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Induction of apoptotic cell death in rat thymus and spleen after a bolus injection of methamphetamine

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Abstract We examined whether methamphetamine (MAP) induced apoptotic cell death in vivo. Male Wistar rats were injected intraperitoneally with 25 mg MAP/Kg body weight and were sacrificed at 4, 8 and 24 h. As early as 4 h after a single dose of MAP, DNA ladder bands representing DNA fragmentation into multiples of the internucleosomal DNA length of about 180 bp were observed by gel electrophoresis in thymic and splenic DNA. DNA from control rats injected with 1 ml physiological saline/Kg body weight showed no ladder band patterns. The proportion of fragmented DNA from the thymus increased in a time-dependent manner up to 8 h and faint ladder band patterns were observed at 24 h, indicating that cell death via apoptosis occurred at an early stage and then apoptotic bodies were scavenged. DNA fragmentation in the thymus and spleen induced with MAP was also confirmed by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method in situ. In control thymus samples, stained cells were numerous in the cortex but sparse in the medulla. At the boundary area between the cortex and medulla, stained cells were seen as a layer. In the MAP-treated rats, stained cells were increased and dispersed equally in the cortex and medulla. In control spleen samples, stained cells were numerous in all areas excluding the germinal centers. Cells at the germinal centers were stained intensively in MAP-treated rat spleen. Light microscopical analyses allowed us to identify lymphocytes during the course of apoptotic cell death. Electron microscopic studies showed morphological landmarks for the process of cellular apoptosis in both organs e.g. lymphocytes with chromatin condensed into crescents at the periphery of the nuclei and apoptotic bodies. These results indicate that MAP induced cell death of the thymic and splenic lymphocytes via apoptosis.

Key words Apoptosis · Methamphetamine · Lymphocytes · Thymus · Spleen

Introduction

Apoptosis is a mode of cell death morphologically and biochemically distinct from necrosis (Kerr et al. 1972; Wyllie et al. 1980; Arends and Wyllie 1991). It is characterized by loss of contact between neighbouring cells, shrinkage and condensation of cytoplasm, loss of plasma membrane microvilli, cleavage of chromosomal DNA into nucleosomal size fragments of 180 bp or multiples thereof caused by activation of endogenous endonuclease (Searle et al. 1982; Cohen and Duke 1984; Wyllie et al. 1984; Arends et al. 1990; Cohen et al. 1993) and degradation of cells to membrane-bound particles (apoptotic bodies) that are rapidly phagocytized by macrophages without an inflammation response. DNA fragmentation is experimentally recognizable by gel electrophoresis as a 180 bp ladder band pattern and as a characteristic pattern of nucleic staining by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) in situ (Gavrieli et al. 1992). In this method, nuclei of apoptotic cells incorporate exogenous nucleotides (dUTP-biotin) in the presence of terminal deoxynucleotidyl transferase. Initiation of apoptotic cell death plays a major role in the control of the shape and size in normal and many pathological conditions and the effect of chemical agents, such as irradiation, ultraviolet exposure, anti-cancer drugs, viral infection and hyperthermia etc. and in a variety of tissues (Arends and Wyllie 1991).

In the studies reported here methamphetamine (MAP), one of the most widely abused stimulants in Japan, was found to induce apoptotic cell death in thymic and splenic lymphocytes in vivo.

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Materials and methods

Animal experiments

All animal experiments were performed in accordance with the Committee for experiments with animals at Nagoya City University, and performed along the guidelines of the Committee. Male Wistar rats weighing 200 g–250 g were maintained on a standard laboratory diet and given water ad libitum. The rats were injected intraperitoneally with methamphetamine (MAP, Dainippon Pharmaceutical, Osaka, Japan) dissolved in sterile 0.85% NaCl at a dose of 25 mg/Kg body weight. Control rats were injected intraperitoneally with 1 ml/Kg of sterile 0.85% NaCl as placebo. The rats were sacrificed with ethyl ether at 4, 8 and 24 h after injection. Blood was collected from the inferior vena cava with a plastic syringe containing 1:10 (vol/vol) of 0.1% sodium heparin solution. Tissues from the brain, heart, lung, thymus, spleen, liver, kidney, adrenal gland and skeletal muscle (quadriceps femoris) were collected.

