SOMETHY Institute of Experimental Animals

GUY OWENS, M.D., AND SAM L. CLARK, M.D.
Departments of Surgery and Anatomy, Vanderbilt University
School of Medicine, Nashville, Tennessee

(Received for publication February 18, 1956)

STUDIES of the influence of enzyme injections on fluid absorption, hematoma dissolution and prevention of excessive fibrosis have been carried on in most areas of the body with the exception of the central nervous system. To extend such studies to this area an investigation of the effects of injecting hyaluronidase† in the subarachnoid space was undertaken.

METHOD AND MATERIALS

To test initially the influence of cerebrospinal fluid on the spreading effect of hyaluronidase in tissues, cerebrospinal fluid in 1 cc. amounts obtained from the cisterna magna of Macacus rhesus monkeys and cats was used to dissolve 150 turbidity reducing units of hyaluronidase. At 30-min. intervals up to 3 hours these solutions mixed with 2 cc. of 0.5 per cent methylene blue or with 100 cc. of saline were injected subcutaneously to provide a visual method of recognizing the spreading effect of the enzyme. Control injections were made simultaneously in symmetrical regions using equal quantities of the same solutions without hyaluronidase. The spreading effect of the hyaluronidase appeared to be unaltered by the cerebrospinal fluid.

A second group of animals anesthetized by intravenous pentobarbital (30 mg./kg.) were studied. Each animal was placed on its right side during the first stages of the experiment. Increments of 1500 units of hyaluronidase were dissolved in 1 cc. amounts of cerebrospinal fluid obtained from the animal being used and injected into the subarachnoid space. Pressure changes were followed for 15 minutes after returning the solution to the cisterna. This procedure was repeated until the total enzyme injected ranged from 6000–9000 units.

Small quantities of cerebrospinal fluid were withdrawn and studied for cellular reactions at the end of 2, 4, 24 and 72 hours by smears stained by Wright’s technique and in some instances with supravital preparations using neutral red and Janus green. Total protein determinations were made at the end of 4, 24 and 72 hours. Alteration in the permeability of the blood-brain barrier was tested in 3 instances by injecting into the peritoneal cavity 1 gm. of trypan blue dissolved in 10 cc. of saline, and the time of appearance of the dye in the cerebrospinal fluid was noted. The 3 Macacus rhesus monkeys used were kept for observation for 36 hours, 1 week and 6 months. The 6 cats were sacrificed at 4 hours, 2, 5, 7, 10 and 12 days. Gross and microscopic studies were made of the brains of all the animals.

† Hyaluronidase supplied by Wyeth Pharmaceutical Co., Philadelphia, Pa. and G. D. Searle Co., Chicago, Ill. with partial support of this project by a grant from G. D. Searle Co.
In a third group 4 cats received daily instillation of 1500 units of hyaluronidase for 4 days into the cisterna magna and 4 other cats for 8 days. Daily cerebrospinal fluid cell counts and neurological observations were made. In 2 of these animals trypan blue was injected intraperitoneally as previously described. Fourteen days following the last hyaluronidase instillation all animals were sacrificed. The vessels to the head were perfused with normal saline followed by 10 per cent formalin, and gross and microscopic studies of the brains were made.

RESULTS

All animals in the second group, which received intrathecal hyaluronidase over a short period of time, demonstrated similar responses with only slight variations in degree. Representative changes of cerebrospinal fluid pressure and cells, and alterations in the blood-brain barrier in these animals are illustrated in Fig. 1. A marked cerebrospinal fluid pressure elevation occurred although there was drainage of a total of from 4 to 6 cc. from each animal over a 4-hour period. Red blood cells were found in varying quantities depending probably on the degree of trauma produced by the needle insertions and subsequent movement. A minimal increase in polymorphonuclear cells was seen in all instances. More impressive were the more numerous large epithelial cells with round densely basophilic nuclei which were found in the cerebrospinal fluid of each animal (Fig. 2). Supravit preparations initially revealed centrally located nuclei but in subsequent smears nuclei lying peripherally were seen. At around 48 hours loss of nuclei of some cells was noted giving to the cell remnant with its open end a basket-like

