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Apoptosis in the developing mouse embryos from T-2 toxin-inoculated dams

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Summary. T-2 toxin (3mg/kg b.w.) was orally inoculated to pregnant mice at 11 days of gestation to examine the effect of T-2 toxin on the developing embryos. At 24 hours after T-2 toxin-inoculation, moderate pyknosis or karyorrhexis was generally observed in some layers of the central nervous system, caudal sclerotomic segment, caudal region of the tongue to pharyngeal- to laryngeal-mesenchyma, trachea and facial mesenchyma. These pyknotic or karyorrhectic nuclei were strongly stained by the modified TUNEL method widely used for the *in situ* detection of apoptotic nuclei and also showed ultrastructural changes characteristic for apoptosis. This is the first report of mycotoxin-induced apoptosis in embryos.

Key words: Apoptosis, Central nervous system, Mouse embryo, Sclerotome, T-2 toxin

Introduction

T-2 toxin is a kind of trichothecene mycotoxin produced by various species of the genus *Fusarium*, which infect corn, wheat, barley, and rice crops in the field or during storage (Yagen and Bialer, 1993). This toxin is associated with mycotoxicoses in farm animals and with illness and death of humans who had ingested moldy agricultural products (Smalley, 1973; Pier et al., 1980). T-2 toxin attacks the organs containing a large number of actively dividing cells such as lymphoid and hematopoietic tissues (Hayes et al., 1980), resulting in lymphopenia and subsequent immunosuppression (Hayes et al., 1980; Schiefer and Hancock, 1984). We first clarified from the multilateral viewpoints that T-2 toxin-induced damage in lymphoid and hematopoietic organs of mice was produced by apoptosis (Li et al., 1997a,b; Shinozuka et al.,1997a,b).

On the other hand, it is recognized that oral or intraperitoneal administration of T-2 toxin to pregnant mice produces maternal toxicity, fetal death and fetal malformations (Stanford et al., 1975; Hood et al., 1978; Rousseaux et al., 1985; Rousseaux and Schiefer, 1987). The purpose of this study was too examine embryotoxicity of T-2 toxin in pregnant mice from the various viewpoints. The present animal experiment was approved by the Laboratory Animal Use and Care Committee of the Faculty of Agriculture, the University of Tokyo.

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Materials and methods

Animals

Nineteen pregnant Jcl:ICR mice (Saitama Experimental Animal Co., Saitama, Japan) were used. They were kept under controlled conditions (temperature, 23±2 °C; relative humidity, 55±5%) using an isolator caging system (Niki Shoji Co., Tokyo) and fed commercial pellets (MF, Oriental Yeast Co. Ltd., Tokyo) and tap water *ad libitum*.

Treatment and sampling

After overnight fasting at 10 days of gestation, ten mice were orally inoculated with 3mg/kg b.w. of T-2 toxin (Lot No. 117F4078, Sigma Chemical Co., St. Louis, MO) dissolved in 20% ethanol in 0.01M phosphate-buffered saline (PBS) at 11 days of gestation. The inoculation volume was adjusted to 10ml/kg b.w. using 0.01M PBS. They were sacrificed by heart puncture under ether anesthesia at 24 hours after T-2 toxin-inoculation. The remaining 9 mice inoculated with 20% ethanol in 0.01M PBS were sacrificed in the same way and served as controls.

At necropsy, all embryos and placenta of each mouse were collected and then living embryos were weighed. The collected embryos and placentas were fixed in 10% neutral-buffered formalin. Paraffin sections (4μm) were stained with hematoxylin and eosin (HE). Some of the paraffin sections were subjected to *in situ* detection of fragmented DNA and immunohistochemical staining for proliferating cell nuclear antigen (PCNA) as mentioned below. The nomenclature for the layers of the brain and spinal cord used here is in accordance with

that used by the Boulder committee (1970), Rugh (1990) and Kaufman (1992).

