Proc. Natl. Acad. Sci. USA
Vol. 78, No. 9, pp. 5838–5841, September 1981
Medical Sciences

Delivery of hexosaminidase A to the cerebrum after osmotic modification of the blood–brain barrier

(reversible osmotic disruption/rodent brain/Tay–Sachs disease)

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Contributed by Roscoe O. Brady, June 1, 1981

ABSTRACT **The present studies were undertaken to evaluate the possibility that hexosaminidase A, the enzyme deficient in Tay–Sachs disease, could be effectively delivered to brain. Previous studies from our laboratory have shown that hypertonic mannitol can be used to osmotically produce reversible disruption of the blood–brain barrier in animals (rat and dog) and man without significant neurotoxicity and that such barrier modification significantly increases the delivery of cytoreductive chemotherapy agents to selected areas of brain. By using the rat model of blood–brain barrier modification and radiolabeled enzyme, increased hexosaminidase A delivery to brain has been demonstrated in more than 85 animals. The time of injection of hexosaminidase A after blood–brain barrier disruption is critical for maximum delivery. Rapid (over 30 sec) intra-arterial administration of hexosaminidase A immediately after blood–brain barrier disruption resulted in a marked increase in enzyme delivery to the brain when compared with controls without prior barrier disruption. When the enzyme was administered 15–20 min after barrier disruption, ≈50% less hexosaminidase A was delivered; when given 60–120 min after barrier modification, the amount delivered was the same as in control animals. This critical time course is very different than that seen in trials of low molecular weight chemotherapeutic agents (methotrexate and adriamycin). These preliminary studies suggest that hexosaminidase A can be delivered to the brain by blood–brain barrier modification and may be indicative of the potential for enzyme replacement in patients who have Tay–Sachs disease.**

Tay–Sachs disease is the most common form of gangliosidosis. It is inherited as an autosomal recessive disorder, and the gene frequency among Ashkenazic Jewish individuals is 1 in 27 (1). The enzyme deficient in Tay–Sachs disease is hexosaminidase A; the defined molecular weight of the enzyme is 100,000. The metabolite that accumulates as a result of the deficiency of this enzyme activity is ganglioside G_{M2} [*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosyl-ceramide]. Biochemical studies indicate that hexosaminidase A cleaves ganglioside G_{M2} and that hexosaminidase B is much less effective in this regard (2).

At present, no specific therapy for Tay–Sachs disease is available. Small quantities of purified enzyme have been produced and success in enzyme replacement *in vitro* has been reported (3). To date, clinical applicability of a replacement therapeutic approach has not been feasible because of the limitation of delivery of the enzyme to the cells in the brain (4, 5).

The major and immediate issues relevant to such delivery include the ability to move the enzyme in a functionally intact state across the blood–brain barrier and into the brain cells. The feasibility of such an approach was provided by the studies of Barranger *et al.* (6), who showed that peroxidase given intravenously after osmotic blood–brain barrier disruption entered the cerebral extracellular space and then the neurons in an enzymatically active form. In addition, cell fractionation studies indicate that enzymatically active peroxidase is incorporated within neuronal lysosomes over a 24-hr period.

These observations, as well as previous experience in increasing drug delivery to the cerebrum and posterior fossa by blood–brain barrier modification (7–12), led to this study. We describe the role of blood–brain barrier disruption in the delivery of radiolabeled hexosaminidase A to brain cells in the rodent.

MATERIALS AND METHODS

Osmotic Blood–Brain Barrier Disruption. Adult female Osborn–Mendel rats weighing 250–300 g were anesthetized with sodium pentobarbital (50 mg/kg), and the carotid artery was exposed through a lateral neck incision. A polyvinyl catheter (inside diameter, 0.023 in, P.E. 50, Clay Adams) filled with 0.9% NaCl containing sodium heparin at 100 units/ml was placed in the right external carotid artery with the catheter positioned 2 to 3 mm distal to the bifurcation of the common carotid and then secured.

