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STRUCTURAL AND FUNCTIONAL CHANGES OF BLOOD-BRAIN BARRIER AND INDICATION OF PRION AMYLOID FILAMENTS IN EXPERIMENTAL AMYOTROPHIC LEUKOSPONGIOSIS

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Summary. – The reproduction of amyotrophic leukospongiosis (AL) agent in the central nervous system (CNS) of guinea pigs was accompanied by local disturbance of blood-brain barrier (BBB), which manifested in passing of horseradish peroxidase through the endothelium of some of capillaries (10–14 %). The disturbance of BBB function coincided with dystrophic changes in a number of pericapillary astrocyte foot processes and in pericytes. In the walls of altered vessels we observed congofilic (amyloid) deposits, which formed immunocomplexes with monoclonal antibodies to the AL agent protein PrP 27–30 kD. By electron microscopic the deposits consisted of masses of filaments with diameter of 5–10 nm located between the basement membrane and endothelium of the vessels.

Key words: amyotrophic leukospongiosis; prion protein; blood-brain barrier; amyloid

Introduction

Amyotrophic leukospongiosis (AL) is a lethal neurodegenerative human disease with preferential lesion of spinal cord motoneurons, astrogliosis and developing of spongiosis in the white matter. AL belongs to the group of transmissive spongiform encephalopathies (Creutzfeldt-Jacob disease (CJD) and scrapie) (Kolomiets *et al.,* 1988). Evidence for the occurrence of prion PrP protein and characteristic infectious scrapie-associated fibrils (SAF) in AL was given in our previous works (Votyakov *et al.,* 1987*a;* Poleshchuk *et al.,* 1990). The nature of the disease agents (prions) has not been investigated sufficiently up to now and much less data have been accumulated about the mechanism(s) how they damage the blood-brain barrier (BBB), including the sensitivity of pericapillar astrocytes and pericytes to their cytodestructive action. There are some communications about the disturbance of transendothelial transport in brain capillaries during prion reproduction (Wisniewski *et al.,* 1983; Lossinsky *et al.,* 1987; Poleshchuk *et al.,* 1990). However, investigations on the role of

pericapillar astrocytes and pericytes in the pathogenesis of disease are fragmentary and, in many respects, contradictory, and as for AL, such analysis was not performed yet.

We investigated the dependence of BBB function on the degree of expression of structural changes in endotheliocytes, pericytes and pericapillar astrocytes when reproduction of AL agent is observed in guinea pig CNS. In addition, we conducted the indication of prion amyloid filaments (aggregates of AL agent protein PrP 27-30 kD) in different cell types.

Materials and Methods

Virus. The AL agent (AL-D strain), was deposited at the State virus collection at the D. I. Ivanovsky Institute of Virology of the C.I.S., Academy of Medical Sciences under Nr 2206.

Experimental animals. The 28 outbred guinea pigs (wt 250-300 g) were divided in two groups. The first group of animals (18) was inoculated retrobulbarly, the second group - intracerebrally as previously described (Kolomiets *et al.*, 1988; Poleshchuk *et al.*, 1988). In the first group (I) characteristic clinical signs - hair shedding, muscle atrophy, extremity paresis and paralysis - developed in 5-6 weeks p.i. (35-50 days). From group I six animals were investigated on day 14 in the stage of preclinical signs (AL agent titre in CNS was 3.5-4.1 log ID_{50}), six animals were investigated on day 28 at the stage of the first clinical signs (titre was 5-5.6 log ID_{50}) and six animals on day 40 p.i. at the preterminal stage (titre was 6-6.2 log ID_{50}).

In animals with intracerebral inoculation (group II) characteristical signs developed by 16-18 weeks after inoculation - the titre of the AL agent in the CNS was 5.2-6.2 log ID_{50}. The brains of animals were removed for investigation in the preterminal stage (96-110 days p.i.).

Control animals (15) were given a 10 % suspension from the brain of healthy individual who died in a car accident: 8 animals were injected retrobulbarly and 7 intracerebrally (group I and II controls, respectively).