In situ detection of fragmented DNA

DNA fragmentation was examined on the paraffin sections of the embryos by the modified TUNEL method first proposed by Gavrieli et al. (1992) with a commercial apoptosis detection kit (ApopTag In situ Apoptosis Detection Kit; Oncor, Gaithersburg, MD). In brief, the procedure was as follows: multiple fragmented DNA 3'-OH ends on the paraffin sections were labeled with digoxygenin-dUTP in the presence of terminal deoxy nucleotidyl transferase (TdT). Peroxidase-conjugated anti-digoxygenin antibody was then reacted with the sections. Apoptotic nuclei were visualized by peroxidase-diaminobenzidine (DAB) reaction. The sections were then counterstained with methylgreen.

Immunohistochemical staining for PCNA

In addition, to evaluate the proliferative activity of cells in the embryo, immunohistochemical staining for PCNA was carried out on the paraffin-sections by the avidin-biotin-peroxidase complex (ABC) method using ABC kit (Vector Laboratories, USA). Anti-PCNA mouse antibody (clone PC10; Novocastra, Newcastle, UK) was used as the primary antibody. The sections were visualized by peroxidase-diaminobenzidine (DAB) reaction and then counterstained with methyl green.

Electron microscope

For electron microscopic examination, small pieces

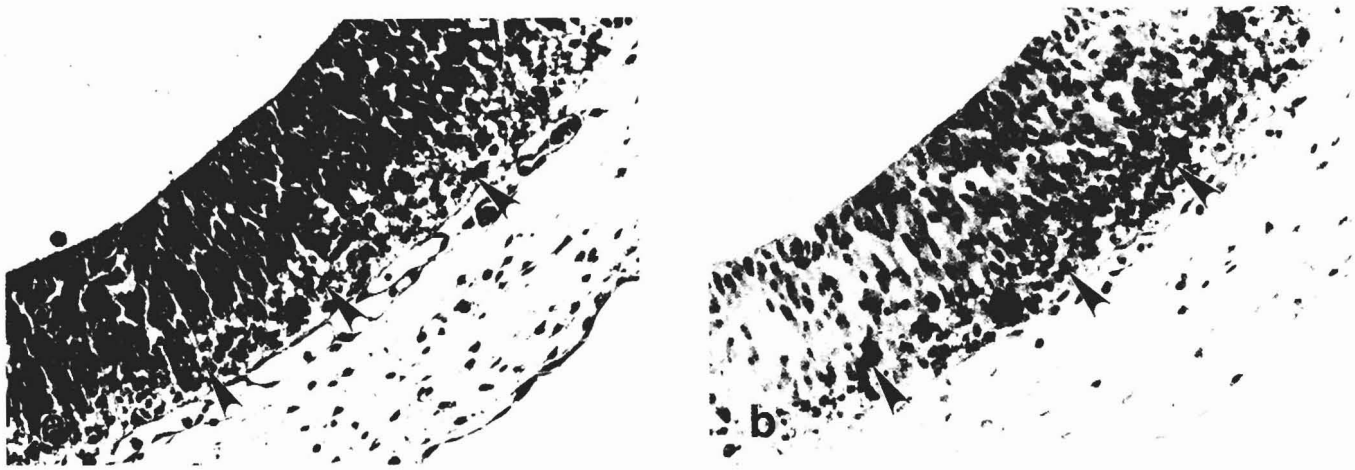

Fig. 1. Telencephalon of a embryo from a T-2 toxin inoculated dam. Many pyknotic or karyorrhectic neuronal cells are seen in the peri-ventricular zone (arrowheads) **(a)**, and almost all of these nuclei are TUNEL-positive (arrowheads) **(b)**. a, HE, x 270; b, TUNEL method, x 270

