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Vascular and Neuroglial Changes in Experimental Herpes Simplex Encephalitis

Ultrastructural Study *

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Summary. Generalized vascular changes and diffused proliferation of reactive microglia were observed in an experimental model of HSV encephalitis of mice. The wide spread of these changes contrasted with the localized character of virus replication and the confined areas of damaged nervous tissue. The vascular and microglial changes were precocious in animals inoculated with concentrated virus suspension ($10^{5.5}LD_{50}$) while they appeared late in mice inoculated with diluted virus suspension (100 LD_{50}). After inoculation with U.V. inactivated virus no changes were seen.

The results obtained in this study suggest that the vascular and microglial modifications are not related to a direct cytopathic effect of the virus but dependent on the amount of virus present in the central nervous system and linked to the virus DNA.

Key words: Herpes simplex encephalitis — Blood-vessels — Reactive microglia — Electron microscopy.

The ultrastructural features of experimental and spontaneous infection of the nervous tissue by herpes simplex virus (HSV) have been extensively described in recent years. The development and spread of virus *in vivo* and *in vitro* (Morgan *et al.*, 1954; Morgan *et al.*, 1968; Nii *et al.*, 1968; Rabin *et al.*, 1968; Leestma *et al.*, 1969; Kristensson, 1970), the relationship between the host and the virus (Feldman *et al.*, 1968; Schwartz and Elizan, 1973a), the aspects of abortive and chronic infection (Spring *et al.*, 1968; Schwartz and Elizan, 1973b) and the neuronal and glial changes in human and experimental HSV encephalitis (Swanson *et al.*, 1966; Kristensson and Haltia, 1970; Baringer and Griffith, 1970; Norris, 1972; Dubois-Dalck *et al.*, 1972; Yamamoto *et al.*, 1973), all have been studied in detail by electron microscopy. However the ultrastructure of the vessels has been reported only occasionally in HSV infection and particular changes have not been referred to: the changes described corresponded to those found in necrotic zones.

The scarcity of information about the vessels and the changes in the activity of alkaline phosphatases and nucleosidephosphatases of the capillary walls and glial cells reported by Farkas-Bargeton *et al.* (1975) in the experimental HSV of

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mice prompted us to investigate a possible correlation between those changes and an ultrastructural modification of the blood-vessels and glia. Particular vascular changes and diffused proliferation of reactive microglia were observed in this study. Thus, experiments were undertaken with the purpose of clarifying the origin of these modifications.

Material and Methods

Cells and Culture Media. Vero cells obtained from the kidney of a normal adult African green monkey and routinely propagated in the laboratory (Unité 43-I.N.S.E.R.M.), were used. Eagle's medium supplemented with 10% calf serum was utilized.

Virus. The Thealy strain of herpes simplex virus Type I was kindly supplied by Dr. P. Lebon (Department of Virology, Saint-Vincent de Paul Hospital, Paris). The cultures were infected at a multiplicity of 10 plaque forming units (PFU). *In vivo* the virus titers were determined by the 50% end-point calculated by the method of Reed and Muench (1938); the virus stock suspension contained aproximately $10^{5.5}LD_{50}$ per 0.01 ml.

Animals. Three weeks old Balb-C mice, weighing 10 g, obatined from Charles River and bred at the Unité 43-I.N.S.E.R.M., were used in the experiments.

Preparation for Electron Microscopy. The central nervous system of mice was fixed by perfusion via the left cardiac ventricle with phosphate buffered mixture of paraformaldehyde and glutaraldehyde. Samples taken from the fronto-parietal cortex, the hippocampus, pons, spinal cord and olfactory bulb were post-fixed in 2% phosphate buffered osmium tetroxide, stained in block with uranyl acetate, dehydrated and embedded in araldite. Sections 1—2 μ thick were cut in Om-U3 Reichert ultramicrotome and stained with toluidine blue. Thin sections from selected parts of the samples were stained with uranyl acetate and lead nitrate and examined in a Siemens 101 electron microscope.