Modification of the blood–brain barrier was accomplished with hypertonic (25%) mannitol (Merck Sharp & Dohme, Canada LTD, Quebec) by the method of Rapoport (6, 13). Paired controls were infused with 0.9% NaCl instead of mannitol. Control and mannitol-treated animals were selected in random order. Hypertonic mannitol or saline at 37°C was infused through the catheter at a rate of 0.12 ml/sec for 30 sec using a constant-flow pump (model 600-000, Harvard Apparatus, Dover, MA). The correct flow rate was determined in initial studies as the rate that completely displaced blood from the circulation of the ipsilateral hemisphere as described (7).

Five minutes before barrier modification 0.5 ml of 2% Evans blue (Chroma-Gesellschaft, Stuttgart, Federal Republic of Germany) was administered intravenously to provide a marker for barrier disruption of the ipsilateral hemisphere. The molecular size of the dye–albumin complex limits its egress from the circulation except following barrier disruption (14). Cerebral staining with the dye was graded, on sacrifice, as 0, no staining; 1+, just noticeable staining; 2+, moderate blue staining; and 3+, deep blue staining. Only those experimental animals with grades 2+ or 3+ staining were used for study.

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Studies of Hexosaminidase A. The temporal pattern and the quantitative aspects of enzyme delivery after osmotic barrier opening were studied. ^{125}I-labeled hexosaminidase A appears to be a suitable tracer for the delivery of enzyme to brain; iodination has little effect on its catalytic activity (15), and the plasma clearance of labeled enzyme is identical to the clearance of unlabeled enzyme as identified by its enzymatic activity. This suggests that labeled enzyme behaves normally in terms of its distribution and interaction with cell surfaces (16).

Human placental hexosaminidase A was purified by a modification of the method of Tallman *et al.* (2) in which concanavalin A-sepharose affinity chromatography and butyl-agarose hydrophobic chromatography steps were added and the ammonium sulfate precipitation step was omitted. The specific activity of the various purified enzyme preparations was 10–15 (mmol/hr)/mg of protein. Enzymatic activity was measured as described (15).

Iodination of hexosaminidase was performed by using Enzymobeads (Bio-Rad) following the manufacturer's instructions. Specific activity of the iodinated protein averaged 1 nmol of ^{125}I/nmol of protein. Iodinated hexosaminidase was separated from free iodine by gel filtration on a Sephadex G-25 (Pharmacia) column equilibrated with 1% human serum albumin in phosphate-buffered saline, pH 7.4. A typical preparation had a specific radioactivity of 20×10^6 cpm/pg, and 97% of the fresh preparation was precipitable with 20% trichloroacetic acid. After barrier modification by mannitol or saline, 10×10^6 cpm of the hexosaminidase A preparation, which was at least 85% Cl_3CCOOH precipitable, was diluted in 1 ml of 0.9% NaCl and infused for 30 sec through the right external carotid catheter.

Serial monitoring of the blood–brain barrier permeability to hexosaminidase A was done by infusing the enzyme at 5 sec, 1.5 min, 3 min, 5 min, 9.5 min, 14.5 min, 19.5 min, 44.5 min, 60 min, and 120 min after mannitol or saline infusion. All animals were sacrificed 1 hr after infusion of either mannitol or saline, except those given enzyme at 60 min and 120 min, which were sacrificed 1 hr after hexosaminidase A administration. Tissue was obtained from both hemispheres at the time the animals were sacrificed, and serum was collected to measure radioactivity.

In a second series of animals, the same amount of enzyme was administered in a volume of 3 ml starting 5 min after either saline or mannitol administration and infused over the subsequent 15 min. These animals were also sacrificed 1 hr after the saline or mannitol infusions.

After the samples were weighed, radioactivity of the samples was measured with a Gamma 4000 spectrophotometer (Beckman). Radioactivity in serum was measured both before and after protein precipitation with 20% Cl_3CCOOH. The amount of enzyme protein was calculated from the specific radioactivity of the labeled hexosaminidase.

