Sequelae of the osmotic blood-brain barrier opening in rats

MICHIYASU SUZUKI, M.D., YUZO IWASAKI, M.D., TEIJI YAMAMOTO, M.D., HIDEHIKO KONNO, M.D., AND HIROKO KUDO, B.S.

Departments of Neurological Sciences and Neurosurgery, Tohoku University School of Medicine, Sendai, Japan

Histopathological sequelae of the osmotic blood-brain barrier opening were studied in 69 adult Wistar rats sacrificed between 2 minutes and 6 days after infusion of 1.6 M mannitol into the unilateral internal carotid artery. The results were correlated with immunohistochemical localization of autologous albumin in the brain parenchyma on paraffin sections. Extravasation of serum albumin was evident in all rats, and the albumin immunoreactivity, commonly localized to the territories of the ipsilateral anterior, middle, and posterior cerebral arteries and contralateral anterior cerebral artery, showed maximum intensity in the rats sacrificed 30 minutes after infusion. The albumin immunoreactivity remained macroscopically visible in the brain parenchyma for 24 to 48 hours, and then gradually faded out. Serum extravasation was accompanied by widening of the perivascular space and focal edema, which largely subsided within 48 hours as the albumin immunoreactivity of the tissue diminished. Although no overt neurological sequelae were seen in the present experiment, minute but definite foci of infarction with focal accumulation of albumin were found in 23 (38%) of 61 rats surviving more than 30 minutes. In addition, ischemic neuronal death of delayed onset was encountered among neurons in the CA-1 region of the hippocampus, in the cerebellum, and in the thalamus in five (25%) of 20 rats sacrificed between Days 4 and 6. Thus, care should be exercised in the practice of this procedure.

KEY WORDS • blood-brain barrier • mannitol • albumin • immunohistochemistry • rat

MODIFICATION of the blood-brain barrier (BBB) has recently been introduced to facilitate drug delivery to central nervous system (CNS) tissue as a new strategy for the treatment of brain tumors and inborn enzyme defects. Following the pioneer work by Rapoport and his colleagues, arterial infusion of hyperosmotic solutions has been most commonly used as a measure to increase the vascular permeability in both experimental and clinical trials, and a number of studies have been devoted to the elucidation of the mechanism and kinetics of BBB opening after infusion of hyperosmolar substances. Although reports on the morphological sequelae of mannitol-induced BBB opening are few, these studies have indicated the common occurrence of brain tissue damage after osmotic opening of the BBB in experimental animals. Direct correlation between cytopathology and BBB opening, however, has not yet been established.

In this report, details of the morphological sequelae of osmotic opening of the BBB in rat brains are correlated with the temporal and spatial sequences of serum protein permeation revealed by immunohistochemical staining of the autologous albumin. The potential risk of CNS tissue damage associated with this procedure is discussed.

Materials and Methods

Adult male Wistar rats,* each weighing 190 to 270 gm, were subjected to mannitol infusion. The animals were placed on a warm pad, and 1.0% to 2.0% halothane inhalation was begun. Under an operating microscope, the right carotid artery was exposed through a midline incision. After careful separation of the external carotid artery from the adjacent tissues and cauterization of its minor branches, a Scoville temporary vascular clip was placed immediately distal to the bifurcation of the common carotid artery. Through a small incision in the external carotid artery, a polyethylene tube (inner diameter 0.58 mm, outer diameter 0.96 mm) filled with heparinized physiological saline was

* Wistar rats were purchased from Funabashi Farm, Shizuoka, Japan.
FIG. 1. Brain sections obtained 2 minutes (a), 30 minutes (b), 2 days (c), and 3 days (d) after infusion of 1.6 M mannitol into the right internal carotid artery to show the temporal sequence of the distribution of albumin-immunoreactive products in rat brains. Paraffin sections stained with anti-rat albumin by the avidin-biotin complex method.

introduced into the bifurcation and was fixed to the arterial wall with Aron Alpha adhesive.† Thus, blood circulation in the common and internal carotid arteries was not compromised during the surgical intervention. After release of the temporary clip, 2.5 ml of a 1.6-M mannitol solution, prewarmed to 36°C, was infused at a rate of 0.083 ml/sec over 30 seconds. The external carotid artery was cauterized after the infusion and the wound was approximated with silk ties. Excluding the animals that were sacrificed immediately, 5 ml physiological saline and 0.025 µg atropine sulfate were given subcutaneously 5 and 30 minutes after mannitol infusion.

