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Relationship between glial reaction to a stab wound and tumor development after receiving transplacental ethylnitrosourea in the rat

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Summary. Fisher 344 rats born from mothers treated with ethylnitrosourea (ENU) 50 mg/kg intravenously were injured at the 1st and 2nd month of extrauterine life by a transcranial stab. The wound affected cerebral cortex, white matter and basal ganglia. The animals were killed 15 and 45 days and 5 months after injury and cell reaction was studied histologically and immunohistochemically. Bromodeoxyuridine (BrdUrd) was administered 1 h before sacrifice and the labeled cells were evaluated. In ENU-treated rats injured at 1 month of age only minor differences were found in comparison with injured controls. In ENU rats injured at 2 months of age and killed 15 days later, a higher number of BrdUrd-labeled cells was found in comparison with controls; 45 days after injury the cell reaction acquired the aspect of a microtumor, however, no microtumor unrelated with the needle track was present. In ENU rats killed 5 months after the injury, there was no difference between injured and not injured ENU-treated rats, as far as the aspect and the number of tumors were concerned. The tumor phenotype was, thus, anticipated by the cell response to trauma in ENU rats. The interpretation is that the additional cell division, in response to trauma, anticipate not only the phenotypic, but also the cell kinetics changes, as indicated by BrdUrd labeling.

Key words: Brain injury – Ethylnitrosourea – rat – Gliomas – Bromodeoxyuridine

The experimental induction of tumors in the CNS of rats by transplacental administration of ethylnitrosourea (ENU) to the mother at the 17th day of gestation is a well-established procedure. ENU acts on the cells of the germinal matrix and its derivatives, radial glia included [10], through alkylation of DNA bases [4, 13]. Cells hit by ENU continue to proliferate, transmit the DNA damage to daughter cells, migrate and differentiate. Tumors appear in sites distant from those where primitive neuroepithelial cells where hit, and after a latency period which varies according to the carcinogen dose [7]. Tumors progressively develop through a series of successive steps, starting from the so-called "early neoplastic proliferation" (ENPs) of the paraventricular white matter [7, 14, 36] and reaching, through microtumors, the stage of fully developed tumors [25].

It can be hypothesized that a glial reaction elicited during the latency period may modify the tumor appearance and, conversely, that the genotypic transformation of developing glia cells may modify glial reaction. Contrasting observations have already been made on the relationship between glial reaction to a stab wound and tumor development [26, 29]. According to Morantz and Shain [29] trauma can act as a cocarginogen and enhance glioma formation. Since reactive gliosis is due mainly to the proliferation of astrocytes [19, 21, 37, 42], earlier and larger tumors should be expected after the elicitation of a glial reaction during the latency period of transplacentally ENU-treated rats. The result of such study are presented in this work.

Materials and methods

Fisher 344 rats born from mothers treated with ENU 50 mg/kg i.v. at the 17th day of gestation were injured, under anesthesia, by a transcranial stab with a 25-gauge needle. The wound affected cerebral cortex, white matter and basal ganglia. One group of 30 rats was injured at the 30th and another group of 30 rats at the 60th day of extrauterine life. Each group of rats was further divided into three subgroups, killed 15 and 45 days and 5 months after injury (Fig. 1).

Normal rats born from untreated mothers were injured, at the same ages and killed after the same times. Same rats born from mothers treated by ENU, as indicated above, were killed at the same ages without being injured.

Bromodeoxyuridine (BrdUrd) 50 mg/kg was administered i.v. to all rats 1 h before sacrifice. The brains were fixed in Carnoy at 0°–4 °C and embedded in paraffin. Five-micron serial sections were stained with hematoxylin-eosin and, immunohistochemically, with antibodies against GFAP (rabbit, Dako, 1/500), vimentin (monoclonal, Dako, 1/100), human Leu-7 HNK 1 (monoclonal, Becton

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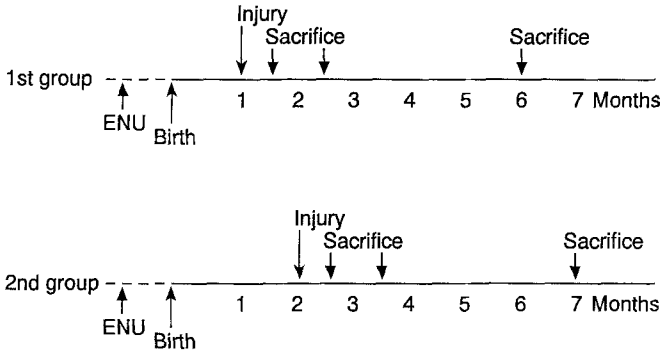