Genomic DNA extraction and gel electrophoresis

After removal of organs small portions were stored at –80° C until use. The stored organs were minced with sterile scissors in sterile phosphate buffered saline (PBS). The suspensions were centrifuged at 3000 rpm for 15 min and the supernatants were removed. The pellets were resuspended in a lysis buffer containing 10 mM Tris-HCl (pH 8.0), 0.1 M EDTA (pH 8.0), 20 µg/ml pancreatic DNAase-free RNAase (Sigma, St. Louis, Mo.) and 0.5% SDS and incubated for 3 h at 50° C with proteinase K (Wako Pure Chemical, Osaka, Japan) at a final concentration of 100 µg/ml. DNA was extracted by phenol and chloroform followed by ethanol precipitation. The pellets were resuspended in TE buffer (Tris-HCl pH 8.0 and 1 mM EDTA) and treated with RNAase for 3 h at 37° C. The DNA was re-extracted by phenol and chloroform followed by ethanol precipitation and finally resuspended in TE buffer. Frozen blood samples were thawed in a water bath at room temperature, transferred to a centrifuge tube, and diluted with 10 vol. of sterile 0.2% NaCl. The suspensions were centrifuged at 3000 rpm for 15 min at room temperature, the pellets resuspended in a lysis buffer, and processed through DNA extraction. The concentration of DNA was measured by spectrophotometry, and 10 µg of each DNA was electrophoretically fractionated on a 2% agarose (NuSieve 3:1 Agarose, Takara, Otsu, Japan) gel with ethidium bromide.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL)

To detect DNA fragmentation in situ the TUNEL method was performed by the method described by Gavrieli et al. (1992) with minor modifications. After removal of the organs small portions were immediately fixed in buffered formalin solution and embedded in paraffin. Sections 5 µm thick were adhered to slides pretreated with a 0.01% aqueous solution of poly-L-Lysine (Sigma, St. Louis, Mo.). Deparaffinized and hydrated sections were incubated with 20 µg/ml proteinase K for 15 min at room temperature, and washed 4 times in distilled water. Endogenous peroxidase was inactivated by incubating the sections with 0.3% H_2O_2 in methanol for 10 min at room temperature. The sections were rinsed with distilled water, and immersed in TdT buffer (30 mM Tris pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TdT and biotinylated dUTP in TdT buffer were then added to cover the sections and incubated in a humid box at 37° C for 60 min. The reaction was terminated by transferring the sections to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at room temperature. The sections were rinsed with distilled water, covered with a 0.5% aqueous solution of skimmed milk (Yukijirushi Nyugyo, Tokyo, Japan) for 10 min at room temperature, rinsed in distilled water, and immersed in PBS for 5 min. The sections were covered with avidin-peroxidase (Sigma, St. Louis, Mo.) diluted 1:20 in water, incubated for 30 min at 37° C, washed in distilled water for 60 min, and stained with 3-amino-9-ethylcarbazole (AEC, Sigma, St. Louis, Mo.) for 20 min at 37° C. Another section from the same organ was deparaffinized, processed through proteinase K, and endogenous peroxidase inactivation as described. The section was then pretreated with DN buffer (30 mM Tris pH 7.2, 140 mM potassium cacodylate, 4 mM $MgCl_2$, 0.1 mM DTT). DNAase (10 mg/ml, Sigma, St. Louis, Mo.) dissolved in DN buffer was added to cover the section. After a 15 min incubation at room temperature, the section was washed extensively with distilled water, and continued to be processed through DNA nick end labeling.

Histological examination

Sections from paraffin blocks of each organ were stained with hematoxylin and eosin. Immediately after the animals were sacrificed, a 1 mm^3 piece from each organ was fixed in Karnowsky solution and postfixed in 1% phosphate-buffered osmium tetroxide (pH 7.4), dehydrated, and embedded in Epon812 (Nisshin EM, Tokyo). Thin sections of 10–20 blocks from each sample were stained with toluidine blue and examined by light microscopy. Ultrathin sections (1–2 Å) were stained with uranyl acetate and lead citrate and examined under a JEOL200CX electron microscope.