Experimental Procedure

Group 1. One hundred mice were inoculated in the right cerebral hemisphere with 100 LD_{50} of HSV (0.01 ml of diluted virus stock suspension). Most of the animals became hyperexcitable to sound and light effects within 48 to 72 hrs and all developed a severe neurological syndrome with paralysis of the limbs, seizures and uncounsciousness on the 4th—5th day following the inoculation. Sixty four mice were sacrificed for electron microscopic study at different time intervals: 4 mice at 24 hrs, 8 at 48 hrs, 8 at 72 hrs, 24 at 4 days and 20 at 5 days after inoculation. From the animals which were inoculated but not processed for electron microscopy, seven died on the 4th day, 10 on the 5th day, 17 on the 6th day and 2 were still alive 1 month later.

Five control mice were intracerebrally inoculated with 0.01 ml of non-infected extract of Vero cell cultures. These animals seemed in good health when sacrificed 5 days after inoculation.

Group 2. Forty mice were inoculated in the right cerebral hemisphere with $10^{5.5}LD_{50}$ of HSV (0.01 ml of undiluted virus stock suspension). The first symptoms of disease were noted 18 hrs after inoculation and all the mice were died within 3 days. Groups of 4 animals were sacrificed at 6, 8, 10, 24, and 72 hrs following the inoculation and processed for electron microscopy.

Group 3. Forty mice were intracerebrally inoculated with $10^{5.5}LD_{50}$ of HSV irradiated by ultraviolet light (U.V.)—total of U.V. irradiation = 18000 ergs. Groups of 4 mice were sacrificed at 6 hrs, 8 hrs, 12 hrs, 24 hrs, 72 hrs, 5 days and 1 month. The surviving animals did not exhibit any disturbance within 2 months.

The assay of the antigenic effect of HSV after U.V. irradiation was determined by two methods: 1) Direct titration of neutralizing antibodies in the sera of mice intraperitoneally inoculated with U.V. irradiated virus; 2) Intracerebral injection of 100 LD_{50} of HSV 1 month after the intraperitoneal injection of U.V. irradiated virus; these animals were compared to a control group intracerebrally inoculated with the same dose of HSV but not previously inoculated with U.V. inactivated virus.

Results

Group 1. Mice inoculated with 100 LD_{50} HSV (0.01 ml of diluted virus stock suspension).

The central nervous system of mice sacrificed 24 hrs after inoculation did not reveal any ultrastructural alteration.

On the second day following the inoculation the nervous tissue was generally well preserved though some neurons and astrocytes in the spinal cord, pons and olfactory bulb showed nuclear changes. These consisted of an excessive nuclear folding and/or coarsening, clumping and margination of nuclear chromatin.

In mice examined on the 3rd day after inoculation changes of the blood capillaries could be recognized in the different regions selected for study. They consisted of an enlargement of the endothelial cells and an increase in the number of free ribosomes which almost completely filled the cytoplasmic matrix (Fig. 1). A slight swelling of the astrocytic processes of the neuropil could also be seen in most of these regions but not in the fronto-parietal cortex and only rarely in the hippocampus.

Nevertheless it was only on the 4—5th day following the inoculation that the ultrastructural changes of the central nervous system became really prominent. At this time striking modifications of the blood vessels were observed in all the areas studied. The endothelium of most of the capillaries was enlarged (Fig. 2a) and endothelial digitations projecting into the lumen were often seen in capillaries (Fig. 2b) and venules. Several arterioles also exhibited severe changes of the endothelium consisting of complete folding of the inner plasma membrane and extremely

