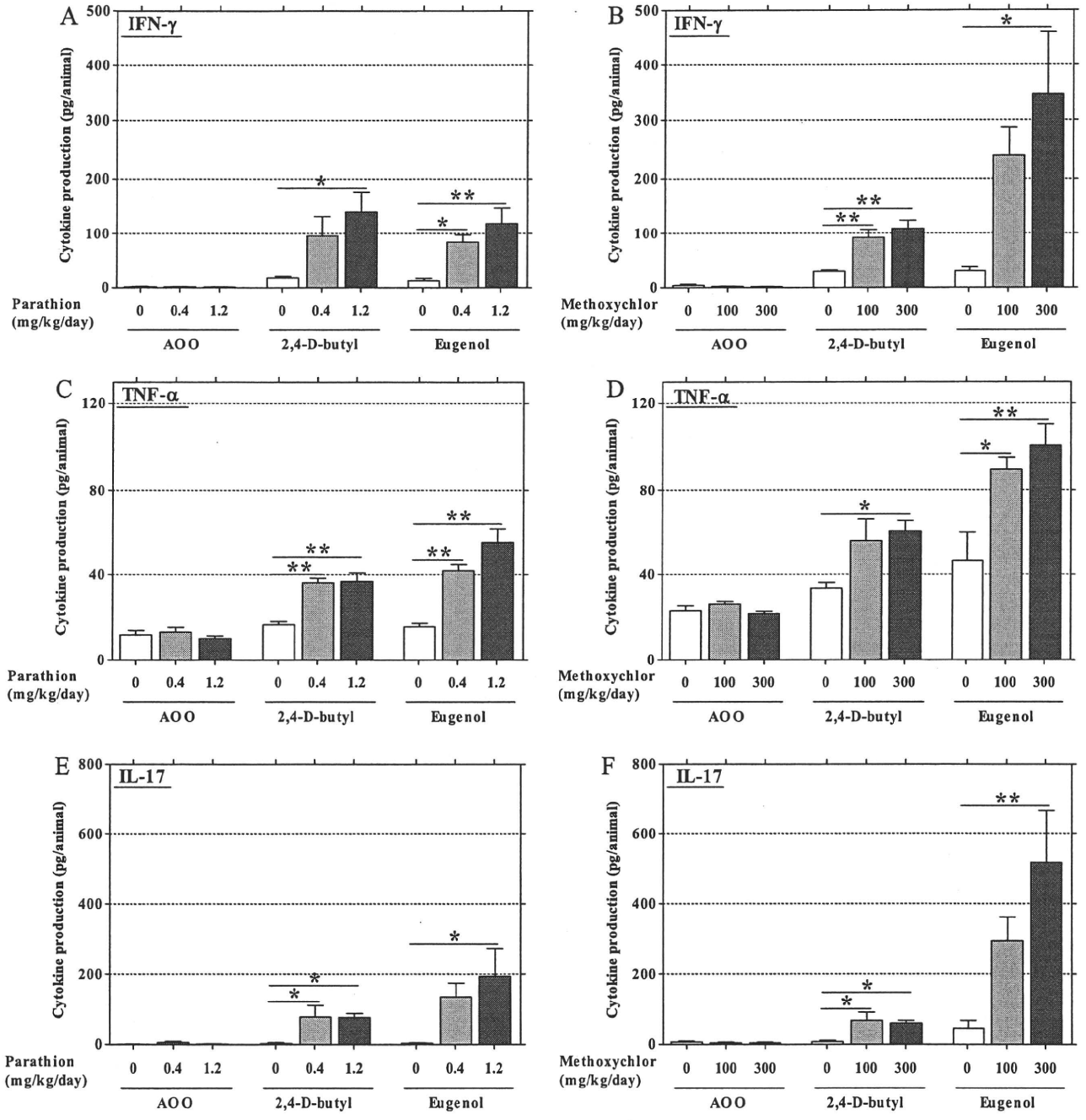

Fig. 4. IFN-γ (A: Parathion; B: Methoxychlor), TNF-α (C: Parathion; D: Methoxychlor) and IL-17 (E: Parathion; F: Methoxychlor) production by auricular lymph nodes of mice treated with test solution of mice. To determine the effects of cytokine production, lymph node cell suspensions were cultured with T-cell antibodies (CD3) for 24 h, and the supernatant was assayed by cytometric bead array. Values are expressed as mean (pg/mouse) ± SD ($n = 6$ per group). Statistical significance is marked by asterisks: $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's multiple comparison test).