![Graph](image_url)

**Fig. 1.** Representative cerebrospinal fluid changes in cat 4 following cisternal instillation of 9000 units of hyaluronidase.
EFFECTS OF HYALURONIDASE INJECTIONS ON CNS

appearance. Nuclei devoid of cytoplasm resembling those in the epithelial cells were occasionally observed. Sheets or plaques of these cells were often seen. The epithelial cells appeared first at about the end of the second hour of the experiment. They increased in number by the end of 24 hours and thereafter progressively diminished. At 72 hours none could be found.

A breakdown of the blood-brain barrier was evidenced by the appearance of trypan blue in the cerebrospinal fluid at the end of 3 hours. Protein determinations on cerebrospinal fluid samples gave values in the range of 300 mg. per cent at the end of 2 hours but had returned to a normal range within 24 hours (15–25 mg. per cent). In vitro addition of 9000 units of hyaluronidase to 1 cc. of cat’s cerebrospinal fluid as a control gave a protein determination of 205 mg. per cent. A prolongation of the usual response to pentobarbital anesthesia for a few hours was observed.

Neurological effects occurred which resembled vestibular disturbance and “cerebellar” seizures. These had become manifest at the end of 24 hours and were most pronounced in the monkey. A tendency for right-sided falling, turning movements, and rigidity of the right extremity was noted. In 1 monkey allowed to survive for 6 months these neurological abnormalities had disappeared within 1 month. The cats showed less severe effects and in 2 allowed to survive for 10 and 12 days little if any disability remained. There were no deaths attributed to the central nervous system changes.

At autopsy gross examination revealed no evidence of inflammatory or hemorrhagic reactions. In several instances there appeared to be a marked
reduction of cerebrospinal fluid present in the cisterna magna. This had been noted previously in the same animals when no fluid could be obtained from this region on successive needle punctures.

In both cats and monkeys denuding of the choroid epithelium occurred with the greatest effects found in the 4th and 3rd ventricles. The most marked loss of epithelium appeared in animals sacrificed at the end of 48 hours (Fig. 3). Partial loss of epithelium was in evidence at the end of the

![Fig. 3. (A) Denuded choroid epithelium in a monkey sacrificed 36 hours following instillation of hyaluronidase (X570). (B) Higher magnification of same (X1080). (C) Choroid plexus of normal monkey (X570).](image-url)
5th day. No areas of epithelial loss were seen in those animals sacrificed at the 7th, 10th and 12th days. Some destruction of the ependyma lining the various ventricles aside from the choroid plexus, edema of the cerebellar cortex, especially about the Purkinje cells (Fig. 4), and congestion of some capillaries were also observed in the animals killed in the first 2 days. There was essentially no inflammatory reaction. No changes in the neuronal or glial elements were observed with the methods used.

Fig. 4. Cerebellum of cat demonstrating edema about Purkinje cells noted at the end of 48 hours (X570).

The animals in Group III receiving daily cisternal instillations of 1500 units of hyaluronidase for successive periods of 4 and 8 days developed a mild cerebrospinal fluid pleocytosis with daily cell counts ranging from 50 to 300/c.mm. These were predominantly polymorphonuclear leucocytes. In no instances were neurological abnormalities observed. At the end of the experimental period gross and microscopic findings of the brain were within normal limits. No evidence of change in the permeability of the blood-brain barrier was observed in the 2 animals with intraperitoneal trypan blue.

DISCUSSION

Cats and monkeys showed definite concentration tolerance of commercially supplied hyaluronidase injected into the subarachnoid space. The pathological changes were limited primarily to structures immediately in contact with the cerebrospinal fluid containing the dissolved enzyme. The occurrence of neurological changes predominantly on the right side, which
in all instances was the dependent region, is of interest. It suggests that effects of the protein substance were concentrated by gravity.