Results

DNA fragmentation induced with methamphetamine

Agarose gel electrophoresis was used to examine the nature of fragments appearing in the DNA from the organs of MAP-treated rats. In spite of the time elapsed prior to sacrifice, the DNA extracted from the control of each rat organ showed a single band close to the loading site of the

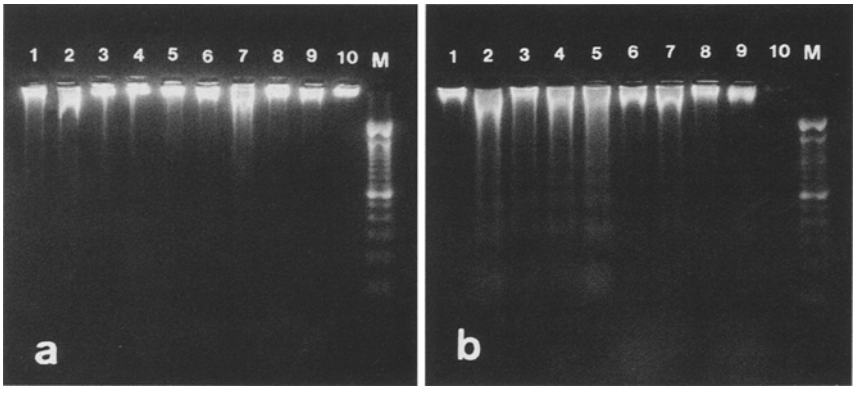

Fig. 1 Agarose gel electrophoresis showing a) absence of internucleosomal cleavage of DNA prepared from organs of control rats 4 h after saline injection and b) DNA fragmentation patterns in MAP-injected rats after 4 h. 1 brain; 2 heart; 3 lung; 4 thymus; 5 spleen, 6 liver; 7 kidney; 8 adrenal grand; 9 skeletal muscle (quadriceps femoris); 10 blood; M 100 bp ladder marker.

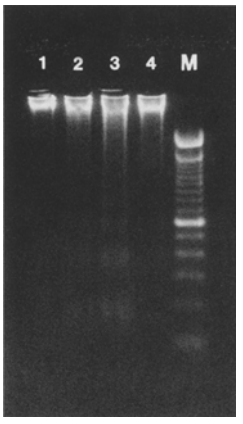

Fig. 2 Agarose gel electrophoresis showing the time course of MAP-injected effects on the electrophoretic behaviour of DNA from rat thymus. 1 control thymus; 2 4 h after MAP injection; 3 8 h; 4 24 h; M 100 bp ladder marker

gel, a typical electrophoretic pattern of high molecular weight nuclear DNA (Fig. 1a). As early as 4 h after a single dose of MAP, DNA ladder bands representing fragmentation into multiples of the internucleosomal DNA of lengths of about 180 bp were observed in DNA from the heart, lung, thymus and spleen (Fig. 1b). The time course of the proportion of fragmented DNA was determined using thymus DNA. The proportion of fragmented DNA from the thymus increased in a time-dependent manner up to 8 h, and faint ladder patterns were observed at 24 h (Fig. 2).

TUNEL method

DNA fragmentation in the thymus and spleen induced with MAP was also confirmed by the TUNEL method described by Gavrieli et al. (1992). In control thymus samples, TUNEL-positive cells were numerous in the cortex but sparse in the medulla. At the boundary area between the cortex and medulla, positive cells were seen as a layer (Fig. 3a). As demonstrated in Fig. 3b, injection of MAP cause a rapid, marked increase in positive cells in the cortex and medulla. The apoptotic cells aggregated in large clusters. In the normal spleen samples, TUNEL-positive cells were numerous in the areas excluding the germinal centers and scattered in the germinal centers (Fig. 3c). Treatment with MAP caused a marked increase in positive cells in the germinal centers (Fig. 3d). The omission of either TdT or the biotinylated substrate gave completely negative results and pretreatment with DNAase I caused an intensive staining of all nuclei in the preparations.

Morphological findings

Microscopical analyses of thin sections from paraffin-embedded MAP-treated rat thymus and spleen allowed us to identify the presence of typical lymphocytes with condensed nuclei stained intensively with hematoxylin (Fig. 4a, b). These nuclei were not detected in sections from control rat thymus and spleen.

