ANALYSIS OF FLUID IN CAPSULES IMPLANTED INTO DOG BRAIN

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Summary Perforated capsules were implanted aseptically into the brain parenchyma of dogs. The tissue and fluid formed within these intracerebral capsules (BC) were analysed for their ions and protein content. The results were compared with those for other perforated capsules implanted simultaneously into the subcutaneous tissue and muscle, respectively (denoted SC and MC). The following results were obtained: 1) In BC and MC, the Na/K ratio was different from that of the respective control tissues (cerebral parenchyma and temporal muscle) but similar to that of both the tissue in SC and control connective tissue. The water content of the three different capsular tissues was almost identical, but in each case different from that of the respective control tissues.

2) The Na/K ratio, Cl and protein content, and electrophoretic patterns of the three capsular fluids were similar to one another, and to serum. However, all (including BC fluid) were different in composition from the CSF (cerebrospinal fluid).

3) Investigations of the effect of time lapse after capsular implantation showed that the Na and K content was generally constant over the observed period, except that the K value was high in the initial few weeks.

4) Based on the results obtained, and on our data for pressure re-equilibration in BC, the nature of the fluid compartment in BC is discussed. It is considered to represent an extension of the narrow space existing physiologically between the blood capillary wall and glial membrane, and not to be part of the true interstitial fluid compartment.

5) The barrier existing between the blood and brain parenchyma may be regarded not as a single membrane but as two membranes of dissimilar nature.

Brain parenchyma is markedly different from other body tissues in the properties and characteristics of its vascular system. For example, when some substance is introduced into the general circulation, equilibrium between the brain

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interstitial fluid and blood is not attained for several hours, although it occurs within about half an hour in other body tissues. This phenomenon was first noticed in 1885 by Ehrlich, and the concept of a "blood-brain barrier" was introduced by Goldmann in 1913. In order to clarify this phenomenon, the permeability of the brain capillaries has been investigated using various substances, and the following results have been obtained: D_2O (BERING, 1952) and blood gases diffuse easily; lipid-soluble substances in general penetrate easily into the brain parenchyma; glucose and certain amino acids are actively transported.

The existence of a blood-cerebrospinal fluid (CSF) barrier was also recognized a long time ago, and an active transport mechanism has been proposed in the formation of CSF, since the concentrations of certain components of the CSF are different from those in plasma. As reviewed by CSERR (1971), many findings over the last decade have helped to define the nature of the blood-CSF barrier. For example, ROUGEMONT *et al.* (1960) succeeded in collecting freshly formed CSF from exposed choroid epithelium; carbonic anhydrase inhibitor (acetazolamide) was found to reduce the production rate of CSF by about 50% (WELCH, 1963; MAREN, 1967; CURL and POLLAY, 1968); the restricted permeability of $^{131}I^-$ from blood to brain and to CSF was found to be due to a slow, passive diffusion across the blood-brain barrier, associated with active $^{131}I^-$ transport from CSF to blood across the choroid plexus (BITO *et al.*, 1966). However, in comparison, our knowledge of the blood-brain barrier is still very limited.

In this connection, the nature of the interstitial fluid of the brain is another important problem to be resolved. Since it is not possible to collect such fluid directly, its composition has been estimated only from data obtained by indirect methods (ROUGEMONT *et al.*, 1960). In a previous paper, we have reported a positive fluid pressure with respect to atmospheric pressure in capsules implanted into the brain of dogs (ADACHI *et al.*, 1974). This contrasts with the negative fluid pressure of subcutaneous and intramuscular capsules, as reported by GUYTON (1963) and confirmed by us. Moreover, we have attempted to evaluate the properties of the intracerebral capsular space based on an analysis of the capsular fluid and capsular tissue formed.

The principal results obtained are reported in this paper. It is believed that the capsule method may offer a useful tool for studying certain of the essential properties of the blood-brain barrier.

MATERIALS AND METHODS

The dogs, capsules, and techniques for capsular implantation into the brain, subcutaneous tissue and muscle, were the same as those described previously (ADACHI *et al.*, 1974). In each case, capsular fluid pressure measurements were made every 1–2 weeks, as described previously. Upon termination of the experiments, the dogs were sacrificed by pentobarbital sodium injection.

The methods for collection and analysis of tissue and fluid samples were as follows.

Tissue samples. Tissue forming within the intracerebral capsules (BC), subcutaneous capsules (SC) and intramuscular capsules (MC) was sampled following sacrifice, together with the following normal tissues as controls; cerebral parenchymal tissue from the intact contralateral hemisphere, subcutaneous connective tissue from the nuchal area, and temporal muscle, respectively. All tissues were subjected to analysis for their water, Na and K content.