Research has shown that some environmental chemicals lead to autoimmune disease and cancer (Schwartz, 2005). Sobel et al. (2005) and Wang et al. (2007) demonstrated that systemic lupus erythematosus in (NZB × NZW) F1 mice was increased by the OC chlordecone. Ward et al. (2009) reported that the incidence of childhood leukemia in industrialized countries was associated with exposure to polychlorinated biphenyls and OCs. Xu et al. (2010) showed that OCs might be associated with an increased risk for breast cancer and prostate cancer. It is important to note, however, that the relationship between allergies and the breakdown of immunological regulation by environmental chemicals is not fully understood. Therefore, we attempted to detect the modulation of allergic potential by prior exposure to immunosuppressive chemicals. We elicited positive reactions using parathion, methoxychlor, 2,4-D-butyl, and eugenol, but whether the same events occur after exposure to other widespread chemicals in the environment remains uncertain. Studies of other endpoints and other types of allergy are currently underway in our laboratories.

Environmental chemicals that lead to immune disorders are thought to fall into three general categories (Rao and Richardson,

1999; Sobel et al., 2005): (1) the chemical alters the self antigen such that it appears foreign to the immune system; (2) the chemical prevents the central tolerance of autoreactive T or B cells; and (3) the chemical alters gene expression. Hormones, such as estrogens, are thought to belong to this last category (Grimaldi et al., 2002), and several studies linking environmental chemicals with immune disorders have noted the chemicals' estrogenic character (Sobel et al., 2005; Ward et al., 2009; Xu et al., 2010). In this issue, 4 weeks of recovery terms were set between oral exposure and allergen sensitization. Therefore, parathion and methoxychlor could not be the self antigen was denied. In terms of relativity of hormones, we elicited positive reactions using parathion, which is not a hormone disruptor. According to our previous in vitro study and results of in this issue, it suggests that the chemical prevents the central tolerance of autoreactive T or B cells. To our knowledge, this is the first study to demonstrate the relationship between allergies and the breakdown of the central tolerance of autoreactive T or B cells by non-estrogenic environmental chemicals. However, the underlying mechanisms are an important issue to be solved.

Conflict of interest

None.

Acknowledgements

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RESEARCH ARTICLE

Detection of thymocytes apoptosis in mice induced by organochlorine pesticides methoxychlor

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Abstract

The thymus has long been known to be vulnerable to atrophy when exposed to variety of stimuli, including hormones, immunosuppressive pharmaceuticals, and environmental chemicals. The organochlorine pesticide methoxychlor (MXC) is an immunosuppressive agent thought to affect thymic atrophy by inducing apoptosis of thymocyte T cells. We sought to develop an experimental protocol to detect *in vivo* thymocyte apoptosis induced by MXC in Balb/c mice. We treated the mice with 150–400 mg/kg MXC. We then measured thymus weight, cell counts, caspase activity (3/7, 8, and 9), annexin V labeling of phosphatidylserine (PS) and DNA fragmentation. In MXC-treated mice we observed decreases in thymus weight and cell counts and increases in caspase activity (3/7, 8, and 9), annexin V PS labeling and DNA fragmentation. These results suggest that MXC induces thymic atrophy caused by thymocyte apoptosis, and that our protocol may be useful for detecting *in vivo* thymocyte apoptosis induced by environmental chemicals in short-time.