Fig. 1. Scheme of the experiments

Dickinson, 1/25), anti-macrophage RPN.701 (monoclonal, Amersham, 1/2)), ferritin (rabbit, Sigma, 1/7), carbonic anhydrase C CA C (rabbit, Behring, 1/250), BrdUrd (monoclonal, Becton Dickinson, 1/25), and with *Ricinus communis* agglutinin 120 RCA-1 (Sigma, 1/100). The immune reaction was revealed by either peroxidase-antiperoxidase (PAP) or avidin-biotin-peroxidase complex (ABC) methods.

BrdUrd labeling index (LI) was calculated as the number of BrdUrd-labeled nuclei expressed as a percentage of the total number of nuclei analyzed. The most heavily labeled areas surrounding the needle tracks were selected.

Results

Rats injured at 1 month of age and killed 15 days later, group A

In control rats the cell reaction extended from the cortex to basal ganglia through white matter (Fig. 2a). It was composed of macrophages, which were strictly confined to the necrotic area, cells with round nuclei and scanty cytoplasm and cells of stellate aspect with long and thick processes which were stained for GFAP and, in lower number, for vimentin. Macrophages contained hemosiderin pigment and were positive for RCA and ferritin. GFAP- and vimentin-positive reactive astrocytes surrounded the lesion in the cortex, were variably diffused in the deep structures and diffused through the whole white matter in the homolateral hemisphere. Mitoses and BrdUrd-positive cells were abundant in the paraventricular matrix (LI = 3%) (see Fig. 5b) and in moderate number in the lesion (LI = 1.5%).

In ENU rats the lesion showed the same cell composition as in control rats, but it was quantitatively less intense (Fig. 2b). The number of reactive astrocytes was definitely lower than in controls. Mitoses and BrdUrd-positive cells were abundant in the matrix (LI = 4%) and very few in the lesion (LI = 0.5%).

Rats injured at 1 month of age and killed 45 days later, group B

In control rats the cell reaction was more circumscribed than in group A (Fig. 2c). It was composed of GFAP- and vimentin-positive reactive astrocytes with very thick processes strictly surrounding the lesion; in the white matter they were more widespread, but not through the whole hemisphere. Pigment-containing macrophages, localized in the needle track, and few cells with round nucleus and scanty, unstained cytoplasm were the other elements. Mitoses and BrdUrd-positive cells were abundant in the matrix (LI = 4%) and absent in the lesion.

In ENU rats the cell reaction showed a similar picture in controls (Fig. 2d), but with a lower number of GFAP- and vimentin-positive astrocytes. Mitoses and BrdUrd-positive cells were present in the matrix (LI = 4%) and not in the lesion (see Table 1). In the paraventricular white matter, independently from the needle tracks, circumscribed lesions of less than 300 μm in size were found, which were characterized by cells displaying features suggestive for oligodendrocytes and astrocytes, similar to those previously found in transplacentally ENU-treated rats [14, 18, 35].

Rats injured at 2 months of age and killed 15 days later, group C

In control rats, the area of cell reaction was larger than in group A (Fig. 3a). Pigment-containing macrophages were localized in the needle track. GFAP-positive and vimentin-positive reactive astrocytes surrounded the lesion in the cortex and were diffused through the whole hemisphere in the white matter. They were intermingled with cells showing round nuclei and scanty and unstained cytoplasm (Fig. 3b). Mitoses and BrdUrd-positive cells were present in the matrix (LI = 6%). In the lesion mitoses were absent and only very few BrdUrd-positive cells were present (LI = 0,3%). In ENU rats, cells with round nuclei and scanty and

Table 1. Labeling indeces of bromodeoxyuridine-positive cells in injured rats

Age at injury	Interval before sacrifice	Controls		ENU rats	
		Matrix	Lesion	Matrix	Lesion
30 days	+ 15 days	3%	1,5%	4%	0.5%
30 days	+ 45 days	4%	–	4%	–
60 days	+ 15 days	6%	0,3%	6%	2,2%
60 days	+ 45 days	6%	–	6%	5%