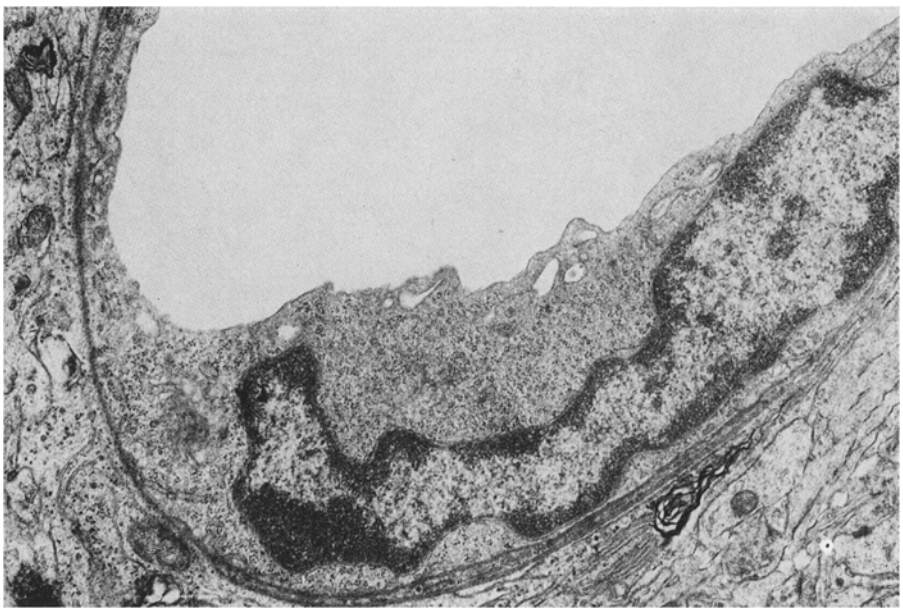

Fig. 1. Blood capillary in the cerebral cortex (3 days after inoculation with diluted HSV suspension). The cytoplasm of the endothelial cell is filled almost completely with numerous free ribosomes. ×14000

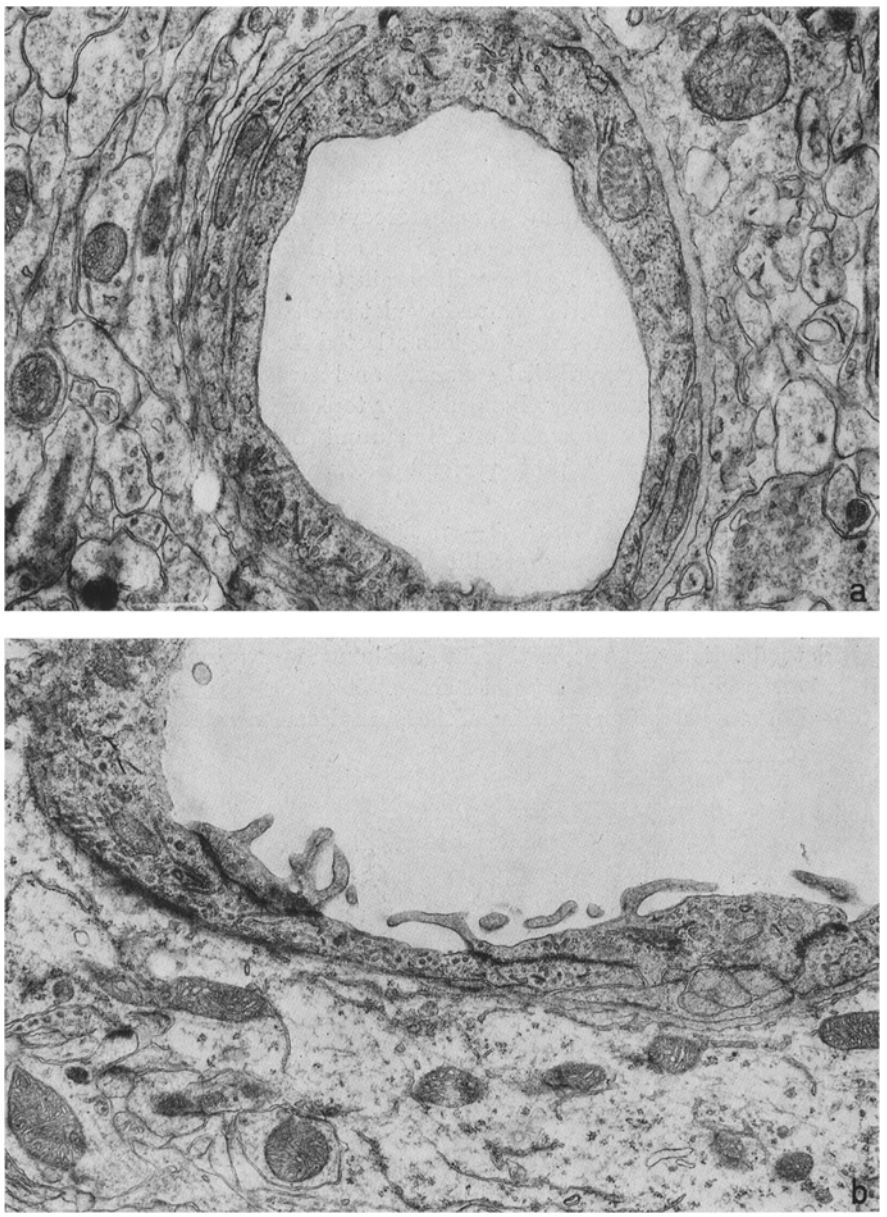

Fig. 2. (a) Blood capillary in the cerebral cortex (4 days after inoculation with diluted HSV suspension). Striking enlargement of the endothelium. ×20000. (b) Blood capillary in the hippocampus (4 days after inoculation with diluted HSV suspension). Numerous flaps projecting into the lumen of the vessel. ×15000