Electron microscopy and histological investigations. Intravascular perfusion of narcotic animals with 4 % glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.2) was performed for 15 min. The brain was removed and cerebrum, cerebellum and spinal cord were cut into pieces of 1-3 mm. The pieces were embedded into paraffin for histological investigation and into Araldit for ultrastructural analysis by routine methods.

Immunocytochemical detection of AL agent antigen in brain sections was made by immune-peroxidase method using monoclonal antibody to PrP protein (Votyakov *et al.*, 1987*b*). Brain and spinal column sections were treated by sodium azid (Na_3N) solution containing hydrogen peroxide (H_2O_2) and then the sections, after being rinsed in salt-phosphate buffer, were treated with the peroxidase-labelled monoclonal anti-PrP antibody. The latter was obtained (Kolomiets, N. D.) from the cell strain ALMA-7 (deposited in C.I.S. National collection of continuous cell lines under N19-2-8). Diaminobenzidine (DAMB) was used as substrate for visualization of the peroxidase. AL agent protein PrP 27-30 was demonstrated in the sections as accumulation of dark brown complexes. The coded preparations were estimated on the scale: -, ±, +, ++, +++.

Histochemical demonstration of amyloid deposits has been performed with the help of Congo red staining by a standard technique in sections (4-5 μm thick). Under polarized light amyloid displayed green-gold birefringence.

Morphofunctional examination of BBB was performed with the peroxidase technique of Gracham and Karnowsky (1966) modified by Wisniewski *et al.* (1983) in 9 animals of group I in different stages of disease, in 3 animals of group II in preterminal stage (96-110 days) and in 3 guinea pigs of control groups I and II. Briefly 50 mg of horseradish peroxidase (HRP) (Sigma Type II) in 0,5 ml of balance salt solution were injected in animal's heart 1.5-2 hr before brain fixation. Then the brain and spinal cord were removed, and cut into blocks about 1x1x3 mm in size. The

blocks were fixed with a mixture consisting of 1 % glutaraldehyde and 2 % paraformaldehyde and the histochemical reaction was made with DAMB tetrahydrochlorid. For HRP control, the reaction was performed in the absence of DAMB. Thereafter, tissue blocks were postfixed in 1 % osmium tetroxide in sodium cacodylate buffer, dehydrated and embedded into Araldit by routine techniques. Histochemical reaction products were seen on ultrathin sections as amorphic or granular electronopaque materials.

Ultrathin sections were studied in electron microscope JEM 100CX-II.