The tissue samples were minced in a moist chamber to form masses of about 100 mg each. The wet tissue masses were weighed, dried to constant weight in an oven ($105 \pm 5^\circ\text{C}$) and then re-weighed. Their water content was estimated from the difference between the wet and dry weights. The dry tissue was incinerated with concentrated nitric acid and the resulting product was diluted to a suitable concentration. The Na and K content of the samples was then measured using an atomic absorption spectrophotometer (Parkin Elmer Model-303). Some of the capsular tissue samples collected were subjected to histological examination.

Body fluid samples. In the case of SC and MC, the capsular fluid was sampled periodically after completion of pressure measurements. Samples from BC, as well as from SC and MC, were also taken at death. Serum and CSF was collected from the animals just before the death for comparison of its Na, K, Cl, and protein content with that of the capsular fluids.

The method for determination of Na and K was essentially the same as in the case of the tissue samples. Cl was titrated against mercuric nitrate according to the method of SCHALES and SCHALES (1941). The protein content of the fluids was estimated with a hand protein refractometer (Hitachi), and patterns for the protein fractions of the fluids were obtained by polyacrylamide gel electrophoresis using the method of DAVIS (1964). The protein concentration of the solutions applied to the gel was controlled, and electrophoresis was carried out in a cold room (0°C). The gel was stained with amido black and the patterns of the protein bands were recorded with a densitometer (Joyce Loebel, Chromoscan).

RESULTS

Intracapsular tissue

Fibrous tissue grew inside SC and MC through the perforations in the capsules, and the entire cavity wall was lined with connective tissue (as also reported by GUYTON, 1963). The air within the capsules was gradually absorbed and replaced by interstitial fluid. Moreover, in some cases the tissue accompanied by blood vessels increased its thickness so as to occupy the entire intracapsular cavity, leaving no room for interstitial fluid. Such a condition was often observed in the case of MC. The tissue was gray in color and hard to the touch. On its inner surface (*i.e.* where the tissue was in contact with interstitial fluid), fibrin-like gel

was formed. This sometimes spread out to form membranes within the cavity, dividing it into a number of fluid-filled compartments.

In BC, unlike SC and MC, the cavity wall was not lined completely by tissue since the perforations were limited to the lower third of the capsules. Tissue growth through the perforations generally reached only up to the level of the upper holes, or slightly higher, leaving adequate space for fluid accumulation above the tissue surface. Occasionally also, when the free surface of the tissue was growing along the capsular wall to form a continuous lining sheet, small fluid-containing cavities were formed at encroaching tissue junctions and incorporated into the growing tissue mass. The tissue on the immediately external surface of the capsule was attached to it rigidly as a firm membranous structure, while the parenchymal tissue formed thin thread-like extensions which penetrated into the capsules through the perforations. The dura was never incorporated into the capsular tissue, since it had been pulled up through the hole in the skull during capsular implantation. (Even in partially faulty cases, the dura formed a thick hard ring on the outer surface of the capsule, a few millimeters above the perforations and clearly separated from the parenchyma.) Most implanted capsules contained both tissue and fluid, although in some cases there was fluid or gel but no tissue; in one case, the entire cavity of the capsule was filled with tissue (see Table 1).

Table 1. Intracerebral capsular contents.

Capsular contents	No. of cases	Duration of implantation (weeks)
Tissue and fluid	18	5, 6, 7(2)*, 8(3), 12, 13, 15, 16, 17(2), 18, 19, 22(2), 26
Fluid		
(in ventricle)	1	13
(in parenchyma)	4	8, 12(2), 22
Gel	2	10 (days), 13
Tissue only	1	17
Total	26	10 days-26 weeks

* Numbers in parentheses indicate multiple occurrences with the same duration.

Histological examination by Nissle's staining and Bodian's silver impregnation techniques failed to reveal nerve cells within the intracapsular tissue, although it was difficult to identify the cells definitively as fibroblast or glial cells (Fig. 1). The tissue contained many connective tissue fibers (Fig. 1B) and wide extracellular space (Fig. 1C).

Water, Na, and K content of the tissues

Each of the different tissues into which the capsules were implanted is known to have distinctive features as regards its water content and Na/K ratio. Com-

Fig. 1. Microscopic (A, Nissle's staining; B, silver impregnation for connective tissue fibers) and electron microscopic (C) photographs of the intracapsular tissue in the brain. Arrows in C indicate extracellular space.

parisons were therefore made in each case to determine whether or not the capsular tissues retained the same features as their corresponding original tissues.