The electron micrographs shown in Fig. 5a–c exemplify the nuclear and cytoplasmic changes that are de-

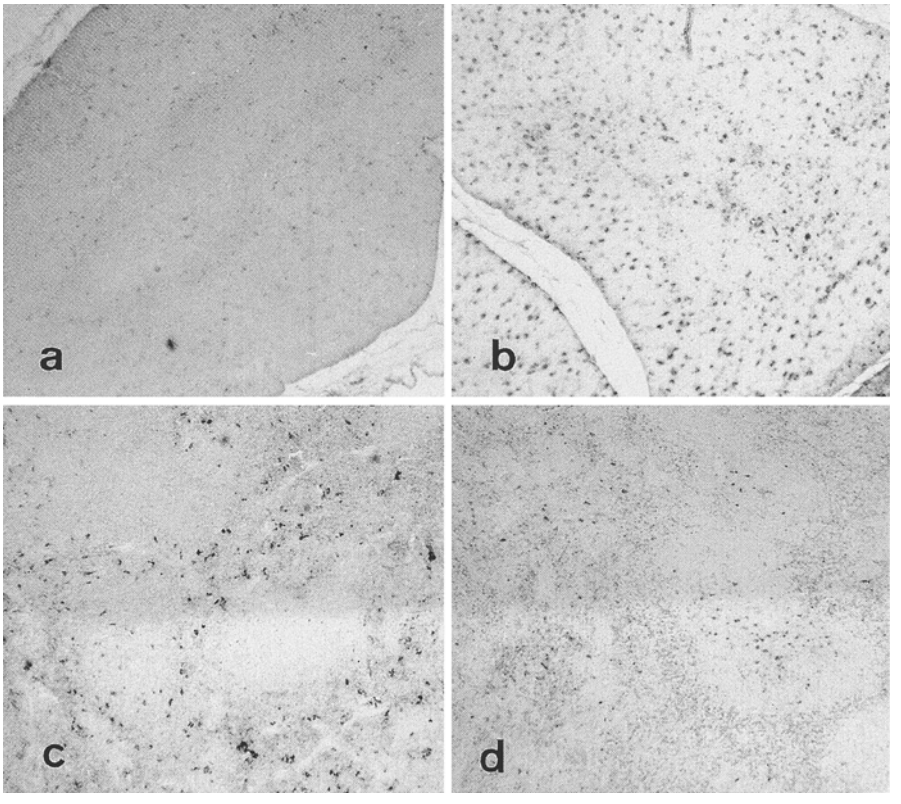

Fig. 3 Detection of apoptotic cells in paraffin-sections of thymus and spleen (4 h after injection of MAP or saline, × 165) with the TUNEL method. a control rat thymus; b MAP-injected rat thymus; c control rat spleen; d MAP-injected rat spleen

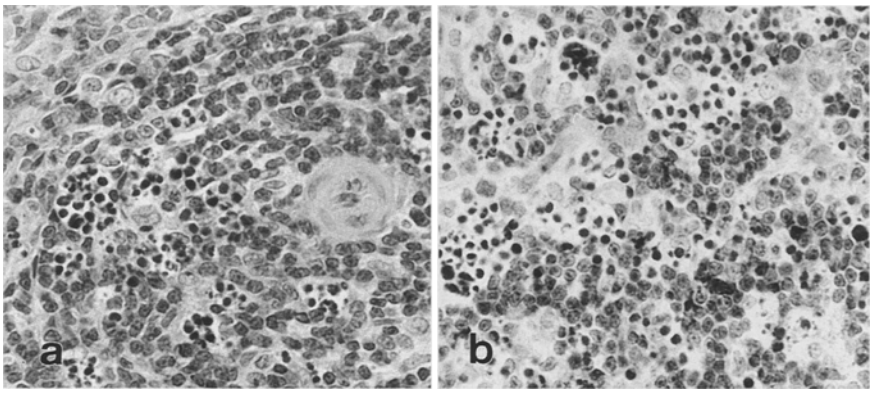

Fig. 4 Light micrographs showing the presence of typical lymphocytes with condensed nuclei stained intensively with hematoxylin (4 h after injection of MAP, × 165). a thymus; b spleen

