ASTROCYTIC PROCESSES: A ROUTE BETWEEN VESSELS AND NEURONS FOLLOWING BLOOD-BRAIN BARRIER INJURY

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ABSTRACT

The fluorescent tracer Evans blue (EB) was studied after circulation time ranging from 30 seconds to 5 minutes following blood-brain barrier (BBB) injuries in 42 dogs. Selective neuronal stainings (SNS), associated with BBB injuries, occurred to a high degree. This phenomenon was examined by modifications in existing fluorescence techniques. The mechanism of SNS was seen to lie in an almost imperceptible transport of EB along astrocytic processes connecting SNS with the adjacent vasculature.

This report presents visual evidence under pathological conditions, and supports the concept of astrocytic processes as the normal route of transport between blood vessels and neurons. This requires long exposure photomicrographs since the fluorescing material is subtle within the neuroglial processes.

INTRODUCTION

One of the fundamental questions in neurophysiology is the mode of transport between capillaries and neurons. It is a process analogous to an irrigation system with vessels slowly leaking nutrients into the neuropile, or does a system of less primitive design exist which parallels the complex anatomical and functional organization of the brain? Distinct from theories of irrigation of the extracellular space (ECS) is that of a more intricate system of microconduits.

The possibility of microconduit transport has been suggested since the advent of histologic techniques demonstrating the morphologic characteristics of neurons and glia. The abundance of astrocytes and their interposition between vessels and neurons has led some investigators to the conclusion that astrocytes provide, in addition to the structural support, a route for transport. The histologic picture easily attracts one to this hypothesis. Maynard et al. estimated that 85% of the capillary vascular surface area is covered by astrocytic processes and suggested that astrocytes serve as transport bridges between capillaries and cells (20). Farquhar and Hartmann expressed a similar view (12). Tschirgi had already suggested this function by astrocytes on a physiological basis (46).

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Roussy, Lhermitte and Oberling suggested metabolism occurring via astrocytes (40). Pomerat and Costero demonstrated pulsatile activity of glia and their processes with tissue culture techniques as well as migration of small particles along neuroglial processes recorded with cinematography (32). Lumsden called astrocytes and oligodendrocytes the “wet nurses” of the neuron (24). Regardless of how attractive this kind of speculation may be, actual transport within astrocytes has never been demonstrated and this fundamental question remains unanswered.

Investigations using the fluorescent and light microscope indicate that the capillary wall is a major component of the BBB system and that neuronal staining subsequent to barrier injury occurs from uptake of tracer materials which reach the neuron by extracellular routes (9, 10, 13, 16, 18-20, 28, 30, 31, 38, 39, 44). More recent electron microscopic studies utilizing horseradish peroxidase seem to confirm these findings and locate barrier sites within capillaries at “tight junctions” between endothelial cells (5-7, 14, 31).

The role of the astrocyte has been minimized (5, 30). Transport from the vascular system to neuronal populations is less speculated upon and left to the assumption that it must be predominately extracellular, since distribution of tracer materials following BBB injury is largely within this space. However, the selective neuronal staining (SNS) following BBB injury without evidence of extracellular tracer extravasation has appeared in several reports (16, 22, 28, 30, 38, 39, 43, 44). This phenomenon still remains without satisfactory explanation (16, 28, 31). In this instance, neurons are stained with fluorescent tracer although extracellular extravasations of dye cannot be found. How can tracer material get from vessels to neurons without some contamination of the ECS? This phenomenon suggests the existence of another route other than the ECS for capillary to neuron transport under these conditions. Recent observations in our laboratory on BBB response to spinal cord injury demonstrate the role of astrocytes in the mechanism which leads to the selective neuronal staining.

TECHNIQUES

Forty-two mongrel dogs 11 to 14 kg were anesthetized with 30 mg/kg of sodium pentobarbital, intubated and placed on a Harvard respirator, monitored for percent end tidal CO2 and cannulated at a femoral artery for pressure and pulse recording. Four-level laminectomies were performed exposing the lower thoracic and upper lumbar spinal cord. 300 gm/cm impaction injuries employing the methods of Albin et al. were carried out with dura intact (1, 2). 25 cc of 2% solution of Evans blue in normal saline was given intravenously at 5 minutes prior to removal of spinal cord in 32 animals and at 30 seconds prior to removal of spinal cord in 10 animals. A 6 cm segment of spinal cord was rapidly and atraumatically removed with dura intact prior to sacrifice of the animals. The spinal cords were removed at 14 intervals from immediately after impaction to 6 days post injury (Table 1). Intravascular perfusion of animals with formalin-saline was not performed in any of the animals reported in this series.