ENU: Ethylnitrosourea treated

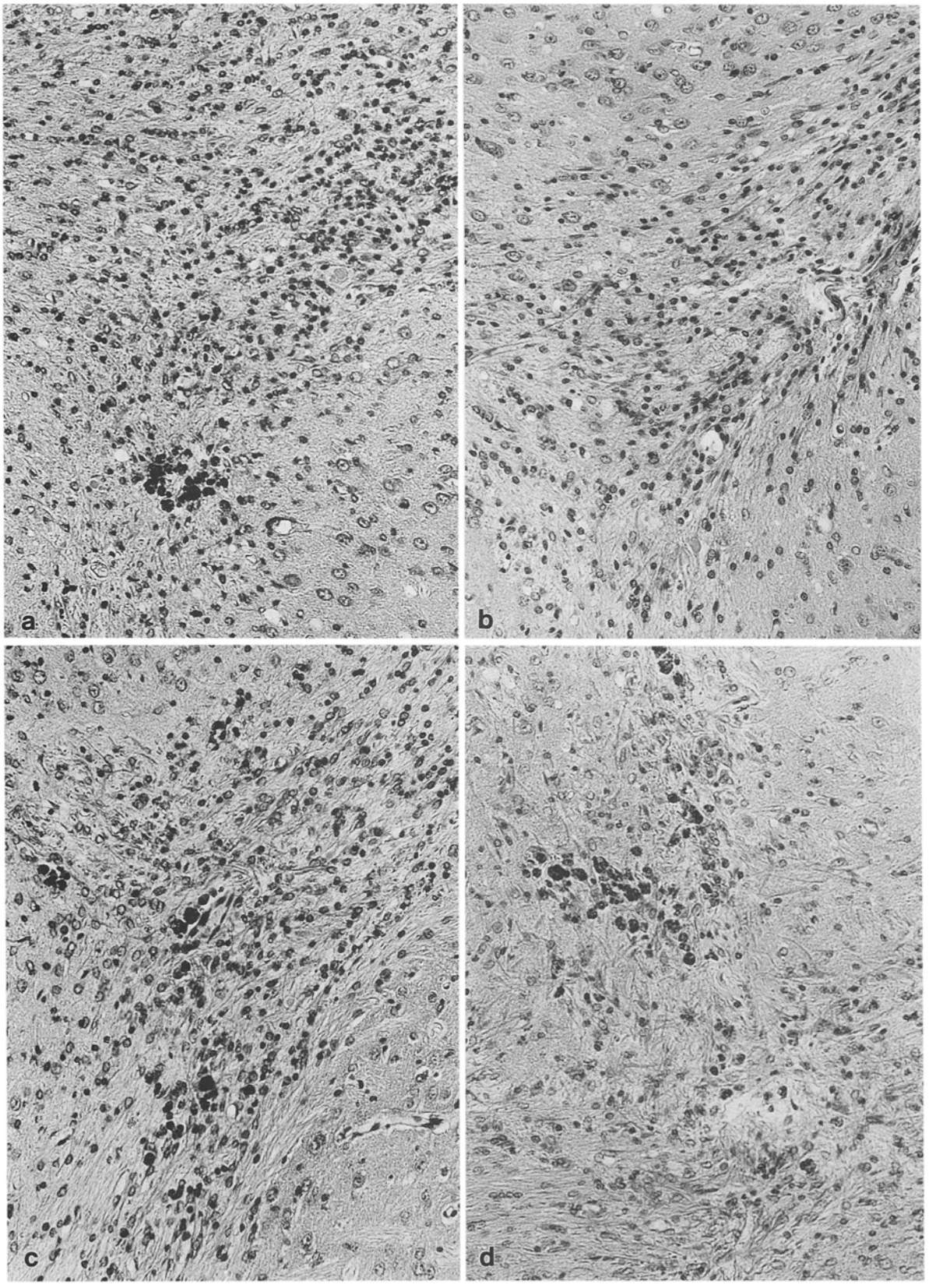

Fig. 2. **a** Control rat and **b** ethylnitrosourea (ENU)-treated rat 15 days after injury performed at the 30 th day of age; **c** control and **d** ENU-treated rat, 45 days after injury performed at the 30th day of age. The reacting cell number is higher in control rat **a–d** H&E, × 250