As described above, samples of intracapsular and control tissues were taken at autopsy. The brain tissue from the intact side was separated into grey and white matter in a moist chamber, and both parts analysed separately. Normally, five tissue masses were prepared from each tissue sample for the water-content determination, of which three were separately incinerated for Na and K measurement. In the case of small BC tissue samples, one or two tissue masses only were prepared for analysis, as appropriate.

1) *Na and K contents.* As shown in Fig. 2A, the Na/K ratio was less than unity in the case of the control brain white matter, grey matter and muscle, and 7.34 on average for control subcutaneous connective tissue. The BC and MC capsular tissues gave values quite different from their corresponding controls, but both were very similar to that for SC capsular tissue. All these latter values were close to that for control subcutaneous tissue.

Fig. 2. Na/K ratio (A) and water content (B) of tissues, and their standard deviations. Rectangles indicate subcutaneous tissue; triangles, muscle; and circles, brain tissue. Open figures with broken lines indicate intracapsular tissue; filled figures with unbroken lines, control tissue. Numbers attached to the bars indicate the number of dogs employed in each case.

On plotting the Na content and K content of the capsular tissues against period elapsed after implantation (see Fig. 3), it was found that all three capsular tissues showed similar tendencies. The Na content remained essentially constant throughout the observed period; the K content was rather high in the initial period but fell sharply by about 10 weeks, and then fluctuated slightly downwards throughout the remainder of the observed period.

2) *Tissue water*. The water content of the capsular tissues did not show any close resemblance to the values for the corresponding control tissues, and all

Fig. 3. Na (filled figures) and K (open figures) contents in the intracapsular tissues, plotted against implantation period. BC, intracerebral capsules; SC, subcutaneous capsules; and MC, intramuscular capsules.

values were clearly different from control subcutaneous connective tissue (see Fig. 2B). However, the three different capsular tissues did show a general similarity as regards their water content. The samples of subcutaneous connective tissue gave a rather wide range of values for water content, possibly due to individual age variation and differences in fat content of the tissue samples.

Composition of capsular fluid

1) *Na, K, and Cl content.* The Na, K, and Cl content of capsular fluid was determined on samples obtained between 6 and 22 weeks after implantation. During this period, no clear tendency for change was observed in the Na and K content of fluid from either BC, SC or MC (see Fig. 4). The Na/K ratio for these capsular fluids was compared with values for serum and CSF (see Fig. 5).

Fig. 4. Na (filled figures) and K (open figures) contents in the intracapsular fluid, plotted against implantation period. SC, MC, BC; see legend of Fig. 3.

Fig. 5. Na/K ratio (unbroken lines) and Cl content (broken lines) in capsular and body fluids. SC, MC, BC; see legend of Fig. 3.

The ratio in all cases (except CSF) was similar, with a value of about 30:1. Clearly, therefore, the Na/K ratio in the intracerebral capsular fluid is significantly different from that in the CSF. Values for Cl content of the capsular and body fluids showed a similar tendency; *i.e.* the values for all fluids were essentially the same, except CSF.

2) *Protein content.* Figure 6 shows a mass plot of all values obtained for the protein content of BC, SC, and MC capsular fluid. As can be seen, in the initial few weeks after capsular implantation, the minimum value for the protein content of the fluid fell gradually to about 1 g/dl after 7 weeks, but thereafter showed no further drop (except in one case of BC fluid where the capsule had penetrated into the ventricle—the filled circle in Fig. 6). Certain individual cases gave rather high value for their capsular fluid protein content, perhaps associated with a state of histogenesis or inflammation. The slopes of the lines for BC and SC fluid, drawn by the method of least squares, were not significantly different (-0.055 and -0.050 g/dl week, respectively).

Electrophoretic patterns obtained for the intracapsular fluid, plasma and CSF are illustrated in Fig. 7. As can be seen, there was no clear separation of protein fractions in the case of CSF, probably due to the rather low protein concentration in the fluid applied to the gel. The patterns obtained for BC, SC,

and MC capsular fluid were in general similar, but partially different from that for plasma in regard to certain of the bands.

Fig. 6. Protein content (P) of the intracapsular fluids, plotted against implantation period. The regression lines for BC and SC were drawn by the method of least squares. (See text for explanation of the filled circle.)

Fig. 7. Electrophoretic patterns for the protein fractions in capsular and body fluids.

DISCUSSION

As described above, following the implantation of capsules into the brain parenchyma of dogs, tissue with blood vessels grew within most capsules, and fluid accumulated in the remaining intracapsular space. The Na, K, and Cl content of this capsular fluid, and the electrophoretic pattern of its protein fractions, were closely similar to those for fluid from other capsules implanted subcutaneously or intramuscularly.

