DISTRIBUTION OF INTRAVENTRICULAR HORSERADISH PEROXIDASE IN NORMAL AND HYDROCEPHALIC CAT BRAINS* †

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ABSTRACT

The distribution of intraventricularly injected horseradish peroxidase (HRP) was examined in the ependymal and subependymal tissue of both normal and experimental hydrocephalic cats by light and electron microscopy. At different time intervals after the intracisternal injection of kaolin, the hydrocephalic ventricles were perfused at various intraventricular pressures with mock cerebrospinal fluid (CSF) containing HRP. The uptake and localization of HRP by periventricular brain tissue was studied with respect to transventricular absorption of cerebrospinal fluid. The results of both light and electron microscopic studies have shown that in normal, acute or chronic hydrocephalic cats perfused at either high or low pressure the distribution of HRP is similar. The enzyme was localized mainly to the intercellular spaces, between ependymal cells, glial cells, and in perivascular spaces (in and across the basement membrane of subependymal blood vessels). HRP did not penetrate endothelial tight junctions even in chronic hydrocephalic cats undergoing significant rates of transventricular absorption of CSF. It is concluded that since the distribution of HRP in brain is determined by diffusion, its use as a tracer for the bulk movement of CSF through nervous tissue is limited. It is, however, an excellent extracellular space marker in electron microscopy.

INTRODUCTION

Previous studies have suggested that transventricular absorption of cerebrospinal fluid (CSF) can occur when normal CSF circulation is obstructed and the fluid is prevented from reaching its normal absorption sites (1, 2). Above a threshold pressure, the rate of bulk absorption of CSF in experimental hydrocephalic animals was shown to vary linearly with intraventricular pressure, although absorption was less responsive to pressure changes than in normal cats (1). However, the mechanism and routes by which CSF returned to the blood is not entirely clear. In studies on the periventricular water content of brains from chronic hydrocephalic cats it was found that an increase in periventricular water content was associated with transventricular absorption of CSF (3). These studies also suggested that fluid from the ventricles penetrated the extracellular space of the brain to a depth of about 600 μ beyond the ependymal

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lining before it was removed by the blood (3). Partial destruction of ependymal cells and looseness of the subependymal areas found in hydrocephalic cats were thought to provide some of the morphological changes associated with transventricular absorption (4).

Attempts to define the extracellular space of the brain have been carried out with the electron microscope, using radiopaque substances such as ferritin, lanthanum, and horseradish peroxidase (5, 6). The distribution of intraventricularly injected horseradish peroxidase into brain tissue of normal animals has shown inter-spaces in brain which are generally patent and which do not interfere with intercellular movement of tracers (5). In the present study, an attempt was made to demonstrate the routes of transventricular absorption of CSF during perfusion of the ventricular system of normal and hydrocephalic cats. Both radiolabelled albumin and horseradish peroxidase were added to the perfusion fluid as tracer substances in order to quantitate absorption of fluid, and to visualize, by light and electron microscopy, the pathways taken by the fluid during absorption.

MATERIALS AND METHODS

Adult mongrel cats weighing 2 to 3 kg were used. Hydrocephalus was produced by injection of kaolin into the cisterna magna as previously described (7). After the development of hydrocephalus (acute stage up to seven days, chronic stage 21 days or more after injection) the ventricular system was perfused from one lateral ventricle to the other (7). Perfusion pressure was monitored during the experiment. The duration of all perfusion experiments was 2.5 to 3 hours. Rates of formation and absorption of CSF were calculated from measurements made during steady-state perfusion (9). As controls, normal cats were perfused from the lateral ventricle to the cisterna magna (8) at pressures varying from −5 cm H₂O (low pressure) to 15 cm H₂O (high pressure) with respect to the interaural line. In each experiment, 100 mg type II horseradish peroxidase (Sigma Chemical Co., St. Louis) was added to the artificial CSF used for perfusion (8). At the end of perfusion the animals were sacrificed by injection through the ascending aorta, first with normal saline (100 to 150 cc) for two minutes and then with 5% glutaraldehyde in cacodylate buffer at a pH 7.4, for 30 to 45 minutes. A total volume of 400 ml of glutaraldehyde was usually employed. Subsequently, the brain was removed, cut in coronal sections, and placed in the same fixative for an additional 2 to 3 hours. The tissues were washed overnight in cacodylate buffer containing 20% dimethylsulfoxide (DMSO) at 4°C according to the method of Zagury et al. (10).