Keywords: *Methoxychlor; apoptosis; thymus; mouse*

Introduction

The immune system is highly sensitive to the toxic effects of several types of chemicals. The thymus in particular has long been known to be vulnerable to atrophy associated with exposure to variety of substances, including hormones, immunosuppressive pharmaceuticals, and environmental chemicals.[1–5] The thymus is a complex organ that is responsible for the maturation and differentiation of most peripheral T cells.[6] Given the complexity of the thymus, compounds that cause thymic atrophy could be acting on a variety of cellular targets and causing atrophy by various mechanisms. Proposed mechanisms include inhibition of thymocyte precursors in the bone marrow or fetal liver and inhibition of intrathymic development of the thymocytes themselves. Such inhibition may result from indirect effects on the supporting stromal elements that produce growth factors and signals or direct mechanisms such as decreased cell proliferation and increased cell death through apoptosis.[5]

Apoptosis is an essential process underlying multicellular organism development and function. In the immune system, apoptosis is required for lymphocyte development and homeostasis. The dysregulation of apoptosis leads to a variety of immune disorders, including immunodeficiency, tumorogenesis, allergies, and autoimmunity.[7] Detection of *in vivo* chemical-induced thymocyte apoptosis is difficult, however, because of the rapid clearance of apoptotic cells by phagocytic cells.[8–12] In addition, it has been taken a long time to detect the apoptosis *in vivo*. Therefore, protocols are needed for the detection of *in vivo* chemical-induced thymocyte apoptosis in short time.

Some recently reported studies have found that organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT) and methoxychlor (MXC) affect the immune system[13,14] as well as the reproductive system. Developed to replace DDT,[15] MXC has a shorter half-life than DDT, lower toxicity in mammals, and greater biodegradability.[15–17] Because of their nature of persistence in the environment, bioaccumulation in the food chain, and possible health effects, the US Environmental Protection Agency restricted and banned the use of most of organochlorine pesticides during the 1970s and 1980s.

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Although organochlorine pesticides are rarely used today, measurable amounts of organochlorine pesticides or their metabolites can still be found in human tissues. Moreover, organochlorine pesticides continue to be heavily used in some developing countries, primarily for mosquito and malaria control.[18,19] Thus, the health effects of organochlorine pesticides exposure remain an important public health concern. In a previous study, we demonstrated that MXC affects the immune system, leading to atrophy of $CD4^+CD8^+$ double-positive (DP) T cells in the thymus.[20–22] In addition, Chapin et al.[13] reported that doses of 150 mg/kg/day in rats led to markedly decreased thymus weights. The mechanisms behind the immunosuppressive action of MXC are not fully understood, however.

In this issue, we attempt to detect thymocyte apoptosis induced by MXC in short time. To that end, we used our original protocol and MXC at rather high doses (about one fourth of the LD50). We sought to determine whether MXC induces thymic atrophy in young mice and whether exposure to MXC triggers *in vivo* apoptosis in thymocytes.

Materials and methods

Chemicals and reagents

MXC (more than 95% pure) was purchased from Sigma-Aldrich (St Louis, MO). Dexamethasone (DEX; more than 99% pure) and ethanol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). MXC was dissolved in ethanol and diluted in corn oil. DEX was diluted in saline. DEX treatment group is referred to as the "positive control" group in this study.

Animals and housing conditions

Female BALB/c mice (age, 4 weeks) were purchased from Charles River Japan Laboratories (Kanagawa, Japan). The mice were housed individually under controlled lighting (lights on from 0700 to 1900 h), temperature (22 ± 2°C), humidity (55% ± 15%), and ventilation (at least ten 100% fresh air changes hourly). Food (Certified Pellet Diet MF, Oriental Yeast Co., Tokyo, Japan) and water were available *ad libitum*.

This study was conducted in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science.[23]

Experimental design

After a 1-week acclimatization period, mice (5 weeks old) were allocated randomly to four treatment groups and one control group ($n = 8$ per group). The experimental protocol used in this study is depicted in Figure 1. As a positive control, one treatment group received a single intraperitoneal injection of DEX (10 mg/kg). The other four treatment groups received three oral doses in 8-h intervals of MXC of 0 (negative control), 150, 300, or 400 mg/kg. All dose amounts of MXC were selected to avoid death and systemic toxicity (particularly in the preliminary test) while still permitting comparisons of the immunological potencies of the chemicals. Eight hours after the last treatment, mice were anesthetized with diethyl ether and killed. Each animal's thymus was carefully removed and weighed. Approximately one-half of the thymus tissue from each mouse was used in a single-cell suspension for analysis of annexin V and caspases 3/7, 8, and 9. The remaining thymus tissue was stored at –80°C until use for the detection of DNA ladder formation.

