
Evans Blue fluorescence permits the rapid visualization of non-intact cells in the perilesional rim of cold-injured rat brain

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Abstract. A focal cold lesion-induced injury, i.e., a model of focal vasogenic brain edema, enhances the permeability of the blood-brain barrier and cell membrane in the perilesional rim. However, non-intact cells can be detected, e.g. by markers of apoptosis, only hours or even days after the injury. The early membrane dysfunction allows extravasated serum proteins to enter the injured cells, which can be readily visualized if the plasma albumin was previously bound to fluorescent tracers, such as Evans Blue (EB). The aim of this study was to demonstrate injured cells that take up the EB/albumin conjugate in the perilesional rim. This tracer was administered 3.5 h after the induction of the injury and the animals were sacrificed 30 min later. With an excitation wavelength of 530–550 nm, the EB-positive cells emitted bright-red fluorescence at >590 nm and were easy to count. No positive cells were observed in the controls. This method provides more information than the classical 2,3,5-triphenyltetrazolium chloride reaction, because it permits an assessment of the density and distribution of cells with non-intact cell membranes in the perilesional area following cerebrocortical injury.

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INTRODUCTION

After a traumatic brain injury, the degree of secondary brain damage is often more important for the prognosis than the primary lesion. In the surroundings of focal traumatic brain lesions, delayed cell injury has been reported to develop over a period of several days or even weeks (Hovda et al. 1992, Morita-Fujimura et al. 1999a,b).

Studies based on various experimental trauma models in rodents, including cortical cold injury in rats, have consistently shown that the volume of tissue affected by focal cortical necrosis increases over time (Eriskat et al. 1994). The brain region tissue that could potentially be rescued by a therapeutic intervention is the perilesional rim surrounding the necrotic domain, also called the penumbra (Plesnila et al. 2003). The time interval in which the perilesional rim persists denotes the therapeutic window (Touzani et al. 2001).

A frequently used focal cortical injury model involves touching the exposed brain surface with a deep-cold metal rod (Klatzo et al. 1958). This is regarded as a classical model of vasogenic edema, and is characterized by an increased blood-brain barrier (BBB) permeability (Gorlach et al. 2001, Morita-Fujimura et al. 1999a). In addition to the dysfunction of the BBB, such a cold lesion impairs the integrity of the cellular membranes, leading to increased intracellular water and calcium contents. These may induce a secondary neuronal loss, which is mainly an apoptotic process, via a mechanism different from that of the primary injury (Murakami et al. 1999).

The primary cortical lesion can be highlighted by the 2,3,5-triphenyltetrazolium chloride (TTC) reaction (Coyle 1987). However, besides detection of the site of the primary cortical lesion, it may be important to visualize the extent of the perilesional rim containing non-intact cells as early as possible. These cells are in potential danger of secondary cell death. As cortical cold injury has been used as a model for vasogenic brain edema (Chan et al. 1991), we presumed that the endangered cells in this perilesional rim could be visualized by means of fluorescence microscopy, following the i.v. administration of Evans Blue (EB). EB is known to bind to serum albumin and has been used as a tracer (Wolman et al. 1981). In the same cold lesion model, Murakami and coauthors (1999) found that the extent of extravasation of the EB/albumin conjugate in the injured hemisphere of the animals was highest at 0.5 h after EB administration. Accordingly, we anticipated that, with

this method, information could be obtained on the size of the cortical area with non-intact cells much earlier than with other immunohistochemical procedures, including the use of markers of apoptosis. Moreover, this method was expected to provide more morphological data concerning the injury and its surroundings than is available from the TTC reaction. The aim of this study was to test this hypothesis.