Frozen sections were then cut (10 to 50 microns thick), collected in 0.3 M sucrose, and soon after, washed with Tris-HCl buffer (0.05 M, pH 7.6) for 30 minutes). The frozen sections were then incubated for peroxidase activity according to the method of Graham and Karnovsky (11) for 30 minutes at room temperature. The incubating media contained: 5 mg of 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis) dissolved in 95 ml of Tris-HCl buffer (pH 7.6). To this was added 0.5 ml of a mixture of 20 ml of Tris-HCl buffer and 1 ml of 3% H₂O₂. As a control, frozen sections, from animals which received no peroxidase, were also incubated in the above media.

Electron Microscopy: After incubation, some sections were washed in Tris-HCl buffer for two hours, placed in Dalton's chrome-osmium fixative (12) for 90 minutes, dehydrated in alcohol, and embedded in Araldite. The appropriate areas desired for thin sectioning were selected and cut under a stereo-microscope when the sections were in liquid Araldite. Propylene oxide was not used at any time during embedding. The sections were left in Araldite under vacuum overnight at room temperature. The Araldite was then changed, and the tissues were cured in the oven at 65°C for 48 hours.
**Light Microscopy:** After incubation, sections were washed in distilled water and either dehydrated in alcohol, passed through xylene and mounted with permount, or mounted wet with glycerine jelly. They were studied and photographed without counterstaining.

**RESULTS**

All the photographs used for illustration were taken from tissues incubated 30 min for horseradish peroxidase. No counterstains were employed for either light or electron microscope preparations. CSF turnover rates were similar to those described earlier. The mean rate of CSF formation in both groups of hydrocephalic animals was 0.0085 ml/min. Characteristically, the acute differed from the chronic hydrocephalic cat by its decreased absorptive capacity (4). At an elevated perfusion pressure, CSF absorption rates were 0.0075 ml/min and 0.045 ml/min in acute and chronic hydrocephalic cats respectively.

**Histochemical Studies:** Horseradish peroxidase (HRP) distribution: Experiments with normal, acute, or chronic hydrocephalic cats perfused under low or high pressure, showed that the distribution of HRP was essentially similar in both light (figs. 1 and 2) and electron microscopic preparations (figs. 3 and 4).

**Light Microscopy:** The four groups of animals showed only slight differences in intensity of enzyme reaction and distance to which the peroxidase staining spread from the ependymal surface into the brain substances. The enzyme seemed to be present in greater concentration and to penetrate deeper into the

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**Fig. 1.** Frozen section of cerebral hemisphere of normal cat perfused under low pressure (−5 cm H₂O). Note dense black horseradish peroxidase enzyme reaction product in periventricular tissues. No counterstain. × 4.

**Fig. 2.** Frozen section of cerebral hemisphere of chronic hydrocephalic cat (28 days after kaolin injection into cisterna magna). Note dense black horseradish peroxidase enzyme reaction product in periventricular tissues. No counterstain. × 4.
Fig. 3. Electron micrograph of ependymal area of lateral ventricle (parietal lobe level) of normal cat perfused at high pressure (15 cm H₂O). The black lines (arrows) indicate sites of deposition of horseradish peroxidase-enzyme reaction product. Vacuole filled with reaction product (V). Unstained. × 12,000.

