Virchows Arch [Pathol Anat] (1986) 408:347–359

Virchows Archiv A

The chronology of lesion repair in the developing rat brain: Biological significance of the pre-existing extracellular space

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Summary. We observed the histological peculiarities of the repair process in a destructive lesion of the developing rat brain during neurogenesis.

Degeneration was induced selectively in certain cells of the proliferating phase in the rat fetal neopallium on embryonic day 16 by transplacental administration of ethylnitrosourea. Successive elimination of necrotic cells and the restoration process were observed.

The repair process was divided into the following steps.

1) Elimination of individually affected cells by phagocytes in the pre-existing extracellular space.

2) Successive restoration of the disintegrated area by cells which differentiated from remaining matrix cells.

No reactive gliosis, fibrosis, abnormal vascularization or infiltration of granulocytes and lymphocytes was observed at any time. The thinned neopallium on postnatal day 21 revealed only a small number and abnormal distribution of the cortical neurons.

It may be assumed that the fetal brain owes its unique repair features to the presence of a vast extracellular space under normal conditions. In this pre-existing extracellular space, every kind of cell seems to exist separately without the intercellular adhesions characteristic of the adult brain. When degeneration occurs in certain cells the phagocytes would be able to eliminate the degenerate cells completely in this space without having to break intercellular adhesions. As a result, after the completion of cell elimination, the injured brain is restored to its original state with no cell reaction, giving the appearance of a small brain with normal-looking histological architecture, save only for the sparseness of cells.

Key words: Developing brain – Extracellular space – Cytotoxicity of ethylnitrosourea – Matrix cell necrosis

* This study was supported by a Grant-in-Aid for Scientific Research (A) 57440050, 60015024 and 60440046 from the Ministry of Education, Science and Culture, Japan

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Introduction

It has been said, in general terms, that the immature central nervous system shows no reactive gliosis or mesenchymal proliferation of the type which is well established in adults in response to destructive lesions (Friede 1975; Larroche 1984). Several factors may contribute to the peculiarities of the response of immature normal nervous tissue to injury; its high water content and loose texture, its different metabolism from that of the adult and the different period of the development of responding cells (Friede 1975). However, there has been no report of the fundamental histological characteristics of the fetal brain being different from the adult brain in terms of the tissue response to injury.

We have reported the possibility of producing constant lesions in the developing fetal brain at a certain period by using ethylnitrosourea (ENU) (Yoshida et al. 1984), one of whose main effects is to block DNA synthesis (Kleihues 1969). In this study, ENU was administered to pregnant rats on gestational day 16. This time involves neurogenesis, at the cytogenetic stage II (Fujita 1966) in the neopallium of rat fetuses (Berry et al. 1964). It is a very significant period since the matrix cells at this time exclusively produce neuroblasts migrating to the cortical plate (Fujita 1966). At this period ENU administration induces selective degeneration of proliferating cells in S phase (Bosh 1977) or S to M phase in the mitotic cycle of the developing brain (Yoshida et al. 1984) and the degree of tissue damage is proportional to the dose of administered ENU (Fujiwara 1980; Yoshida et al. 1984). With the purpose of observing the mechanism of elimination of affected cells and the restoring process within the brain tissue, a single intravenous injection of ENU 60 mg/kg body weight was administered, in order to keep the external limiting membrane over the marginal zone and the junctional complex of matrix cells intact.

We demonstrate here the histopathological appearance of lesion repair in the developing brain at the stage of neurogenesis. The biological basis for the lack of glial or mesenchymal reaction is discussed.

Materials and methods

Animals. Four-month-old Wistar albino rats, weighing 250 g to 300 g, were used. Virgin females were mated with males overnight, and vaginal smears were examined at 10 a.m. every day. The day on which sperm appeared in the smears was taken as the embryonic day 1 (E1).

Each of the 23 pregnant rats was given a single injection of ENU, 60 mg/kg body weight, freshly dissolved in physiological saline, in the tail vein at 2 p.m. on E16. The time of completion of injection was designated as 0 h. From E17 fetuses were resected sequentially by Caesarean section from the dams anesthetized by ether, 1 per hour for 24 hours and 1 per 24 h thereafter. The day of birth was designated as postnatal day 1 (P1). Postnatal examination was performed on P1, 2, 3, 7, 14 and 21. A pair of fetuses or newborns from two different dams were sacrified at the same time for the purpose of ruling out individual variability in the development or the effectiveness of the drug. The fetuses and newborns of 18 other rats served as age-matched controls.