Fig. 3. Arteriole in the hippocampus (5 days after inoculation with diluted HSV suspension). The endothelium is deeply folded all along its inner contour. Note the wavy outline of the basement membrane. × 11000

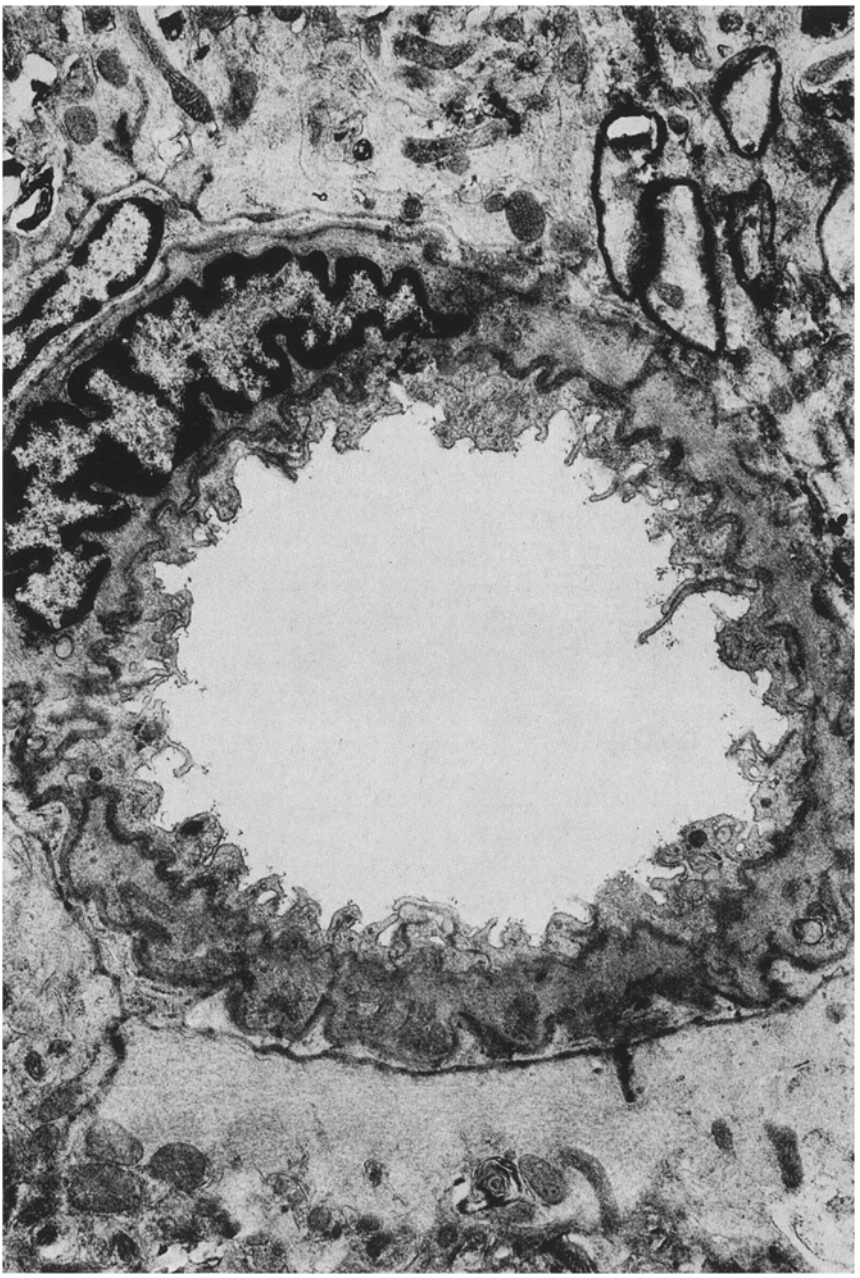

Fig. 3

numerous flaps giving to the vessel an embryonic appearance (Fig. 3). The endothelial projections contained frequently numerous pinocytotic vesicles and a fibrillar osmiophilic material running perpendicularly to the basement membrane

