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EFFECTS OF CHLORAMBUCIL ON THE ULTRASTRUCTURE OF NEUROEPITHELIAL CELLS OF NEURULATING MOUSE EMBRYOS

(mice/neuroepithelial cells/chlorambucil)

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The effects of chlorambucil on the neuroepithelial cells of mouse embryos are described ultrastructurally. Pregnant ICR mice were treated with oral administration of 3 mg/kg chlorambucil at 7th day of gestation. Embryos were collected 6, 12, 24, 36, and 48 hours after treatment and used for ultrastructural study. At 6 hours after treatment, many pyknotic cells were observed in the neural fold, which were thought to be the result of the direct cytotoxic effect of chlorambucil. At 24 hours, electron-dense protrusions of cytoplasm into the lumen of neural tube, which we call cytoplasmic clump, were observed. Intranuclear inclusions, which seemed to originate from invagination of the inner membrane of the nuclear envelope, were also observed. They were thought to be the characteristic findings in hypermetabolic cells induced by chlorambucil. At 36-48 hours pyknotic cells had almost disappeared, indicating that repair had taken place.

Chlorambucil (CA) is an alkylating agent and its characteristic cytotoxic action is due to its ability to cross-link the twin strands of the DNA molecule, thus preventing its replication. CA is used in cancer chemotherapy, although it has been demonstrated to be teratogenic in rats (2), and in mice (12). These studies included descriptions of the skeletal malformations, limb defects and cranial and tail defects, which were thought to be the result of the direct cytotoxic effect of CA, resulting in the cell death of involved tissues. CA is reported to be also teratogenic in humans (13), causing agenesis of the left kidney and ureter on the CA exposed fetus.

Recently, Yoshioka *et al.* (16) reported that oral administration of 3 mg/kg CA at 7th day of gestation caused a high incidence (42%) of exencephaly in ICR mice.

The aim of this study was to investigate the teratogenicity of CA on the developing mouse CNS with special reference on the pathological effects on the neuroepithelial cells at the ultrastructural level.

MATERIALS AND METHODS

Timed pregnant females of ICR mice (day of vaginal plug = 0 day) were given an oral administration of 3 mg/kg CA, which was dissolved in cotton seed oil, at approximately 9:00 a.m. on day 7 of gestation. Litters were removed at 6, 12, 24, 36, and 48 hours after treatment. Control treatment was done with cotton seed oil in the same manner. According to Yoshioka *et al.* (16), exencephaly occurred mainly at the level of prosencephalon-mesencephalon; we therefore studied the pathological effects of CA on the neuroepithelial cells of these levels. A total of 137 embryos (treated group; 107, control group ;30) were fixed in a Karnovsky's fixative (8) for 2-3 hours at 4°C; this was followed by rinsing with 0.1M phosphate buffer containing 8% sucrose (290 mOsm) overnight. They were postfixed with 2% OsO4 in 30 mM PIPES buffer containing 4% sucrose (290 mOsm) at 0-4°C. After degraded dehydration series, they were embedded in Epon 812 (TAAB). Semithin sections and ultrathin sections of 3 embryos of each group were obtained with ultramicrotome (Reichert OmU4), then stained by toluidine blue for histological examination and uranyl acetate and lead nitrate to be examined with HS-9 transmission electron-microscope (HITACHI, Japan).