Histology. Observations were performed of the neopallium of each animal. The animals were sacrificed by perfusion through the heart or aorta with 0.1 M phosphate buffer (pH 7.3) con-

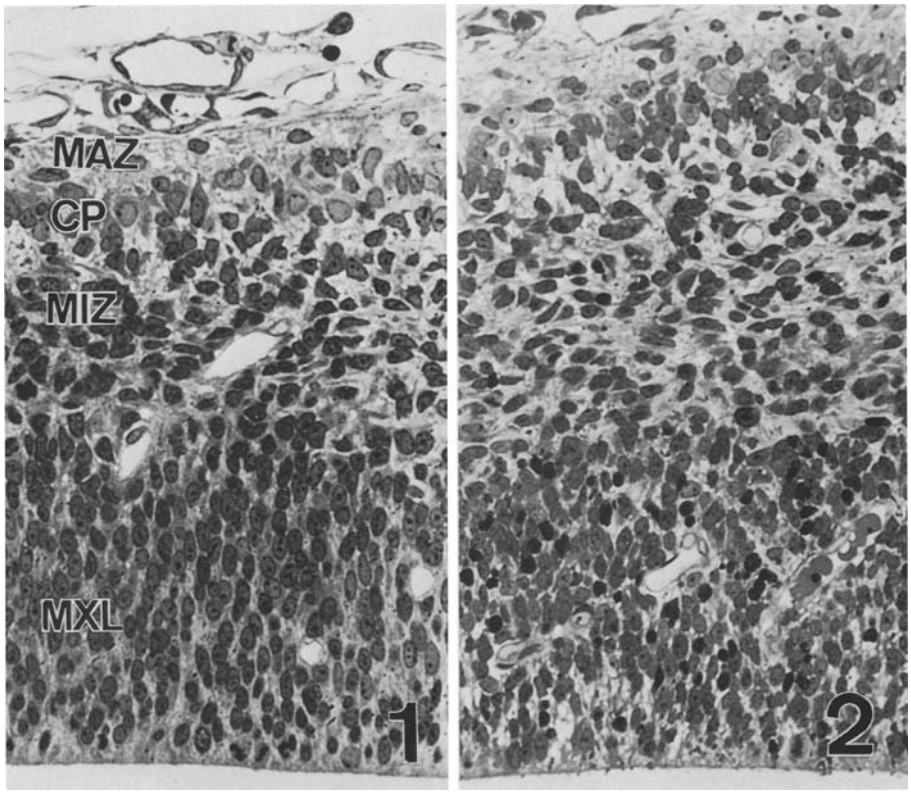

Fig. 1. The neopallium of a normal E16 rat fetus showing many mitotic figures at the paraventricular surface. *MXL:* matrix cell layer. *MIZ:* migrating zone. *CP:* cortical plate. *MAZ:* marginal zone. Epon embedded semithin section stained with toluidine blue and saffranin. × 300

Fig. 2. The neopallium four hours after ENU treatment. Scattered degenerative cells with pyknotic nuclei and darkly stained cytoplasm exclusively in the deeper zone of the matrix cell layer. Toluidine blue and saffranin preparation. × 330

taining 1% heparin followed by 1% glutaraldehyde – 1% paraformaldehyde in the 0.1 M phosphate buffer, pH 7.3. Brain tissue was removed and immersed into 1% glutaraldehyde – 3% paraformaldehyde in 0.1 M phosphate buffer, then transferred into 1% osmium tetroxide. Blocks of tissue were dehydrated in graded ethanol and embedded in epon 812 after changes of propylene oxide. Semithin (1 μm) and ultrathin sections were prepared for light and electron microscopic examinations. Semithin sections were stained with toluidine blue and saffranin. Ultrathin sections were contrasted with uranylacetate and lead citrate and viewed in an electron microscope, HITACHI HU-11B or HS-7D.

In addition, some other fetuses and newborns were sacrified and fixed in 10% formalin. Tissue blocks were embedded in paraffin and sectioned at 4 μm. The sections were stained with H & E, Klüver-Barrera, PTAH or PAS, and examined with a light microscope.

Terminology. During the last two decades, the terminology of the developing neopallium was revised several times (Fujita 1962; Boulder committee 1970; Sidman and Rakic 1973).

In this paper, the embryonic and newborn neopallium is divided into four basic zones which are stratified parallel to the ventricular surface.