Results

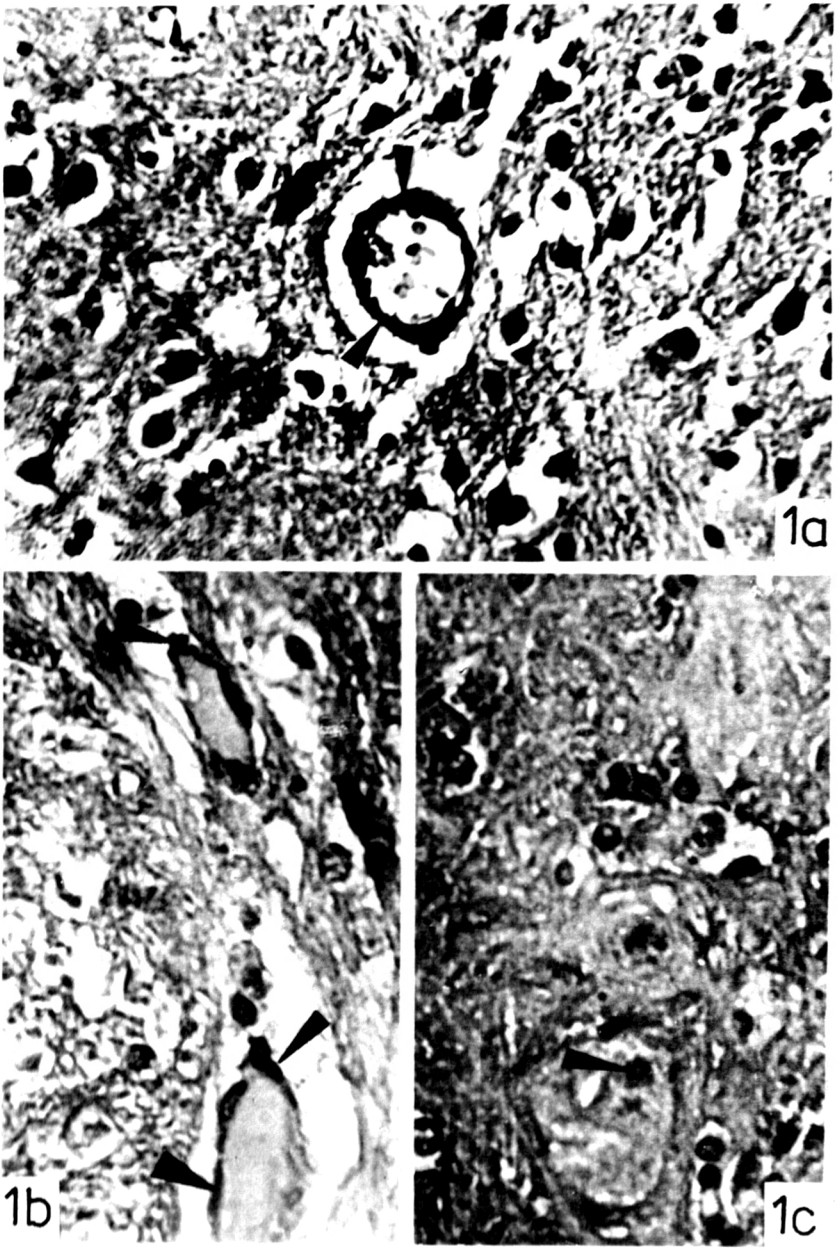
In histological preparations we observed no dystrophic or inflammatory changes in endotheliocytes, pericytes and basement membrane of animals group I neither in the preclinical stage nor in the stage of the first clinical signs. In these stages the degenerative changes were seen in neurons, accompanied with astrogliosis and formation of spongious cavities in white matter (Kolomiets *et al.,* 1988). The hypertrophic endotheliocytes of the capillaries in limited areas of CNS were observed in animals of both groups I and II at the preterminal stage of disease. Only these changes were observed in different portions of brain and spinal cord, more often they were detected in areas of neuronal damage (in arterior horns of spinal cord and in the cortex of cerebrum and cerebellum).

Histochemical staining with Congo red showed that the guinea pigs of groups I and II in preterminal stage of disease had a few congofilic (amyloid) deposits in the walls of certain small and large vessels (Fig 1a). In a part of sections larger amyloid masses were found forming specific deposits below the internal elastic membrane of arterial walls in pia mater. No amyloid plaques were seen directly in the CNS tissue.

Using antibodies to AL PrP 27–30, a little amount of AL protein PrP 27–30 was detected in the nervous tissue of group I animals before the development of the clinical signs of disease. However, in this stage we found no differences in staining of the walls of the vessels as compared to controls. Intensive brown staining of small areas in the walls of certain vessels (Fig. 1b) was noted in the majority of animals with the appearance of the first clinical signs in group I and II (78–90 %) at the preterminal stage of disease. Remarkably, formation of a large antigen-antibody complexes in these stages took place also in the nucleus and perikaryons of some degenerating neurons (Fig. 1c).