Specimens were immediately placed in 10% formalin-saline and left undisturbed for 1-4 days, after which the dura was removed and the spinal cord sectioned in 2-3 mm slices. 12 micron thick sections were then cut on a freezing microtome. These sections were then dried
TABLE 1
Timetable for Evans Blue Administration After Spinal Cord Contusion in Dogs

<table>
<thead>
<tr>
<th>Interval Post Trauma</th>
<th>EB Circ. for 5 min.</th>
<th>EB Circ. for 30 sec.</th>
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<tbody>
<tr>
<td></td>
<td>No. of dogs</td>
<td>No. of dogs</td>
</tr>
<tr>
<td>0 sec.*</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>30 sec.</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>5 min.</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>15 min.</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>30 min.</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>1 hr.</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>2 hr.</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>3 hr.</td>
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</tr>
<tr>
<td>4 hr.</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>8 hr.</td>
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</tr>
<tr>
<td>12 hr.</td>
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</tr>
<tr>
<td>1 day</td>
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</tr>
<tr>
<td>2 days</td>
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<td>-</td>
</tr>
<tr>
<td>6 days</td>
<td>2</td>
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* 0 Sec. indicates that Evans blue was administered immediately before impact.

for 5-10 minutes at 60°C, mounted in Entellan (nonfluorescent mountant) and a coverslip applied.

Sections were examined from all levels of the lesion and adequate controls were obtained at either end of these long spinal cord segments as well as from nontraumatized spinal cord. A Leitz fluorescence microscope with BG 12 exciter filter, BG 35 heat filter and Kodak Gelatin #12 barrier filter was used for examination of slides. High speed daylight Ektachrome film, ASA 160, was used for photomicrography. Hematoxylin and eosin stains were performed on all specimens for light microscopy.

RESULTS

Intravascular administration of Evans blue (EB) produces a red fluorescence filling blood vessels in tissue sections examined by fluorescent microscopy. BBB disruption is detected by EB extravasation into the neuropile with subsequent red staining of the ECS, glia, or neurons. Three phases of vascular response following trauma with attendant destructive changes in spinal cord segments are described in another report (49). Histochemical staining of specimens in formaldehyde detected fluorescent catecholamine-like perivascular extravasations. This autogenously produced injury tracer has been previously reported (48).

A marked degree of SNS was observed at the periphery of lesions at early intervals and throughout entire lesions at later intervals. The uptake of EB leading to SNS followed a definite sequence. Four distinct stages of uptake of EB by nucleus and cytoplasm were apparent. Stage 1: The nucleus stained red, while there was little or no detectable EB in the cytoplasm (Fig. 1). Stage 2: The nucleus stained distinct red and the cytoplasm stained a faint pink to orange color. Stage 3: Intense red staining of the neuronal cell body and its processes obliterated distinction between cytoplasm and nucleus. Stage 4:
Cellular damage with poor cytoplasmic uptake of EB and no nuclear staining. The nucleus at this point became brown.

The microvasculature within SNS areas was intact and there was no evidence of EB extravasation into the ECS. Multiple timed exposures of these areas ranging from seconds to over 5 minutes were routinely studied by large screen projection following microscopic evaluation. Hidden in the greenish autofluorescent background were numerous fine red processes connecting the neuron with nearby EB-filled vasculature. The processes are usually less than or equal to 1 micron in diameter. They originate from points on vessel walls that correspond to astrocytic foot plates (Fig. 2). En route to the neuron they were frequently seen to course over the surfaces of pale green astrocytic cell bodies in a serpiginous fashion, then trail off to end on the cell body or dendritic process of an abnormally stained neuron (Fig. 3).

These features cannot be seen with the naked eye under the fluorescence microscope and cannot be demonstrated on photomicrographs of brief exposure time with dark backgrounds. In addition, barrier filters which transmit significant amounts of blue light tend to obscure this detail. Kodak Gelatin #12 filters produced optimal contrast.