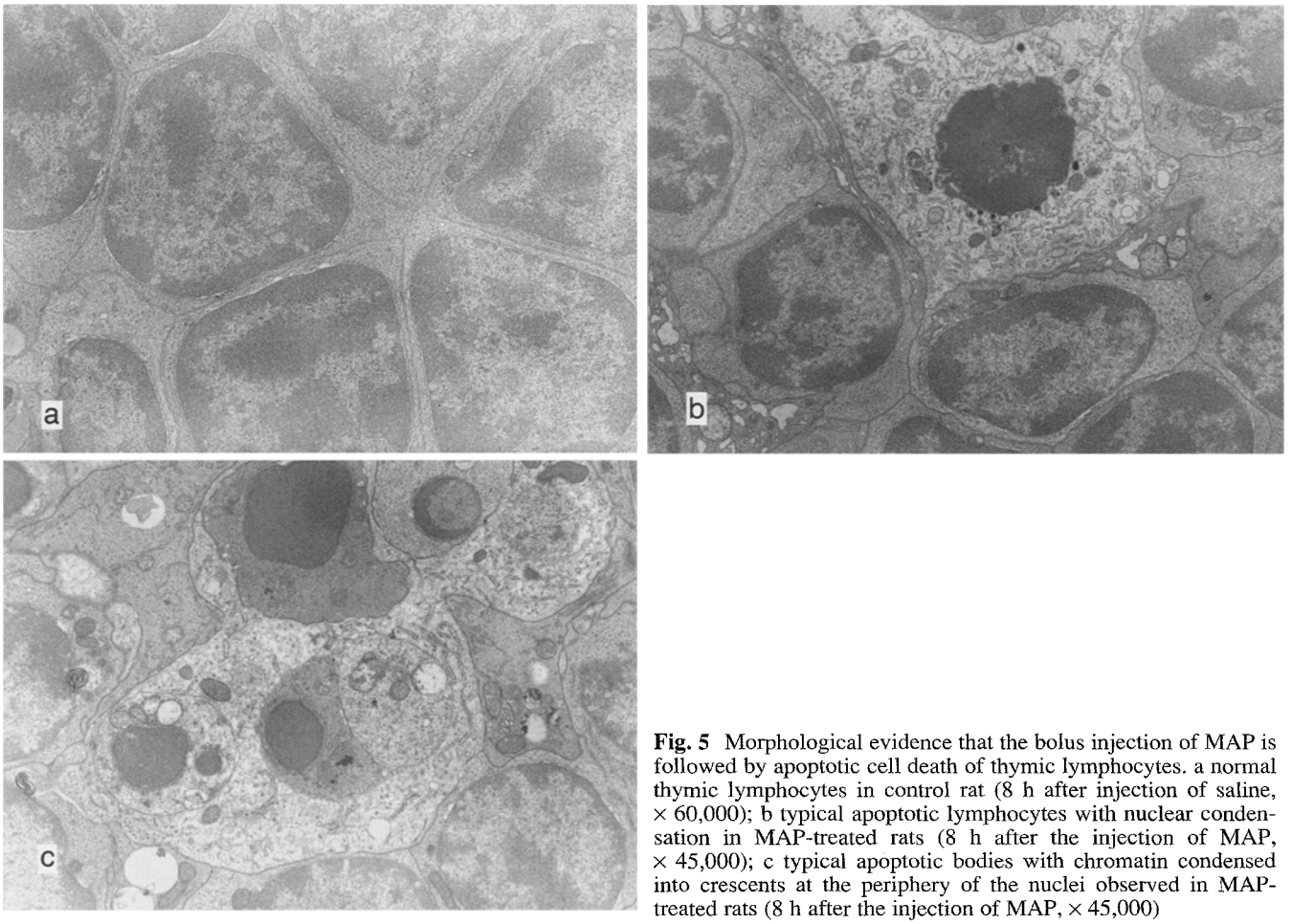

Fig. 5 Morphological evidence that the bolus injection of MAP is followed by apoptotic cell death of thymic lymphocytes. a normal thymic lymphocytes in control rat (8 h after injection of saline, × 60,000); b typical apoptotic lymphocytes with nuclear condensation in MAP-treated rats (8 h after the injection of MAP, × 45,000); c typical apoptotic bodies with chromatin condensed into crescents at the periphery of the nuclei observed in MAP-treated rats (8 h after the injection of MAP, × 45,000)

tected in the MAP-treated rat thymus and spleen. These changes, which are characteristic of apoptosis, include lymphocytes with condensed chromatin and apoptotic bodies. These alterations were not presented in the control samples.

Discussion

During the last decade, about 15,000–23,000 persons have been arrested on suspicion of amphetamines abuse in Japan. Abusers usually administer the compound by intravenous injection because this produces an enhanced effect. The injection route of administration leads to a sequence of medical problems. Transmission by contaminated needles is a major carrier for HIV and other infectious diseases, such as bacterial endocarditis and hepatitis (Balster 1991). With reference to infectious diseases, there are a few reports on the immunological response to amphetamines. Freire-Garabal et al. (1991) reported that mice chronically injected with amphetamine showed a decrease in thymus and spleen T-cells, and a blastogenic response of spleen cells to Con A. They also showed that amphetamine inhibits the development of immunity to

Listeria monocytogenes and reduces the capacity of mice to the passive transfer of immunity to *Listeria monocytogenes*. The findings in the present study that MAP induces apoptotic cell death in thymic and splenic lymphocytes simplify the interpretation of their results, as amphetamines cause apoptotic cell death of the lymphocytes responsible for the reduction in immunological responses.