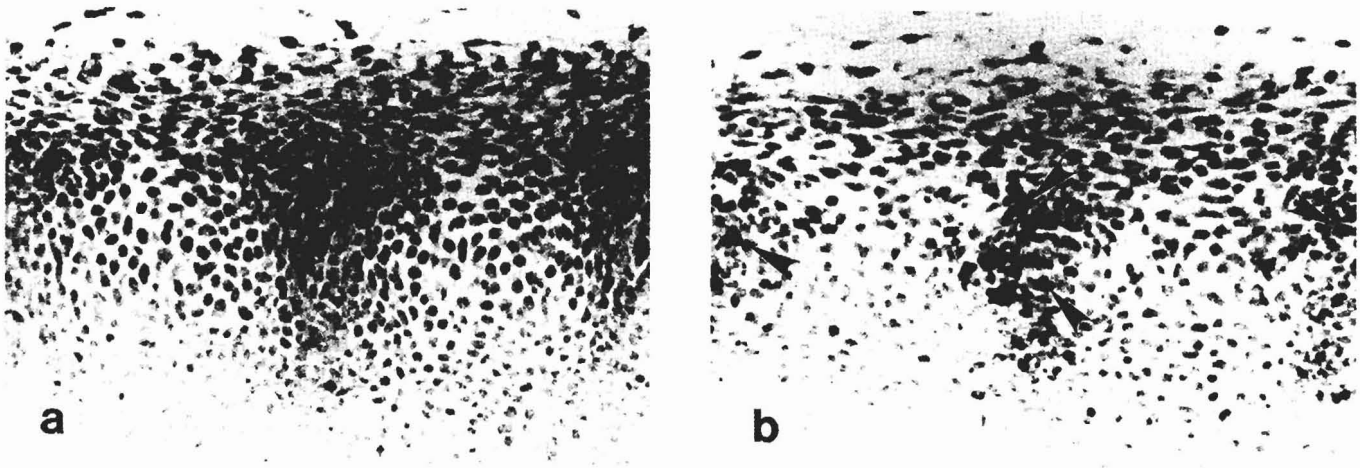

Fig. 2. Caudal sclerotomic segment of a embryo from a control dam **(a)** and a embryo from T-2 toxin-inoculated dam **(b)**. Many TUNEL-positive cells (arrowheads) are seen in (b) while almost no positive cells are found in (a). TUNEL method, x 300

of the brain were fixed in 2.5% glutaraldehyde in 0.1M PB (pH7.4), postfixed in 1% osmium tetroxide in the same buffer, and embedded in Epok 812 (Ohken Co. Ltd., Tokyo). Ultrathin sections were double stained with uranyl acetate and lead citrate and observed under a JEM-1200 EX electron microscope (JEOL Co. Ltd., Tokyo).

Results

In the T-2 toxin-group, two mice died during the observation period, and these two and one other living one mouse showed vaginal hemorrhage. As to the embryos, 37.5% of them (60/160 embryos) died and/or resorbed, but the embryonal death and/or resorption rate was different among dams. On the other hand, in the control group, the total death and/or resorption rate was 4.4% (6/135 embryos). In the T-2 toxin-group, embryonic weight loss was observed as compared with the control group (control group: 0.108g, T-2 toxin-group: 0.079g).

Light microscopically, in the control-group, only a few cells in the liver and mesenchyma showed pyknosis or karyorrhexis. In the embryos from 8 dams in the T-2 toxin-group, moderate pyknosis or karyorrhexis was generally observed in the telencephalon (Fig. 1), diencephalon, myelencephalon, caudal sclerotomic segment (Fig. 2), caudal region of the tongue to pharyngeal-, laryngeal-mesenchyma, trachea and facial mesenchyma. Almost all of these nuclei were strongly stained by the modified TUNEL method, and the incidence of TUNEL-positive cells was 5 to 30% (Fig. 3). Similar but less severe changes were also observed in the mesencephalon, metencephalon, spinal cord, esophagus, intestine, heart, liver, lungs, adrenal primordium, metanephron, pancreatic primordium, dorsal root ganglions, trigeminal ganglions and other parts of mesenchyma. The number of TUNEL-positive cells was somewhat different between embryos from the same dam.