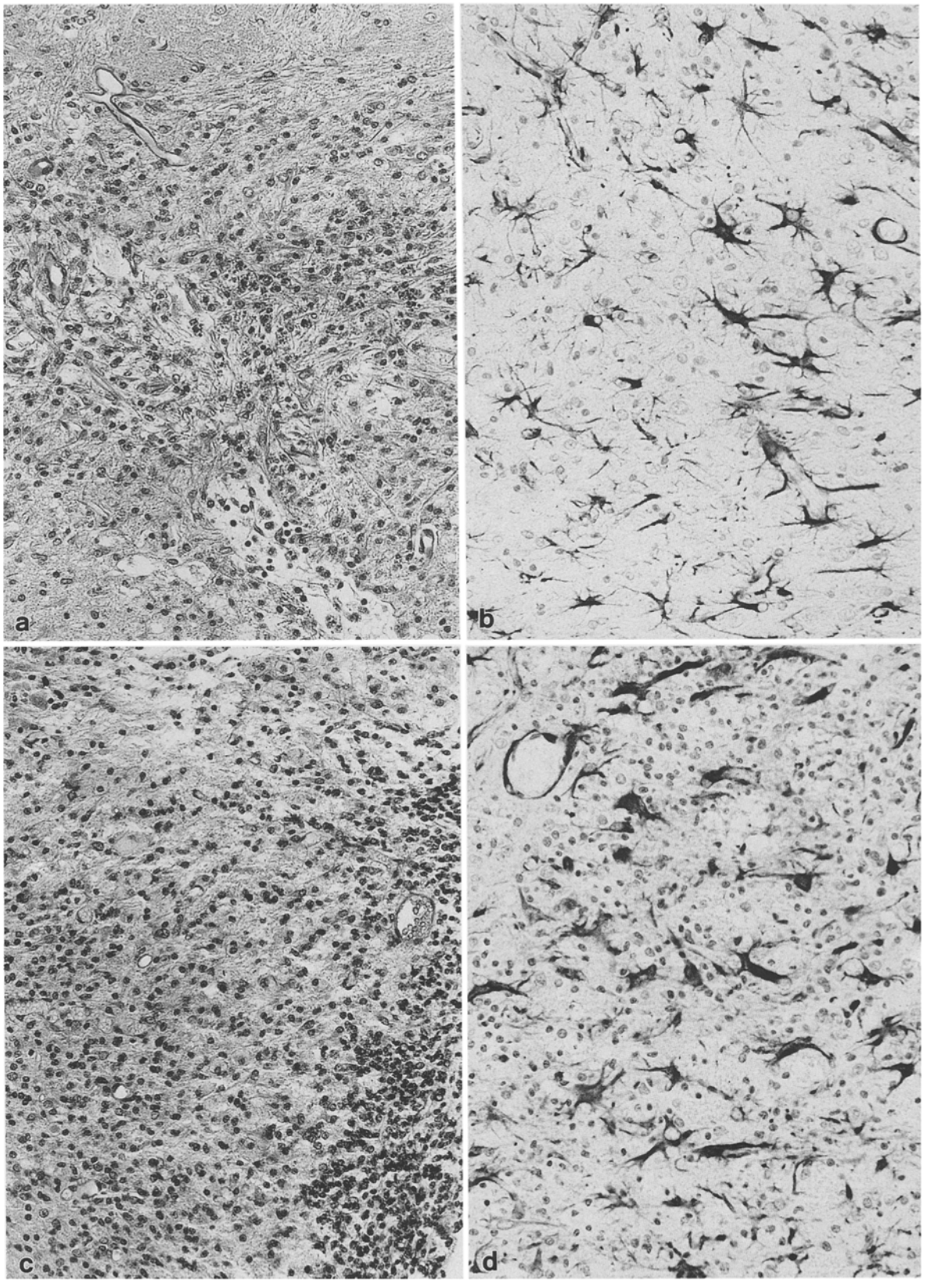

Fig. 3a–d. Injury at the 60th day of age and sacrifice 15 days later; **a** control rat, H&E; **b** control rat, GFAP; **c** ENU-treated rat, H&E; and **d** ENU-treated rat, GFAP. Note the higher number of unstained cells in ENU rat than in control rat. **a–d** × 250

