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Cytoarchitectonic investigation of the rat spinal cord following ethylnitrosourea administration at different developmental stages*

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Summary. We examined the histological findings and cytoarchitectonic alterations in the rat spinal cord following matrix cell degeneration caused at different developmental stages, from neural plate formation through neuroblast generation. Ethylnitrosourea (ENU) 20 mg/kg body weight was administered transplacentally to the fetuses on the 10th embryonic day (E10) to 14th. The observations were made until the 21st postnatal day. Normally, mitoses were present scatteredly in the matrix cell layer of the neural plate or neural tube on E10 or E11, and gradually restricted to the dorsal portion of the alar plate as development occurred. The localization and number of degenerative cells as well as the site and degree of neuronal decrease in the completed dysgenetic spinal cord seemed to correlate with the topography and frequency of the mitoses in the matrix cell layer at the time of ENU administration. Disorder in the pattern of cytoarchitecture of neurons was not observed. The degree of hypoplasia of the white matter was proportional to the intensity of decrease of the spinal neurons. Aberrant myelinated fibers were not seen. No reactive gliosis, fibrosis or abnormal vascularization was observed at any time.

Key words: Developing spinal cord – Cytotoxicity of ethylnitrosourea – Matrix cell necrosis – Neurogenesis – Cytoarchitecture

Introduction

It has been said that the specific patterns of malformations in the central nervous system are closely related to the developmental stages at which tissue injury occurred. The critical periods for malformations in the central nervous system have been considered as the periods of neural tube closure, of neurogenesis and of functional and morphological differentiation in neural growth (Hicks 1953). Many extensive experimental studies have been performed on the cerebrum and cerebellum to elucidate the histological peculiarities of various anomalies caused at each critical period (Hicks 1953; Hicks et al. 1959; Altman et al. 1969; Phemister et al. 1969; Altman and Anderson 1971; Hallas and Das 1978; Morrissey and Mottet 1983; Yamano et al. 1983; Ferrer et al. 1984; Yoshida et al. 1984; Oyanagi et al. 1986).

However, most experimental studies of the developing spinal cord have been made on the failure of neural tube closure which relates to meningomyelocele or spina bifida. Experimental studies on the disturbance of neurogenesis in the spinal cord have been performed at the embryonic stages of 16-cell (Jacobson 1981), neural tube (Wenger 1950; Holtzer 1951; Watterson and Fowler 1953) or neuroblast formation (Hicks et al. 1959; Kirby 1980; Houle and Das 1983). However, no precise chronological observation from ocurrence of tissue injury until completion of anomaly formation nor investigation on the alteration in the neuronal cytoarchitecture of the spinal cord has been presented.

In the developing spinal cord, the generation of large neurons is earlier than that of small neurons and there exists a ventral-first dorsal-last temporal cytogenetic gradient in neuron production (Kanemitsu 1971; Nornes and Das 1974; Altman and Bayer 1984). It is essential to examine the cytoarchitectonic alteration and temporal changes in histological findings in the spinal cord following tissue injury occurring at each period of neuron

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production, in order to elucidate the pathogenesis of spinal cord anomalies showing abnormal cytoarchitecture (Friede 1975; Ludwin and Norman 1985). The spinal disorders in newborns or infants such as those with arthrogryposis multiplex congenita (Drachman and Banker 1961; Clarren and Hall 1983) or Werdnig-Hoffmann disease (Oppenheimer 1984; Schoene 1985) also need to be studied in this way. This type of work is essential for understanding the basis of normal development of the spinal cord.

A single injection of ethylnitrosourea (ENU), which is cytotoxic immediately after administration (Kleihues 1969; Kleihues and Patzschke 1971; Kleihues et al. 1973; Bosch 1977b; Fujiwara 1980; Yoshida et al. 1984; Oyanagi et al. 1986), was administered transplacentally to rat fetuses at different developmental stages, from neural plate formation through neuroblast generation, and the resultant histological alterations were observed.