METHODS

Animals, anesthesia, and cold lesion

Wistar rats weighing 300–350 g ($n=41$) were used. The principles of laboratory animal care approved by the Hungarian Health Committee (1998) and the European Communities Council Directive of 24 November 1986 (86/609/EEC) were followed. Rats were anesthetized by the intraperitoneal administration of a mixture of 10 mg/100 g BW ketamine (Ketanest, Parke Davis, Berlin, Germany) and 0.8 mg/100 g BW xylazine (Rompun, Bayer, Leverkusen, Germany). The animals were secured in a stereotaxic headholder (David Kopf Instruments, Tujunga, CA, USA) and craniotomy was performed on the left hemisphere over the motor cortex. The dura mater was kept intact.

Cold injury was produced in the primary motor cortex (MI) of 28 animals by applying a copper cylinder (diameter 2 mm) cooled to -78°C by filling it with an acetone/dry ice two-phase system (for details, see Gorlach et al. 2000 and Hortobagyi et al. 2000). The duration of cooling was 30 s. The rectal temperature was maintained at $37 \pm 0.5^{\circ}\text{C}$ with a body temperature-controlled infrared lamp during the surgical preparation and until the end of the anesthesia. Sham-operated animals ($n=13$) served as controls. The procedure in the sham-operated animals was the same as described above, except that the copper cylinder thermode otherwise used to evoke cold injury was at room temperature.

Lesion detection (TTC reaction)

Four h after the induction of the lesion, 6 animals were deeply anesthetized and killed by decapitation. Thereafter, 0.4-mm-thick slices were cut throughout the ischemic region with a vibratome (Campden Instruments, Sibley, UK). The injury was visualized by using a 1% TTC (Sigma, Munich, Germany) solution dissolved in artificial cerebrospinal fluid [its composition

Table I
Numbers of animals in the different experimental groups

	Animals treated with			Σ
	TTC	EB	Vehicle	
SHAM	4	6	3	13
Cold lesion	6	13	9	28
Total				41

is given in Juhasz-Vedres and others (2006)]. When the reaction was complete, the slices were fixed with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer on coverslips for 15 min, and subsequently kept overnight in the same solution. Four of the 13 sham-operated animals were also stained with TTC (Table I).

Evans Blue labeling

Two-percent EB (Sigma) (0.2 ml) was administered through the tail vein to 13 of the 28 cold-lesioned animals 3.5 h post-injury. As the control of the labeling procedure, 0.2 ml saline was administered to 9 cold-lesioned animals (vehicle in Table I). The animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer 30 min after EB administration.

The brains were removed and post-fixed overnight in the same fixative. Coronal sections (50 μm) encompassing the EB-labeled area were cut with a Vibratome (Campden). The location of extravasated EB on the sections was observed with a fluorescence microscope (Olympus BX51) with an excitation wavelength of 530–550 nm and an emission wavelength of >590 nm. In the 9 cold-lesioned animals without EB labeling, the autofluorescence of the tissue was recorded with the same excitation/emission parameters (vehicle in Table I).

RESULTS

Cortical lesion

The sections from sham-operated (non-lesioned) animals did not reveal any sign of injury in the corresponding cortical area, either with TTC ($n=4$ animals) or with EB staining ($n=6$ animals). Similarly, no sign of labeling was observed in the vehicle-treated sham-operated animals ($n=3$). The standardized cortical lesion within the MI region did not result in any significant change in the measured physiological parameters, as described earlier (Gorlach et al. 2000, 2001, Hortobagyi et al. 2000). The lesion visualized with the TTC reaction was characteristic: this region appeared as a white, non-stained area with a thin rim of transition to the surrounding red-stained brain tissue (Fig. 1A).