Fig. 4. Normal cat. Ependymal area of lateral cerebral ventricle. The black lines between ependymal cells and cell processes mark the sites of deposition of horseradish peroxidase. Unstained. × 24,000.
brain of the chronic than of the acute hydrocephalic or the normal cat (figs. 1 and 2). In addition, a greater accumulation and distribution of peroxidase was visible to a greater distance from the surface of the ventricle which contained the perfusion needle during ventriculocisternal perfusion (fig. 2). Whenever the peroxidase had reached the cranial subarachnoid space over the cerebral cortex of the normal cat it spread through the pia matter into the neuropil (fig. 2). No evidence of enzyme reaction was noted in tissues taken from control animals which did not receive peroxidase.

**Electron Microscopy:** Tissues from both normal and hydrocephalic animals examined with the electron microscope showed the black, granular precipitate, enzyme reaction product of horseradish peroxidase (figs. 3 to 7). This was localized to the intercellular spaces of the ependymal cells, at the ependymal glial cell boundary, between glial cells, between myelinated axons and in the perivascular spaces. The peroxidase enzyme reaction product was also seen in and across basement membrane of subependymal blood vessels (figs. 5 and 6). This extended up to the endothelial tight junction, but was not seen to go through it. Some ependymal cells contained membrane bound vacuoles filled with enzyme reaction product (fig. 3). Similar vacuoles were also seen in the endothelial cells of subependymal blood vessels. The junctional complexes of ependymal cells especially the terminal bars showed no obvious reaction product. HRP appeared in most cases (normal and pathological) at a maximal depth of about 2 mm from the ependymal or cortical surfaces. Although all tissues showed HRP in some ependymal and subependymal areas, there were areas where no HRP was noted. HRP was especially intense in some intersynaptic spaces of the gray matter. Only rarely was some HRP seen in the cytoplasm of neurons and glial cells. Some endothelial cells, and ependymal cells showed a dark finely granular appearance of their cytoplasm indicating some diffusion of HRP into the cells. This was prominent in detached ependymal cells lining the ventricles of chronic hydrocephalic animals. The areas of rarefaction due either to frozen section artifact or to pathological changes seen in the subependymal areas of hydrocephalic animals, also contained HRP, usually in the bordering zone outlining the defects (fig. 4).

In both acute and chronic hydrocephalic, as well as in normal cats, the distribution of HRP was essentially the same. This was also true in normal animals perfused at a very low pressure (−5 to 0 cm H₂O). The peroxidase reaction product was seen up to 2 mm beyond the ependymal surface, even in the acute hydrocephalic cat in which transventricular absorption of CSF was not detected during perfusion.

**DISCUSSION**

Evidence for transventricular absorption of CSF as an alternate pathway for the removal of CSF has been provided by a number of observations made during the development of experimental obstructive hydrocephalus. In acute hydrocephalic cats, the increased intraventricular pressure was found to be due to a marked decrease in the CSF absorptive capacity (18). In these animals,
Fig. 5. Normal cat. Lateral cerebral ventricle. Subependymal area about 1 mm beneath the ependymal surface. Note the horseradish peroxidase reaction product in between myelinated axons and other cell processes (arrows) and in the basement membrane (BM) of a blood vessel (BV). Unstained. × 20,000.

Fig. 6. Acute hydrocephalic cat (7 days). Ependymal and subependymal areas of lateral cerebral ventricle. Note horseradish peroxidase reaction product (arrows) in the extracellular space and in the basement membrane (BM) of a blood vessel (BV). × 8,000.
the water content of the periventricular white matter was normal and not affected by the perfusion pressure. In chronic hydrocephalic cats the return of the intraventricular pressure to normal was attributed to the 7-fold increase in the rate of CSF absorption (18). Since there was no communication between
the ventricular system and the subarachnoid space, the removal of CSF was thought to occur by transventricular absorption. Data to support this contention was obtained from the CSF-derived increase in water content measured in periventricular white matter during CSF absorption (3). Moreover, it was predominantly the straight sinus, as opposed to the sagittal sinus in normal cats, that contained the radiolabeled tracer during CSF absorption (19). In the chronic hydrocephalic cat CSF absorption began at a higher threshold pressure than in a normal cat and was preceded by ventricular dilatation (20, 21).