Materials and methods

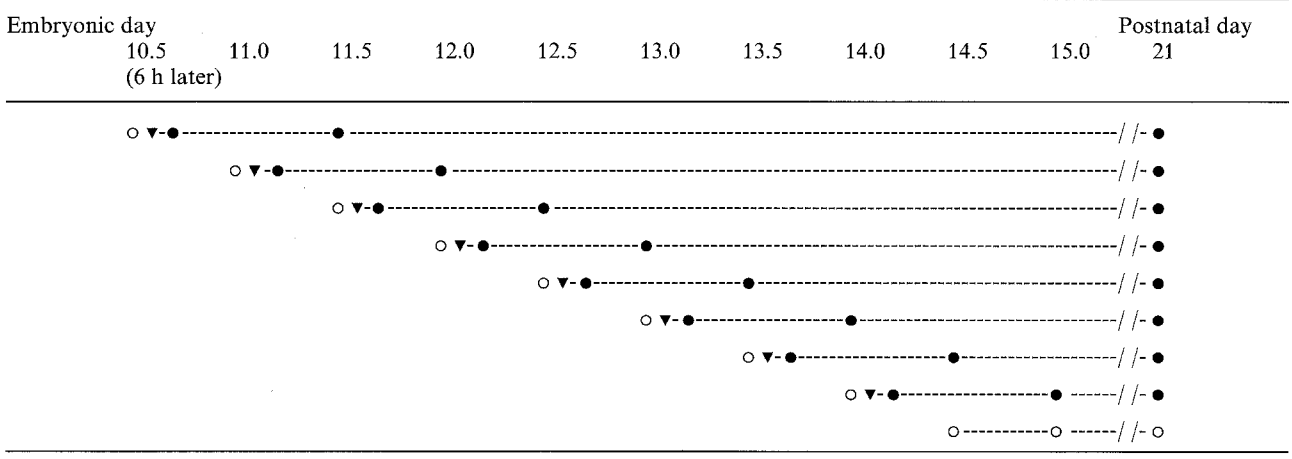
Four-month-old Wistar albino rats, weighing 250 g–300 g, were used. Virgin females were mated with males for 6 h from 0:00 am to 6:00 am approximately, and vaginal smears were examined at 6:00 am. The day on which sperm appeared at 10:00 am in the smears was designated as the first embryonic day (E1.0), and 10:00 pm as E1.5. Four pregnant rats were used for every experimental group. In order to study the histological findings and cytoarchitectonic alterations following matrix cell degeneration, from E10.5 till E14.0, every 12 h the pregnant rats were given a single injection of ENU freshly dissolved in physiological saline in the tail vein, after resection of a fetus as a normal control from each of the 4 pregnant rats of each experimental group. Based on our experiences using ENU (Yoshida et al. 1984; Oyanagi et al. 1986), a fetus of each pregnant rat was resected 6 and 24 h after treatment in order to examine the histological peculiarities of the degenerative cells and of the eliminating mechanism. The remaining fetuses were permitted to survive to maturity, and sacrificed on postnatal day 21 (P21). A total of 128 rat fetuses and pups was used for the experiment. As control rats for E14.5, E15.0 and P21, 4 other normal fetuses and pups were sacrificed on each day (Table 1).

The degree of tissue damage in the developing central nervous system is proportional to the dose of administered ENU and to the mitotic activity of the matrix cells (Koyama 1970; Fujiwara 1980; Yoshida et al. 1984). In order to determine the amount of ENU suitable for injection on rats E10.5 to E14.0, ENU of 10, 20 or 40 mg/kg body weight was administered transplacentally and the degree of tissue degeneration was observed after 6 and 24 h and on P21.

Based on the results of these preliminary experiments, a single intravenous injection of ENU 20 mg/kg body weight, freshly dissolved in physiological saline, was administered to each pregnant rat.

For histological study the fetuses were immersed in 1% glutaraldehyde – 1% paraformalde-

Table 1



Experimental schedule. Each o and • indicates normal or ENU treated 4 fetuses or pups respectively. (▼) indicates intravenous injection of ENU 20 mg/kg body weight. After resection of a fetus as a normal control respectively from 4 pregnant rats of each experimental group, the pregnant rats were given a single injection of ENU. A fetus of each pregnant rat was resected 6 and 24 h after the treatment and a pup, P21

hyde in 0.1 M phosphate buffer (pH 7.3). Two rats of each experimental group of P21 were sacrificed by perfusion through the heart with the 0.1 M phosphate buffer containing 1% heparin followed by 1% glutaraldehyde – 1% paraformaldehyde in the 0.1 M phosphate buffer. The tissue was fixed in 1% osmium tetroxide. Blocks of the tissue were dehydrated in graded ethanol and embedded in epon 812 after changes of propylene oxide. For light and electron microscopic examination semithin (1 μm) and ultrathin sections were prepared at the neural plate or neural tube at the site of the future cervical segment in fetuses and at cervical enlargement of P21 rats. Semithin sections were stained with toluidine blue and saffranin. Ultrathin sections were contrasted with uranylacetate and lead citrate and viewed in an electron microscope.

The remaining two rats of each experimental group of P21 were perfused with 0.1 M phosphate buffer containing 1% heparin followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Tissue blocks of the 5th cervical segment were embedded in paraffin and sectioned at 4 μm. The thickness of the sections was verified by manipulating the fine adjustment drum of the light microscope. The sections were stained with H&E, Klüver-Barrera, PTAH, PAS or thionine, and examined with a light microscope.

In order to visualize and elucidate the topographic distribution of the neurons of P21 spinal cord treated with ENU on E11.5, E12.0 and E14.0 according to their sizes, thionine stained sections were examined and compared with the controls. The unilateral spinal cord of each section was surveyed under 1,000-fold magnification. Neurons were identified by the presence of Nissl substance and prominent nucleoli. The longest diameter of the nucleus (A) and the largest dimension perpendicular to the diameter (B) were measured with an ocular micrometer, and the nuclear area (S) was calculated according to the formular $S = \pi$ AB/4 (Oyanagi et al. 1983). Spinal neurons were classified into 6 groups according to their nuclear areas. The first group was composed of neurons whose nuclear areas were smaller than 50 μm^2; the 2nd group, 51–100 μm^2; the 3rd group, 101–150 μm^2; the 4th group, 151–200 μm^2; the 5th group, 201–300 μm^2; and the 6th group, greater than 301 μm^2. Each group of the neurons was expressed by dots of graded sizes and was approximately plotted on the trace of the spinal gray matter magnified 100 times. We defined in this study large neurons as those with a nuclear area greater than 201 μm^2, medium-sized neurons, 101–200 μm^2, and small neurons, less than 100 μm^2.