Ultrastructure of the CNS vessels did not reveal any changes in the CNS of the group I animals at 14 day. Among microtubules, mitochondrions and other organells pinocytic vesicles (5–6 per section of one cell) were seen in the cytoplasm of endothelial cells. The basal membrane of capillaries consisted of amorphous material with low electron density.

Astroglial sheath of capillaries showed mostly no visible structural alterations. Only in a little part (up to 5–7 %) of astroglial foot processes minor dystrophic changes were revealed, such as englihtment of the matrix, mito-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chondrial hyperthrophy with destruction of the part of membrane and appearance of osmiophilic inclusions. At day 28 p.i. still no structural changes of CNS vessels occurred. The rate of capillars with disturbance of astroglial sheath remained the same or slightly increased (from 8–11 to 10–14 %) but the number of foot processes with dystrophic signs such as almost full disappearing of matrix, disruption of mitochondrions and appearance of myelinlike structures abruptly increased. Distinct astroglial foot processes showed swelling, loss of structural components and gained the appearance of "empty" vacuoles. Despite of destructive changes the gap junctions between astroglial foot processes remained intact. Along with dystrophic changes in a portion of foot processes changes occurred such as accumulation of filamentous structures (diameter of 7–10 nm) arranged at random or, rarely, in parallel. We failed to determine the nature and fine structure of filaments because of their limited number and scantiness of the method of ultrathin sections.