RESULTS

Intravenous administration of 1×10^3 ng of hexosaminidase A into animals in which there was no barrier modification (saline-infused controls) resulted in a mean level of enzyme of 236 pg/gm ($n = 4$) in the ipsilateral hemisphere. Intra-arterial administration of enzyme in controls resulted in a slightly higher level in the ipsilateral hemisphere [560 pg/gm ($n = 2$)]. Serum values at sacrifice were $1.0–6.0 \times 10^3$ pg/ml, and 45–70% of the serum enzyme could still be precipitated with Cl_3CCOOH.

These enzyme values are higher than can be accounted for by the activity of tracer remaining in the brain vascular compartment at the time of sacrifice. The brain blood volume is generally ≈2 ml/100 g of brain (17). In addition, when 2-, 3-, or 4-fold incremental doses of hexosaminidase A were given after intracarotid saline infusion (Fig. 1*A*), there were no increases in brain enzyme delivery. This suggests that some small fixed amount of the enzyme (*i*) nonspecifically binds to the brain vascular endothelium, (*ii*) is pinocystosed by it, or (*iii*) gains access to brain in regions where the blood–brain barrier is incomplete. This background is constant and does not change with increasing enzyme dose size. Further, it represents only a small fraction of the activity that is delivered to the brain after barrier disruption. Thus, in nonbarrier-modified control animals (i.e., no Evans blue–albumin staining of brain), these observations suggest that no significant amount of enzyme is delivered across the blood brain barrier, as has been reported previously (4).

By contrast, hexosaminidase A delivery to the brain after barrier modification was directly related to amount of enzyme given. In fact, not only was the absolute amount of hexosaminidase A increased, but the fraction of infused enzyme delivered to brain increased with increasing dose size (Fig. 1*B*). Enzyme delivery corrected for the above defined background after an infused enzyme dose of 500 ng was 0.01%; after a dose of 1000 ng, it was 0.24%; and after a dose of 2,000 ng, it was 0.59%. Thus, the percent delivery (i.e., enzyme that penetrated the blood brain barrier and is still present at 1 hr) appears to increase with increasing enzyme dose size. In the contralateral hemisphere, no increase in enzyme delivery was observed with increasing amounts of administered enzyme (Fig. 1).

Intra-arterial hexosaminidase A administration given rapidly (30 sec) and immediately after osmotic blood–brain barrier disruption resulted in a greater increase in enzyme delivery to the brain when compared with intra-arterial administration by rapid infusion 15–20 min after barrier modification. This observation led to more detailed studies of the importance of the timing of enzyme administration after barrier modification. The results showed that any delay in rapid bolus infusion of enzyme resulted in less enzyme delivered to brain (Fig. 2). Rapid bolus infusion of enzyme 120 min after barrier modification by mannitol

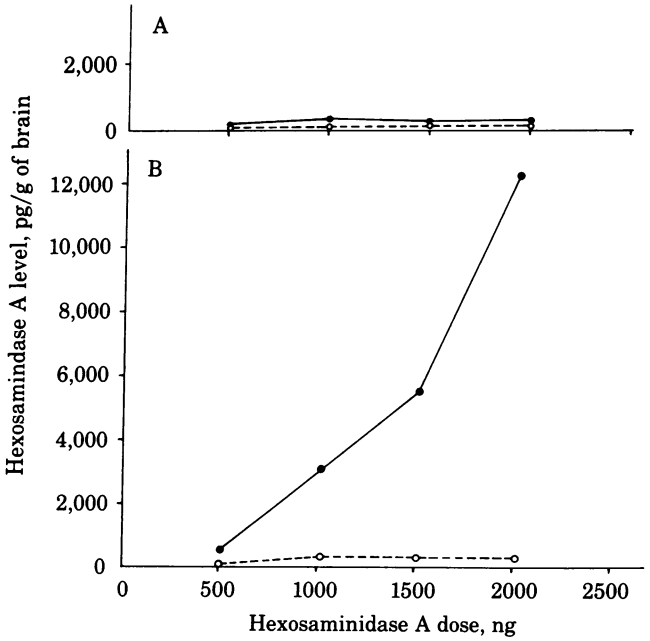

FIG. 1. Delivery of various doses of hexosaminidase A after osmotic blood–brain barrier disruption. Hexosaminidase A was given intra-arterially over a 30-sec period 5 sec after either normal saline (*A*) or mannitol (*B*) infusion. None of the saline-infused animals ($n = 4$) demonstrated any Evans blue–albumin staining, whereas all mannitol-infused animals ($n = 4$) displayed 3+ Evans blue staining of the ipsilateral hemisphere. ●, Ipsilateral hemisphere; ○, contralateral hemisphere.