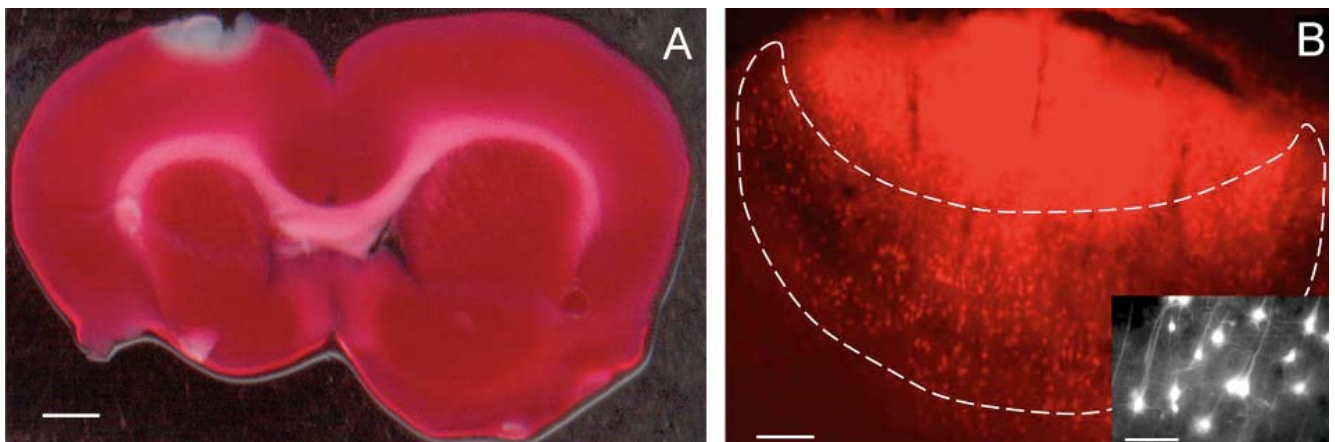


Fig. 1. Representative photomicrographs showing a cold-induced lesion in the primary motor cortex of a rat. (A) The lesioned area visualized by the TTC (2,3,5-triphenyltetrazolium chloride) method. The primarily lesioned cortex appears as an unstained white area with a narrow rim of transition to the “intact” part of the brain, appearing red. (B) The lesioned cortical area (core) and the affected cells in the perilesional rim (denoted by a dashed line) visualized by fluorescence microscopy following the intravenous injection of Evans Blue. Insert: Labeled cells in the perilesional rim at higher magnification. The labeled pyramidal cells exhibit discernible apical and basal dendrites. Scale bars: (A) 1000 μm ; (B) 200 μm ; (C) 50 μm in the insert.

Detection of cells with EB fluorescence

EB leaks through the disrupted BBB and stains the brain where the BBB is disrupted (Fig. 1B, see the intensely labeled core region). However, EB may also enter cells with damaged cell membranes in the perilesional rim. Indeed, EB-positive cells were detected in the perilesional rim (Fig. 1B). These cells emitted bright-red fluorescence and could easily be counted in 50- μm -thick slices. The cell bodies and the apical and basal dendrites of the affected cells could be clearly seen at higher magnification (Fig. 1B, insert). The boundaries of the cortical area with the EB fluorescent cells extended beyond the pale transition zone on the corresponding slides stained with TTC.

Control of the autofluorescence of the tissue

As demonstrated above, EB administration is suitable for the visualization of non-intact cells with some histological details in the perilesional rim of the injured cortex (Fig. 1B). Since autofluorescence from tissue components may contribute to these images, control processes were applied. First, fluorescent images were taken from the perilesional rim of the cortex of cold-lesioned animals without i.v. EB application. Comparison of image pairs (taken with the same excitation/emission and exposure parameters) of sections obtained from labeled and unlabeled animals revealed a clear EB staining in the perilesional rim (Fig. 2A,B). Alternatively, image pairs from the ipsilateral and contralateral sides were recorded with the same exposure parameters from individual sections from either EB-labeled or vehicle-treated animals. In each section, the image of the contralateral side served as the control for the background fluorescence. Subtraction of the images obtained on the contralateral side from those on the operated side clearly indicated that significantly different images were obtained only in the EB-labeled animals (Fig. 2C,D).