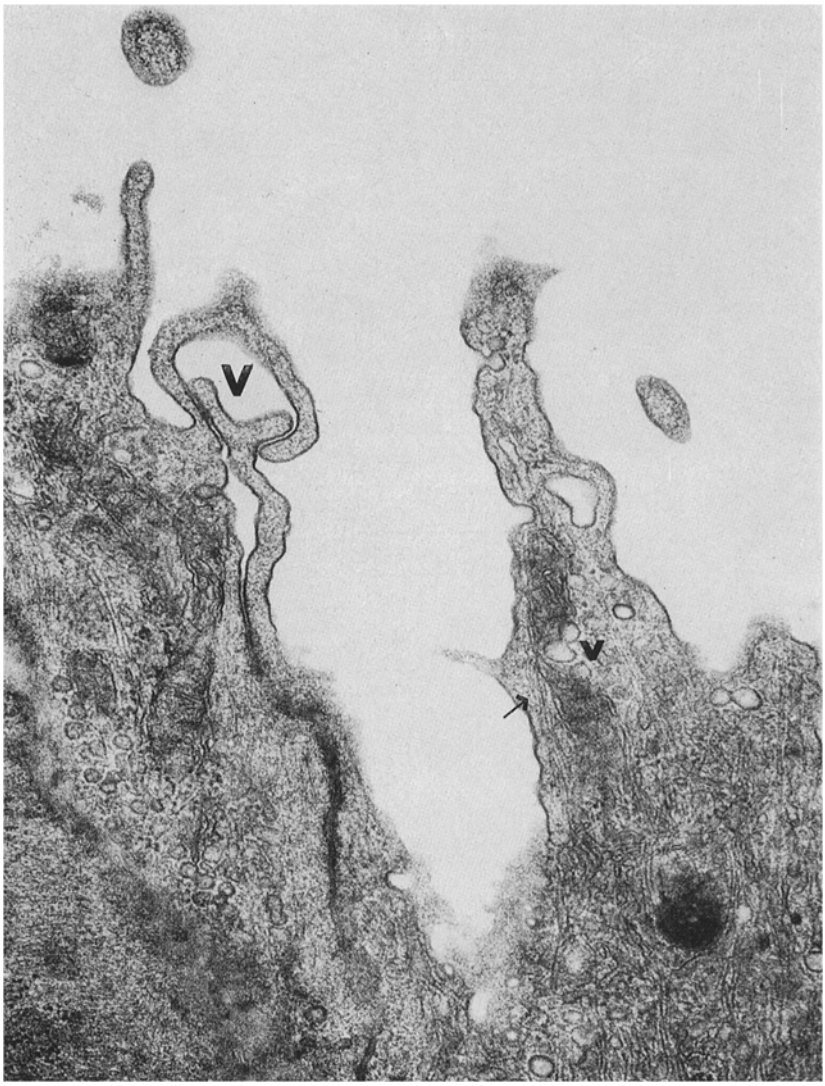

Fig. 4. Digitations of the endothelium into the lumen of an arteriole in the cerebral cortex (4 days after inoculation with a diluted HSV suspension). A false vacuole (*V*) is formed by the imbrication of two endothelial digitations. The endothelial projection on the right contains a fibrillar material (arrow) and some large pinocytotic vesicles (*v*). × 40000

(Fig. 4); sometimes the imbrication of neighbouring flaps formed voluminous false pinocytotic vesicles (Fig. 4). Occasionally the basement membrane of the vessels showed a waving contour or exhibited some extensions which spread into the surrounding nervous parenchyma.