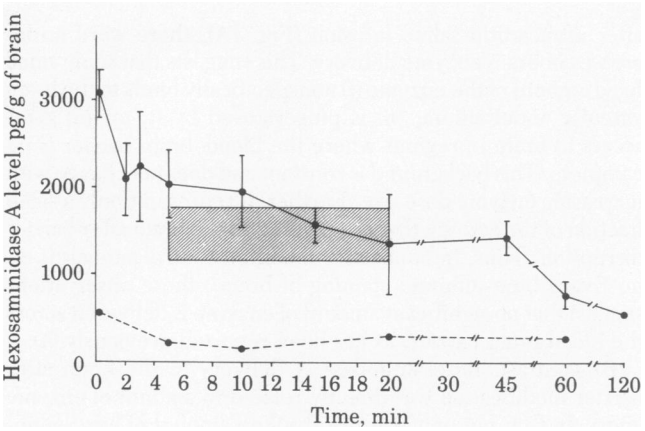

FIG. 2. Brain hexosaminidase A levels after using two different methods of intra-arterial administration in the rat. ▨, Enzyme levels (mean ± SEM) in rats given hexosaminidase A for 15 min starting 5 min after blood–brain barrier disruption; ——, rats were given a constant amount of hexosaminidase A by a 30-sec bolus infusion at the indicated time points after mannitol infusion; ----, saline-infused control rats were given hexosaminidase A by a 30-sec bolus infusion at the indicated time points. Results are mean ± SEM; n = 3–6.

yielded essentially the same enzyme delivery as seen in the control (saline-infused) animals.

The amount of hexosaminidase A delivered to the brain after administration by rapid bolus infusion was then compared with the amount of enzyme delivered when enzyme was given by constant infusion over a 15-min period starting 5 min after barrier modification. The amount of enzyme delivered to brain by such an infusion protocol was only 50–60% of that delivered when rapid bolus infusion immediately followed barrier modification (Fig. 2).

The amounts of hexosaminidase A in the contralateral hemisphere after either intracarotid saline or mannitol infusion followed by intracarotid hexosaminidase A infusion were virtually identical to the values shown in Fig. 1*A* in the ipsilateral hemisphere after saline infusion. As noted above, this indicates that there was no enzyme delivery across the blood brain barrier in the contralateral hemisphere.

DISCUSSION

In this study, we have explored the feasibility of enzyme delivery to brain to achieve replacement for the treatment of patients who have Tay–Sachs disease. The delivery of enzyme to the central nervous system is blocked by the existence of the blood brain barrier. This barrier, created by tight junctions between the endothelial cells of brain capillaries, excludes molecules in blood from entering the brain on the basis of molecular weight and lipid solubility (10, 13). The blood brain barrier normally prevents passage of molecules of $M_r > 180$. An even more important factor is lipid solubility; for instance, methotrexate (M_r, 454) is lipid insoluble and normally has a cerebrospinal fluid:plasma concentration ratio of 0.02 (12).

Adequate enzyme replacement therapy requires that the enzyme reach the brain cells catalytically active and in significant amounts. The rationale for such an approach is supported by *in vitro* studies by Brooks *et al.* (3) who showed that cerebellar cells from a fetus having Tay–Sachs disease exposed to concanavalin A incorporated exogenous hexosaminidase A and that the hexosaminidase A was capable of mobilizing stored ganglioside G_{M2} substrate. Those observations suggest, therefore, that a critical step is the delivery of the enzyme from the blood to the brain.