By day 40 p.i., as a rule, in capillars, surrounded by modified dystrophic astrocyte foot processes and occasionally also in capillaries without such visible structural disturbance of their astrocytic sheath, the endothelial changes consisted of increasingly translucent hyaloplasm with destruction of some of cytoplasmic organells, increasing of matrix density of some mitochondria and with accumulation of pinocytotic vesicles (2–3 fold increasing in amount). The vesicles were arranged along the luminal plasmatic membrane grouped in small chains or bands. The increased number of vesicles was mainly observed in the endothelium of capillaries, which were surrounded by dystrophic astrocytic foot processes (destruction of cell organells, fragmentation of plasmolemma with disappearance of gap junctions between the astrocytic foot processes).

At this stage involvement of a few pericytes into pathologic changes deserves attention. Some of the pericytes became swollen, membraneous and osmiophilic inclusions accumulate in their cytoplasm due to destruction of cell organelles. Occasionally the dystrophic changes in the pericytes were more profound up to disintegration of all cell ultrastructure with obvious nucleolysis (Fig. 2). In such capillaries inclusions formed between the endothelium and basement membrane. They consisted of bands (arranged regularly or at random) of amyloidlike filamentous structures (diameter of 5–10 nm) (Fig. 2b).

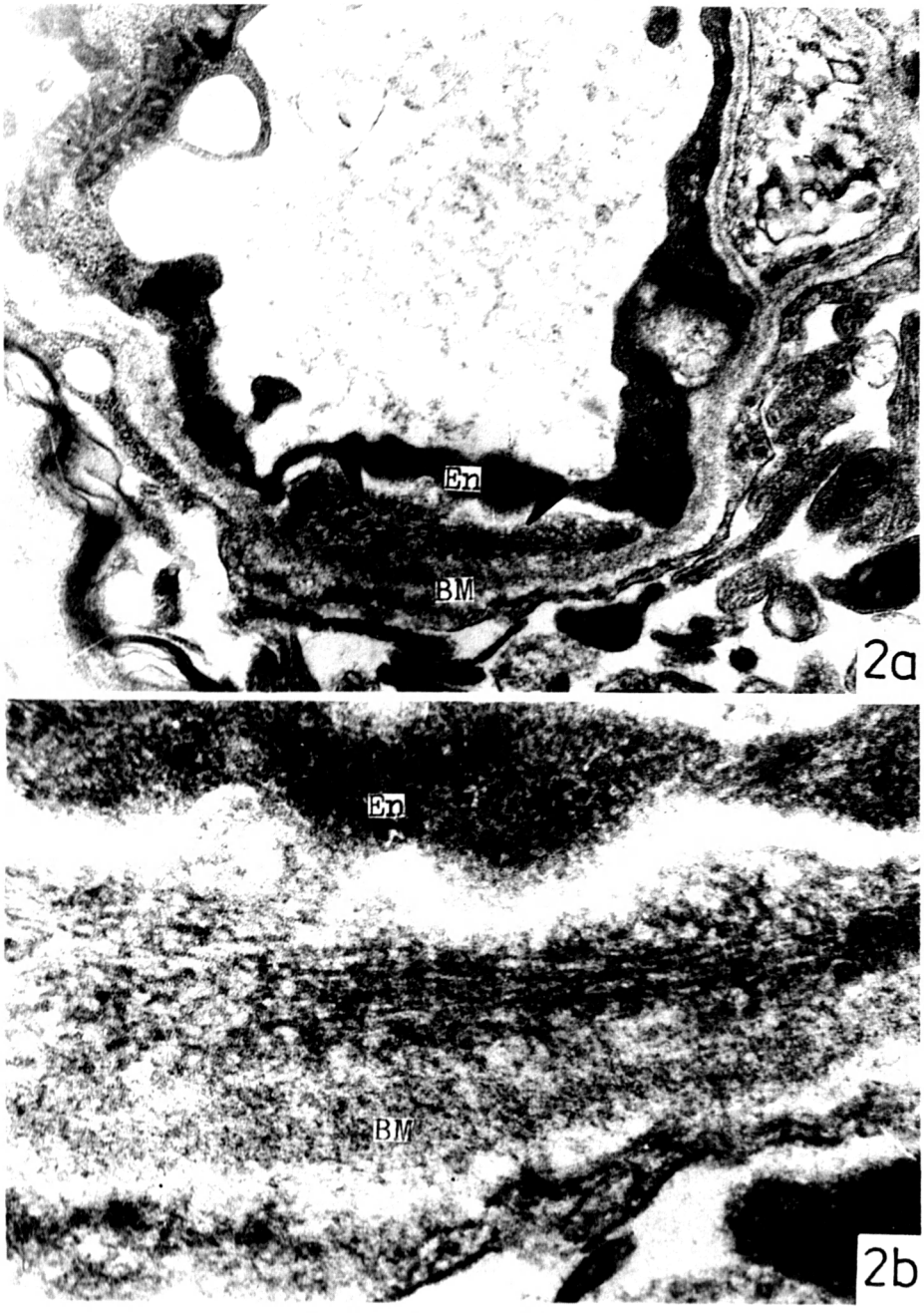
Fig. 1

Changes of guinea pig neural tissue in experimental AL

1a: Brain cortex, amyloid in the vessel wall (arrows), Congo red staining, magn. x 400.

1b: Spinal cord white matter, thoracal segment Th-1. The AL agent antigen in the vessel wall (arrows). Indirect immunoperoxidase method with monoclonal antibody to the 27–30 PrP protein of AL agent. Magn. x 700.

1c: Spinal cord anterior horn, thoracal segment Th-1. Antigen-antibody complexes in the nucleus (short arrow) and in cytoplasm (long arrow) of neurons. Indirect IP method, magn. x 640.