Another prominent feature detected in all the areas selected for this study was the occurrence of a great number of reactive microglial cells (Fig. 5) representing

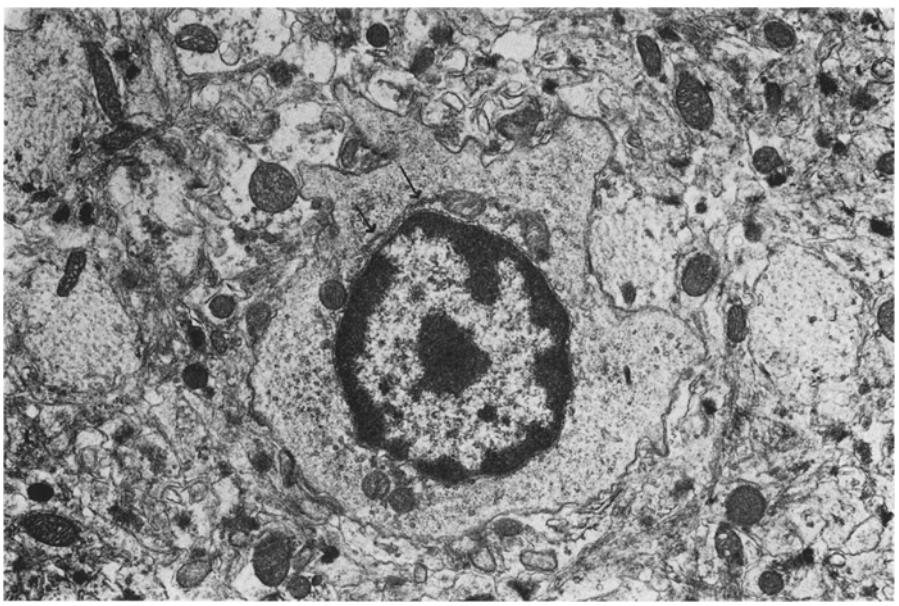

Fig. 5. Microglial cell in the cerebral cortex (4 days after inoculation with a diluted HSV suspension). The chromatin is disposed in clumps broadening the inner nuclear membrane; a large mass of chromatin lies in the middle of the nucleus. A single stringy cisternae of granular endoplasmic reticulum (arrows) overlies the top of the nucleus. The cell membrane has an indented contour. ×11000

about 10% of the cell population observed in our material. These cells, which spread throughout the nervous parenchyma, showed a very sinuous outline. They had a clumped nuclear chromatin broadening the inner nuclear membrane. The granular endoplasmic reticulum consisted of a single or few long and stringy cisternae disposed around the nucleus. Lipid bodies were rarely observed within the cytoplasm.

The generalized distribution of vessels showing the above mentioned changes and the widespread occurrence of reactive microglial cells were in sharp contrast with the localized character of changes undergone by the neurons, astrocytes and oligodendrocytes. In fact, while severe and extensive lesions of the nervous tissue were seen in the spinal cord, the pontine basis and olfactory bulb, a well preserved ultrastructure of the nervous parenchyma was observed in the fronto-parietal cortex and hippocampus, excepted in a very restrict zone of the pyramidal band.

In the severely affected areas a large number of neurons and oligodendrocytes were infected by the HSV showing numerous virus particles within the nucleus. Astrocytes were also infected but virus were not detected in the vessels neither in the endothelial cells nor in the pericytes. In these areas, zones of necrosis with dilated extracellular space, degenerating neurons, swollen astrocytes and blood vessels exhibiting pale and swollen pericytes, were frequently observed. In the neighbourhood of these necrotic zones monocytes included in/or adjacent to the vessel walls were frequent.