Preparation of cell suspension

Single-cell suspensions of thymocytes in 10 mL PBS (Gibco, Carlsbad, CA) supplemented with 5% heat-inactivated fetal calf serum (Gibco) were prepared by passage through a stainless-steel screen. The number of thymocytes was determined with a Coulter counter Z2 (Beckman Coulter, Tokyo, Japan).

Caspase 3/7, 8, and 9 analysis

From each cell suspension, 100 µL (1×10^5 cells) was seeded in duplicate into opaque-walled 96-well plates (Nulge Nunc International, Tokyo, Japan). Caspases 3/7, 8, and 9 in each well were measured with a luciferin-luciferase system (caspase-Glo 3/7, 8 and 9 assay, Promega, Tokyo, Japan). We used a microplate luminometer (TR717, Berthold Japan, Tokyo, Japan) to measured the activity of each caspase in relative light units (RLU).

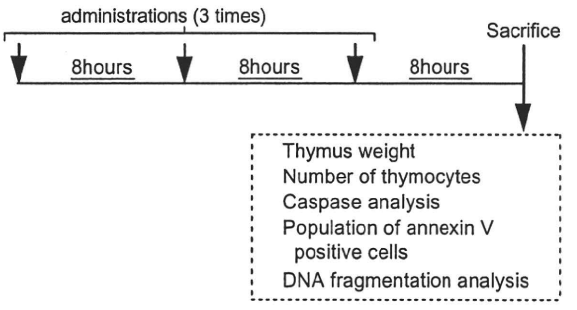

Figure 1. Experimental protocol used to administration BALB/c mice with methoxychlor (MXC). Eight hours after the last treatment, mice were anesthetized with diethyl ether and euthanized. Each animal's thymus was carefully removed and weighed. Approximately one-half of the tissues were pooled per mouse in phosphate-buffered saline (PBS, Gibco, Tokyo, Japan) analyzed for annexinV, caspase-3/7, caspase-8, and caspase-9. The other half of the thymus was kept at –80°C until used for the detection of DNA ladder formation.

Cell staining and flow cytometric analysis

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment.[24] Because externalization of PS occurs in the earlier stages of apoptosis, annexin V staining can enable identification of apoptosis at an earlier stage than assays that measure nuclear changes.

Flow cytometric analysis was performed by staining thymocytes with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide. To assay the thymocytes we used an annexin V: FITC Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA) in accordance with the manufacturer's protocol. Briefly, thymocytes (1×10^6 cells/animal) were pelleted by centrifugation and resuspended in 1 mL of annexin V-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$). We then incubated 100 µL of cell suspension (1×10^5 cells) with FITC-conjugated annexin V and propidium ioide for 15 min at room temperature in the dark. We added 400 µL of annexin V-binding buffer and then performed analysis with a FACSCalibur flow cytometer (BD Pharmingen) with the Cell Quest program (BD Pharmingen). For each sample, 10,000 events were collected and analyzed for the expression of antigens to annexin V and propidium ioide.

DNA fragmentation analysis

Isotissue® (Nippon Gene, Tokyo, Japan) DNA extraction kit was used to extract DNA from the frozen thymus samples. Each sample was placed in a microfuge tube with extraction buffer and proteinase K and incubated at 55°C until completely dissolved. We then added 5 M NaCl and centrifuged the mixture for 20 min (12,000*g* at 4°C. The supernatant was placed in a microfuge tube and incubated for 15 min at 55°C with RNase A (0.4 mg/mL) and then incubated for another 15 min at the same temperature with proteinase K (0.4 mg/mL). After being washed with washing buffer and 70% ethanol, DNA was eluted with Tris-EDTA (pH 8.0) buffer. The eluate was used as the DNA sample for agarose gel electrophoresis.