In areas with more severe injury an intricate network of fine red processes coursing between vessels and abnormally stained neurons was revealed (Fig. 4). In areas where gross extravasation of EB filled the ECS this detail was obliterated (Fig. 5).

In the adjacent areas of spinal cord beyond the site of injury none of these red processes could be demonstrated. However, the pale green autofluorescent neuroglia could be seen. Neuroglial processes as well as foot plates were identifiable at their multiple attachments to vascular surfaces. EB staining of astrocytic processes occurred only in areas of BBB damage.

It was consistently observed in areas of injury where the neurons were stained, the astrocytic processes were also stained with EB (Fig. 6). In absence of injury, staining was not detectable. In areas of slight injury, red stained processes coursed over the surfaces of neuroglial cell bodies without staining the cytoplasm. In areas of heavier injury the neuroglial cell bodies frequently stained an intense red.

Neuronal staining without free extracellular dye and with fine red glial processes linking them to the vascular system was demonstrable in sites of BBB injury after circulation of EB for only 30 seconds (Figs. 7 & 8).

**DISCUSSION**

Ehrlich's observation over 85 years ago that a vital dye stained all tissues of the body except that of the brain has led to a proliferation of theories regarding the nature and location of a BBB (11). Bodenheimer and Brightman's report of peroxidase studies on barrier function gives a review of three modern theories of the anatomical location of the BBB (5). One theory has been that:

"substances are able to cross the capillary endothelium but are impeded from entering the parenchyma by a layer of perivascular glial end feet (Schalten-
brand and Bailey, 1928; Tschirgi, 1952). The hypothesis of a glial barrier was strongly reinforced by the observation that brain capillaries are entirely invested by glial processes (Wolff, 1963) and that the junctions between the adjoining glial processes are sealed (Gray, 1961; Peters, 1962). However, it is now clear that there is no continuous barrier of tight interglial junctions around capillaries (Kuffler and Nichols, 1966; Reese and Karnovsky, 1967). The patency of clefts between perivascular glial end feet has been demonstrated by the movement of peroxidase through the clefts in the brain of the mouse (Brightman, 1967) and of necturus. The perivascular glia are, therefore, unlikely to present an effective barrier to different substances that are able to cross the endothelium” (5).

The inherent assumption, however, on which this conclusion is based is that transport to neurons occurs predominately via an extracellular route. Patency of clefts between perivascular glial feet does not on the other hand detract from the hypothesis that transport occurs within the astrocytic process.

Electron microscopic observations of an ECS of 3–5% within the brain have been challenged by demonstration of a larger space with rapid freezing techniques of Van Harreveld et al. (47). Confirmation of a larger space would obviate theories suggesting that the BBB exists because of absence of a significant ECS, but neither supports nor contradicts the hypothesis that transport from the vascular system to neurons occurs primarily by the extracellular route.

**Fig. 1.** Neuron (N) from an injured spinal cord segment. Example of stage 1 uptake of EB first staining the nucleus (Nu) red. Arrow points to fluorescent granules. Astrocytic processes (AP) filled with EB are faintly seen adjacent to this neuron (× 1000).

**Fig. 2.** Spinal cord. Longitudinal section of blood vessel (BV) with BBB injury. Outpouring of EB into astrocytic processes (AP) at their multiple attachments along vessel wall. Fixation artifact produced gap along one segment of central surface where astrocytic processes are stripped off vessel wall (× 650).

**Fig. 3.** EB-filled astrocytic process (AP) seen in oil immersion photomicrograph. Other structures out of the focal plane are: astrocytic cell body (A), dendrite of a neuron (D), blood vessel (BV). The neuron of which only the dendrite can be seen in this picture has a densely stained red nucleus and minimal staining of its cytoplasm with EB (× 1000).

**Fig. 4.** Spinal cord. Longitudinal section showing relationship of blood vessel (BV) with its myriad of astrocytic processes (AP) coursing through white matter (WM) to adjacent gray matter (GM). EB circulated for 30 sec. before cortectomy which was performed at 5 min. post impaction. This photomicrograph demonstrates the instant release of EB into astrocytic processes at site of BBB injury (× 100).