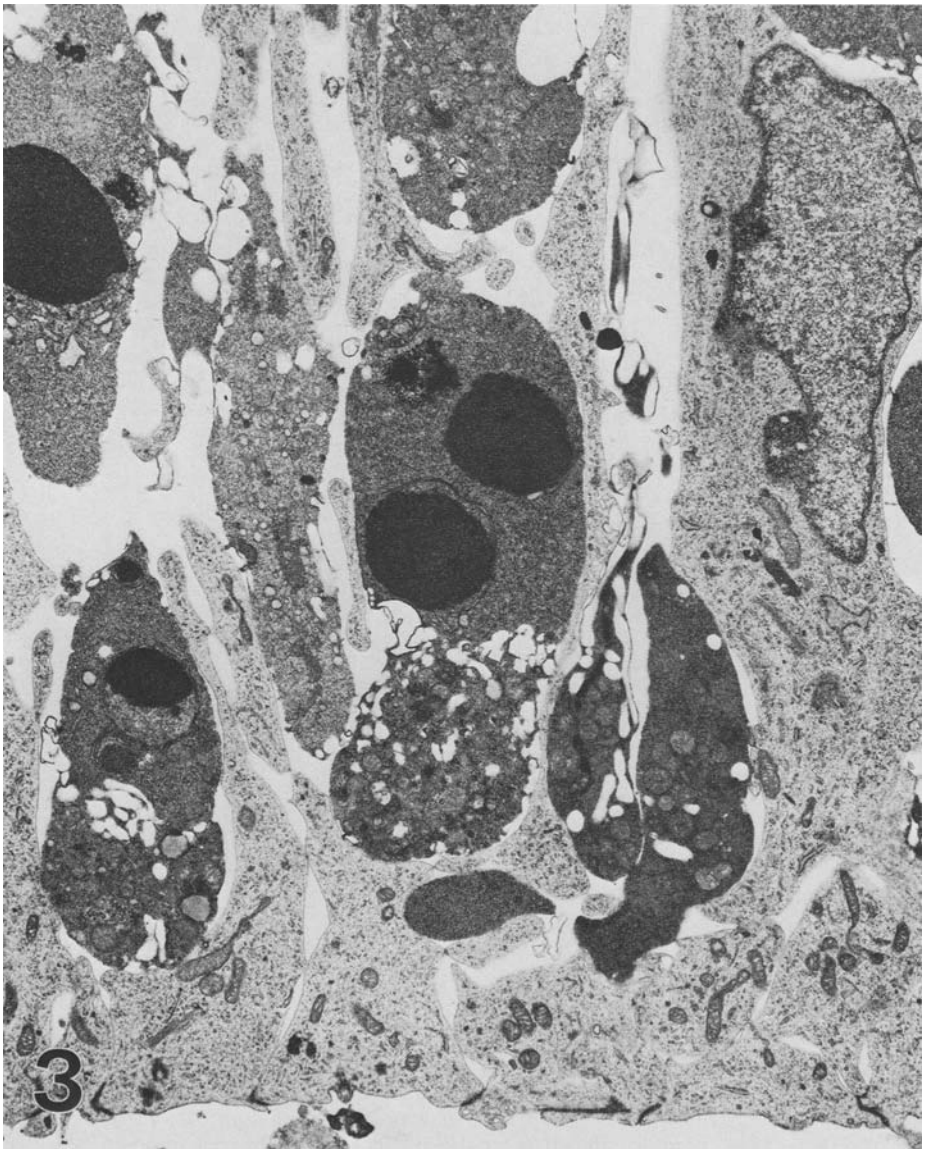

Fig. 3. Electron micrograph of the matrix cell layer six hours after the treatment. The degeneration is noted in the individual cells separately migrating in the wide extracellular space. The cytoplasm of these degenerative cells contains abundant free ribosomes and many vacuoles, and the pyknotic nuclei show condensed chromatin masses. Well preserved zonulae adherentes. ×7,600

The matrix cell layer (Fujita 1962) is a proliferating zone lining the ventricles. *The migrating zone* is composed of migrating cells and their processes. *The cortical plate* (Boulder committee 1970) is a mass of neuroblasts differentiating into neurons. *The marginal zone* (Boulder committee 1970) is a cell sparse zone consisting of numerous cell processes.