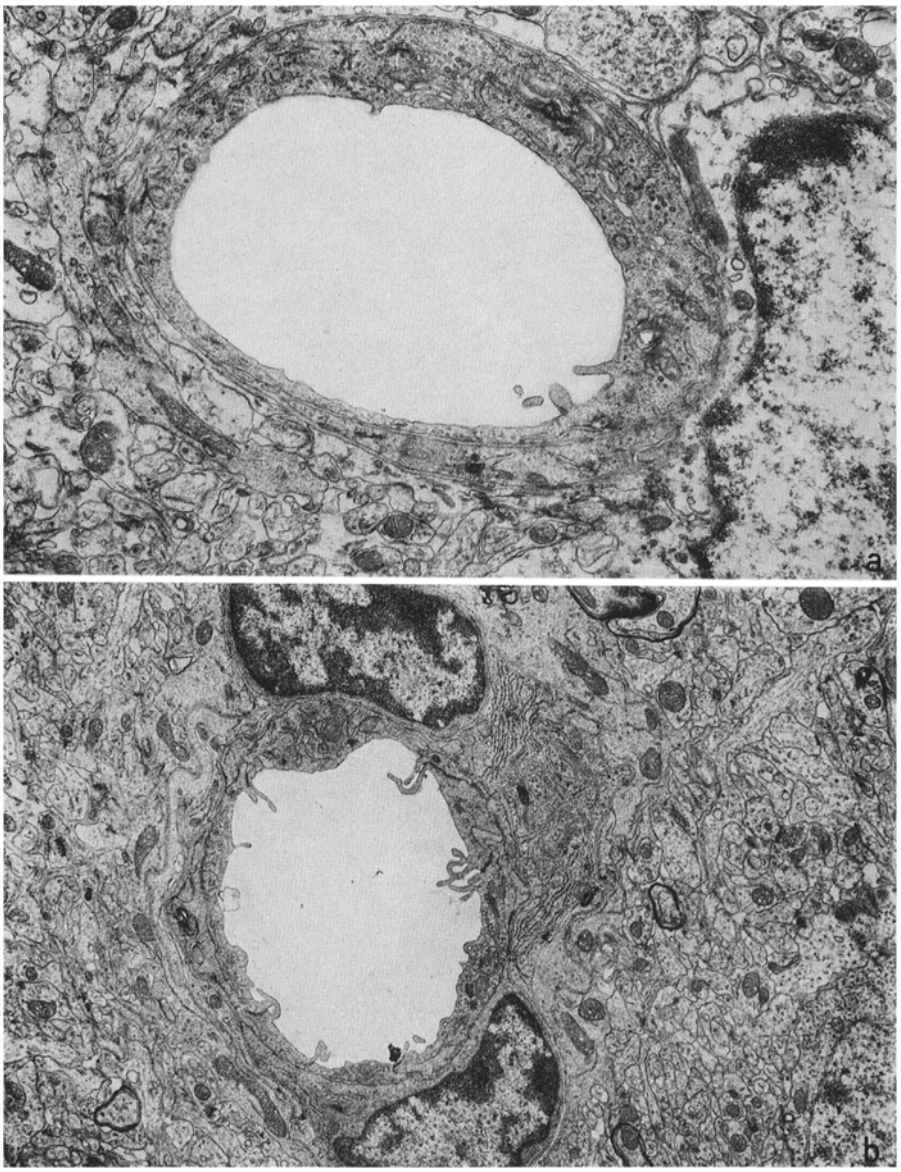

Fig. 6. (a) Blood capillary in the hippocampus (8 hrs after inoculation with undiluted HSV suspension). The cytoplasm of the endothelial cell is enlarged and contains numerous organites. ×11500. (b) Venule in the hippocampus (8 hrs after inoculation with undiluted HSV suspension). Numerous digitations of the endothelial cells project into the lumen of the vessel. The outline of the pericytes is very sinuous. The neuropil surrounding the vessel did not show any change. ×10000

Virus particles and infiltration of the vessel walls by hematogenous cells were not observed neither in the fronto-parietal cortex nor in the great part of the hippocampus.

The ultrastructural study of the central nervous system of the five control mice did not show proliferation of microglial cells and the blood vessels did not exhibit any alteration. Occasionally the arterioles showed prominences of the endothelium into the lumen but they never reached neither the extent nor the number of the endothelial projections observed in mice inoculated with HSV.

Group 2. Mice inoculated with $10^{5.5}LD_{50}$ HSV (0.01 ml of undiluted virus stock suspension).

Six hrs after inoculation the central nervous system did not show any change and at 8 hrs the nervous tissue appeared yet generally well preserved: only a slight swelling of some glio-vascular end-feet was present in the spinal cord, olfactory bulb and the hippocampus. Virions were not seen in any of the regions studied at 8 hrs after inoculation. Nevertheless striking changes of the vessels were already observed in this group of mice at 8 hrs after inoculation. These changes were similar to those seen 4—5 days after inoculation with the diluted virus suspension. They consisted of: 1) enlargement of the capillary endothelium (Fig. 6a); 2) presence of numerous flaps projecting into the lumen of the capillaries and venules (Fig. 6b); 3) prominent folding of the inner plasma membrane of the arterioles. Reactive microglia was also present at 8 hrs and monocytes included in the vessel walls were occasionally seen.