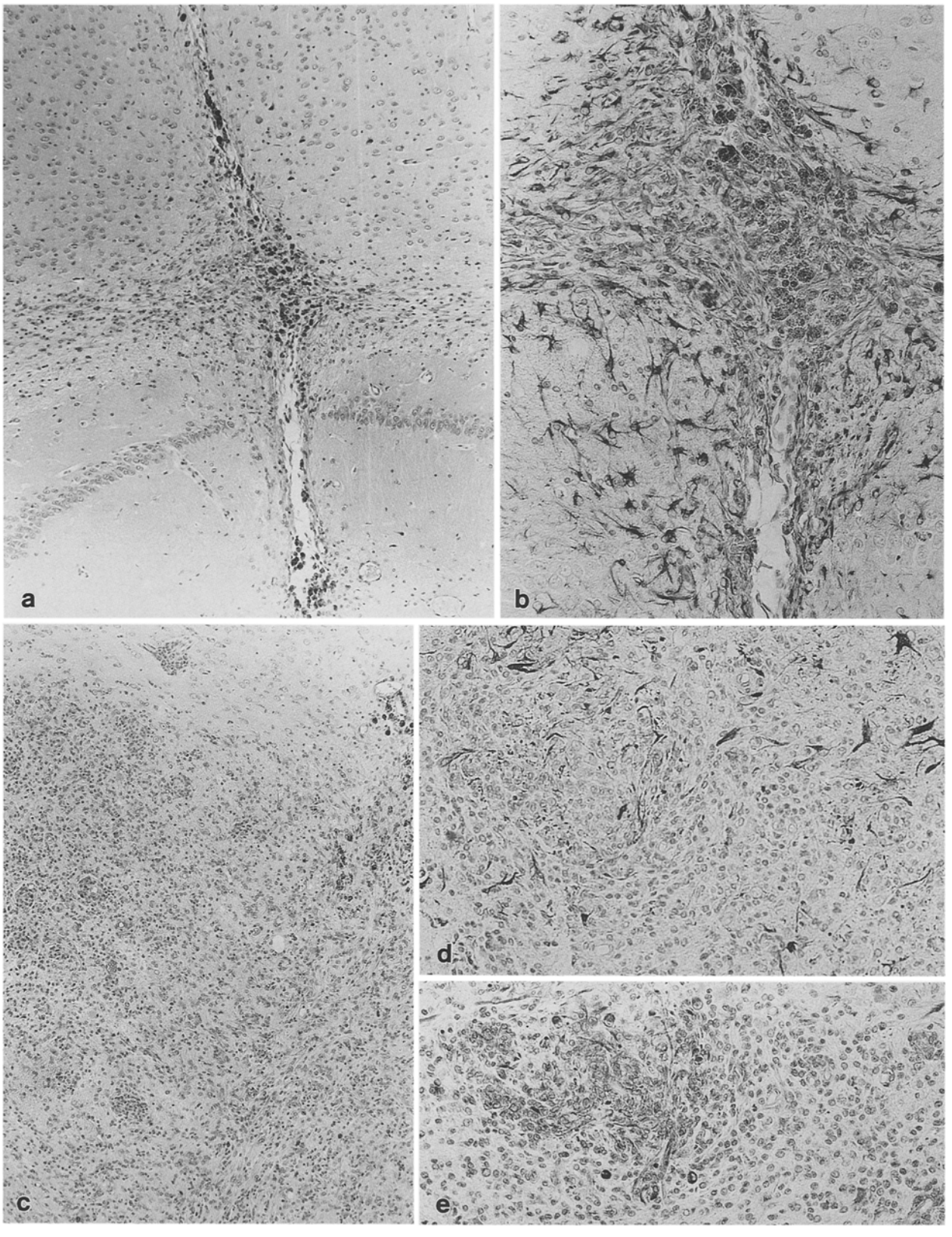

Fig. 4a–e. Injury at the 60th day of age and sacrifice 45 days later; **a, b** control rat, **a** H&E, and **b** GFAP; **c–e** ENU-treated rat, **c** H&E, **d** GFAP and **e** vimentin, Note in ENU-treated rat the character of the microtumor of the lesion and cells of proliferative centers which are GFAP negative and vimentin positive. Reactive astrocytes are positive for both markers. **a, d, e** × 250; **b** × 400; **c** × 150