The animals were sacrificed immediately (less than 2 minutes), 5 or 30 minutes, 6, 12, or 24 hours, or 2, 3, 4, 5, or 6 days after the infusion of mannitol. Under deep intraperitoneal pentobarbital anesthesia, they were transcardially perfused with 200 ml of physiological saline, and then with 500 ml of 10% formalin under a pressure of 80 to 90 cm H₂O. After in situ fixation overnight at 4°C, the brain was removed from the skull and fixed in 10% formalin for 2 or 3 days. Multiple coronal sections of the brain were dehydrated and embedded in paraffin. Sets of consecutive 3-µm paraffin sections were processed for hematoxylin and eosin (H & E) staining and immunostaining with anti-rat albumin antibody to assess the extent of the BBB breakdown. Some sections were also subjected to immunostaining with antibody against glial fibrillary acidic protein (GFAP) for astrocytes and anti-ML-1 serum to identify mistletoe lectin bound to microglia.

Rabbit anti-rat albumin antibody‡ was preadsorbed twice with brain powder and used at a dilution of 1:2000. Rabbit anti-GFAP antibody and anti-ML-1 serum were diluted 1:2000 and 1:1000, respectively, in phosphate-buffered saline containing 0.3% Triton-X.§ For microglia staining, deparaffinized sections were first incubated with ML-1 for 48 to 72 hours at 4°C. For GFAP and ML-1 immunohistochemistry, the sections were incubated with antibodies for 48 hours at 4°C, and then processed according to the modified avidin-biotin complex method as described previously.¹⁰

To estimate the sensitivity of albumin immunohistochemistry, slices of normal mouse brain were incubated overnight in rat serum diluted to 1:25, 50, 75, 100, 150, 200, 300, or 1000 in phosphate-buffered saline, and then fixed in 10% formalin, paraffin-embedded, and processed for albumin immunohistochemistry as described above.

---

† Aron Alpha adhesive manufactured by Sankyo Co. Ltd., Tokyo, Japan.
‡ Rabbit albumin antibody was purchased from Cooper Biomedical, Malvern, Pennsylvania.
§ Rabbit anti-GFAP antibody was purchased from DAKO (Denmark), and ML-1 and anti-ML-1 rabbit serum were kindly supplied by Dr. H. Franz, Staatliches Institut für Immunopreparate und Nahrmittel, Berlin, DDR.
Osmotic blood-brain barrier opening in rats

**FIG. 2.** Correlation of histopathology with immunoreactivity to autologous albumin in consecutive paraffin sections of a rat brain fixed immediately after the infusion of 1.6 M mannitol into the right internal carotid artery (high-power views of Fig. 1a). At this stage, all neurons appear normal by hematoxylin and eosin stain and are albumin-negative even in the tissue with albumin permeation (white spots in the dark neuropil in d). Occipital cortices ipsilateral (b and d) and contralateral (a and b) to the side of mannitol infusion. H & E (a and b) and immunostain of rat albumin (c and d), × 100.

**Results**

*Temporal Tissue Permeation of Serum Albumin*

In sham-operated control rats, only the pineal body was macroscopically albumin immunoreactive and, under a microscope, the immunoreactivity was localized to the choroid plexus, the subpial tissue, and parts of the paraventricular tissue.

In five of eight rats sacrificed immediately (less than 2 minutes) or at 5 minutes after mannitol infusion, numerous discrete albumin immunoreactive foci were macroscopically identified (Fig. 1a), while in the remaining three rats, albumin immunoreactivity was confined to the perivascular tissue of small vessels. In the H & E preparations (Fig. 2a and b), the most conspicuous change immediately after infusion was a marked widening of the pericapillary and perivenular spaces. On the side of infusion, there were numerous minute pale edematous spots often containing a capillary wall in the center. Astrocytes with a swollen pale nucleus were commonly seen in the vicinity of these pale spots but other cellular elements appeared normal. Corresponding to these histopathological changes, there were numerous strongly albumin-immunoreactive spots where albumin-negative cell bodies and neurites stood out clearly in the dark albumin-positive neuropil (Fig. 2d). In the albumin-positive zones, the walls of many of the small vessels were severely distorted by perivascular edema (Fig. 3a).

Diffuse tissue permeation of serum albumin was evident in all nine rats sacrificed 30 minutes after mannitol infusion. In many sections, the entire hemi-
sphere was deeply stained as if the tissue had been soaked in an albumin solution (Fig. 1b), and unstained cellular elements were scattered throughout the dark background. In addition, circumscribed darker areas were often seen adjacent to the wall of relatively large veins. In the periphery of diffuse albumin permeation, often in paramedian structures of the contralateral hemisphere, albumin-immunoreactive products appeared as dendritic structures in the perivascular tissue, possibly localized to astrocytic processes (Fig. 3b). In the H & E preparations, widening of the perivascular spaces was more pronounced, and irregular edematous spots were randomly distributed in the ipsilateral hemisphere. The vesicular appearance of the astrocytic nuclei became more conspicuous and some had a watery swollen cytoplasm.