Results

The group treated with ENU 10 mg/kg body weight showed quite faint morphological alterations with a few degenerative cells in the matrix cell layer. However, in the rat fetuses treated with ENU 40 mg/kg body weight during E10.5–E11.5, most matrix cells degenerated 6 h later. Half of the fetuses expired *in utero* and the rest died within 3 days after birth.

The preferable dose of ENU seemed to be 20 mg/kg body weight in order to observe the histological findings after matrix cell degeneration, occurring on E10.5 through E14.0. Under those conditions, intrauterine fetal death was rarely seen, and postnatally half of newborns treated with ENU on E10.5 through E12.0 were dead within 3 days, but those remaining developed until 21 days.

Normal E10.5 rat fetuses showed neural plate with scattered mitoses in the matrix cell layer (Fig. 1a). Six hours after ENU treatment, the neural tube was well formed and scattered degenerative cells with pyknotic nuclei and darkly stained cytoplasm were found, relatively more in the alar plate than in the basal plate (Fig. 1b). Those degenerative cells were eliminated totally 24 h later and the size of the neural tube was severely reduced. The thickness of the matrix cell layer was reduced and the number of matrix cells was small (Fig. 1c) when compared with those of controls (Fig. 1d).

The P21 spinal cord treated with ENU on E10.5 was severely hypoplastic in general. Spinal nerve roots were slender but normal in number. Quite severe decrease of large and medium-sized neurons in the whole spinal gray matter but not in the anterior horn was observed. The white matter was severely hypoplastic. Meningomyelocele or syringomyelia was not observed (Fig. 5a).

In the normal E11.0 rat the neural groove was seen and 6 h after ENU treatment the neural tube was formed. Degenerative cells were distributed equally in the basal and alar plates. There were no degenerative cells in the matrix cell layer 24 h after the treatment. At that time, the thickness of the matrix cell layer was reduced and the number of matrix cells was small. The differentiating neuroblasts in the anterior horn looked to be reduced in number when compared with controls.

The P21 cervical cord treated with ENU on E11.0 revealed diffuse decrease of neurons and hypoplasia of the white matter. The degree of change was less severe than that in rats treated with ENU on E10.5, and was intermediate between those of rats treated with ENU on E10.5 and E11.5.

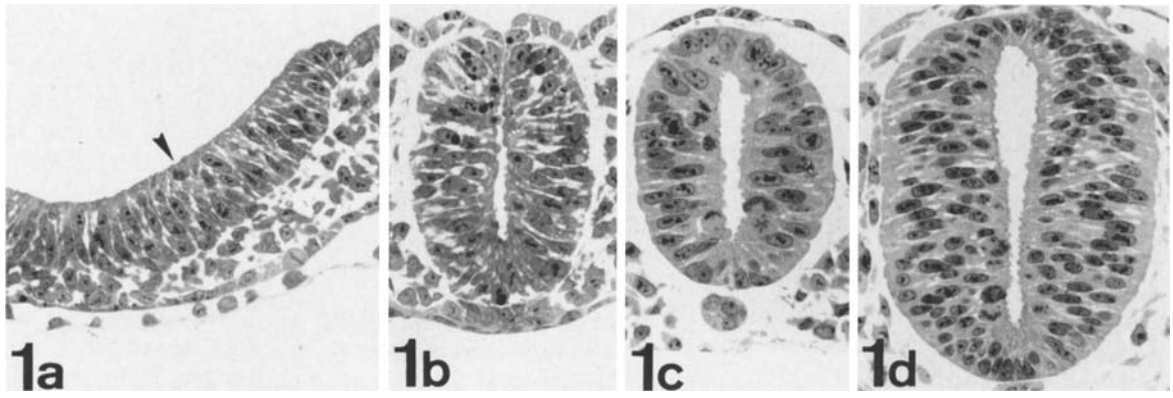


Fig. 1a–d. Toluidine blue and saffranin preparation of neural plate or neural tube at the level of cervical. ×240. **a** Normal, E10.5. Neural plate with scattered mitotic cells. (➘). **b** 6 h after ENU treatment on E10.5. Neural tube with scattered degenerative cells relatively more in the alar plate than in the basal plate. **c** 24 h after ENU treatment of E10.5. Severely hypoplastic neural tube and absence of degenerative cells. **d** Normal, E11.5