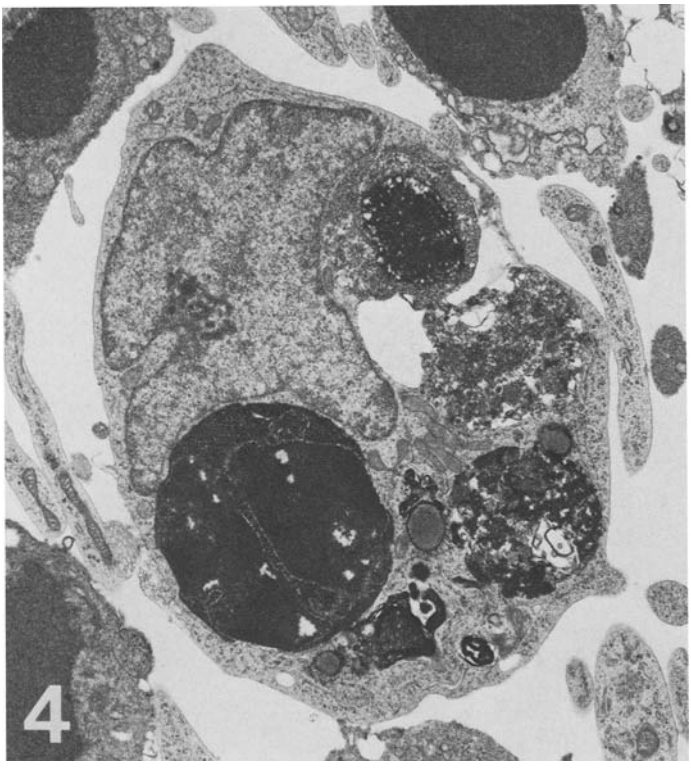

Fig. 4. Electron micrograph of a phagocyte in the matrix cell layer 6 h after the treatment. The cytoplasm contains necrotic cell debris, phagosome, mitochondria, rough endoplasmic reticulum and occasional Golgi apparatus and filamentous component. × 7,500

Results

In the neopallium of normal E16 rat fetus, the matrix cell layer revealed rows of matrix cell nuclei arranged perpendicularly to the paraventricular surface and many mitotic figures were observed at the paraventricular surface. The migrating zone was composed of several layers of cells and the cortical plate, of two or three layers. The thin marginal zone was recognized in the outermost portion (Fig. 1).

The brains of six control fetuses serially resected hourly from one dam showed no significant degenerative changes, suggesting that the artifact in this procedure is negligible.

Until 3 h after ENU treatment, no degenerative cells were observed.

Four hours later, scattered degenerative cells with pyknotic nuclei and darkly stained cytoplasm were found exclusively in the deeper zone of the matrix cell layer on the toluidine blue and saffranin preparation (Fig. 2). Electron microscopically, the degeneration was noted in the individual cells migrating separately in the wide extracellular space. The cytoplasm of these degenerative cells contained abundant free ribosomes and many vacuoles

