Effect of Thiamine Deficiency on the Competence of the Blood-Brain Barrier to Albumin Labeled with Fluorescent Dyes.

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Recent ultrastructural studies have emphasized the fact that intracellular edema, notably involving glial cells and their perivascular processes, is a conspicuous early change in the brainstem lesions of experimental thiamine deficiency in rats.\(^1,2\) Later, frank necrosis, often hemorrhagic, supervenes, and at this stage there is marked expansion of the extracellular space as well.\(^3-5\)

Since the early edema is intracellular, and since it occurs in the absence of morphologic evidence of parenchymal or vascular necrosis, it was suggested that it might represent a defect of transport directly related to the deficiency state. In contrast, edema occurring during the necrotic phase could be analogous to the reactive increase in capillary permeability known to occur in relation to mechanical,\(^6\) heating,\(^7\) freezing,\(^8-10\) severe anoxic,\(^11\) neoplastic,\(^12,13\) and inflammatory\(^14\) lesions associated with tissue destruction and termed vasogenic edema by Klatzo.\(^15\)

This experiment was designed to test the hypothesis that the blood-brain barrier remains intact with respect to plasma proteins in the early edematous lesion, but becomes permeable to protein in the necrotic stage. This demonstration was considered essential for subsequently evaluating transport of and barriers to other substances in the pathogenesis of lesions in thiamine deficiency.

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Materials and Methods

Immature female rats of the Long Evans strain (Blue Spruce Farms Inc, Altamount, NY) or the Wistar Furth strain (Microbiological Associates Inc, Bethesda, Md) were caged individually in a ventilated room at 68–70 F. The details of the procedure used in this laboratory for inducing acute thiamine deficiency have been published. Briefly, a synthetic thiamine-free diet (Nutritional Biochemical Corp) was fed ad libitum to the experimental group. Every fifth animal, the control, received the same diet in an amount corresponding to the average consumed by 4 experimental rats on the previous day; each control rat was given a daily intraperitoneal injection of thiamine hydrochloride USP, 40 μg/100 g body weight.

Bovine albumin conjugated with fluorescein isothiocyanate (FLA) was obtained commercially (Nutritional Biochemical Corp, Cleveland, Ohio); the dose administered was 1.0 ml/100 g body weight.

Evans blue was complexed to bovine albumin (EBA) in the following manner: To 100 ml of a 10% w/v solution of albumin in normal saline, 4 g of Evans blue powder was added, with constant stirring. The mixture was then filtered through Whatman No. 1 paper. The dosage was 0.25 ml/100 g body weight.

The albumin-dye complexes were administered intravenously at all stages of thiamine deficiency from day 28 to 46. Animals were killed 30 minutes later by intracardiac perfusion of saline for 3 minutes, and either phosphate-buffered 10% formaldehyde or phosphate-buffered 3% glutaraldehyde for 20 minutes.

Brains were carefully removed and the brainstem sectioned 1 mm caudal to the acoustic tubercles.

Transverse frozen sections of the brainstem and cerebellum obtained at the acoustic tubercle level were mounted unstained in Fluormount and examined in ultraviolet light, using a darkfield condenser. The exciter filter was BG12, 2 mm thick; the barrier filter for FLA sections transmitted light only above a wavelength of 490 μm, and that for EBA sections only above 530 μm. Adjacent sections were stained with H&E for histologic evaluation.

In some animals, sections of liver, kidney or lung were also examined by fluorescence microscopy. Paraffin sections prepared in the routine manner for histologic study of the lesions were found unsatisfactory for fluorescence study because quenching of EBA fluorescence occurred.

Of 134 rats in the initial groups, 56 were suitable for evaluation. The 51 animals dying spontaneously, 5 perfusion failures and 22 technical failures were not evaluated.