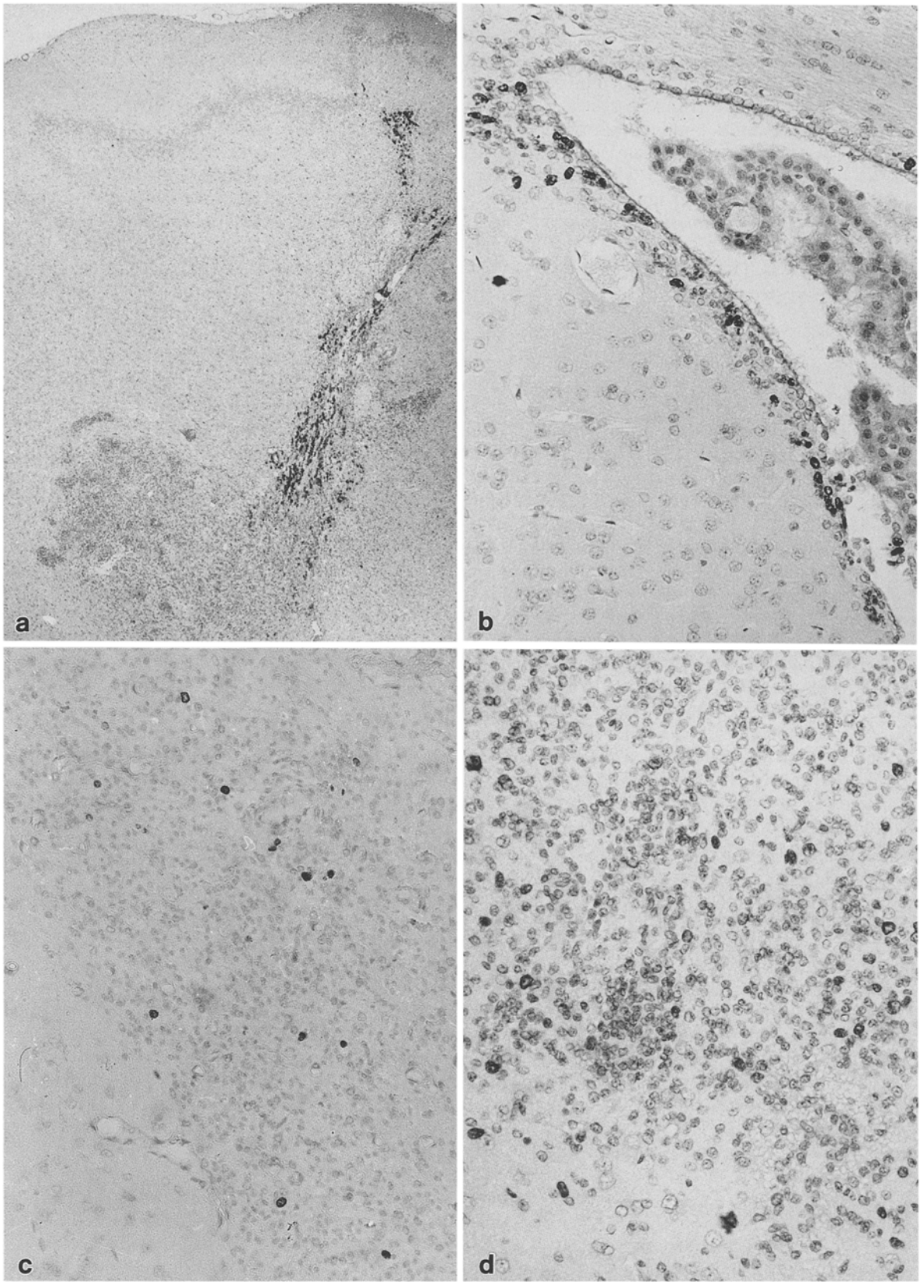

Fig. 5a–d. ENU-treated rats injured at 2 months of age; **a** microtumor associated with a needle track, 5 months after injury, H&E; **b** bromodeoxyuridine (BrdUrd)-labeled cells in the periventricular matrix; **c** BrdUrd-labeled cells in the lesion 15 days and **d** 45 days after injury. **a** × 150; **b** × 400; **c, d** × 250

unstained cytoplasm were found in higher number than in control rats (Fig. 3c). GFAP- and, in lower quantity, vimentin-positive reactive astroytes, were distributed as in control rats, whereas unstained cells were much more numerous than in controls (Fig. 3d). Mitoses and BrdUrd-positive cells were present in high number both in the paraventricular matrix (LI = 6%) and in the lesion (LI = 2.2%) (see Fig. 5c). In rats where the needle track affected the paraventricular matrix, there was a proliferation of matrix cells with many mitoses and BrdUrd-positive cells.