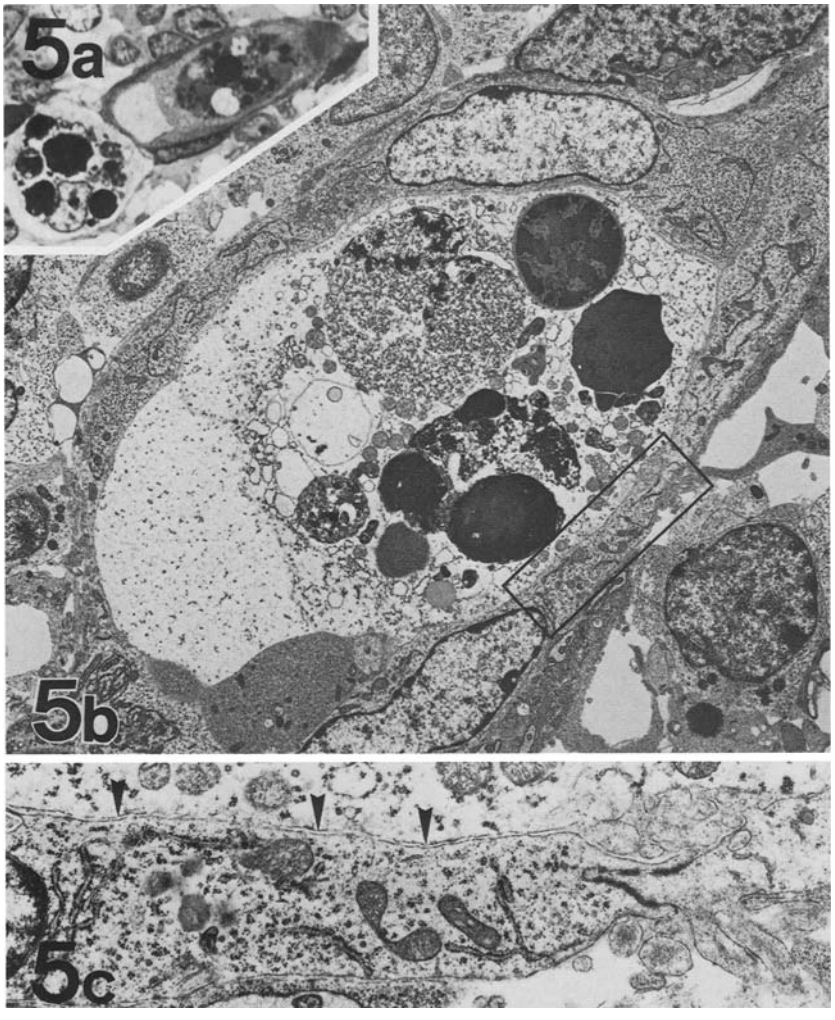

Fig. 5a. Phagocytes in a blood vessel and adjacent to the vessel in the matrix cell layer. Toluidine blue and saffranin preparation. × 830
b Electron micrograph of a phagocyte in the vascular lumen containing much necrotic cell debris. Section of adjacent to **a**. × 2,800
c The plasma membrane (▼) of the phagocyte. Higher magnification of the framed area in **b**. × 10,000

of various sizes, and the pyknotic nuclei showed condensed chromatin masses (Fig. 3).

The number of the degenerative cells in the matrix cell layer seemed to reach its maximum 8 h after the treatment, after which the number of the degenerative cells decreased in the matrix cell layer but increased in the migrating zone. Starting at 10 h after the treatment the degenerative cells showed karyorrhexis or vacuolation of the cytoplasm. The migrating

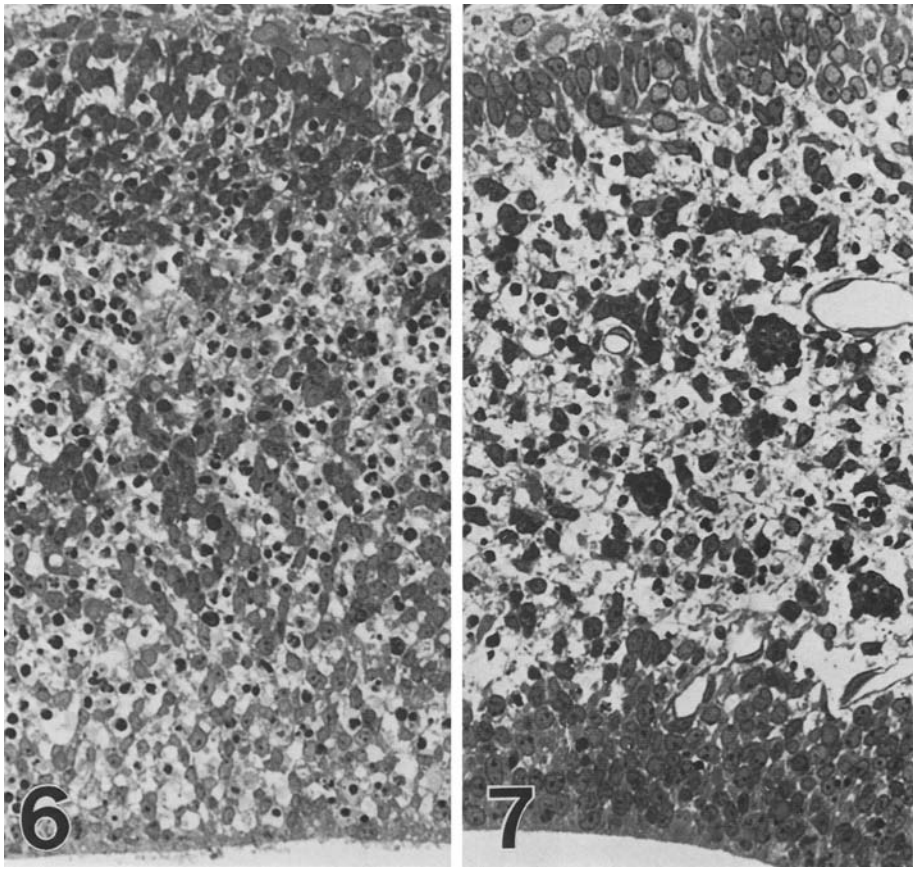

Fig. 6. Fifteen hours after the treatment. Disintegration of the migrating zone and the matrix cell layer. Toluidine blue and saffranin preparation. × 320

Fig. 7. Twenty-four hours later. Huge phagocytes containing many degenerative cells in the disintegrated migrating zone. No remarkable infiltration of granulocytes or lymphocytes. No degenerative cells in the cortical plate formed before the treatment. Toluidine blue and saffranin preparation. × 320

zone appeared to be disintegrated after fifteen (Fig. 6) to twenty-four hours (Fig. 7).

Following the appearance of degenerative cells, 5 h after the treatment, cells with light nuclei and prominent nucleoli, probably phagocytizing the degenerative cells, were recognized in the matrix cell layer. Electron microscopically, fine chromatin granules were observed at the periphery of the nucleus. The cytoplasm contained many degenerative cell fragments and phagosomes. Mitochondria, rough endoplasmic reticulum, vacuoles, occasional Golgi apparatus and filamentous components were also recognized. Wide extracellular space surrounding those phagocytes was also observed (Fig. 4).