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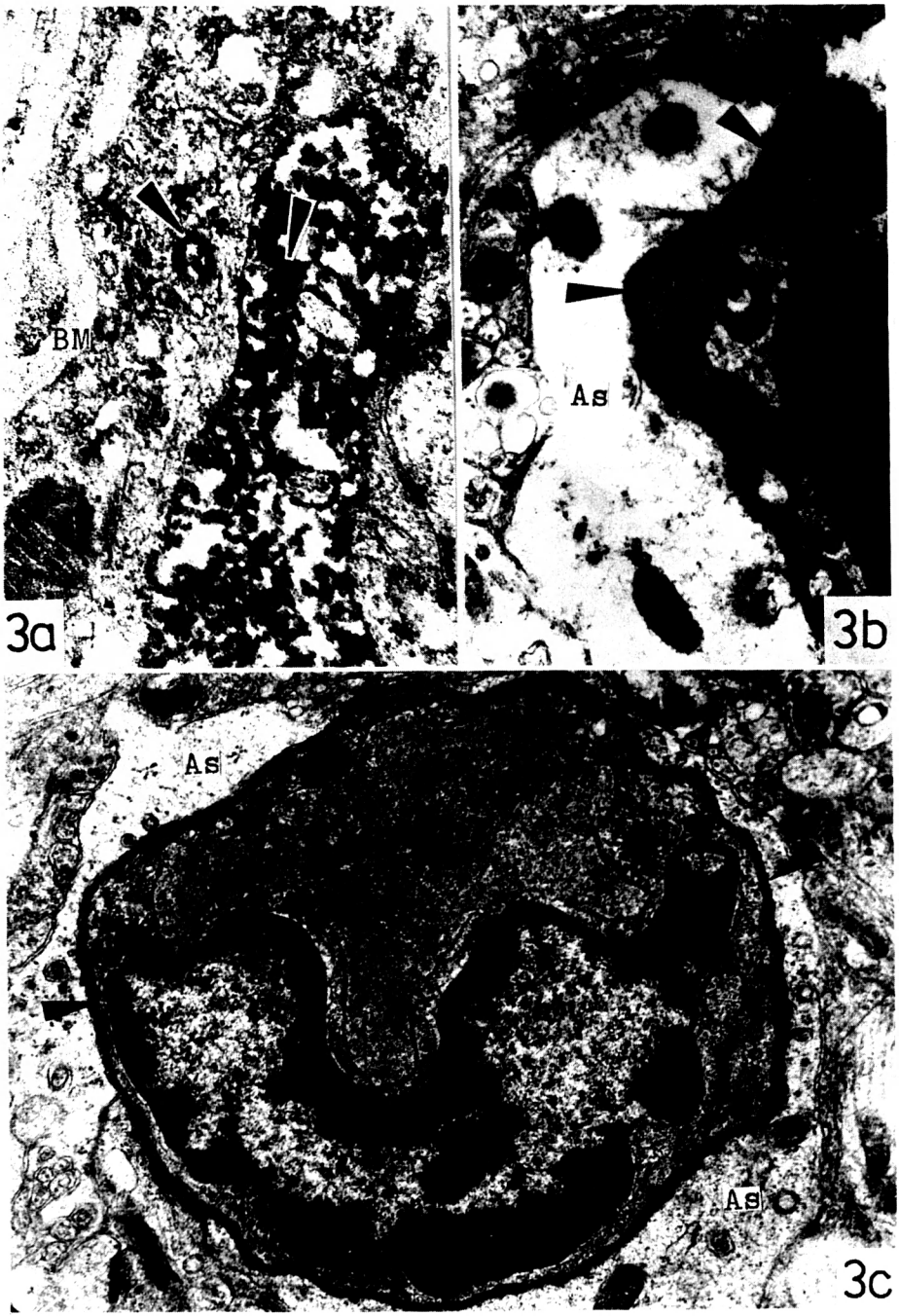

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Despite of deep dystrophic changes in the pericytes we did not observe an opening of tight junctions between endothelial cells. At the same time in some cases separate erythrocytes passed through BBB into neurophil.

Ultrastructural investigation of group II guinea pig brains at preterminal stage of disease (intracerebral inoculation) showed disturbances of astroglial coat, endothelial cells and pericytes, which were similar to that of animals of group I by day 40 after retrobulbar inoculation.

Ultracytochemical analysis of BBB permeability to HRP allowed, only in preterminal stage, to detect essential differences in the distribution of enzyme reaction products as compared to controls. We observed no passage of HRP through BBB in intact animals as well as in the group I and II animals on days 14–28 p.i. Granular products of cytochemical reaction were seen exceptionally in vessel luminas and, rarely, in separate pinocytic vesicles of endothelial cells (Fig. 3a). On the contrary, in group I and II animals with preterminal stages of AL HRP was absent in vessel luminas, in endothelial cells and the basement membrane of some of capillaries showed higher electron density because of impregnation with HRP (Figs. 3-b, 3-c). In addition, some astrocyte processes with dystrophic changes contained diffuse masses of HRP. It is necessary to underline, that HRP passed mainly through the endothelium of capillaries, surrounded by astrocyte foot processes with structure-modification changes as well as of capillaries with dystrophic changes of their pericytes.

Discussion

Many investigators consider the endothelium of cerebral vessels for the morphological basis of BBB, not denying significance of basement membrane, pericytes and pericapillar astrocytes, which coat the surface of the majority of

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Fig. 2

Ultrastructural changes of the CNS capillary in experimental AL of guinea pigs

2a: Spinal cord anterior horn, thoracal segment Th-1. Complete destruction of the pericyte (pe) and inclusion formation (arrows) between endothelial cell and basement membrane. Contrasted with uranil acetate and lead citrate, magn. x 25 200.

2b: A part of ultrathin section of the same capillary at higher magnification. An inclusion body located between the endothelial cell(s) and basement membrane contains filamentous structures with a diameter of 5–10 nm. Magn. x 90 000.