DISCUSSION

The cold lesion model is a useful and established means of investigating the mechanism of brain injury involving vasogenic brain edema. The injury induces not only cortical necrosis, but also a rapid breakdown of the BBB, causing vasogenic brain edema (Murakami et al. 1999), thereby resulting in secondary

tissue damage. As the EB content in the injured hemisphere is highest at 0.5 h after its administration (Murakami et al. 1999), we injected the dye 0.5 h before sacrifice. The animals were killed at 4 h post-injury. This is a short survival time, but sufficient to give rise to detectable expression levels of early markers of cell injury in other models, such as HSP70 (Pavlik et al. 2003).

It has long been known that the primarily lesioned brain area can be visualized with the TTC method (Coyle 1987). However, in its surroundings a number of cells are injured, which may result in delayed cell death occurring over several days (Hovda et al. 1992). This surrounding perilesional rim is the main target of neuroprotective interventions. This area cannot be reliably detected by the TTC method. Although the TTC method reveals a transition zone between the unstained core and the red viable brain tissue, the pale staining, at least in part, is probably due to the edema and is not a consequence of cellular damage. In contrast, the perilesional rim appears larger and better defined with the EB-based method and is more consistent with the penumbra zone of poor perfusion.

A potential problem of this EB-fluorescence-based method is the non-specific (auto)fluorescence of the tissue. This problem is particularly important if the aim is to perform quantitative analysis. A solution to this problem has been described by Murphy and Lever (2002), who used a ratiometric method to eliminate the autofluorescence signal from the images. For rapid qualitative analysis, however, our approach of using vehicle-injected animals or the contralateral brain region for control purposes seems to be appropriate (Fig. 2). The affected cells in various models of focal brain injury can be identified by demonstrating the alterations in gene expression related to metabolic disturbances and delayed cell death (Hermann et al. 2004). For this purpose, widely used markers are the immediate-early genes, such as the activator protein-1 gene *c-jun* and mitogen-activated protein kinase phosphatase (*mkp*)-1 (Hata et al. 2000). Similarly, the *de novo* expression of *caspase-3* may contribute to the injury evolution (Hermann et al. 2004), and this may also be a good marker of apoptosis. Additionally, there are several conventional astrocytic and microglial markers, such as GFAP and CD68, respectively. Preliminary studies suggest that both neuronal and glial cells are involved in delayed damage (unpublished observations). However, they are not suitable for