**Fig. 5.** Gray matter in area of heavy BBB injury with staining of all elements of the neuropile. Network of astrocytic processes (AP) nearly obscured by gross extravasation of EB into the extracellular space. Extracellular dye (ECD). Grade 3 staining of neuron (N) which almost obliterates distinction between nucleus (Nu) and cytoplasm. Arrow shows discrete yellow fluorescent granules of the neuron (× 400).

**Fig. 6.** A nest of EB-filled astrocytic processes (AP) seen in an area of BBB injury adjacent to gray matter. It should be noted that these features cannot be detected with the naked eye under the fluorescent microscope. Exposure time for this photomicrograph was 2 min. Selectively stained neuron (N) (× 400).

**Fig. 7.** Spinal cord. Longitudinal section showing instantaneous release of EB from blood vessel (BV) into myriad of astrocytic processes (AP). EB circulated for 30 sec. Cortectomy performed at 5 min. post injury (× 100).

**Fig. 8.** This is a silver stain of dog spinal cord, not a fluorescent slide. This photomicrograph demonstrates the morphologic characteristics of networks of astrocytic processes (AP) and their interposition between blood vessels (BV) and neurons (N). Nuclei of astrocytes (A) are also shown. Note the correlation between the dark silver stained processes in this picture and the red EB filled astrocytic processes in Fig. 7 and Fig. 4. (× 100).
Current opinion holds that a major component of the BBB system is the capillary endothelium and its junctions. Brightman and Reese have redefined endothelial appositions and suggest that the BBB to peroxidase results from these anatomical barriers (8). Studies employing horseradish peroxidase show disruption of these sites following chemical or mechanical injury with subsequent spillage of tracer into the parenchyma. However, it has been shown that tracer material may appear in the parenchyma in areas without evidence of disruption to the endothelial junctions (15, 31). When experimental injury is of a magnitude to physically disrupt the capillary wall and/or its anatomical junctions, then horseradish peroxidase can be seen to traverse the sites proposed for the BBB. Whether one can equate these pathways with what occurs under physiologic circumstances or when there is subtle injury is a question which remains to be answered.

This same phenomenon has appeared in BBB studies with the fluorescent microscope; that is, fluorescent tracers are frequently observed within neurons in areas where no gross damage can be demonstrated to the vessel wall nor extravasations of fluorescent tracer seen in the ECS. This SNS has occurred repeatedly and has eluded adequate explanation. The explanation for neuronal staining when dye is observed in the adjacent ECS has been that injured neurons take this material up from the neuropile. How cells stain when no ECS dye is present raises two immediately obvious questions. How can EB get from injured blood vessels to neurons, producing heavily concentrated stains which fill these cell bodies, without contaminating the ECS? Secondly, how can neurons which supposedly stain by taking up extravasations from the ECS extract all traces of dye from the ECS's and neatly fill their cell bodies? Extraction to this degree does not seem likely unless an artifact is assumed, caused by the fixation, which could wash out EB from the ECS. However, such an artifact has not been suggested in previous reports of SNS. This obvious improbability is supported by previous reports. Limiting circulation times of fluorescent tracer to 5 minutes as was done in 32 of our animals and to 30 seconds in 10 animals makes the rapid extraction from the ECS's unlikely. This report demonstrates on long exposure photomicrographs of areas of SNS that astrocytic processes are discretely filled with EB and link stained neurons with the adjacent vasculature. When the phenomenon of SNS is reexamined in light of the finding that astrocytic processes are filled with EB and connect stained neurons to the vascular source of dye, then transport through the astrocytic system is the most plausible explanation for the occurrence of this phenomenon.

A review of the literature in which BBB's have been studied with fluorescent microscopic techniques reveals that the majority of these studies utilized relatively high degrees of injury (i.e., chemical, anoxic, or mechanical injuries that produced gross extravasation of tracer, staining both intra- and extracellular areas) and/or long circulation times with the tracer substance (30 minutes to hours). This insured maximum demonstration of barrier injury; however, studies using light injury and not perfused excessively with tracer substances have produced more of the unexplained phenomenon mentioned
above. In the study of Johansson et al. the mechanism of injury was an induced hypertension which approximates physiologic injury (16). Vascular integrity as evidenced by minimal "parenchymatous" extravasation of tracer was combined with the curious result of SNS. Some of the animals in their series were sacrificed after only 10 minutes of circulation with EB.