In the central nervous system (CNS) of the T-2 toxin-group, the incidence of TUNEL-positive cells was 10-20% in the neuroepithelia of the peri-ventricular zone of the telencephalon (Fig. 4a). A somewhat lower number of pyknotic cells were detected in the ventricular and intermediate zones of the diencephalon, mesencephalon, metencephalon, and the junction of ependymal and mantle layers of the spinal cord. In the CNS of

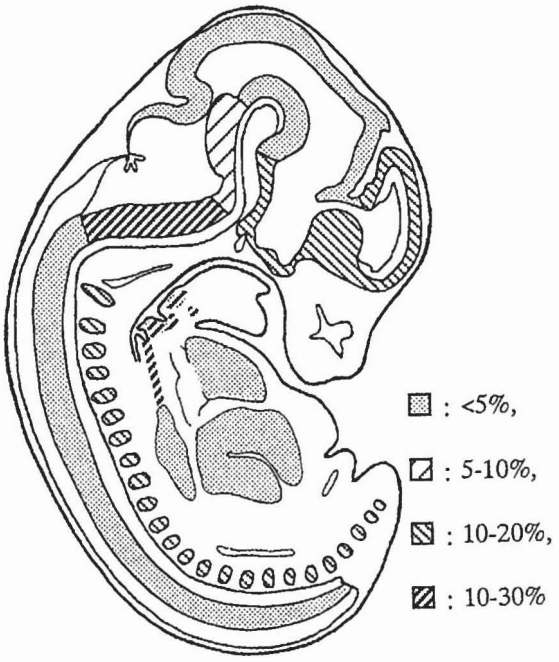

Fig. 3. Distribution and incidence of TUNEL-positive cells in embryos from T-2 toxin-inoculated dams.

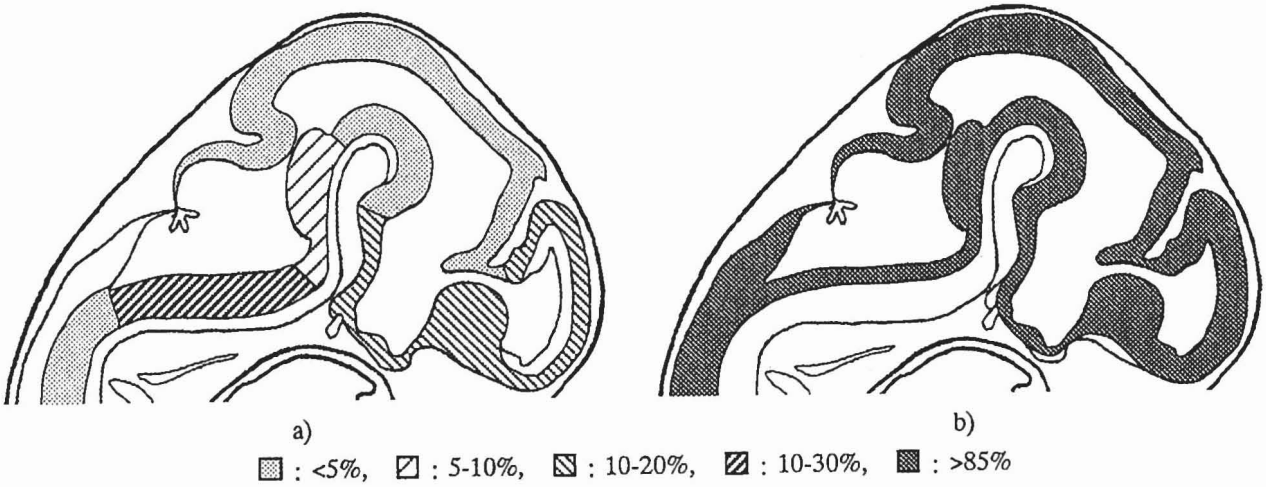

Fig. 4. Distribution and incidence of TUNEL-positive neuronal cells in the central nervous system of embryos from T-2 toxin-inoculated dams **(a)** and those of PCNA-positive ones of embryos from control dams **(b)**.