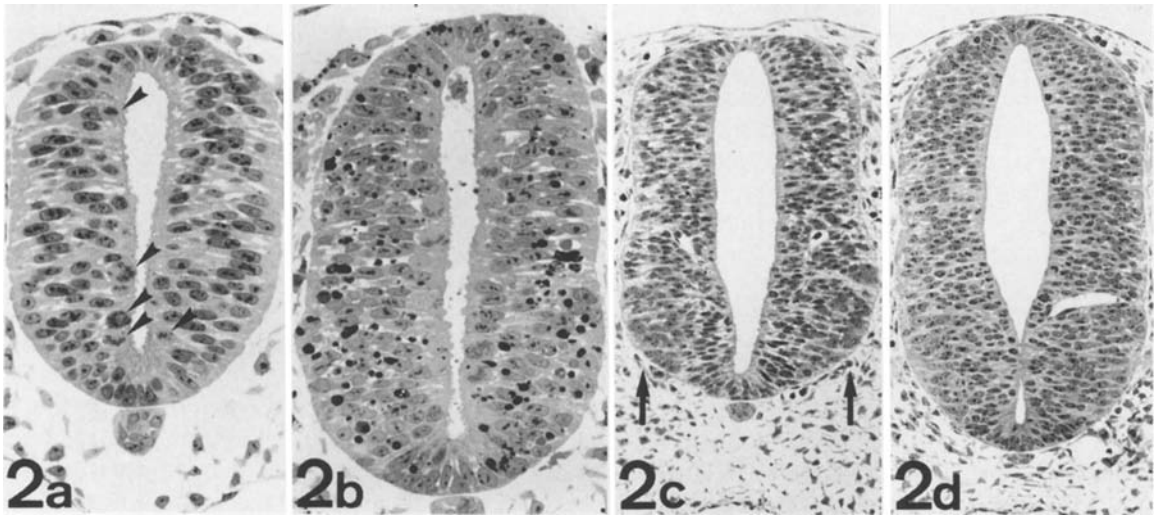


Fig. 2a–d. Toluidine blue and saffranin preparation of neural tubes at the level of cervical. **a** Normal, E11.5. Scattered mitotic figures (➘) with relative predominance in the basal plate. ×240. **b** 6 h after ENU treatment on E11.5. Degenerative cells more in the basal plate than in the alar plate. ×240. **c** 24 h after ENU treatment on E11.5. Generally hypoplastic neural tube with reduced number of differentiating neuroblasts (➙) in the anterior horn. ×120. **d** Normal, E12.5. ×120

The normal E11.5 rat revealed a neural tube showing scattered mitotic figures in the matrix cells, a change more marked in the basal plate than in the alar plate (Fig. 2a). The degenerative cells were diffusely distributed with slight predominance in the basal plate 6 h after ENU treatment (Fig. 2b). After 24 h the thickness of the matrix cell layer and the number of matrix cells were generally reduced, and the number of differentiating neuroblasts having rounder and paler nuclei and lightly staining perikarya (Altman and Bayer 1984) in the anterior horn looked decreased (Fig. 2c).

The P21 cervical spinal cord treated with ENU on E11.5 revealed marked decrease of large and medium sized neurons (4% of that of the control for large cells and 25% of the control for medium size) in the whole spinal gray matter (Fig. 6a). However, disorder in the pattern of cytoarchitecture and aberrant myelinated fibers were not observed.

Normal E12.0 and E12.5 rats revealed clusters of differentiating neuroblasts in the anterior horn and mitotic figures of the matrix cells diffusely distributed sparing the ventral portion of the basal plate. Six hours after the treatment, degenerative cells existed scatteredly in the matrix cell layer sparing the ventral portion of the basal plate. Thickness of the matrix cell layer and number of matrix cells were reduced equally 24 h after the treatment, but the number of neuroblasts in the

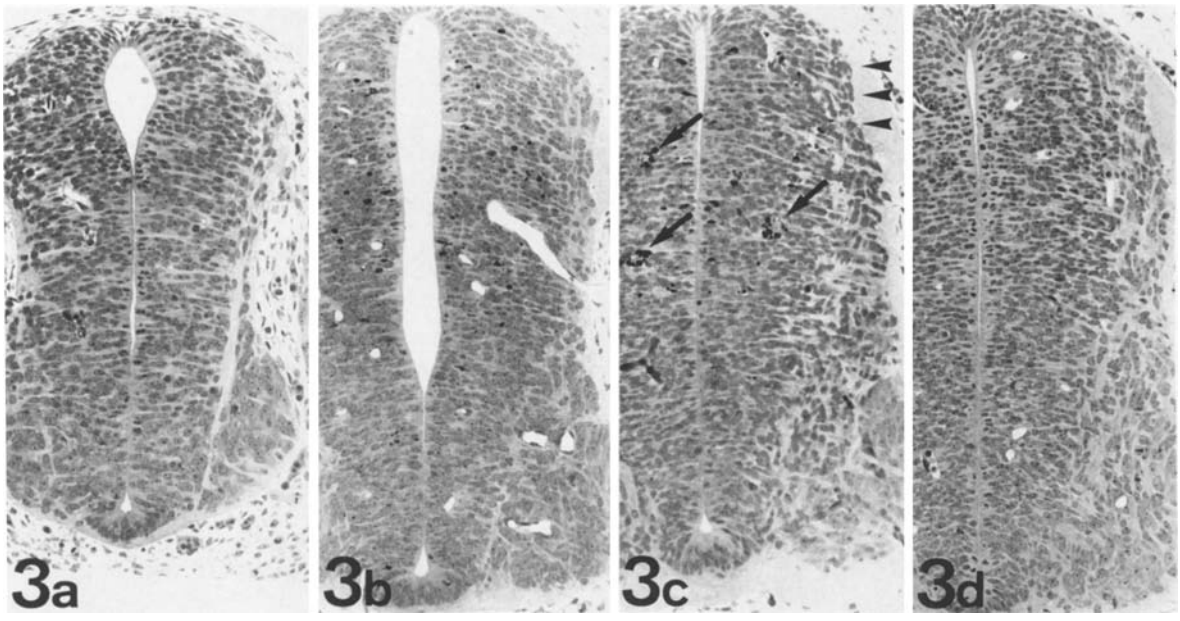


Fig. 3a–d. Toluidine blue and saffranin preparation of neural tube at the cervical. × 120. **a** Normal, E13.0. Clustering of neuroblasts in the posterior horn as well as anterior horn. Mitotic figures are seen in the alar plate exclusively. **b** 6 h after ENU treatment on E13.0. Exclusive appearance of degenerative cells in the alar plate. **c** 24 h after ENU treatment on E13.0. Reduced thickness of matrix cell layer in the alar plate and decreased number of differentiating neuroblasts (➘) in the posterior horn. Presence of several phagocytes (➡) containing cell debris in the alar plate. **d** Normal, E14.0

anterior horn looked to be relatively well preserved. Phagocytes containing degenerative cell debris were still seen in the matrix cell layer with relative sparing of the ventral portion of the basal plate.