Six hours after the infusion, the perivascular spaces of some vessels remained widened, and deposits of amorphous eosinophilic substance were occasionally seen in perivascular tissues. By 12 hours after the infusion, except for focal ischemic lesions with groups of pyknotic neurons in the edematous neuropil and occasional perivascular edema, histological abnormalities were hardly discernible and astrocytes also appeared normal in the H & E preparations. Albumin immunoreactivity, however, remained strongly positive for another 12 to 36 hours, and tended to diffuse out more homogeneously in the gray and white matter.

By 48 hours after mannitol infusion, diffuse hemispheric albumin immunoreactivity was markedly reduced but it was still macroscopically discernible in six of the nine rats sacrificed on Day 2 (Fig. 1c). In the 27 rats sacrificed on Days 3 to 6, including seven rats sacrificed on Day 3 (Fig. 1d), albumin-immunoreactivity was not macroscopically detectable with the exception of focal staining in ischemic lesions and their vicinity (Fig. 4). Perivascular edematous changes in small vessels and the swelling of astrocytes also became inconspicuous in accordance with the loss of albumin-immunoreactivity on Days 2 and 3. As reminiscence of albumin extravasation, astrocytes with dark granular dendritic processes were occasionally encountered in close approximation to small vessels in the weakly albumin-positive zones. From Day 4 on, these astrocytes with albumin-positive cell processes were found only in the vicinity of ischemic lesions.

The lowest albumin concentration detected in the present study by immunohistochemistry of mouse brain tissues soaked in the rat serum with various concentrations was estimated to be 1:200 to 1:300 of serum concentration, being slightly lower than the albumin concentration in the cerebrospinal fluid (CSF).

**Topography of Albumin-Immunoreactive Areas**

In most of the rats, albumin-permeated areas were confined to the territories of the ipsilateral anterior, middle, and posterior cerebral arteries and to that of the contralateral anterior cerebral artery, which included the anteromedial and anterolateral cerebral cortices, occipital cortex, hippocampus, basal ganglia, midbrain, and cerebellar hemisphere on the side of mannitol infusion, and the contralateral anteromedial cortex and caudoputamen (Fig. 1). There was a certain individual variation in the distribution of albumin-positive areas, however. For instance, in all of the nine rats sacrificed 30 minutes after mannitol infusion, the ipsilateral anteromedial cortex, basal ganglia, occipital cortex, midbrain, and cerebellar hemisphere were positive for albumin while the territory of the bilateral anterior cerebral artery was spared in two rats. In the midbrain, the ipsilateral dorsal quadrant was always severely involved. Albumin permeation in the cerebellum was commonly seen in the ipsilateral hemisphere, but it might extend to the vermis and, on rare occasion, to the contralateral hemisphere. The pons and medulla oblongata were always spared.
Osmotic blood-brain barrier opening in rats

FIG. 4. Correlates of histopathological sequelae (a, c, and e) and albumin immunohistochemistry (b, d, and f) in consecutive paraffin sections obtained 4 days after mannitol infusion from the right frontal cortex. a and b: A small infarct in the cortex appears pale on the H & E-stained specimen (a) and is heavily labeled with anti-albumin antibody (b) indicating persistent leakage of serum components at the site of infarction. c to f: High-power views of a and b showing that the area with a group of pyknotic neurons intermingled with reactive cells (right lower portion in c) is strongly albumin immunoreactive (d) and at the margin of this area, a line of albumin-positive pyknotic neurons is present (long arrows in d). Some of the normal-appearing neurons in their vicinity are also labeled with anti-albumin antibody (short arrows in d), but sparing of the nucleus clearly distinguishes them from necrobiotic neurons. Neurons stained deeply pink are inconspicuously located in the grossly normal III cortical layer (e) and are heavily labeled with anti-albumin antibody (f). H & E stain (a, c, and e), albumin immunostain (b, d, and f); × 25 (a and b), × 150 (c to f).