Observations

The clinical behavior of animals conformed to those in published descriptions on the development and progression of symptoms in acute experimental dietary thiamine deprivation. Experimental animals commenced to lose weight in the third week and fur in the fourth week. During the fifth and sixth weeks, distinct neurologic signs appeared. At the time of sacrifice, animals were graded into three categories on the basis of neurologic manifestations. To the early group belonged those which had merely lost weight and some fur, but were still active. The next category included those animals that also exhibited flaccidity, reduced motor activity and some postural abnormality. The last group comprised animals with severe ataxia, tremors, loss of righting reflexes and seizures.
Histologic sections were graded according to the degree of spongy edema, and the presence of congestion, hemorrhages, tissue necrosis and neuronal loss (Table 1). The brainstems of all control animals appeared normal. (Fig 1). Slight edema qualified for inclusion in group A (Fig 2); more marked spongy reticulation, frequently accompanied by vascular congestion, characterized group B; and hemorrhages, tissue disintegration and neuronal fallout were requisite for inclusion in group C (Fig 3 and 4).

In general, there was a good correlation between severity of clinical signs and histologic grade.

Control and thiamine-deficient animals in all categories demonstrated coarsely granular, white to yellowish autofluorescent perivascular cells in all parts of the brainstem, chiefly about larger vessels. In some control and thiamine-deficient rats, the elastica of the basilar and larger arteries in the subarachnoid space, the choroid plexus stroma, the walls of arteries of other viscera and the blood within some unperfused vessels fluoresced brightly with the distinctive color of the albumin-dye complex administered. In control animals, specific fluorescence of the neuropil—that is, tissues beyond the vascular confines, was never seen.

In the series given fluorescein-labeled albumin, 8 of 14 animals in group C exhibited an extravascular yellowish-green fluorescence in the neural parenchyma of the dorsolateral pontine tegmentum (Fig 5). None of the 8 in groups A and B demonstrated this feature.

In the series given Evans blue-labeled albumin, 4 of 8 animals in group C (Fig 6) and 1 of 9 animals in group B exhibited an extravascular red to reddish-orange fluorescence in the vestibular nuclear complex.

The specific fluorescence beyond vascular confines was restricted to the immediate vicinity of vessels or was evident more diffusely. There appeared to be no selective localization of fluorescence in relation to

<table>
<thead>
<tr>
<th>Fluorescence</th>
<th>FLA</th>
<th>EBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>FLA</td>
<td>EBA</td>
</tr>
<tr>
<td>Severity of lesion*</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>A</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Controls</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

* See text for definition.
the size of individual vessels, or to hemorrhage around them. However, the presence of hemorrhages in the dorsolateral pontine tegmentum was the most useful criterion for assessing the severity of the morphologic lesion.

Because extravascular fluorescence was observed chiefly in group C, an analysis of the data suggested that combining groups A and B would be advantageous. Likewise, extravascular fluorescence reflects the extravasation of albumin, for which the fluorescent dyes served merely as convenient labels for ease of demonstration. Thus, the results of the two dye series can be combined (Table 2).

Chi-square analysis confirmed, at a high level of significance, an association between occurrence of protein extravasation and the presence of a severe structural lesion.

**Discussion**

The value of anionic dyes in the study of neural vascular permeability is well established. Although the dyes used are strongly and rapidly bound by serum albumin, preformed dye–albumin complexes were administered to avoid the possibility of free dyes entering the neuropil. In essence, the dyes thus serve as sensitive and convenient markers for circulating albumin. By fluorescence microscopy, the behavior of the blood-brain barrier to this protein could then be assessed.

We predicted that, in the late necrotic lesions of thiamine deficiency, increased permeability to plasma proteins would be found, as it is in several other models in which necrosis is a feature; this was demonstrated in 54% of animals with these lesions. The permeability change is interpreted as being nonspecific, occurring as a consequence of the underlying lesion.

**Table 2—Two by Two Contingency Table Relating Extravascular Fluorescence to the Severity of Lesion**

<table>
<thead>
<tr>
<th>Extravascular fluorescence</th>
<th>Severity of lesion</th>
<th>Group C</th>
<th>Groups A and B</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>12</td>
<td>1</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>10</td>
<td>23</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>24</td>
<td>46</td>
<td></td>
</tr>
</tbody>
</table>

\[ \chi^2 = 14.38 \quad \chi^* = 12.98 \]

\[ P < 0.001 \quad P < 0.001 \]

*Yates' correction.*
In contrast, the finding that the blood-brain barrier is intact with respect to albumin in the early lesions is of more significance. Although previous ultrastructural studies showed no morphologic vascular changes at this stage, this observation was not considered to be an adequate demonstration of functional competence. However, this study, together with the morphologic evidence that the fluid accumulation is intracellular, presents evidence that the edema is truly "cytotoxic" in the sense that it results from a defect in cell membrane transport rather than a vascular "leak" of the inflammatory type.