Despite demonstration that the fluid spreading reaction in subcutaneous areas is unaltered by contact with spinal fluid up to 3 hours and presumably also in the meninges, it is difficult to attribute clinical and pathological changes to specific enzymatic actions directly on the neurons. High osmotic concentrations and foreign protein substances might be able to create local capillary reactions which would produce similar changes. The alteration of the blood-brain barrier as shown by early appearance of trypan blue may be an indication of change in capillary permeability or it could be in part attributed to the change in the barrier by loss of epithelium of the choroid plexus. Spatz in study of the blood-brain barrier demonstrated in dogs that trypan blue appeared in the cerebrospinal fluid in minute amounts only after repeated daily intraperitoneal injections or following intravenous instillation of 30 cc. quantities of a saturated solution of the vital dye. In our experiments the trypan blue used was much less than the above and it appeared in the cerebrospinal fluid in rather profuse amounts within 3 hours following intraperitoneal administration.

Epithelial loss from the choroid plexus began within 2 hours following the instillation of the maximum concentration of the enzyme and apparently reached its peak at around 48 hours. By the 5th day minimal epithelial denuding was still evident and at the end of 7 days no pathology was observed. Regeneration had apparently been accomplished but no direct evidence as to the method was obtained. No mitotic activity was seen. More studies at shorter intervals during the critical period for cytological study of the choroid plexus are planned.

The identification of epithelial cells in the cerebrospinal fluid has not been reported previously. The presence of such cells in this study indicates the possibility of similar changes occurring in various disorders of the central nervous system. Without careful study of them in stained smears under proper magnification such cells could easily be mistaken for leucocytes.

Two hours following instillation of hyaluronidase the cerebrospinal fluid protein ranged from 396 to 338 mg. per cent. These figures are significantly greater than the protein (205 mg. per cent) found in the control sample made of 1 cc. of cerebrospinal fluid to which 9000 units of the enzyme were added. During the initial stages of each experiment, if the rise of intracranial pressure is allowed as evidence, there is probably an increased amount of cerebrospinal fluid which would serve to dilute the added protein. And so the protein in the cerebrospinal fluid of the experimental animal is relatively high. The recorded differences must therefore represent an addition of protein from a source within the animal. Speculation as to the source implicates the choroid plexus since there is simultaneous evidence of cellular damage and loss. It is possible that the increased protein could be derived from the denuded cells, themselves, but at present this has not been determined. A more probable explanation is that the additional protein came from the blood
EFFECTS OF HYALURONIDASE INJECTIONS ON CNS

serum through the altered blood-brain barrier. Within 24 hours after the hyaluronidase injection the protein in the cerebrospinal fluid had dropped and remained at relatively normal levels (15–25 mg. per cent). This rapid return to normal remains unexplained.

Recent reports of the effective suppression by hyaluronidase of torula organisms studied in vitro and demonstrations of such in the bacteriological laboratory here are of interest. At concentrations of 8 to 10 turbidity reducing units the inhibition of torula growth was noted. As demonstrated effectively in this study concentrations of 1500 turbidity reducing units/cc were tolerated without undue reactions when inserted into the cisterna magna of experimental animals. It is suggested therefore that clinical trial at high concentrations should be feasible.

SUMMARY

Responses of the central nervous system to various concentrations of hyaluronidase introduced via the cisterna magna have been studied in cats and monkeys. Concentrations of 6000–9000 turbidity reducing units produced temporary clinical changes related to the animals' position during the initial study and perhaps to the local cerebellar alterations. Occurrence of choroid epithelial cells was noted in the cerebrospinal fluid. Microscopic studies revealed denuding of the choroid plexus and partial loss of ependyma. Tolerance of daily injections of 1500 turbidity reducing units without significant microscopic pathological or clinical changes was noted. It has been suggested that high concentrations of hyaluronidase would be tolerated clinically without undue side effects. It is suggested that the presence of previously unrecognized epithelial cells in the cerebrospinal fluid may occur in clinical disorders.

REFERENCES

3. Knight, V. Unpublished data (from the Department of Clinical Bacteriology, Vanderbilt Medical School).