The same circumscribed lesion in the paraventricular white matter found in group B occurred.

Rats injured at 2 months of age and killed 45 days later, group D

In control rats the lesion was similar to that described in group C, as regards cell composition, but it had the appearance of a circumscribed glial scar (Fig. 4a, b). Mitoses and BrdUrd-positive cells were present in the matrix only (LI = 6%).

In ENU rats the lesion showed a definite tumoral aspect (Fig. 4c). A proliferation of small cells with round nuclei and unstained cytoplasm predominated. Clusters of these cells were stained, however, with vimentin and not with GFAP (Fig. 4d, 4e). Macrophages were still present in the needle track. GFAP- and vimentin-positive reactive astrocytes were intermingled with the above-mentioned cells or they surrounded the lesion. Such proliferations were not present independently from the needle track. Mitoses and BrdUrd-positive cells were present in the matrix (LI = 6%) and were abundant in the lesion (LI = 5%) (Fig. 5d). Circumscribed lesions in the white matter, similar to ENPs, were found independently from needle track.

Injured ENU rats killed after 5 months, group E

ENPs, microtumors and tumors were found either in associated with (Fig. 5a) or separately from needle tracks. They did not differ qualitatively and quantitatively from the tumoral lesion of non-injured rats of the same age transplacentally treated with ENU at the same dose.

The distribution of BrdUrd LI is illustrated in the Table 1. No cells in the reaction areas were positive for anti-Leu-7. C.A.C. stained oligodendrocytes in the cortex and white matter, but not in the reactive and tumor areas.

Discussion

Our observations demonstrate that in rats transplacentally treated by ENU the cell reaction to a stab wound is scarcely modified, in comparison with controls, if the injury is performed in 1-month-old rats. If the injury is performed in 2-month-old rats the differences compared with controls are clearly evident. The distribution of reactive astrocytes after a stab wound follows different patterns in the cerebral cortex, white matter and deep structures. In the cortex glial reaction, initially limited to the vicinity of the wound, spreads in time to the entire ipsilateral cortex and then it regresses. By the 20th day it is again limited to the wound [23]. This is confirmed by our experiments. It must be added that the diffuse reaction has a longer duration in the white matter and it regresses at the 45th day. In fetal and neonatal brains the glial reaction is much less pronounced and shows characteristics different to those found in adult brains [3, 27], but these observations are not relevant to our experiments which have been performed on adult rats. However, the mechanisms through which hyperplasia and hypertrophy of astrocytes take place have a paramount importance. The old concept that hyperplasia is accomplished through amitotic divisions [11] is definitely encompassed by the observations of mitoses or of [^{3}H]thymidine in reactive astrocytes around wounds [1, 5, 19, 21, 30, 37, 42]. In our experiment the occurrence of mitoses and of BrdUrd-positive cells 15 days after wounding confirms the limited duration of the hyperplastic cell response. A fundamental question is the origin of hyperplastic astrocytes. In our previous experiments [37] carried out with different models, a distinction was made between simple hypertrophic astrocytes, occurring both at a distance from the lesion and in its vicinity, and hyperplastic astrocytes which are limited to the lesion area. Vimentin-positive staining prevailed in the latter. Some hypotheses have been suggested to explain vimentin in astrocytes around the lesion: either it is a marker of immaturity [6, 32] or of motile ability [20]. Other experiments with different models indicated different possibilities. The diffuse astrocytic reaction in the cortex could be due to cell division in the molecular layer and white matter and to cell migration in the II and VI cortical layers.

Vimentin would be expressed in cells which underwent division [42]. Event though the reactive astrocytes distant from the lesion are better interpretable as only hypertrophic and those in in the vicinity of lesion as hyperplastic [37], the demonstration that distant astrocytes are of hyperplastic nature given by Takamiya et al. [42] seems to be unopposable. According to some observations reactive astrocytes could derive from preexistent astroblasts [8]. Normally [^{3}H]thymidine-labeled cells can be found in the cortex [12, 31, 39, 41], even though there is no demonstration that they are astroblasts.