P21 spinal cord treated with ENU on E12.0 and E12.5 revealed a moderate decrease in medium-sized neurons (to 55% of the control value) in the whole spinal gray matter with slight loss of large anterior horn cells (to 80% of the control value) (Fig. 5b, 6b).

Normal E13.0, E13.5 and E14.0 rat cervical segments revealed clustering of neuroblasts not only in the anterior horn but also in the posterior horn. The mitotic figures of the matrix cells were observed exclusively in the alar plate (Fig. 3a). Degenerative cells were selectively observed in the alar plate 6 h after the treatment (Fig. 3b). The thickness of the matrix cell layer in the alar plate and the number of neuroblasts in the posterior horn seemed reduced 24 h after the treatment. Phagocytes encasing cell debris were still observed in the alar plate (Fig. 3c).

The P21 cervical spinal cord treated with ENU on E13.0 through E14.0 revealed decrease of medium-sized and large neurons (to 50% of the control value) and small neurons (to 80% of the control value) in the posterior horn and slight decrease

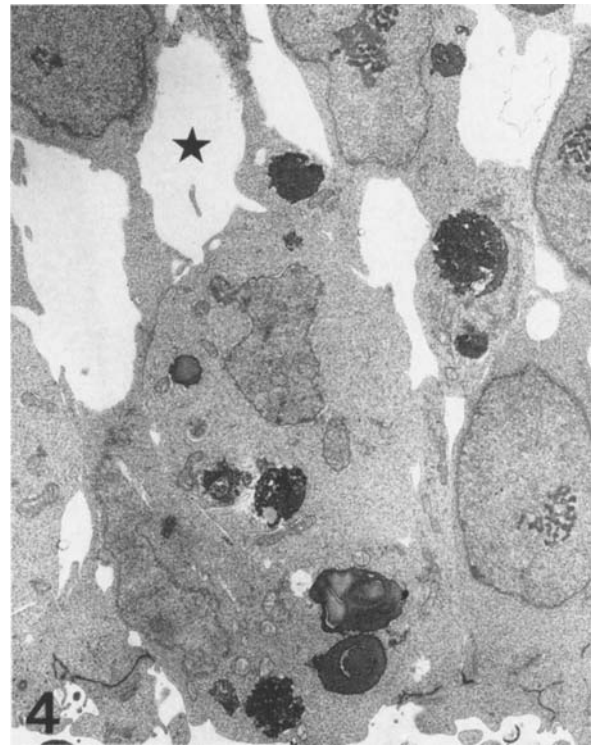


Fig. 4. Electronmicrograph of the matrix cell layer 6 h after ENU treatment on E11.0. Debris encased in the matrix cells and presence of extracellular space (★). The extracellular space seems to be irregularly widened because of immersion fixation. However extracellular space is definitely present. × 3500