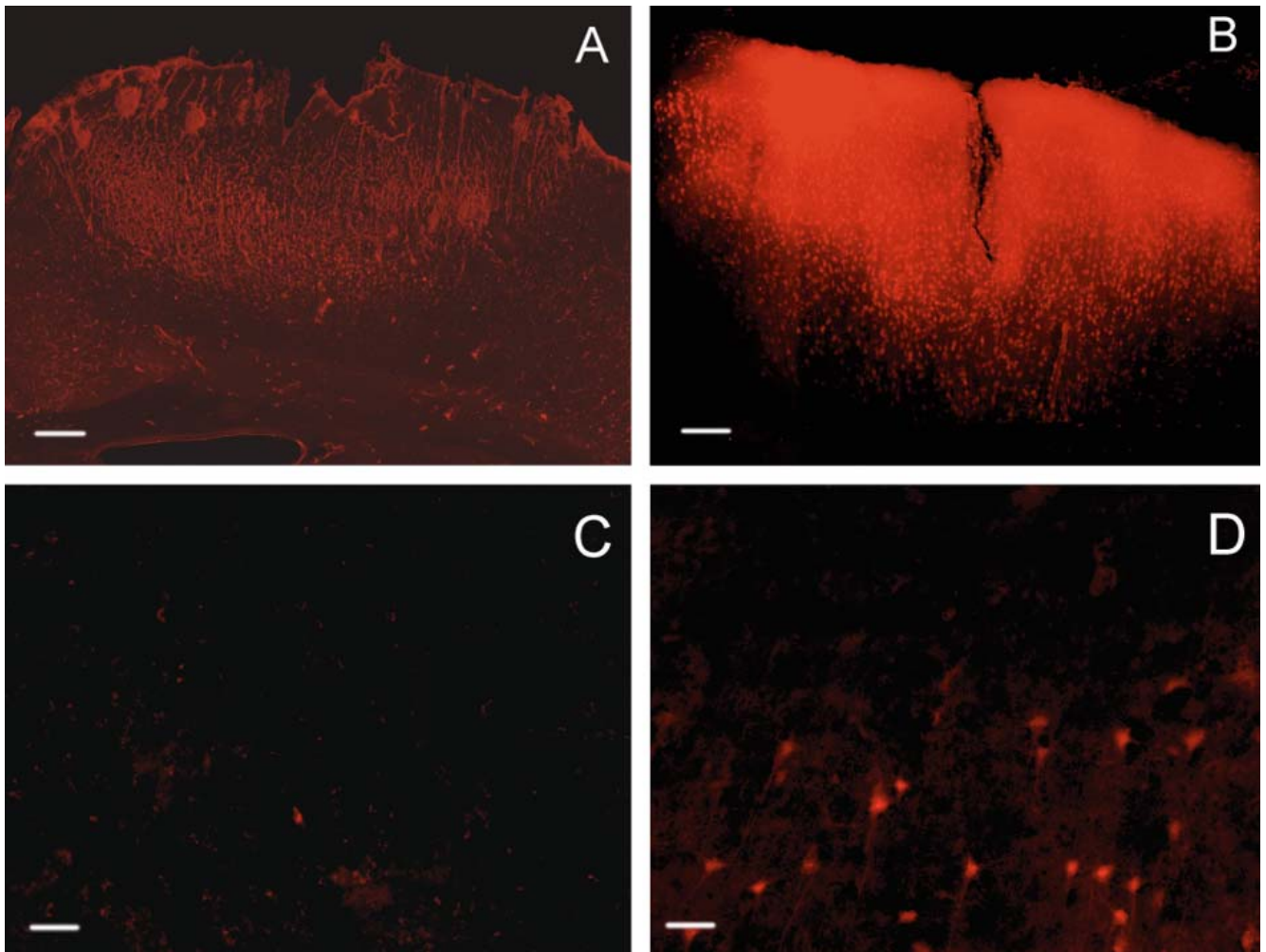


Fig. 2. Correction of the autofluorescence of the tissue to Evans Blue fluorescence images. EB administration to animals resulted in bright details in images taken from a cold-lesioned cortical area (B) as compared with similarly lesioned brain samples from animals injected with vehicle alone (A). When the background fluorescence was calculated from the cortical region on the unoperated side in each section, and then subtracted from the corresponding image on the operated side, no discernible image was obtained when the animals were treated with vehicle alone (C), whereas labeled neurons could be recognized in the perilesional rim when the animals were injected with EB (D). Scale bars: (A, B) 200 μm ; (C, D) 50 μm .

the early demonstration of the extent of the perilesional rim.

In contrast with these immunocytochemical procedures, the detection of extravasated EB proved suitable for visualization of the extent of the perilesional rim as early as 4 h after the injury. Though, it has long been known that injured neurons and other cells of the nervous tissue take up EB (Sasaki and Schneider 1976), this is the first presentation with fine details of labeled pyramidal cells, which exhibit discernible apical and basal dendrites. Moreover, this method proved useful in recent pilot experiments to characterize the neuroprotective effects of candidate molecules/agents in the

cold lesion model (Juhász-Vedres et al. 2006) and to demonstrate the impairment of the BBB (Hawkins and Egleton 2006).

Further studies, which are beyond the scope of the present paper, are needed to clarify which cells take up EB: apoptotic, necrotic, or degenerate, i.e., dead or sub-lethally damaged cells.

CONCLUSION

Our study provides a simple and cost-effective tool for a rapid estimation of the size of the perilesional rim and the density of non-intact cells in this region.

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