Neuronal staining has been studied by fluorescent techniques for a number of years. The present study demonstrates that examination of specimens under the fluorescent microscope alone is only the first step in evaluating an area of BBB injury. Important events are invisible to the naked eye under the fluorescent scope. They require appropriate barrier filters and time exposure photomicrographs which should be studied by large screen projection after reproduction. Otherwise, many subtle events will go undetected.

A common feature of CNS injury is the prompt reactive swelling within astrocytes preceding other histologic changes (3, 14, 21, 23, 35, 45). Glycogen accumulation within astrocytes and their processes is another sensitive indication of early changes shown to accompany various types of CNS insult (27-29, 42). Another reactive feature of this cell is the capacity of the astrocyte to show pronounced elevations of oxidative enzymes shortly after injury which exceed that of any other cell in the nervous system. A sensitive indication that BBB damage has occurred when experimental injuries are studied with fluorescent tracer is SNS (16, 22, 28, 30, 38, 39, 43, 44). The subtleties of injury reflected on the one hand by changes occurring first in astrocytes as seen in electron microscopic studies, by glycogen accumulation and rapid enzymatic elevations determined by other techniques, and by SNS when fluorescence microscopic techniques are employed, become related findings if the hypothesis is accepted that SNS results from transport of fluorescent tracer within the astrocytic system.

A report by Klatzo et al. suggests that astrocytes could be of essential importance in determining the permeability of the BBB (21). The observations herein reported which suggest direct transport of EB along astrocytic processes to injured neurons not only support their findings but suggest that astrocytic foot processes are more than barrier envelopes; they may be gateways of transport to the neuron under normal conditions.

In this report and others reference is made to "the injured neuron." It is not unreasonable to consider the "injured neuroglia" and the degree of this damage as perhaps being a factor and determinant of reversibility of injuries within the CNS. If astrocytic links to the microcirculation are destroyed, neurons may become metabolically isolated. The astrocytic system embraces most elements within the CNS (capillaries, venules, neurons, other glia, the ependymal lining of the ventricles, and the subarachnoid space). Transport observed in part of the system as shown in this study leads to the logical question of whether similar transport occurs in its other ramifications. This consideration is particularly interesting in light of the fundamental response of the CNS to injury, i.e., astrocytic proliferation. This phenomenon has been an index of disease since the very foundation of neuropathology. Their reaction to injury, their proliferation in sites of repair, their role in BBB function, their increase in sites of heightened
metabolic demand, are all in keeping with an ability of astrocytes to directly transport materials between the vascular bed and the neuron.

Recent fluorescent and electron microscopic investigations have produced much valuable information regarding the BBB and the microstructure of the capillary wall. However, elucidation of pathways that open into the ECS rather than into the glial capillary investment may more accurately reflect the magnitude of experimental injury than those naturally occurring routes for transport within the central nervous system.

SUMMARY AND CONCLUSIONS

Neuronal staining with EB following BBB injury is divisible into four stages of sequential uptake of this tracer material. The phenomenon of SNS occurs by an almost imperceptible transport of EB within the astrocytic processes. Under circumstances of injury astrocytic foot plates accept vital dye and by transport along their processes produce neuronal stains. Barrier injuries of a magnitude to disrupt vascular walls stain, in addition to this, the ECS. Astrocytic foot processes at some point following injury (usually in association with distortion and swelling of the foot plates) accept vital dye only poorly or not at all.

This report demonstrates an essential point regarding one of the most abundant cellular structures in the CNS: that the astrocyte can transport materials directly as a conduit from capillaries to neurons. This possibility has been suggested by investigators for years based upon the architectural predisposition of these cells with their processes to provide such a function. Whether these avenues are truly functional to a greater or lesser degree for vessel to neuron transport under normal or pathological conditions cannot be answered by this study. It does, however, raise this question which may be a key in understanding the nature of astrocytic proliferation. The possibility that the astrocytic system represents another circulatory network within the CNS may lead to experimental efforts to define it in these terms. Study of the dynamics of such a system would be an interesting concept in many neuropathologic conditions.

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