system: a calomel-saturated KCl electrode (SCE) as a reference electrode, an auxiliary electrode (platinum foil) separated from the main compartment by a glass frit, and the working electrode, which is a disc electrode made from a 3 mm-diameter glassy carbon cylinder. The working potential for hydroquinone detection was 350 mV/saturated-calomel electrode. This technique allows measurement of glucose oxidase activity in biological samples. The assay procedure was carried out as follows: 100 µl of glucose oxidase test solution was added to a mixture of 1 ml of 2.5 M-glucose solution and 2.5 ml of 10 mM-benzoquinone solution in 0.1 M-phosphate buffer, pH 6.5, completed with 0.1 M-phosphate buffer, pH 6.5, for a final volume of 5 ml. The difference in slopes of the curves obtained in the presence and absence of glucose oxidase was directly proportional to the enzyme concentration. For glucose oxidase activity determination in the nerve samples, the whole sample was homogenized in 100 µl of phosphate buffer and introduced without further modification into the electrochemical cell.

RESULTS

Characterization of the conjugate

The glucose oxidase derivative containing 11 groups of 2-pyridyl disulphide per molecule was allowed to react with a thiolated B-II_b fragment preparation containing 2.5 readily available thiol groups per molecule. The glucose oxidase/B-II_b fragment concentration ratio was 1:3. Under these conditions,

Fig. 2. Section of the sciatic nerve proximal to the ligature

Staining was with 3,3'-diaminobenzidine. A dark-field photomicrograph is shown; magnification $\times 66$.

Fig. 3. Section of the sciatic nerve distal to the ligature

Staining was with 3,3'-diaminobenzidine. A dark-field photomicrograph is shown; magnification $\times 66$. The orange-brown precipitate of oxidized 3,3'-diaminobenzidine demonstrates the presence of glucose oxidase activity.

Fig. 1. Elution profile at 280 nm of the reaction product (100 μ l of sample; 5 mg/ml) between pyridylated glucose oxidase and thiolated B-II_b fragment of tetanus toxin on an FPLC 300 SW column (Pharmacia)

Peaks were identified by control experiments with native proteins.

65% of the thiol-disulfide exchange occurred within 30 min. Chromatography of an aliquot of the reaction mixture on a 300 SW column showed heterogeneity and, in particular, the presence of high-molecular-mass molecules that were considered to be the conjugate (Fig. 1). The enzyme activity yield was 2%. The conjugate preparation contained 6.2 mg of protein and 540 units of glucose oxidase activity/ml.

Qualitative study of retrograde axonal transport

Histological slices were performed proximally and distally to the ligature. Since no staining was observed after 2.5 h of staining, the immersion period was extended to 6 h (Figs. 2 and 3). Precipitated oxidized 3,3'-diaminobenzidine, indicating the presence of glucose oxidase, was detected only in the distal fragment.

Quantitative study of retrograde axonal transport

This study was conducted on three mice (mean weight 31 g). After ligating the sciatic nerve, each mouse was injected with 16 μ l of conjugate supplemented with catalase at one site of the gastrocnemius muscle. The nerve was removed 24 h later, and distal and proximal parts were separated for activity measurements. Results are shown in Table 1.

The fact that some glucose oxidase activity was also present in the nerve segment proximal to the ligature may be due to an incomplete blockage of axonal transport as has been observed by Dumas *et al.* [24]. The control samples (controlateral nerves of the injected mice and the ligated nerve of a non-injected mouse) exhibited no glucose oxidase activity. In addition, crushed nerve does not interfere with the enzyme-activity measurement. When a 4-fold larger glucose oxidase activity was injected intramuscularly, no enzyme activity was found in the nerve.

Distribution of the native enzyme and conjugate in the organism

Although previous results reported that one-tenth of the 125 I-labelled toxin was still present at the injection site after 46 h [25], we found less than 1% residual activity when glucose oxidase activity was measured 6 h after the injection of the conjugate or native enzyme. About 4–10% of the total injected enzyme activity was recovered in the liver. We were unable to detect activity in the spleen or lungs.

DISCUSSION

In order to treat storage diseases due to enzyme deficiencies, we investigated the feasibility of transporting an enzyme to the central nervous system by its coupling to a peptide fragment which is known to undergo retrograde nerve transport. Using the B-II_b fragment of tetanus toxin, because it allows both recognition by the nerve-cell endings and retrograde axonal transport toward the spinal cord, and glucose oxidase as a marker, we showed that the B-II_b fragment could serve as a vector for glucose oxidase transport to the central nervous system. It was also shown that the transported enzyme retained its activity.