The hyperplastic cell reaction is obviously more important than the astrocytic hypertrophy as far as the present problem is concerned. In control rats mitotic count and BrdUrd labeling confirm that hyperplasia is regressing 15 days after trauma, in line with the finding of Takamiya et al. [42], and completely absent after 45 days. Leaving aside macrophages or cells of blood vessel origin that at these times are no longer dividing [3] only astrocytes, oligodendrocytes or their precursor cells can be responsible for the labeling. In injured ENU rats these cells may contain alkylated DNA and, because of

the previous action of ENU on matrix cells, their tumor transformation may occur [13, 17].

If ENU rats are injured at the age of 1 month, the only modification of cell reaction observed is a smaller number of reactive astrocytes and BrdUrd-labeled cells in comparison with control rats. The cytocidal action of ENU on matrix cells [4] may be responsible for the temporarily lower hyperplastic response. Both in ENU-treated rats and in the ENU-treated and injured rats small circumscribed lesions are visible in the paraventricular white matter, which correspond to those described as ENPs [14–16, 35]. This lesions are the earliest visible morphological changes after ENU administration. Their interpretation is not easy [16]. The astrocytic component is hypertrophic, strongly GFAP positive, and problably reactive in nature [22]. ENPs usually appear by the 2nd month after an ENU dose of 50 mg/kg.

If ENU rats are injured at 2 months of age, i.e., at the end of the latency period of ENU tumors [36], the cell response to the wound after 15 days shows a greater quantity of cells with round nuclei and scanty cytoplasm, and the number of dividing cells, as evidenced by BrdUrd labeling, was much higher than in controls. After 45 days the proliferation associated with the needle track assumed the characteristics of a classically defined microtumor [14, 15, 35]. The cell reaction thus evolved into a microtumor. Since microtumors were only found in association in with needle tracks in these rats, trauma must have acted not only by promoting but also by anticipating the tumor appearance. The additional cell divisions in response to trauma may be responsible for the phenotypic and cell kinetic changes.

After more than 3 months from the time of injury there was no difference between injured and non-injured rats, as far as tumor composition and frequency were concerned, as alread observed by Mennel at al. [26]. Also the cellular composition of established tumors is similar in injured and non-injured rats. The exact cell of origin of transplacental ENU-induced tumors is not known, even if many hypotheses have been put forward. From the present results some speculations can be tentatively made concerning the histogenesis of these tumors.

In the cell response of ENU rats injured at 2 months of age there is a higher number of cells with round nuclei and scanty and unstained cytoplasm than in control rats. These cannot be confused with macrophages, which contain hemosiderin pigment and are strictly localized in the needle track.

They must, therefore, be oligodendrocytes or precursor cells, as it has already been suggested in ENPs [16, 35, 36]. In this regard the observation of Ludwin [21] on the oligodendroglial participation in the cell hyperplastic response to trauma is particularly interesting, even though it concerns the acute cell response and the whole hemisphere. The oligodendroglial interpretation of these cells cannot be supported by immunoistochemical evidence because of the lack of positive staining in the sections for specific markers; however, it must be taken into account that oligodendrogliomas are the most frequent tumors in transplacental ENU experiments [24, 35]. The absence of staining for CAC, which stains normal oligodendrocytes in the cortex and white matter, is probably related to the immaturity of tumoral oligodendrocytes [9].

Although Schuller-Petrovic et al. [38] claimed that Leu-7 specifically stains rat oligodendrocytes, the early stages of transplacentally induced tumors were negative for this antigen [40, 43]. Anti-Leu-7 is considered of little value in the classification of ENU-induced neural tumors [33].

Another interesting observation is that in ENU rats 45 days after trauma, cells with round nuclei express vimentin, even though their morphology is different from that of reactive astrocytes, which show long thick processes. These vimentin-positive cells could correspond to the vimentin-, but not GFAP-, positive cells found in clusters of microtumors and tumors induced by transplacental ENU, interpreted previously as undifferentiated cells [9, 34] or even as neoplastic derivatives of radial glia [10]. Recent observations demonstrated that at the moment of ENU administration, i.e., the 17th day of extrauterine life, GFAP-negative and vimentin-positive radial glia is still present [10]. The tumor anticipation in consequence of the reaction of transformed glia cells is relevant to the debated relationship between trauma and human gliomas, as discussed by Morantz and Shain [29] and Morantz [28].

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