After 10 hrs virus particles within the nucleus and cell necrosis were detected in the same regions as they were observed in the mice of group 1. The number of vessels exhibiting particular changes as well as the reactive microglia were strikingly increased. Numerous monocytes included in the vessel walls were observed around the necrotic zones.

Group 3. Mice inoculated with $10^{5.5}LD_{50}$ HSV irradiated by U.V. light.

The ultrastructural study of samples taken at different time intervals after the inoculation did not show changes neither in the nervous tissue nor in the vessels. Reactive microglia was not observed in this group.

The direct assay of anti-sera indices in mice inoculated with U.V. irradiated virus demonstrated the presence of neutralizing antibodies in the serum.

75% of mice intracerebrally injected with 100 LD_{50} HSV 1 month after the intraperitoneal injection of U.V. irradiated virus were alive 3 weeks after the intracerebral inoculation; on the contrary all of the animals of the control group in this experiment died within 6 days.

Discussion

This study demonstrated particular vascular changes and proliferation of reactive microglia during experimental herpes simplex encephalitis. The wide diffusion of these vascular and microglial modifications was in contrast with the limited spread of cytopathic effect of the virus. The areas in which HSV replication was detected were similar to those described by Yamamoto *et al.* (1968) during the herpetic infection in 3 weeks old mice. The precocity of the diffused vascular and microglial changes was dependent on the amount of virus inoculated in the brain. These changes were absent when U.V. irradiated viral sead was employed.

The great number of reactive microglial cells seen in our material may be related to the increased inosine-diphosphatase activity in the glial cells referred to by Farkas-Bargeton *et al.* (1975) in a similar model of HSV encephalitis. The origin of this reactive microglia which was present both in the damaged and preserved areas was impossible to determinate in the present study. However, the frequence of mononuclear cells included in the wall of the vessels or lying close to them in the altered zones suggests an hematogenous origin of reactive microglia in these regions (Adrian and Walker, 1962; Konigsmark and Sidman, 1963; Blackmore, 1969; and Kitamura *et al.*, 1972). In the preserved areas, in which passage of monocytes throughout the vessels was not detected, an indigenous origin of the reactive microglia appears as more probable (Vaughn and Peters, 1968; Vaughn, 1969; Privat and Leblond, 1972; and Davidoff, 1973).

The particular vascular changes observed in this study may be correlated with the diffused modifications of alkaline phosphatase activity in the vessel walls described by Farkas-Bargeton *et al.* (1975). Regarding the origin of these changes, which were quite different from the degenerating vascular alterations exhibited by the blood vessels in the severely damaged zones, several hypothesis may be considered.

A cytopathic effect of the virus appears unlikely since in the experiments with undiluted HSV suspension these changes were already present 8 hrs after inoculation, that is before the HSV replication had occurred. Moreover we never observed virus particles within the nucleus of the endothelial cells and both Yamamoto *et al.* (1968) and Kristensson and Sourander (1969) based in their immunofluorescent studies emphasized the absence of viral antigens in cerebral vessels of 3 weeks old infected mice. Likewise an effect of cytolitic products of the degenerated neuronal and glial cells is highly improbable since the particular modifications of the blood vessels were present in well preserved regions. Furthermore these alterations were already seen 8 hrs after inoculation in the animals of group 2 when the nervous parenchyma did not show any severe alteration.

The vascular changes are probably not due to neutralizing antibodies induced by the virus since similar changes were not observed with U.V. irradiated HSV suspension. In fact, irradiation with the U.V. light damages the DNA of the virus, preventing therefore its replication, but it does not interfere with its possibility to induce neutralizing antibodies as has been demonstrated.

Finally from the present study two conclusions can be drawn: 1) The late appearance of particular vascular changes and proliferation of reactive microglia in mice inoculated with a low amount of virus and their early presence when the animals were inoculated with a HSV suspension of high titre, indicate that those changes were dependent of the amount of virus present in the nervous parenchyma. 2) The absence of these modifications in mice inoculated with U.V. inactivated virus suggests that they may be atributed to the synthesis of antigens governed by the viral genome. These antigens will be probably non-structural antigens early produced during the cycle of virus replication.

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