DNA samples were electrophoretically separated on 2% agarose gel (Mercury Heat & Pour Agarose, Pretech Instruments KB, Sollentuna, Sweden) in 90 mM Tris-boric acid-EDTA solution (Nippon Gene) at 8.5 V/cm for 2 h. After electrophoresis, the gels were stained with Vistra Green nucleic acid gel stain (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), and the nucleic acids were visualized with a fluorimager (FluorImager 585, GE Healthcare UK Ltd. Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England).

Statistical analysis

We transformed the data logarithmically to homogenize the variances and used analysis of variance (ANOVA) to evaluate the difference between test (MXC and DEX) and vehicle treatment group. When ANOVA was significant, the differences between groups were assessed by means of ANOVA followed by the Dunnett's multiple comparison test. A value of $P < 0.05$ was considered to be significant. Values are expressed as the mean ± standard deviation (SD).

Results

Thymus weights and numbers of thymocytes

In the MXC treatment groups thymus weights decreased in a dose-dependent manner, and statistically significant differences were found between the group that received a dose of 400 mg/kg and the other groups ($P < 0.01$) (Figure 2A). The total numbers of thymocytes were

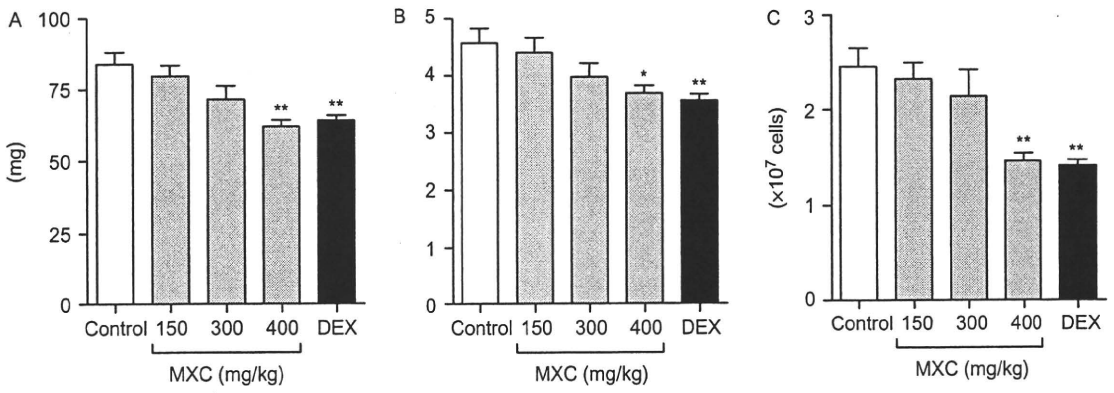

Figure 2. Absolute thymus weight (A) relative thymus weight (B) and total thymocytes count (C) of mice treated with test solution. Absolute thymus weights are expressed as means (mg/mouse) ± SD. Relative thymus weight are expressed as means (absolute thymus weigh, mg/body weight, g) ± SD. Thymocyte counts are expressed as means (×10^7 cells/mouse) ± SD. Statistical significance is marked by asterisks: **$P < 0.01$ (Dunnet's *t*-test).

lower in the 400 mg/kg group, but the decrease was not statistically significant (Figure 2B). Absolute thymus weight and number of thymocytes in the MXC 150 mg/kg treatment group were 79.63 ± 10.8 mg and 2.32 ± 0.5 × 10^7 cells, and in the MXC 300 mg/kg treatment group were 71.50 ± 13.8 mg and 2.14 ± 0.8 × 10^7 cells. Results for both of these groups were similar to those in the control group (absolute thymus weight, 83.88 ± 12.0 mg; number of thymocytes; 2.45 ± 0.5 × 10^7 cells). In the MXC 400 mg/kg treatment group, absolute thymus weight was 62.00 ± 6.7 mg, approximately 74% of that in the control group; this is a 26% decrease, and the number of thymocytes was 1.45 ± 0.2 × 10^7 cells, approximately 60% of the cell numbers in the control; this is a 40% decrease. (Figure 2).