The use of horseradish peroxidase as so-called nondiffusible indicator substance was an attempt to demonstrate transventricular absorption of CSF by the localization of this enzyme (accumulation, and penetration beyond ventricular surface) in brain tissue. Both acute and chronic hydrocephalic cats were studied since CSF absorption readily occurs only in the latter animals. Accordingly it was also thought possible to determine whether the marked resistance to the bulk flow of fluid in the acute hydrocephalic cat was due to: (a) limited capacity of the brain tissue to take up fluid, (b) slower flow of fluid through narrow extracellular channels (c) relative inability of the fluid in the tissue to gain access to the blood through capillaries. Subsequently, these results would be compared to those obtained in normal cats where CSF absorption is known to occur distal to the fourth ventricle.

However, the distribution of HRP in the periventricular gray and white matter of all animals in the present study was similar. This included brain tissue from normal cats where the bulk absorption of CSF is thought to occur via the arachnoid villi, as well as hydrocephalic cats, some of which (chronic hydrocephalic) demonstrated transventricular absorption, and other (acute hydrocephalic) with virtually no measurable absorption. The penetration of HRP into the brain tissue has been shown to increase with time. Brightman and Reese (5) suggested that both the concentration and movement of HRP in brain tissue are primarily determined by diffusion. The present study supports this view. Previous studies by Sahar et al. (13) have also shown that the difference in uptake of radiolabeled albumin in the periventricular white matter of both normal and hydrocephalic cats is dependent on the perfusion time and not on perfusion pressure. From those experiments it was also concluded that CSF absorption probably takes place within 2.5 to 3 mm from the ventricular surface. With the use of more refined techniques, however, and based on the water content of brain slices, absorption sites were brought to within 600 µ from the ventricular surface (3).

An electron microscopical study (4) of acute hydrocephalus in cats showed a correlation between flattening and stretching of ependymal cells with minimal rarefaction of ependymal tissue on one hand, and a markedly decreased rate of CSF absorption on the other. The chronic hydrocephalic cat with significant rates of CSF absorption showed detachment of ependymal cells, significant rarefaction of subependymal tissue, and marked increase in subependymal extracellular space. These findings are similar to those of Weller et al. (16), in dogs with silicone oil-induced hydrocephalus, and to those of McLone et al.
(17) in mice with naturally occurring hydrocephalus. However, Weller et al. (16) observed re-establishment of the ependymal lining in chronic hydrocephalic dogs with a return of normal intraventricular pressure.

Although not alluded to specifically, the diffusibility of HRP in brain tissues has been shown by Becker, et al. (6) and Shabo and Maxwell (14). Eighteen to twenty-four hours after intracerebral implantation of a pellet of HRP into the cerebrum of the rat, the former authors noted that peroxidase moved a considerable distance from the implantation site via intercellular spaces of both gray and white matter. Through electron microscopic observations of the fate of HRP injected into the cranial subarachnoid space, the latter authors were able to demonstrate the movement of HRP across the pial-glial basement membrane into the neuropil; the present studies with normal cats confirm this work. The peroxidase was prevented, however, from passing into the lumina of intracerebral as well as subarachnoid blood vessels, or through the arachnoid membrane by tight endothelial junctions or junctional complexes (terminal bars).

The combined use of ventricular perfusion technique and electron microscopy in the present work was an attempt to quantitate the clearance of HRP and determine its distribution in cerebral tissue with respect to the transventricular CSF absorption pathways. However, since the distribution of HRP as found here appears to be determined primarily by diffusion, its use as a tracer for the bulk movement of CSF through nervous tissue is doubtful. From previous studies on the uptake and diffusion of inulin (15) and albumin (8) by the brain during ventricular perfusion experiments, the amount of HRP visualized in the present electron microscopic study probably represents only a small percentage (1%) of that contained in the perfusate. Horseradish peroxidase does seem, however, to be an excellent extracellular space marker for electron microscopy even though its concentration in brain is limited by the CSF-brain barrier.

REFERENCES