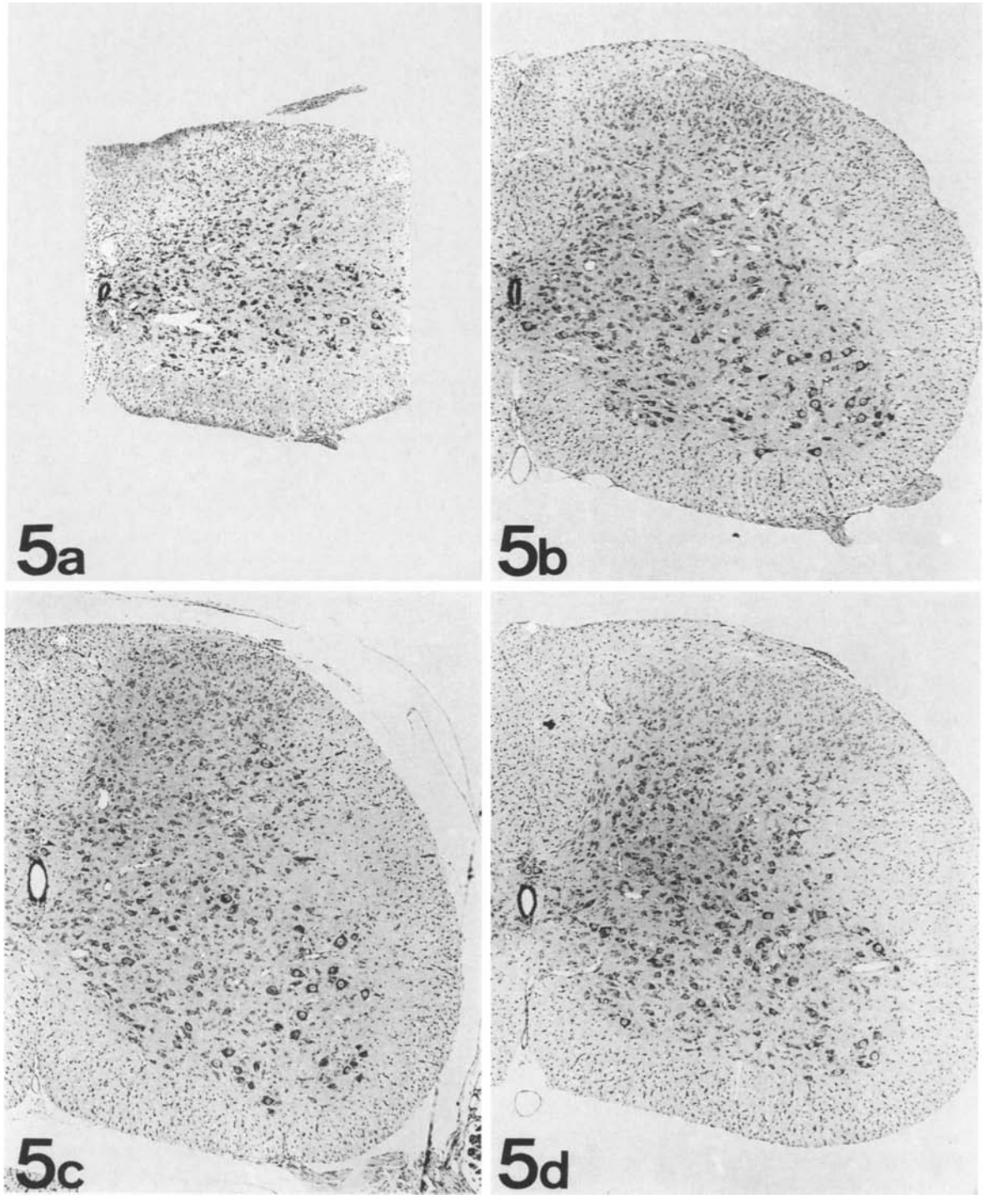


Fig. 5a–d. Four μm thick thionine preparation of cervical enlargement of P21 rat. ×42. **a** Treated with ENU on 10.5. Severely hypoplastic spinal cord showing severe decrease of neurons in the whole gray matter and hypoplastic white matter. **b** Treated with ENU on E12.0. Moderate decrease of medium sized neurons in the whole spinal gray matter with slight loss of anterior horn cells. **c** Treated with ENU on E13.0. Decrease of neurons in the posterior horn. **d** Normal, P21