In the DEX treatment group, statistically significant decreases were noted in both thymus weight and total number of thymocytes ($P < 0.01$) (Figure 2). The absolute thymus weight and total number of thymocytes were 64.25 ± 4.7 mg and 2.19 ± 0.2 × 10^7 cells, approximately 75% of those for the control group (Figure 2).

Caspase 3/7, 8, and 9 analysis

Caspase 3/7, 8, and 9 activities induced by 400 mg/kg MXC treatment were significantly higher than those of the control group ($P < 0.01$) (Figure 3). Caspase 3/7, 8, and 9 activities in the MXC 150 mg/kg treatment group were 3854 ± 574, 19,603 ± 2599, and 45,325 ± 6659 RLU, and were similar to the control group values of 4241 ± 623, 22,011 ± 2443, and 50,514 ± 5406 RLU, respectively (Figure 3). In the MXC 300 mg/kg treatment group, caspase 3/7, 8, and 9 activity values were 33,378 ± 50,723, 23,285 ± 9147, and 63,915 ± 39,268 RLU, respectively, approximately 800, 105, and 130% of those of the control group (Figure 3). In the MXC 400 mg/kg treatment group, caspase 3/7, 8, and 9 activity values were 155,655 ± 47,397, 46,950 ± 11,576, and 163,973 ± 47,386 RLU, respectively, approximately 3600, 200, and 300% of those of the control group (Figure 3).

In the DEX treatment group, statistically significant increases were noted in the activity of all caspases ($P < 0.01$) (Figure 3). The activity values of caspases 3/7, 8, and 9 were 196,822 ± 13,049, 52,918 ± 3723 and 182,498 ± 14,771 RLU, approximately 4600, 240, and 360% higher, respectively, than those in the control group (Figure 3).

Flow cytometric analysis

To evaluate the state of apoptotic cells after treatment with MXC, flow cytometric analysis was performed with thymocyte staining by FITC-conjugated annexin V and propidium iodide (Figures 4 and 5). The real cell numbers of annexin V-positive thymocytes were represented. The number of annexin V-positive thymocytes