GUYTON (1963) observed tissue growth and fluid retention in capsules implanted into the subcutaneous tissue of dogs, and reported free communication between the capsular and interstitial fluids. He concluded that the fluid pressure in the capsules was in equilibrium with that of the surrounding interstitial fluid. Although this conclusion is entirely acceptable for SC, further investigations are necessary to confirm whether or not it is entirely applicable to our intracerebral capsules, *i.e.* whether the fluid retained in BC is actually the interstitial fluid itself or not.

The amount of CSF that might drive from the ependymal surface of brain was estimated at almost 33% in rabbits (POLLAY and CURL, 1967) and 30% in dogs (SATO and BERING, 1967). ROUGEMONT *et al.* (1960) compared newly formed fluid at the choroid plexus with fluids from the cisterna magna and from the cisterna pericallosa and concluded the latter fluid was formed through the same mechanisms as its formation at the choroid plexus. From these observations, it is deduced that CSF consists of fluid secreted from the choroid plexus plus from extrachoroidal sources in about one third, and that the composition of both components may not be widely divergent. The extrachoroidal fluid production probably occurs through a slow flow of interstitial fluid from the brain into the CSF. Consequently, if the fluid retained in BC is in fact the interstitial fluid itself of the brain, some conflict arises with the reports referred to above.

In our experiments, the blood vessels invading the intracapsular tissue of BC had their origin in the vessels of the brain parenchyma, and fluid retained in the capsules was thus filtrated from the same blood supply. On the other hand, the tissue growing within the capsules lacked identifiable nerve cells and did not show the typical morphological characteristics of glial cells. Also, its Na and K content was essentially similar to that of tissue from SC and MC. In fact, judging from their hardness, *etc.*, these capsular tissues all resembled the connective tissue that forms as scar tissue at points of damage or injury. Moreover, the BC and MC capsular tissue showed characteristics different from their respective control tissues; their Na/K ratios were very close in value to that for normal connective tissue.

In a previous paper (ADACHI *et al.*, 1974), we reported that the half-time for pressure reequilibration in BC, following the injection or removal of a small amount of fluid, was considerably prolonged. Since similar results were also

obtained immediately after death, the retarded restoration of pressure equilibrium is ascribed to the restricted flow of fluid through the surrounding interstitial space. It is known that capillary endothelium in the brain is entirely enveloped in astrocyte end-foot, leaving in general an intercellular space of only about 200Å. However, electron microscopic observations of our intracapsular tissue have not revealed any comparable structures.

In conclusion, the intracapsular fluid cannot be considered as entirely corresponding to the true interstitial fluid of the brain. Because, the glial membrane does not contribute in its production, though the fluid space in the capsule lies outside of the capillaries, and fluid accumulating in it is filtrated through their walls. Further, based on the observation of only restricted flow between the capsular fluid and surrounding interstitial fluid, the fluid space of BC is perhaps best considered as a relatively independent one, *i.e.* as an expanded form of the restricted space existing physiologically between the capillary wall and glial membrane.

Since the composition of the fluid accumulating in BC was closely similar to that of other capsular fluids, the selective transport mechanism of the brain can hardly be attributed to the vascular wall. Moreover, if the interstitial fluid of the brain were closely similar in composition to the CSF, the glial membrane enveloping the vascular wall could produce interstitial fluid from the filtrate by an active transport mechanism, as does the choroid epithelium. Fluid would then flow through the interstitial space and enter the CSF.

It is generally known that the passage of substances into the brain parenchyma occurs only with difficulty. According to the pore theory, the effective pore radius in cat muscle capillary has been estimated at 30–45 Å by PAPPENHEIMER (1953). For the brain, FENSTERMACHER and JOHNSON (1966) have given a figure of 7–9 Å based on experiments for water loss in the brain, assuming the single membrane as a rate-limiting barrier. However, if the cerebral capillary wall and glial membrane are considered as separate barriers to the passage of substances, then the value obtained for them would be attributable to the combined effect of two membranes and not to the capillary wall alone. On the other hand, BRIGHTMAN and REESE (1969) have reported that tight junctions were primarily observable only on the endothelium of brain parenchymal capillaries and the epithelium of choroid plexus. Thus, on this basis, it can be expected that the capillary permeability of BC tissue will be less than that of SC tissue. To clarify the capillary permeability the authors are working on radioisotope, ^{22}Na and ^{125}I .

In conclusion, therefore, it is suggested that although many details remain to be clarified, our capsular implantation procedure may represent a very profitable model situation for studying the blood-brain barrier, especially since the parenchymal capillary wall can, so to speak, be isolated from the remainder of the blood-brain barrier.

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