In general, it is accepted that macromolecules that are pinocytosed or endocytosed by plasma membrane follow one of three subsequent intracellular pathways [26]: (1) bulk-phase or adsorptive transport; (2) receptor-mediated endocytosis as part of the regulation and recycling of receptors by the cell; or (3) transcellular transport. Macromolecules that are not bound selectively to the cell surface, such as horseradish peroxidase, serum albumin and native ferritin, follow the first pathway. They are pinocytosed and transferred from endosomes to lysosomes, where they are ultimately degraded. The second major class of endocytosed macromolecules includes those which bind to specific cell-surface receptors and are subsequently internalized. Ligands such as nerve growth factor or wheat-germ agglutinin are transferred to endosomes and may ultimately reach lysosomes [27,28]. The third intracellular pathway fulfills the more specialized function of transporting the ligand; such as tetanus toxin, from one side of a polarized cell to the other.

Tetanus toxin, as well as its B-II_b fragment, is taken up and transported in all neuron types, namely motor, sensory and autonomic neurons in the peripheral nervous system [29] and by several fibre tracts in the central nervous system [30]. By contrast, transport of nerve growth factor [29] and antibodies to dopamine β -hydroxylase [31] is restricted to fewer types of neurons. In peripheral nerves, although tetanus toxin travels mainly within the axons, it has also been shown that the toxin travels in the epineurium, perineurium and to some extent in the endoneurium. However, it seems that only retrograde axonal transport allows access of tetanus toxin (or its fragment B-II_b) to the central nervous system [32].

Table 1. Glucose oxidase activity on either side of the ligature of the sciatic nerve

The enzymic activity was measured 24 h after injection of the conjugate glucose oxidase-B-II_b fragment in the gastrocnemius muscle. Retrograde axonal transport of the conjugate is blocked by the ligature, so that the glucose oxidase activity can be detected in the distal part of the nerve.

Parameter	Mouse...	1		2		3	
		Proximal	Distal	Proximal	Distal	Proximal	Distal
Activity (nmol/min per litre)		1.90	26.40	0	1.33	0	1.74
Proportion of the injected activity (‰)		0.02	0.03	–	0.015	–	0.02

The toxin-fixing capacity of specific gangliosides, particularly to types GD1b and GT1b [33,34], has led to their being considered as part of the receptor for tetanus toxin. However, the tetanus-toxin receptor might be something other than gangliosides [21,35,36]. A toxin-cell membrane interaction via ganglioside-independent sites has been proposed, since a trypsin-sensitive receptor component was shown to facilitate binding [37]. Moreover, it was found that B-II_b fragment, or its equivalent C fragment, which contains the ganglioside-binding site of the toxin, only partly competed with the labelled fragment for binding to the nerve plasma membrane [38–40].

A double-receptor model for the binding of tetanus toxin has been postulated by Montecucco [41]. In this model, it is hypothesized that binding sites for different cellular structures on the toxin molecule are carried by different toxin segments. Fragment B-II_b (equivalent to C) binds to a ganglioside-dependent receptor [association constant K_{lipids} (step 1)]. This step is followed by a lateral random search on the two-dimensional solvent plasma membrane to encounter an integral membrane protein, only present on nerve-cell endings, that acts as toxin protein receptor (step 2). The toxin is now in a high-affinity binding state which is the result of the product of two association constants. At this stage the binding of the toxin to the cell is to be considered as effective, since it is followed by internalization of the toxin and subsequent poisoning of the cell. The binding site to the second receptor would be carried on the *N*-terminal half-portion of the heavy chain.

One hopes that it may be possible to produce a vector, more complex than fragment B-II_b, that possesses the same recognition properties as tetanus toxin but which is non-toxic. In any case, since fragment C (another name for fragment B-II_b) has been cloned and expressed in *Escherichia coli* [42], an atoxic vector (without risk of incomplete purification of the toxin from *Clostridium tetani*), is available.

A rate of transport of the toxin in excess of 75 mm/day was estimated on the basis of the rate of appearance of tracer in the nerve-cell body [25]. In our experiments, the conjugate is detected distally to a ligature 24 h after the injection, an optimum time that was determined by Weller *et al.* [40]. The ligature technique is the easiest way to concentrate a product in a nerve and demonstrate its retrograde axonal transport. However, most of the injected preparation is taken up by lymphatic drainage and is no longer available for endocytosis by nerve ends. The variability of the intramuscular injection could explain at least in part the variability of the response of different mice (Table 1). More studies will be necessary to clarify the mode of endocytosis, as well as the routes and rate of intracellular degradation, which are critical for the fate of these conjugates after they are internalized by neurons.

In conclusion, macromolecules can travel rapidly from one compartment of the human organism to another only at the expense of energy. Uptake by nerve endings and retrograde axonal transport of an exogenous enzyme covalently linked to fragment B-II_b of tetanus toxin have been demonstrated. It would now be interesting to carry out a similar study with a therapeutically useful enzyme such as hexosaminidase A.

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