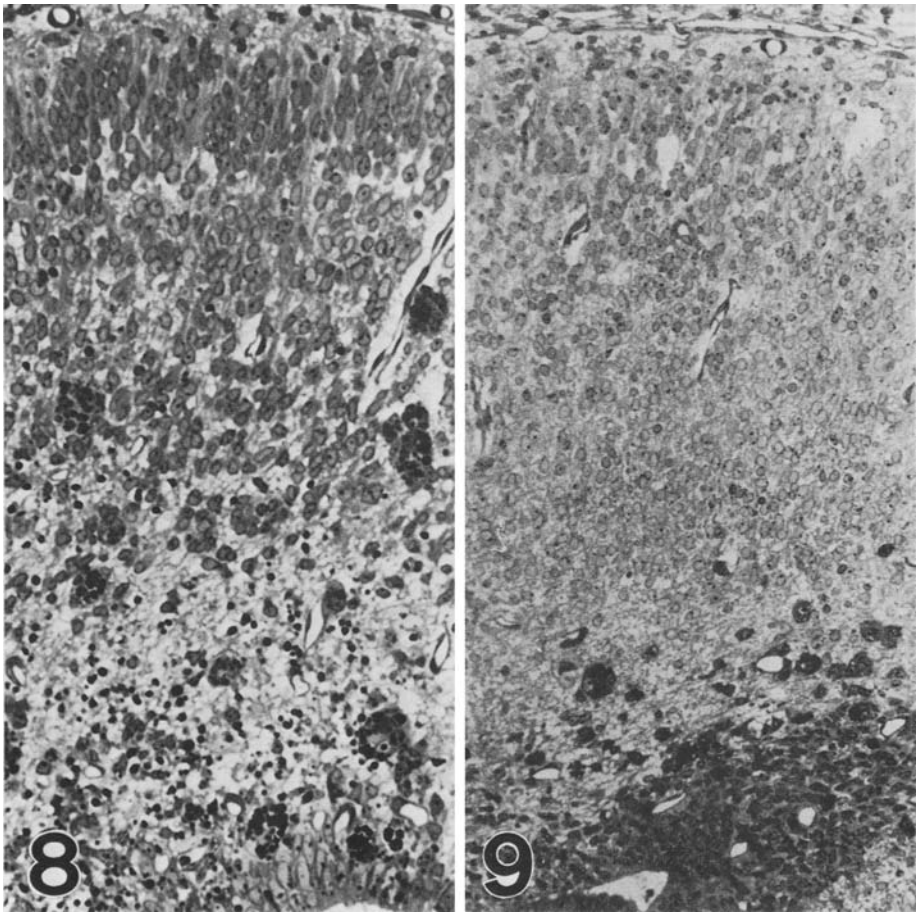

Fig. 8. Forty-eight hours later. The phagocytes containing fragments of degenerative cells tend to aggregate around blood vessels near the ventricle. The disintegrated migrating zone is gradually filled up by the migrating cells newly derived from the matrix cells. Toluidine blue and saffranin preparation. ×230

Fig. 9. Seventy-two hours later. Decreased number of phagocytes and increased thickness of the neopallium. Toluidine blue and saffranin preparation. ×170

The number of those phagocytes in the matrix cell layer rapidly increased, and a few phagocytes containing necrotic cell debris were observed even within the blood vessels in the matrix cell layer 7 h after the treatment (Figs. 5a, b, c).

Nine hours later, phagocytes containing necrotic cell debris were seen in the migrating zone, rather than in the matrix cell layer, corresponding to an increase of degenerative cells in the migrating zone.

Twenty four hours later, many huge phagocytes containing many degenerative cells were observed in the completely disintegrated migrating zone