Focal Ischemic Lesions Associated With Mannitol Infusion

Two distinct types of focal ischemic lesions were identified. Minute infarcts with focal accumulation of serum albumin were seen in 23 (38%) of 61 asymptomatic rats sacrificed from 30 minutes to 6 days after mannitol infusion. These lesions were randomly distributed but were more frequently seen in the basal ganglia and midbrain than in the cerebral cortex. The neocortex was spared in 10 of 23 rats with such infarction. In


425
M. Suzuki, et al.

most instances, the lesions were identified as a discrete minute vesicular lesion with degenerating cells, occasionally accompanied by a thrombosed capillary in the center of the lesion. Groups of degenerating cells were readily identified by their diffuse strong albumin immunoreactivity even 30 minutes after mannitol infusion. Among these 23 rats, relatively large foci of infarction were encountered in the neocortex of two rats and in the basal ganglia and the hippocampus of another two rats (Fig. 4a to d). In such lesions, albumin immunoreactivity remained strongly positive at least 6 days after the insult, and the albumin-positive zone was often demarcated by groups of albumin-positive neurons (Fig. 4d, short arrows). Although accumulation of albumin in apparently normal neurons was not rare in the area of albumin permeation (Fig. 4a, long arrows) even as early as 2 minutes after the BBB opening (not shown), these cells were clearly distinguished from diffusely albumin-positive necrobiotic neurons by the absence of nuclear stain.

In addition, deeply eosinophilic neurons with karyorrhectic nuclei were found in normal-appearing tissue in the neocortex of one rat, in the CA-1 region of the hippocampus of two rats, in the Purkinje cell layer of two rats, and in the thalamus of one rat among the 20 rats surviving more than 4 days (25%). These severely degenerated neurons were intermingled with normal-appearing neurons and were accompanied by no appreciable cellular reaction (Fig. 4e) but were strongly albumin-immunoreactive and stood out in the albumin-free neuropil (Fig. 4f). In four of five rats, this type of lesion coexisted with infarction. The overall incidence of ischemic lesions was estimated to be 39% (24 rats) of the 61 asymptomatic rats surviving more than 30 minutes.

Reactions of Astrocytes and Microglia

In general, glial reactions after mannitol infusion were surprisingly subtle considering the extent and the intensity of tissue permeation of serum components. Throughout the course of the experiment, there was no appreciable difference in the immunoreactivity to GFAP and ML-1 antibodies in the tissues with and without albumin permeation (Fig. 5). The glial reaction, both of astrocyte and microglia, was confined to the focal ischemic lesions and it became apparent in animals sacrificed on Day 2 and thereafter.

Discussion

The present experiments clearly showed that an arterial infusion of 1.6 M mannitol is a reliable measure for temporary breaching of the BBB in the CNS. Extensive permeation of serum albumin to the CNS tissue was seen in all of the 69 rats examined. The albumin immunoreactivity, initially localized to the vicinity of
Osmotic blood-brain barrier opening in rats

small parenchymal vessels, rapidly spread to the neighboring tissue in both gray and white matter, and the extent of the albumin-immunoreactive area reached maximum within 30 minutes. The highest intensity of immunoreactivity was also seen in the rats sacrificed 30 minutes after the infusion, and it persisted for 24 to 48 hours, when it gradually disappeared and became undetectable by Day 3. Although Rapoport, et al.,17 observed the presence of an Evans blue dye-albumin complex in the brain parenchyma at least 2 weeks after infusion of hyperosmotic urea, we found that the persistence of albumin immunoreactivity occurred only in the ischemic lesions. Thus, the levels of extravasated serum proteins in the undamaged CNS parenchyma seem to return to those of normal CSF within 48 hours after a single infusion of mannitol if the assessment of the sensitivity of the technique in vitro is applicable to an in vivo situation.

The duration of osmotic BBB opening after a single infusion of hyperosmotic solution has been estimated by several investigators. Chiueh, et al.,4 reported that the permeation of an Evans blue-albumin complex into the brain diminished 30 minutes after infusion of 1.58 M arabinose and was undetectable 4 hours later. Tomiwa, et al.,21 suggested that the BBB remained open for 60 minutes in all rats subjected to infusion of 1.4 M mannitol. Nagy, et al.,12 observed that small arteries and veins were persistently leaky to horseradish peroxidase up to 3 hours after 1.8 M mannitol infusion. On the other hand, Houthoff, et al.,9 reported that extravasation of tracer proteins seemed to be completed within 5 minutes. The results of the present study with immunohistochemical demonstration of autologous albumin largely agree with those of Chiueh and Tomiwa and their colleagues.