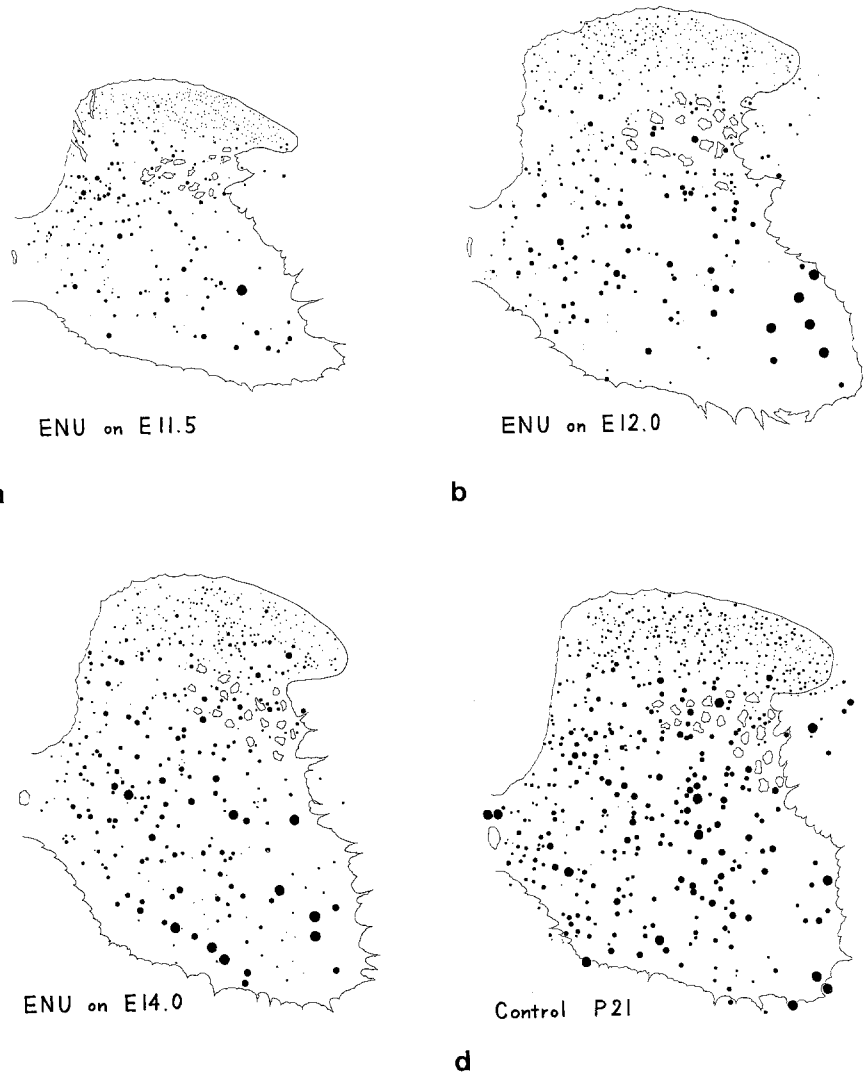


Fig. 6a–d. Topographic distributions of neurons according to the nuclear size in the 5th cervical segment of P21 rats. **a** Treated with ENU on E11.5. Marked decrease of large and medium-sized neurons in the whole spinal gray matter. Absence of disorder in the pattern of cytoarchitecture. **b** Treated with ENU on E12.0. Moderate decrease of medium-sized neurons in the whole spinal gray matter with slight loss of large anterior horn cells. **c** ENU treated on E14.0. Moderate decrease of small neurons as well as medium-sized and large neurons in the posterior horn and slight decrease of medium-sized neurons in the intermediate gray matter. **d** Normal, P21

of medium-sized neurons in the intermediate gray matter (to 80% of the control value) (Fig. 5c, 6c).

Thus in normal developing spinal cord, the mitoses were present scatteredly in the matrix cell layer of the neural plate or neural tube on E10 or E11, and gradually restricted to the dorsal portion of the alar plate as the development elapsed. There existed ventral-first dorsal-last temporal cytogenetic gradient of neuroblast production.

The localization and number of degenerative cells 6 h after ENU treatment as well as the site and degree of neuronal decrease in the cervical segment of the spinal cord of P21 rats seemed to correlate with the topography and frequency of mitoses of the matrix cell layer at the time of ENU administration.

No evident disorder in the pattern of neuronal cytoarchitecture was seen. Decrease of neurons restricted in the anterior horn (Drachman and Banker 1961; Clarren and Hall 1983), empty cell bed, neuronophagia, "ghost" cells or glial bundle in the spinal nerve roots (Oppenheimer 1984; Schoene 1985) were not seen. Spina bifida, meningomyelocele or syringomyelia was not observed. The size of the nucleus of spinal neurons of each experimental group as well as the control seemed to correlate with the size of perikaryon.

The spinal white matter of ENU treated P21 rats revealed various degree of hypoplasia proportional to the intensity of the decrease of the neurons. Aberrant myelinated fibers were not observed.

In the process of elimination of degenerative cells, the debris was encased within the normal looking matrix cells (Fig. 4) and was completely eliminated within 24 h after ENU treatment from E10.5 to E11.5 (Figs. 1c, 2c). In rats treated on E12.0 and after, however, several phagocytes con-

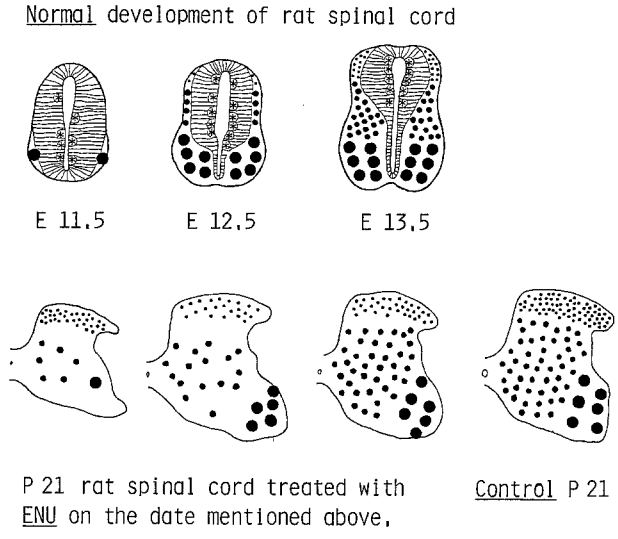


Fig. 7. Schematic presentation of the stages in the neuroblast production of normal developing spinal cord and the pattern of decrease of the neurons in the completed dysgenetic spinal cord following ENU administration at each stage. (*) indicates mitosis in the matrix cell layer and (•) indicates neuroblast or neuron. The normal developing spinal cord reveals scattered existence of mitoses in the matrix cell layer at earlier stages and gradual restriction to the dorsal portion of alar plate as the development elapsed. There exists ventral-first dorsal-last temporal cytogenetic laminal gradient of neuroblast production. The site of decrease of neurons in the completed dysgenetic spinal cord are clearly correlative to the topography of the mitoses in the matrix cell layer at the time of ENU administration

taining many degenerative cells remained in the matrix cell layer 24 h later (Fig. 3c).

The extracellular space was observed in the fetuses under normal condition as well as pathological states at 6 and 24 h after ENU administration in each experimental group (Fig. 4).

No reactive gliosis, fibrosis, abnormal vascularization or infiltration of granulocytes and lymphocytes was observed at any time.

Discussion

Ethylnitrosourea (ENU) is known to be a neurogenic resorptive carcinogen (Ivankovic and Druckrey 1968; Druckrey et al. 1972; Bosch 1977a), and is also known to have cytotoxic properties immediately after administration. Following systemic administration, ENU damages cellular DNA synthesis by alkylation of the bases, and has been shown to decompose in the intact animal with a half-life of less than 10 min (Kleihues 1969; Kleihues and Patzchke 1971; Kleihues et al. 1973).

ENU administration to rat fetuses induces selective degeneration of proliferating cells in S-phase (Bosch 1977b) or S to M phase in the mitotic cycle of the developing brain (Yoshida et al. 1984). The degree of tissue damage is proportional to the dose of administered ENU, and anomalous brains showing various degree of reduced number of neurons and of abnormal cytoarchitecture were formed (Koyama 1970; Fujiwara 1980; Yoshida et al. 1984).