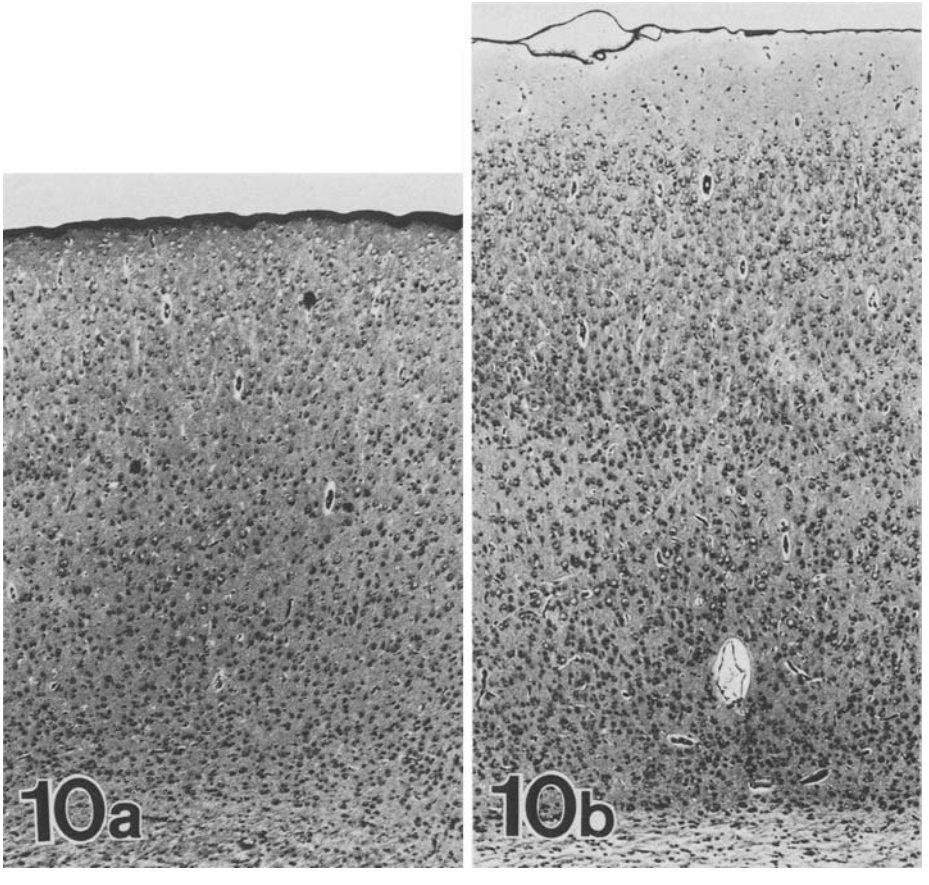

Fig. 10. ENU treated P21 rat neopallium (**a**), which is thinner than the normal control (**b**), with fewer neurons and abnormal distribution. H & E preparation. × 54

(Fig. 7). Even at this time the blood vessels in the lesion showed no definite proliferation, nor remarkable infiltration of granulocytes or lymphocytes. In the thin cortical plate, which had been formed before the ENU treatment, no degenerative cells or phagocytes were observed. The matrix cells were arranged closely and the ventricular wall looked smooth.

Forty-eight hours later, huge phagocytes containing numerous fragments of degenerative cell debris tended to aggregate around blood vessels near the ventricle (Fig. 8). The degenerative cells and phagocytes decreased in number (Fig. 9) and disappeared from the neopallium at P3.

Following the elimination of degenerative cells by phagocytes, the disintegrated migrating zone was gradually filled up by the migrating cells newly derived from the matrix cells from forty-eight hours after ENU administration (Fig. 8). The number of cells and the thickness of the neopallium increased (Fig. 9).

The neopallium of the ENU treated rat on P21 (Fig. 10a) revealed a

small number and abnormal distribution of the cortical neurons, compared with the control (Fig. 10b). It was noteworthy that the neopallium shows no abnormal capillary proliferation, infiltration of lymphocytes and granulocytes, fibrosis nor reactive gliosis at any period from one hour after the ENU treatment through P21.

Discussion

In various cerebral injuries of adults, it is well known that the extracellular space fluid retention, oedema, is inevitably induced. Astrocytes also show various morphological changes in response to every type of injury or disease in the central nervous system (Miquel et al. 1982). However, the role of the oedema fluid in lesion repair has not been discussed, and the relationship between the extracellular space fluid retention and the morphological changes of the astrocytes is unclear. Ikuta et al. (1983, 1984) were the first to stress the biological significance of oedema fluid in lesion repair. If an injury is made in the adult brain, the extracellular space is forced to expand by oedema fluid of serum origin. Macrophages move around in this space and phagocytoze and eliminate necrotic cell debris. Astrocytes undergo mitosis while immersed in the protein rich fluid, and then exhibit migrating activity. The astrocytes migrate to the remaining neurons, synapses, blood vessels or cysts and cover them again as reactive astrocytes. At this moment the repair of the lesion is completed.

In immature animals, however, systemic morphological examination of the tissue response in the lesion repair has been made only in new born animals. Spatz (1921) reported the limited reactivity of glial tissue in a traumatic lesion of the rabbit spinal cord, and Sumi and Hager (1968) stressed that the lack of glial scar after the injury in the rat brain is related to the paucity of swollen astrocytic processes with glycogen particles at the early stage of the injury. These observations were made in the central nervous system in lesions produced at the stage of gliogenesis (Berry et al. 1964). The biological basis of the repair is unclear in the reports, and the morphological peculiarities in the lesion during neurogenesis has not been reported.