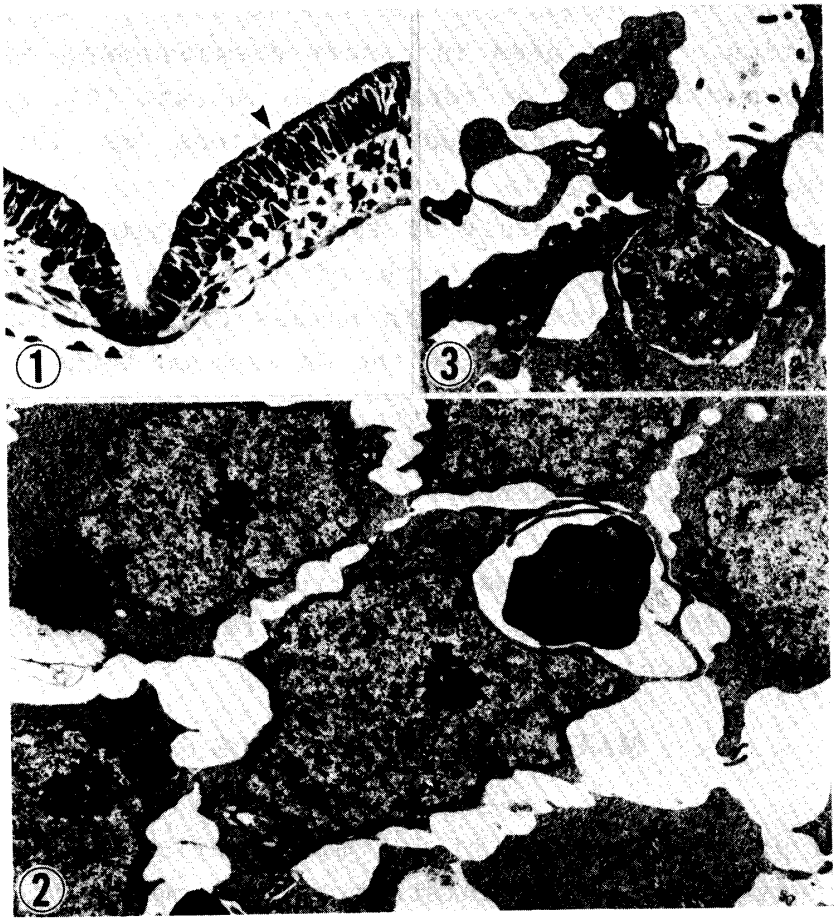

Fig.1. 6 hours after treatment (toluidine blue staining). Pyknotic cells (arrowheads) were scattered in the neural folds. (X 150)
Fig.2. 6 hours after treatment. A small-sized cell with electron dense cytoplasm and nucleoplasm is encircled by the cytoplasmic processes of the neighboring neuroepithelial cell. (X 7,500)
Fig.3. 12 hours after treatment. Degenerating cell is protruding into the lumen of the neural tube. (X 16,000)

RESULTS

At 6-12 hours after CA treatment many pyknotic cells and/or pyknotic materials were seen in the neuroepithelium throughout the examined CNS (Fig. 1). Ultrastructurally, these were degenerating cells with electron-dence cytoplasm and nucleoplasm. Some of such materials were encircled by the thin cytoplasmic processes and some were already taken in the cytoplasm of the neighboring neuroepithelial cells (Fig. 2). Occasionally, it was