Apoptosis is a term first used to describe a physiological process of cell death distinguishable from necrosis (Kerr et al. 1972). This process may be part of the homeostatic mechanisms that function to control cell number in a wide variety of tissues (Wyllie and Duvall 1992). In the immune system, a mode of apoptosis is used to delete autoreactive T-cell clones during thymic maturation and to delete splenic B cells for clonal abortion (Cohen 1991). Recently Surh and Sprent (1994) using the TUNEL method used in the present study, reported direct evidence that the excess cells of the thymus die in situ by the process of apoptosis and are cleared by thymus macrophages. TUNEL-positive cells in normal spleen were limited to isolated cells in the germinal centers. Positive cells in normal mouse thymus were sparce in the medulla but were found scattered thoughout the cortex. Our findings in control rat thymus showed close agreement with their observations, supporting that deletion of autoreactive T-cell clones occurs at the boundary area between the cortex and medulla (Roitt 1994). In the control spleen, we observed many TUNEL-positive cells lying outside the germinal centers in contradiction to their results. It has been known that T- and B-lymphocytes in the spleen are largely separated into different anatomical compartments: B-cells are distributed in the follicle, germinal center and marginal zone and T-cells are in the peri-arteriolar lymphocyte sheath and marginal zone (Roitt 1994). However, our discrepancy is unresolved and under consideration. TUNEL-positive cells which increased in the germinal centers after MAP-injection are considered to be B-lymphocytes. They also showed the distribution of TUNEL-positive cells in the mouse thymus with apoptotic cell death induced by anti-CD3 antibody or irradiation. These treatments caused an increase of apoptotic cells in the cortex but not in the medulla. In the present study, we observed an increase of the TUNEL-positive cells in both cortex and medulla in rat thymus with MAP-induced apoptosis. These findings suggest that there are differences between MAP and anti-CD3 antibody or irradiation in the mechanisms inducing apoptosis of thymic lymphocytes.

Possible mechanisms could be surmised to account for the induction of apoptosis of thymic and splenic lymphocytes with MAP. 1) MAP and/or its metabolites might directly induce apoptosis of target cells. 2) It was reported that dopamine induced apoptotic-like cell death in cultured chick sympathetic neurons (Ziv et al. 1994), and similarly MAP-enhanced catecholamine could induce apoptosis. 3) MAP and/or its metabolites could be the ligand of the Fas antigen on lymphocytes as described by Yonehara et al. (1989). 4) MAP could activate the hypothalamic-pituitary-adrenal axis as described in morphine-induced apoptosis of thymocytes (Fuchs and Pruett 1993) followed by excess production of glucocorticoids responsible for the induction of apoptotic cell death of lymphocytes (Cohen and Duke 1984). 5) It was reported that apoptosis in thymocytes could be induced using low doses of calcium ionophore (Wyllie et al. 1984) and a cytosolic calcium increase by amphetamines (Hurd and Ungerstedt 1989) might activate endonucleases which cause chromatin cleavage (McConkey et al. 1989). 6) A sustained and moderate rise in cytosolic calcium is associated with a cascade of induction of new messenger RNA species such as certain oncogenes associated with apoptosis (Williams 1991). Induction of the proto-oncogene, c-fos, with amphetamine in striosome-matrix compartments and limbic subdivisions of the striatum has been reported (Graybiel et al. 1990), suggesting that certain oncogenes induced with MAP might act as a trigger for apoptosis. Finally, MAP might activate as yet unknown mechanisms. The mechanisms of MAP-induced apoptosis of thymic and splenic lymphocytes are under investigation.

In conclusion, MAP-induced cell death via apoptosis of the thymic and splenic lymphocytes suggests that apoptotic cell death of lymphocytes is added to the conventional physical effects of acute and chronic MAP toxicity and the problems of infectious diseases transmitted by contaminated needles. Apoptosis of other organs induced by MAP is under investigation.

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