With regard to the topography of albumin permeation in the rat brain, it should be noted that the extent of protein permeation was not restricted to the ipsilateral cerebral hemisphere. The contralateral anteromedial cortex and caudoputamen in the territory of the contralateral anterior cerebral artery were frequently involved, and albumin permeation in the territory of the ipsilateral posterior cerebral artery extended often to the midbrain and ipsilateral but rarely to the contralateral cerebellar hemisphere. These results are unlikely to be due to elevated arterial pressure during mannitol infusion, since the infusion rate was not high enough to cause hypertension,4 and the common carotid artery was left open during the infusion. Thus, the variable distribution of albumin-positive areas seems to reflect anatomical variations in the circle of Willis, which give rise to considerable diversity in the extent of the anterior and posterior circulations. Therefore, comparison of cerebral hemispheres may not be appropriate for assessment of the efficacy of the unilateral osmotic BBB opening.22

Although an Evans blue-albumin complex is widely used as an indicator in studies of the BBB,23 demonstration of albumin immunoreactivity in paraffin sections has been shown to be a sensitive measure for assessing the extent of BBB breakdown in experimental allergic encephalitis and experimental spinal cord injury.10,11 The immunocytochemistry of autologous albumin is also known to be a useful tool for studying the integrity of cell membrane and the viability of cells.3,10 The obvious advantage of this technique is that it allows for correlation of histopathological changes with the extent of BBB breakdown as demonstrated in the present study.

A high incidence of neuronal damage after osmotic BBB opening has been reported and has been attributed to either compromised blood flow in the common carotid artery or embolization of cerebral vessels with mannitol precipitates. Rapoport, et al.,17 reported an experiment with monkeys infused with 2 to 3 M urea at a flow rate of 0.4 to 1.67 ml/sec after ligation of the unilateral common carotid artery; nine of 11 monkeys so treated developed muscle weakness of differing severity on the contralateral limbs, and avoidance of carotid ligation in subsequent experiments diminished the neurological sequelae. Tomiwa, et al.,21 reported the development of organic brain changes including edematous changes and focal necrosis in 70% of rats subjected to a 1.4-M mannitol infusion; they noted substantial reduction in the incidence of organic changes when they strained the mannitol through a 0.45-μ filter.

Although in the present study, care was taken to avoid these risk factors and no gross neurological deficits were seen, there was also a high incidence of minute but permanent tissue damage. In 61 rats surviving more than 30 minutes, two distinct types of tissue damage were identified. One was microinfarction and the other was so-called "ischemic neuronal death" of delayed onset.16 The foci of microinfarction were identified by the presence of groups of neurons with strong diffuse albumin immunoreactivity as early as 30 minutes after mannitol infusion. The distribution of such neurons was very similar to that of diffuse Evans blue-fluorescent neurons in the study with Evans blue dye.24 Therefore, even though the presence of diffuse fluorescence neurons has been often interpreted as a good indication of successful BBB opening and subsequent cellular uptake of the substance delivered,21 it may merely be an indication of the development of cellular damage. Such microinfarction was found in 23 of 61 rats sacrificed between 30 minutes and 6 days after mannitol infusion, and was localized to the ipsilateral cerebral neocortex, hippocampus, thalamus, midbrain, and cerebellar hemisphere. Albumin immunoreactivity persisted in the neuropil of these lesions even on Day 6, and was often accompanied by increased immunoreactivity of GFAP in astrocytes and of ML-1 in microglia on Day 2 and thereafter.

Ischemic neuronal death of delayed onset has been characterized by the occurrence of shrunken neurons with deeply eosinophilic cytoplasm and a karyorrhectic nucleus among normal-appearing neurons in grossly

J. Neurosurg. / Volume 69 / September, 1988 427
normal neuropil.\textsuperscript{10} Cellular reaction was usually unremarkable in H \& E preparations but increased GFAP and ML-1 immunoreactivity could be detected in the lesions. This type of lesion was usually found in rats with microinfarction but, in one rat sacrificed on Day 6, the neuronal death in the CA-1 region was the only lesion. Local edema around small vessels and the development of astrocytes with pale swollen nuclei were seen in all rats sacrificed in earlier stages. These changes were apparently reversible and mostly subsided between 12 and 48 hours after a single infusion of mannitol in accordance with the disappearance of albumin immunoreactivity from tissue.

Considering the extent and intensity of tissue permeability of serum components, the tissue damage following the osmotic BBB opening was relatively mild and mostly reversible, and unaltered GFAP and ML-1 immunoreactivity also suggested that the overall insult to the brain parenchyma was subtle. The high incidence of microinfarction in asymptomatic animals, however, warrants caution to minimize the potential risk of permanent neuronal damage with the use of this procedure.

References


Manuscript received December 1, 1987.
Address reprint requests to: Yuzo Iwasaki, M.D., Department of Neurological Sciences, Tohoku University School of Medicine, 1-1 Seiryoi-Machi, Sendai 980, Japan.