Among the histological peculiarities of the normal fetal brain, the presence of the large extracellular space was recently confirmed (Sumi 1969; Nakanishi 1983), and Ikuta et al. (1983, 1984) elucidated that this space and the fluid filling it, play an important role in the production and migration of neuroblasts.

In our observation of lesion repair at the stage of neurogenesis, 4 h after the ENU treatment, degenerative cells appeared exclusively in the matrix cell layer, and appeared in the extracellular space which was also observed in the normal developing state. Five hours later, phagocytic cells engulfing the necrotic cell debris emerged.

The cytological features of these phagocytes including the folding of plasma membrane, the appearance of many endocytic vacuoles, lysosomes, phagosomes and mitochondria are indistinguishable from those of normal

macrophages previously reported in adults (Goldberg and Rabinovitch 1983) except for the paucity of the Golgi apparatus. And recently Matsumoto and Ikuta (1985) demonstrated that a certain number of macrophages obviously exist in the matrix cell layer and migrating zone of normal mouse fetal brain in the cytogenetic stage II. Furthermore, as observed in our study, the presence of phagocytes in the blood vessels may suggest that macrophages play a definite role in the eliminations of degenerative cells in the neopallium at cytogenetic stage II.

Our results reveal that the number of macrophages in the neopallium is maximum 24 h after the treatment, when the elimination of degenerative cells is almost completed, leaving a few residual macrophages until 72 h later. In the adult rat brain the number of macrophages is maximum 3 days (72 h) after a stab wound (Imamoto and Leblond 1977) or cold injury. Thus a certain number of macrophages in the brain of cytogenetic stage II may quickly respond to the appearance of degenerative cells and may be more active in eliminating the necrotic cell debris than those of adults.

It was evident in our study that a pre-existing extracellular space and many mitoses in the matrix cell layer are both observed under normal conditions. The cell degeneration due to ENU treatment was caused to individual cells in the proliferating phase migrating in the extracellular space and the presence of the extracellular space is recognized during and after the elimination of degenerative cells by macrophages. The most important point to elucidate is that after the elimination of degenerated cells by macrophages, the tissue is in a state identical to the original with a decrease in the number of cells. Under such conditions it may be that there is no necessity for the occurrence of a cellular reaction such as gliosis or fibrosis, regardless of whether the glial precursor cells are present or not in cytogenetic stage II (Fujita 1963, 1966; Bignami and Dahl 1974; Levitt et al. 1981, 1983; Bignami et al. 1982). After the elimination of degenerate cells the extracellular space is restored by newly derived neuroblasts from the matrix cell layer.

The observations performed up to P21 revealed a decreased number and abnormal arrangement of the neurons in the neopallium without any abnormal capillary proliferation, infiltration of granulocytes and lymphocytes, fibrosis or reactive gliosis. This result shows that the glial cells produced after the elimination of degenerative cells do not react as reactive astrocytes.

Even though the constituent cell elements are quite different in repair in the developing brain from those in adult brain, the presence of the extracellular space appears to be essential for the elimination of debris by macrophages in both fetal and adult brains. It is also necessary for the restoration of neuroblasts in the fetal brain and for the reaction of astrocytes in the adult brain.

We conclude that the fetal brain owes the unique features of its repair process to the presence, under normal conditions, of a vast extracellular space which in the adult brain would apparently be pathological. In this pre-existing extracellular space fluid comparable with oedema fluid in the adult brain (Ikuta et al. 1983, 1984) all kinds of cell, including neuroblasts and phagocytes, seem to exist individually without such intercellular adhe-

sions as are seen in the adult brain. Should degeneration occur in certain cells in this space phagocytes would be able to eliminate the degenerate cells completely in the space without having to break intercellular adhesions. It can be assumed that when the phagocytes have completed cell elimination the developing brain is restored to its original state, without any necessity for cell reaction. This gives the appearance of a small brain with normal-looking histological architecture, save only for the sparseness of cells.

Acknowledgements. The authors are grateful to Dr. Y. Matsumoto of our department of his kind suggestion of cooperation based on his experiences. They also wish to thank Dr. K. Watabe and Dr. A. Nishiyama for the help, and Mr. S. Egawa, Mr. T. Ichikawa, Mr. K. Kobayashi and Miss S. Sekimoto for their technical assistance during this observation and Mrs. Y. Tanahashi and Miss Y. Takahashi for their aid in this study.

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Accepted October 1, 1985