In order to examine the cytoarchitecture of spinal neurons according to sizes, a certain number of serial sections should be examined in man (Kanemitsu and Ikuta 1977; Oyanagi et al. 1983). However, the neuronal density in the rat spinal cord on P21 is higher than that of the man, therefore the examination of the spinal neurons of one section with 4 μm thick may demonstrate the correct cytoarchitecture of rat spinal neurons.

In normal condition, a fixed correlation has been found between the size of the nucleus and the perikaryon of various neurons (Cruz and Lison 1963; Kanemitsu 1977; Oyanagi et al. 1987), and the volume of perikaryon correlates with total cell volume including dendrites and axons (Mannen 1966). Although, an antimitotic agent, ENU, was administered in our study, the size of the nucleus of spinal neurons seemed to correlate with the size of perikaryon. We decided to examine the cytoarchitecture of rat spinal cord in relation to the area of the nucleus.

In restitution after tissue degeneration in the developing spinal cord, Rohon-Beard neurons develope from an adjacent substitute cell after extirpation of an ancestral cell of the neurons in the 16-cell frog embryo (Jacobson 1981). However, extirpation of a part of the neural tube induced spinal cords showing defects at the extirpated area (Wenger 1950; Holtzer 1951; Watterson and Fowler 1953). Thus the remaining cells at the 16-cell stage have the capability for restitution after extirpation of certain cells, but this is not present at the stage of the neural tube.

Our experiments clearly revealed that degeneration of matrix cells from the period of the neural plate on induces a decrease in neuroblasts and neurons in the spinal cord. The results indicate that the matrix cells are specified in phenotype at the stage not only of neuroblast formation and neural tube but also of at the neural plate stage. The degree of neuronal decrease seemed to depend on the mitotic activity of matrix cells at the time of ENU administration.

In the normal rat fetuses of E10.5 or E11.0 a neural plate or, later a neural groove was observed. The neural tube was formed in spite of appearance of many degenerative cells 6 h after the treatment of ENU 20 mg/kg body weight.

Meningomyelocele or syringomyelia was not observed on P21. This result shows neural tube formation may occur even if the degree of cell degeneration seen in our study was induced at this stage.

According to Nornes and Das (1974), in normal rat spinal cord at the cervical segment, most of large neurons in the anterior horn and lateral aspect of dorsal horn generate on E11 and E12 (this date corresponds to E10 and E11 of our experiment), medium-sized neurons in the Rexed's (1952, 1954) laminae IV, V, VI, VII and VIII generate on E13 (E12, our experiment), and small neurons in the laminae I to VIII generate from E12 to E15 (E11 to E14, our experiment).

Anomalous spinal cords showing a reduced number of neurons in the whole gray matter have been reported in rats administered ENU on E14 and 15 (E13 and 14, our experiment) (Houle and Das 1983). This period falls in the stage of neurogenesis at the posterior horn and intermediate gray matter (Nornes and Das 1974). In the case of ENU administration on earlier stages, the fetuses were killed in the uteri (Houle and Das 1983) and the findings have therefore not been reported.

In our experiment on E10.5 to E11.5 not only large neurons in the anterior horn but also medium-sized neurons in the whole spinal gray matter were decreased in number. Thus ENU, which was given at the period of production of the anterior horn cells, damaged not only the matrix cells producing neuroblasts of the anterior horn cells but also the other proliferating matrix cells destined to produce neurons in the intermediate gray and posterior horn (Fig. 7). In the group treated on E14.0, the number of large neurons in the Rexed's laminae IV to VIII as well as the small neurons in the substantia gelatinosa was reduced. These large neurons may generate from E14.0 on.

Even though severe decrease of neurons was observed in the whole spinal gray matter in the rats administered ENU on E10.5 through E11.5, disorder in the pattern of cytoarchitecture or ectopia of the neurons was not seen. These findings were quite different from those in the cerebrum and cerebellum showing disorganized cytoarchitecture of the neurons following matrix cell degeneration (Hicks 1953; Hicks et al. 1959; Altman et al. 1969; Altman and Anderson 1971; Hallas and Das 1978; Yamano et al. 1983; Ferrer et al. 1984; Oyanagi et al. 1986). The difference seemed to result from the ventral-to-dorsal laminae formation in the spinal cord due to ventral-first dorsal-last temporal cytogenetic gradient of neuron production with different neuronal sizes (Kanemitsu 1971; Nornes and Das 1974; Altman and Bayer 1984) when compared with inside-first outside-last laminal formation in the cerebrum (Fujita 1964; Smart and Smart 1982) (Fig. 7).

The P21 rat spinal cord treated with ENU revealed no abnormal capillary proliferation, infiltration of lymphocytes and granulocytes, fibrosis nor reactive gliosis. We suggest that the fetal spinal cord owes these unique features to the presence, under normal conditions, of a vast extracellular space (as in the fetal neopallium Ikuta et al. 1983, 1984; Oyanagi et al. 1986). When degeneration occurs in matrix cells or neuroblasts migrating individually phagocytes have completed cell elimination and the developing spinal cord is restored to its original state without any necessity for cell reaction, regardless of whether glial precursor cells are present or not.

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