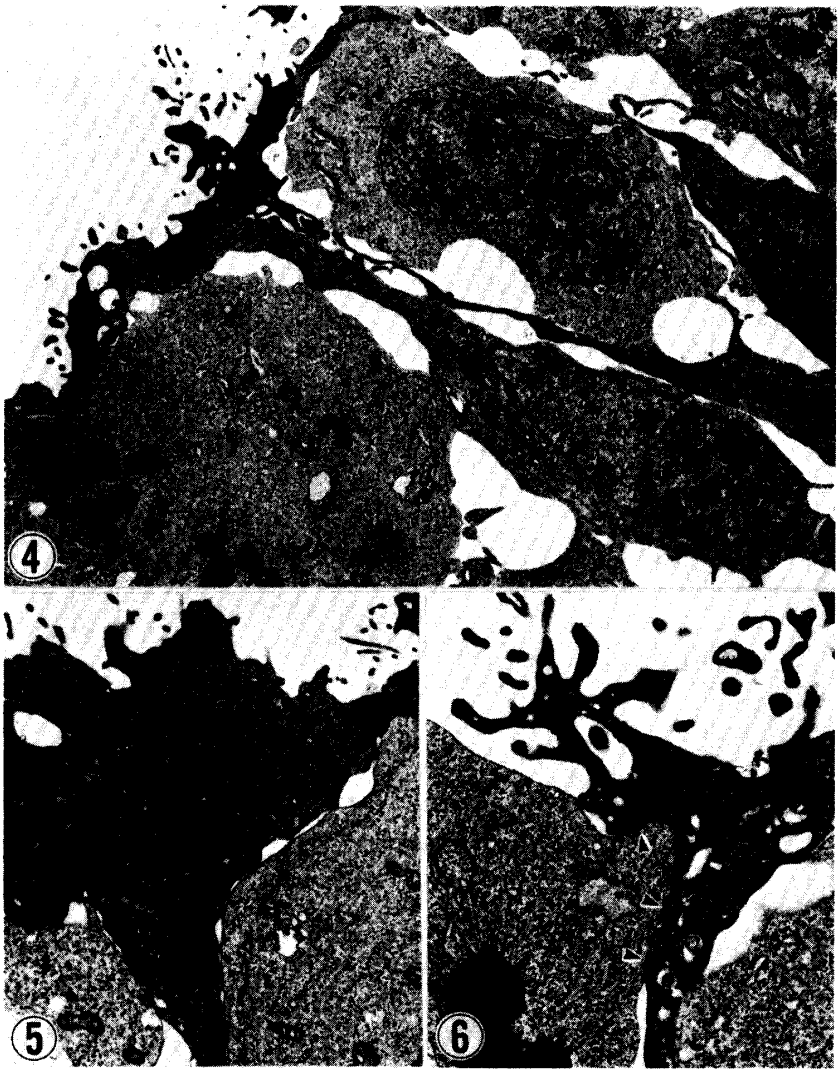

Fig.4. 36 hours after treatment. Cytoplasmic clumps of various electron densities are present along the luminal surface of the neural tube. They are continuous with the basally located nuclei by thin cytoplasmic segments. (X 7,000)
Fig.5. 24 hours after treatment. Within the cytoplasmic clumps are seen mitochondria, rough surfaced endoplasmic reticulum and vesicles. (X 12,000)
Fig.6. 36 hours after treatment. Some cytoplasmic clumps had the microvilli-like processes. Vesicles of various size are also present within it. Note the well preserved cell to cell junctions (arrowheads). (X 16,000)

observed that degenerating cells were protruding, as if pinched off into the lumen of the neural tube with the intracellular organelles in the electron-dense cytoplasm (Fig. 3).

At 24 hours after treatment, the intracellular inclusions, which were phagocytosed materials, were still observed in the neuroepithelium of the neural tube. Some neuroepithelial cells

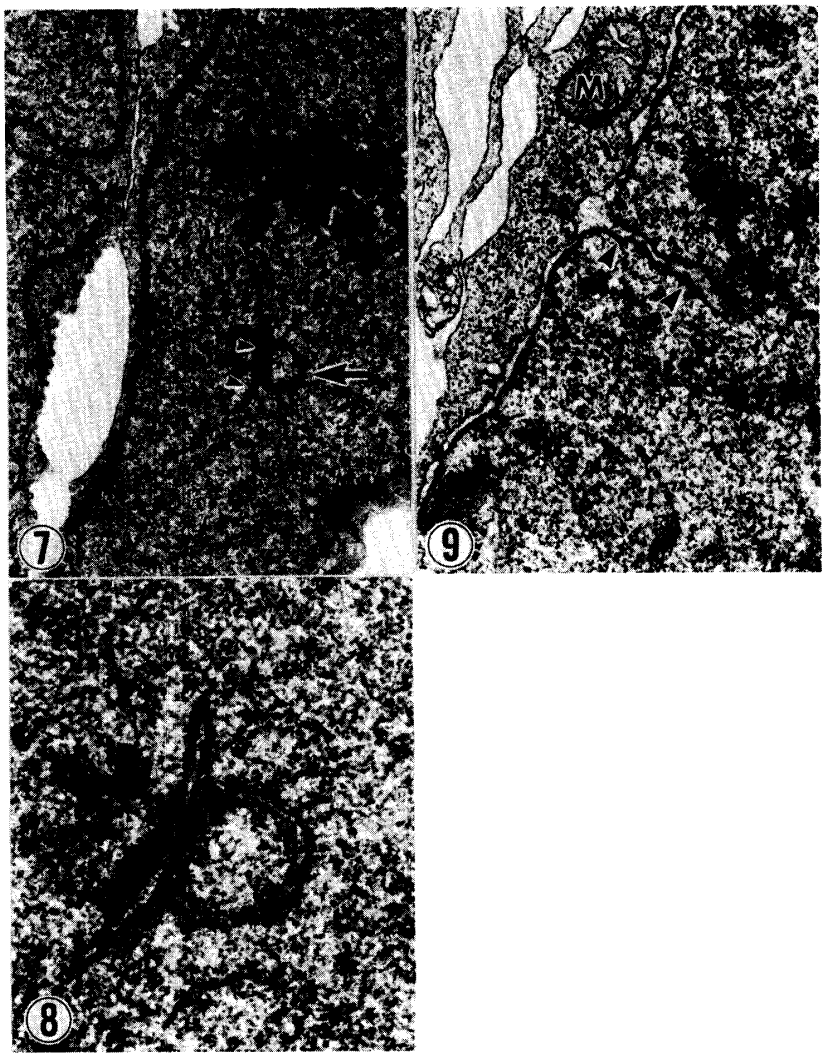

Fig.7. 24 hours after treatment. Intranuclear inclusions observed in the present study. Round shaped structure (arrow) is double membrane bound, whereas the tubular one (arrowheads) is single membrane bound. On the left is the invagination of nuclear envelope. (X 13,000)
Fig.8. Higher magnification of intranuclear inclusion shown in Fig.7. (X 50.000)
Fig.9. 24 hours after treatment. Invagination of the inner membrane of the nuclear envelope (arrowheads). M = Mitochondria (X 25,000)

were acetabulum-like shaped and their luminal cytoplasm was deeply stained by toluidine blue. These cytoplasmic clumps were more numerous than in the control group. Ultrastructurally, they were electron-dense and were continuous with the nucleus located basally by the thin segment of cytoplasm (Fig. 4). Intracellular organelles such as mitochondria, rough surfaced endoplasmic

reticulum and vesicles of various sizes were present within those cytoplasmic clumps (Fig. 5). These cytoplasmic clumps of varying electron densities were observed along the lumen of the neural tube and some had microvilli-like cytoplasmic processes and the structure of cell junctions with the neighboring neuroepithelial cells seemed to be well preserved (Fig. 6).