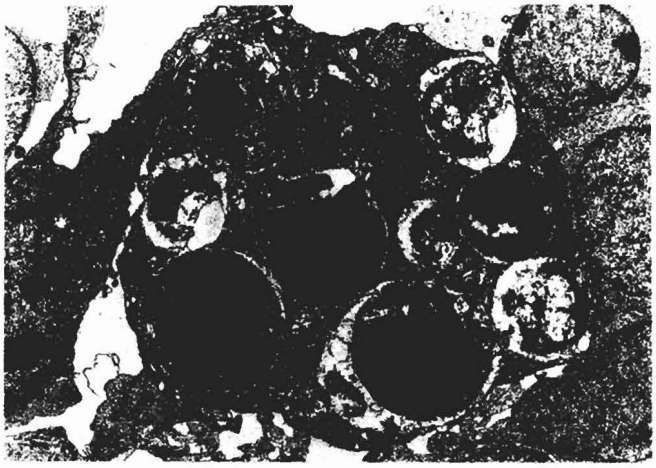

Fig. 5. Telencephalon of a embryos from a T-2 toxin-inoculated dam. Neuronal cells showing prominent condensation of nuclear chromatin are ingested by macrophage. x 3,400

control-group, PCNA-positive cells were observed practically restricted to the ventricular zone of the brain and the ependymal layer of the spinal cord, and the incidence of PCNA-positive cells was more than 85% in the neuroepithelia of the ventricular zone (Fig. 4b). The regions containing TUNEL-positive cells in the T-2 toxin-group were consistent with those containing PCNA-positive cells in the control group on the whole. However, a small number of TUNEL-positive cells were also detected in some regions where PCNA-positive cells were not found.

Ultrastructurally, in the brain, neuroepithelial cells characterized by shrinkage of the cell body, condensation of nuclear chromatin and/or margination of condensed chromatin along the nuclear membrane were observed (Fig. 5). Some cell nuclei were fragmented into small pieces, and these small pieces were sometimes ingested by adjacent neuroepithelia and macrophages.

Discussion

In this study, toxic effects of T-2 toxin on mouse embryos were examined at 24 hours after the inoculation to pregnant mice at 11 days of gestation. In the T-2 toxin-group, dead cells showing pyknosis or karyorrhexis were observed in the CNS, viscera and mesenchyma, and these nuclei were strongly stained by the modified TUNEL method which detects fragmented DNA *in situ*. Electron microscopically, the dead cells were characterized by shrinkage of the cell body, condensation of nuclear chromatin and nuclear fragmentation. These morphological characteristics are well consistent with those of apoptosis (Kerr et al., 1972).

Previous reports have also shown that T-2 toxin induced maternal and fetal death, decrease in number of fetuses, fetal weight loss, fetal malformations, placental hemorrhage and necrosis of the fetus brain as observed in the present study (Stanford et al., 1975; Hood et al., 1978; Khera, 1984; Rousseaux et al., 1985; Blakley et al., 1987; Rousseaux and Schiefer, 1987). However, there have been no reports indicating that T-2 toxin-induced embryonic cell death might be apoptosis. Similar changes in the fetus brain has also been reported in the case of Ochratoxin A intoxication (Hood et al., 1978; Szcsech and Hood, 1981; Fukui et al., 1987), and neuronal cell death in that case also seems to be apoptosis.

The regions containing TUNEL-positive cells in the T-2 toxin-group were consistent with those containing PCNA-positive cells in the control group. Therefore, T-2 toxin is considered to induce apoptosis in the actively proliferating cells in embryos probably through its radiomimetic effect as observed in lymphoid and hematopoietic organs (Li et al., 1997b; Shinozuka et al., 1997a,b). However, it should not be neglected that a small number of TUNEL-positive cells were also observed in some regions where PCNA-positive cells were not detected.

It is well known that oral administration of T-2 toxin to pregnant mice produces bone malformations such as incomplete ossification, absence of bones, wavy bones and fused bones (Rousseaux and Schiefer, 1987), and it is hypothesized that such fetal bone malformations are secondary to maternal toxicity of T-2 toxin (Khera, 1984; Rousseaux and Schiefer, 1987). In the present study, apoptotic cell death was prominently observed in the caudal sclerotomic segment in the T-2 toxin-group. This suggests that at least a part of T-2 toxin-induced fetal bone malformations might be initiated by apoptosis in the caudal sclerotomic segment.

To our knowledge, this is the first report of apoptosis in the developing embryos induced by mycotoxin. Now, further study on the mechanism of T-2 toxin-induced apoptosis in the mouse embryos is in progress to distinguish between direct effects of T-2 toxin on embryos and indirect ones secondary to maternal toxicity.

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