Rattazzi *et al.* (18) have already begun to approach the problem. They observed that delivery of hexosaminidase A to the brain can be enhanced by blocking the rapid hepatic uptake of the enzyme with mannans. They also demonstrated a qualitative increase in blood–brain barrier permeability to hexosaminidase A by using hyperbaric oxygen. However, Gruenau *et al.* (19), using a quantitative technique, were unable to document increased blood–brain barrier permeability under the experimental conditions reported by Rattazzi.

This study has shown that it is possible to increase the delivery of hexosaminidase A to the central nervous system parenchyma when the enzyme is given intra-arterially after osmotic blood brain barrier disruption. Intra-arterial administration without barrier modification results in little enzyme delivery across an intact blood–brain barrier as shown by two observations: (*i*) intracarotid hexosaminidase A infusion only slightly increased enzyme delivery to brain when compared with intravenous infusion and (*ii*) progressively increasing doses of intracarotid hexosaminidase A did not increase enzyme delivery to the brain. On the other hand, with smaller molecular weight molecules (adriamycin and methotrexate) intracarotid administration or the use of progressively larger doses does increase drug delivery despite an intact barrier (unpublished results).

The best mode of hexosaminidase A delivery was intracarotid infusion as a bolus within the first 30 sec after barrier disruption. However, even when given under these conditions, only 0.6% of the administered dose of enzyme (2000 ng) entered the brain. Using this delivery fraction, we estimate that ≈100 milligrams of enzyme would be required to achieve a normal concentration of enzyme in brain (20). It is probable, however, that this degree of enzyme replacement may not be necessary to achieve significant mobilization of the substrate (G_{M2}) ganglioside in the pathological state. Moreover, it appears that enzyme delivery increases with increasing doses of administered enzyme. It seems clear that we have not reached maximum delivery.

One inherent problem in replacement therapy is that hexosaminidase A in the circulatory system is rapidly cleared by the liver (4). This loss from the potential pool for central nervous system delivery to brain has been managed in animal studies by the administration of agents to block hepatic uptake (18, 21). It is of interest that mannose-containing moieties do not block enzyme binding to synaptosomes (16, 22). Thus, the membrane receptor for hexosaminidase A in the central nervous system appears to be different from the one in the liver. Moreover, mannans (23), bovine pancreatic ribonuclease, and other related mannose-rich moieties previously used to block hepatic clearance *in vivo* can be toxic. Other strategies need to be examined if incorporation into the liver must be reduced.

Another pertinent observation derived from the present studies is that the regimens used to optimize delivery of various molecules after osmotic blood–brain barrier modification are not necessarily the same. For instance, we have found that, for a chemotherapeutic agent (adriamycin) administered as a rapid bolus immediately after barrier modification or as a slow infusion starting 5 min after barrier modification and infused over a 15-min period, there was little difference in amount of drug delivered to brain (unpublished results). On the other hand, there is a clear difference in the amount of hexosaminidase A that penetrates the barrier if given by rapid bolus infusion as opposed to a slow infusion (starting 5 min after barrier modification).

We have shown here that hexosaminidase A can be delivered across the blood–brain barrier. Whether the enzyme, once across the barrier, can enter the cellular lysosomes *in vivo* in a form that enables it to mobilize accumulated substrate is not known. As the pathological substrate in Tay–Sachs disease ap-

parently accumulates slowly, as evidenced by the delayed age of onset of the clinical manifestation of the disease, intermittent enzyme replacement therapy after barrier modification may be beneficial. This may be practical providing that the delivery of enzyme can be improved. We are encouraged by the findings presented here that this may eventually be possible. Finally, it should be noted that, even with the prospect of genetic engineering as another approach to treatment in genetic diseases involving the central nervous system, the obstacle of the blood brain barrier to a functional genome must still be dealt with.

We wish to acknowledge the expert secretarial assistance of Mrs. Jolene McBride. This work was supported in part by the March of Dimes Birth Defects Foundation (Grant 1-720), the National Cancer Institute (Grant CA 27191), and the American Cancer Society (Grant PDT-175).

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