Another interesting finding in the present study was the appearance of intranuclear inclusions (Figs. 7, 8) in the treated group from 24 hours after administration and these were not detected in the control group. It was a single membrane bound structure within the nucleus, which seemed to originate from the inner membrane of the nuclear envelope. Perinuclear space dilatation and invagination of inner membrane of nuclear envelope were observed in some cases (Fig. 9).

At 36-48 hours after treatment, intranuclear inclusions and cell debris had almost disappeared, suggesting that repair had taken place. However, the invagination of the inner membrane of the nuclear envelope was still observed in many neuroepithelial cells.

DISCUSSION

Many investigators have suggested that in neural tube formation, the neural plate acts as an integrated epithelial sheet and the disruption of this integration may lead to neural tube defects. Teratogens, which are thought to induce neural tube defects, affect the individual neuroepithelial cells, for example, causing cell death or morphological change of junctional complexes and disruption of cytoskeletal elements; and they lead to the failure of the neural folds to close and fuse. Many intracellular inclusions at 6 hours after treatment seemed to be the results of cell death and they were morphologically identical to those induced by various agents such as cadmium (14), suggesting that it is due to the direct cytotoxic effect of CA. Cytochalasins have been reported to cause damage on the microfilament of the closing neuroepithelial cells, which is thought to be involved in purse-ring constriction of the neuroepithelial cells and lead to rolling up and closing of the opposing neural folds.

Godman *et al.* (7) used the term "zeiosis" to describe the projection of knobby protuberance at the cell surface of the Hela

cells, which was induced by the treatment of cytochalasin D. Wiley *et al.* (15) speculated that the principal target of cytochalasin B and D is the apical neuroepithelial membrane, resulting in zeiosis.

We have used the term cytoplasmic clump to designate the electron-dense cytoplasm which protrudes into the lumen of the neural tube; these clumps were continuous with nucleus located basally by the thin segments of cytoplasm. Occasionally they had microvilli-like processes and contained intracellular organelles such as mitochondria and rough surfaced endoplasmic reticulum, retaining cell junctions with neighboring neuroepithelial cells and apical microfilaments well preserved. These cytoplasmic clumps observed in the present study are acetabulum-shaped and electron-dense, which seem to be different from zeiosis which means cluster of many club-shaped cytoplasmic protrusions with slender stalks from the cell surface. Linford *et al.* (9) reported that CA affects the membrane of the red cell and removes a simple chemical component from the cell surface, resulting in hemolysis. These cytoplasmic clumps were observed from 24 hours after treatment, that is at 8 days of gestational age, the period when the rolling up of the neural folds most actively takes place. The developing mechanism of such a structure can not be well explained, although it supports the hypothesis that the disruption of integrity of the neuroepithelial layer, which is probably caused by the decrease in cell numbers, will induce the misled closing mechanism of the neural folds.

Another interesting finding in the present study is the intranuclear inclusion. Intranuclear inclusion consists of pseudoinclusion and true inclusion (6). The former arises from the result of nuclear invagination, bounded by a well-defined double membrane resembling the perinuclear membrane. The intranuclear inclusions observed in the present study are not double-membrane bound; they are single-membrane bound tubular structures, indicating that they are not the pseudoinclusions. Moreover, they seem to originate from the invagination of the inner membrane of the nuclear envelope. These true intranuclear inclusions are reported to appear not only in normal cells such as human endometrial cells (1, 4), but also in tumorous cells, including murine pulmonary tumor cells (5), Yoshida ascites hepatoma (10) and intracranial Rous sarcoma (3). Parry (11) reported single-membrane bound structures, which were thought to

originate from the inner membrane of the nuclear envelope within the nuclei of rat and mouse liver cells after short exposure in vitro to sodium tetraphenyl boron. He speculated that they are not the agonal change of dying cells but the response of the living cells, suggesting that they appear as the result of rapid synthesis or rearrangement of the nuclear envelope. As described above, intranuclear inclusions in the present study seem to be the characteristic finding in hypermetabolic cells, giving a clue to the formation of the the aberrant cells induced by CA.

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