Fig. 3

Ultrastructure of the HRP transport across the BBB in AL

3a: Control animal. HRP in the lumen of a vessel and in a cytoplasmic vesicle (arrow). Basement membrane unstained, magn. x 46 000.

3b: Experimental AL. Transfer of HRP across the basement membrane (arrow); degeneration of astrocyte foot processes (As); magn. x 25 500.

3c: Experimental AL. Transsudation of HRP across the capillary basement membrane. Astrocyte foot processes show no obvious alterations. Magn. x 20 700.

vessels by foot processes (Reese, Karnovsky, 1967; Nemechek, 1978; Bradbury, 1988). Here we determined that reproduction of the AL agent in CNS was accompanied by both the progressive dystrophic changes in neurons and by developing of local disturbances of BBB. As in other prion infections (Wisniewski, 1983; Lossinsky, 1987), BBB became permeable for HRP in result of the damage of barrier function of endothelium, but not because of opening of tight junctions. The latter did not change in all stages. At the same time we revealed two periods in disturbance of glia-capillar complex BBB: a period before appearing of structural changes in endothelium, when changes in astroglial sheath of vessels predominated, and a period of developing in some pericytes of dystrophic changes, which coincided with structural changes in endothelium. At the same time HRP started passing through endothelium and accumulated in basement membrane and also in pericapillary space, that indicated on disturbance of BBB function (Wisniewski, 1983).

In all structural-functional changes described remarkable is the early envolvement of pathologic changes of some astrocytes, which are considered as facultative immunocytes. They are able to present antigen to T-lymphocytes, and to participate in phagocytosis reactions. They also control the structure and barrier function of endothelial cells by producing of some cell factors (Risau, 1987; Kato, Nakamura, 1989). It is possible that astrocytes, being the "target" cells of AL agent, lose their ability to regulate the barrier function of endothelial cells.

We consider most important our first data on pericytes involved in pathologic changes. It is possible that amyloid plaques in the walls of brain vessels are of prion origin. However, we have to consider this data as preliminary because the cell peroxidase activity can nonspecifically increase the extent of positive findings.

Ultrastructural investigation allowed us to determine the localization of amyloid in the vessel's wall. Filamentous masses are located between basement membrane and endothelium. There is no universal opinion about amyloid filament morphogenesis. Formation of filaments can be connected with different cell types: neurons, astrocytes and microglial cells (Hikita *et al.,* 1989; Szumanska *et al.,* 1986; Brown, 1989). Localisation of amyloid filaments mainly in capillaries and their close connection with pericytes witness about possible participation of pericytes in amyloid genesis. On the other hand, on the basis of presented data, one can presume that primary production of specific amyloid component - probable of protein PrP 27-30 kD - takes place in other cell types (neurones or astrocytes) and then it is polymerased and accumulated by means of receptor-mediated phagocytosis in the pericytes. Some of pericytes later degenerate. Pericyte is polypotential mesenchymal cell, which is able to differentiate into different cell elements. Involvement of pericytes in pathologic changes indicates to disturbance of cell regeneration in AL and is in agreement with conception of Sarkisov *et al.* (1990), that blood flow system supports not only blood supply and tissue metabolism, but it is one of central

structures, around which different pathologic events can develop as around a core.

It is known that amyloid extracted from brains of patients with spongiform encephalopathy (scrapie and CJD) consists of characteristic helically twisted protofilaments with a periodicity of 40–110 nm, called scrapie-associated fibrills (SAF) (Merz *et al.*, 1984; DeArmond *et al.*, 1985). In contrary to Alzheimer's amyloid filaments, SAF in cases of AL, CJD and scrapie contain PrP 27–30 kD protein and are infectious (DeArmond *et al.*, 1985; Poleshchuk *et al.*, 1990*b*). Thus, our experiments show that dystrophic changes in CNS with AL are developing both in neurons and in some pericapillar astrocytes and pericytes. At this time, there is correspondence between involvement in pathological changes of some of pericapillar astrocytes, pericytes and accumulation amyloid and also BBB function disturbance. In contrary to CJD and scrapie, experimental AL in guinea pig may be used for modeling of viral cerebral amyloidosis, when deposition of amyloid plaques in brain tissue does not take place.

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