Related evidence for a defect in selective transport mechanisms was provided by Warnock and Burkharter who showed that circulating $^{14}$C-labeled pyruvate, which normally is actively excluded from adult brain tissue, passed readily into the brain of thiamine-deficient rats, although trypan blue did not. They postulated that an alteration in thiamine-dependent glial metabolism underlay this loss of selectivity.

Two recent studies of the functioning of the blood-brain barrier to protein in thiamine-deficient pigeons are pertinent to this work. Autoradiographs were prepared after $^{125}$I-albumin, and evidence of extravasation of ferritin was sought using electron microscopy; no evidence of leakage of either protein was found, although 25% of the deficient animals (number not specified but fewer than 36) had developed necrotic lesions. These findings could be interpreted as being consistent with our own, in the early lesions. However, their failure to demonstrate protein extravasation in the late lesions might indicate a lack of sensitivity of the technics chosen or might be a chance finding in a small number of animals.

Thiamine deficiency results in a reduced activity of a number of thiamine pyrophosphate-dependent enzymes, including pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and transketolase, and in an inhibition of oxidative metabolism. A possible consequence is depletion of high-energy phosphates required for, among other things, active transport of electrolytes across cell membranes. Astrocytes and their perivascular foot processes are thought to have a major role in these functions. McCandless et al. found levels of ATP to be normal in several areas of the brain of thiamine-deficient rats; however, their samples of the entire brainstem would contain a large admixture of uninvolved tissue with the comparatively small lesions in the vestibular area. In this regard, Dreyfus demonstrated clearly the necessity for careful selection of involved tissue for biochemical, as well as morphologic study. Although the experiment demonstrates that increased permeability to protein is not a feature of the early edematous lesions,
it does not provide direct evidence of defective transport mechanisms; these will be the subject of further studies.

**Summary**

Studies of the functioning of the blood-brain barrier with respect to circulating albumin in the brainstem lesions of thiamine-deficient rats showed that in the early, edematous phase there was no extravasation of protein, whereas in the late, necrotic lesions, increased permeability to protein occurred. This provides further evidence that the early edema, which is predominantly within glial cytoplasm, reflects disturbances of active transport directly related to the thiamine deficiency. In late stages, the protein extravasation represents a nonspecific, reactive edema common in destructive cerebral lesions.

**References**


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Fig 1—Neurons, myelinated fibers and neuropil arranged compactly in lateral vestibular nucleus of control animal (rat No. 295, celloidin, hematoxylin-van Gieson (HVG), \( \times 375 \)).

Fig 2—Spongy edema in lateral vestibular nucleus qualifies this lesion for inclusion in group A (slight lesion). Neurons exhibit normal cytologic features (rat No. 274, celloidin, HVG, \( \times 145 \)).

Fig 3—Advanced lesion (group C) in lateral vestibular nucleus. There are extensive spongy reticulation, degenerative changes in neurons and perivascular and more diffuse hemorrhages (rat No. 269, paraffin, hematoxylin-phloxine-saffran, \( \times 50 \)).

Fig 4—Extensive necrosis of lateral vestibular nucleus, with neuronal loss, reticulation of neuropil and pyknosis of glial nuclei. Two recently necrotic neurons are present at margin of the destructive lesion (right upper corner) (rat No. 266, celloidin, HVG \( \times 70 \)).
Fig 5—Diffuse extravascular fluorescence of extravasated fluorescein-labeled albumin within superior vestibular nucleus. Spongy reticulation, well-perfused empty vessel lumina and some freeze artifact are also apparent (rat No. 114, frozen section, unstained; darkfield fluorescence microscopy, x 100).

Fig 6—More limited perivenous fluorescence of extravasated Evans blue-labeled albumin within lateral vestibular nucleus (rat No. 182, frozen section, unstained; darkfield fluorescence microscopy; x 250).