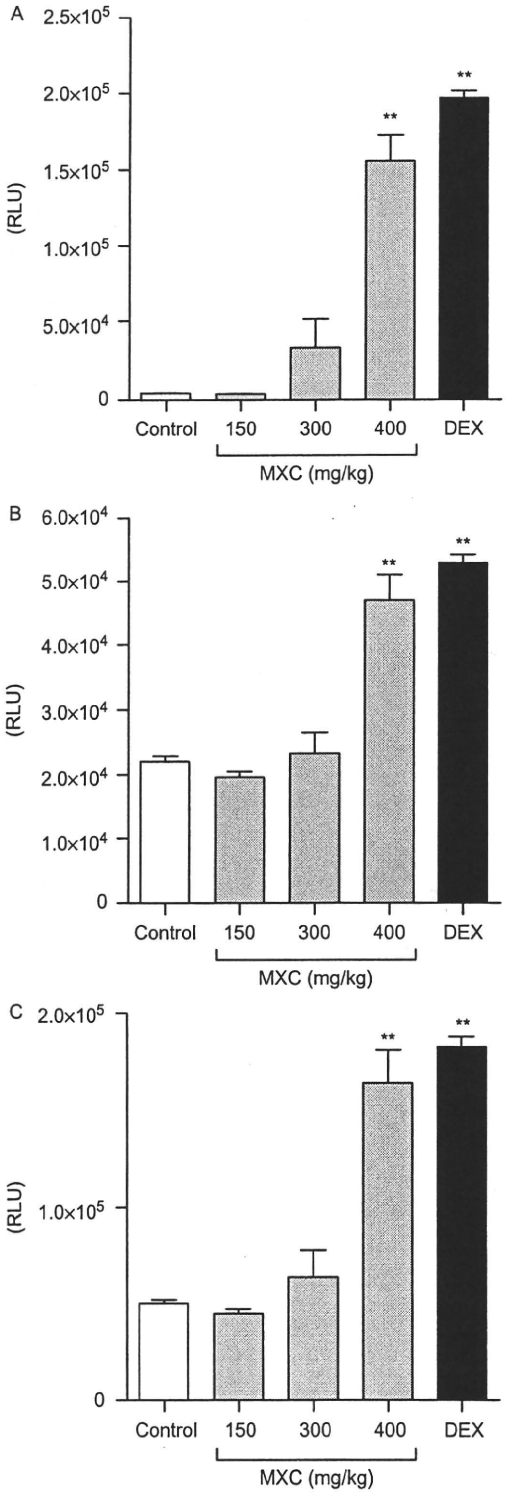

Figure 3. Caspases activities (A: 3/7; B: 8; C: 9) in the thymus of mice treated with test solution. These activities are expressed as means (RLU) ± SD. Statistical significance is marked by asterisks: **$P < 0.01$ (Dunnet's *t*-test).

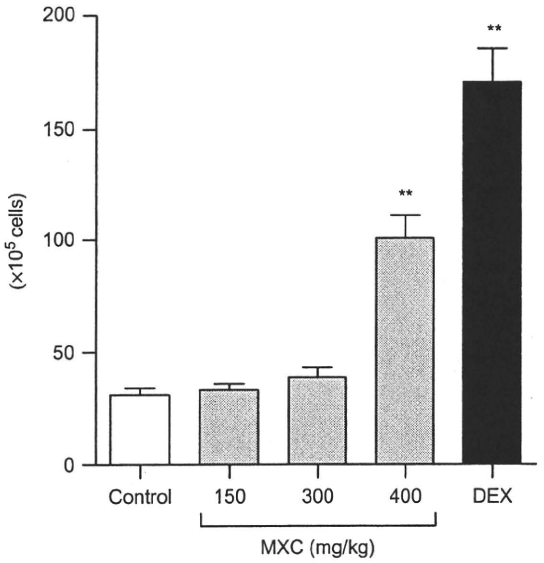

Figure 4. The populations of annexin V-positive cells (A: 3/7; B: 8; C: 9) in the thymocytes of mice treated with test solution. The populations of annexin V-positive cells are expressed as means (×10⁵ cells/mouse) ± SD. Statistical significance is marked by asterisks: $^{**}P < 0.01$ (Dunnet's *t*-test).

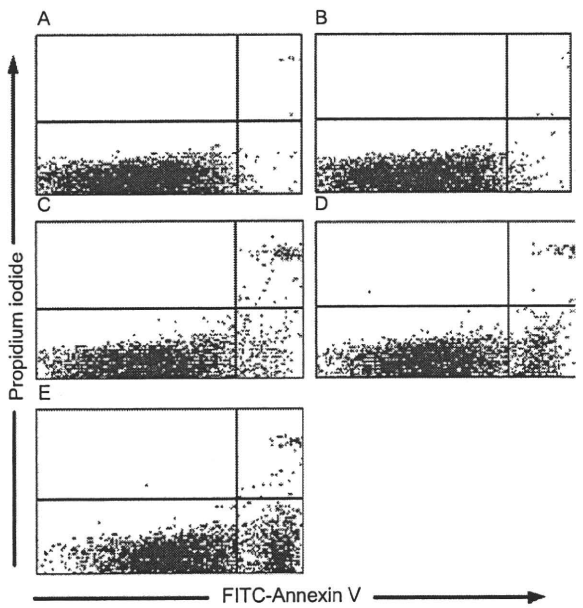

Figure 5. Characteristic exemplification of dot-plots of annexin V-positive cells (A: control; B: MXC 150mg/kg; C: MXC 300mg/kg; D: MXC 400mg/kg; E: DEX) in the thymocytes of mice treated with test solution. (See colour version of this figure online at www.informahealthcare.com/ipi)

was significantly higher in the 400 mg/kg MXC-treated group than in control group ($P < 0.01$) (Figure 4). In the MXC 150 mg/kg treatment group, the number of annexin V-positive thymocytes was $33.13 \pm 7.9 \times 10^5$ cells, approximately 107% of the control group value of $30.88 \pm 8.7 \times 10^5$ cells (Figures 4, 5A and 5B). In the MXC 300 mg/kg treatment group, the number of annexin V-positive thymocytes was $38.89 \pm 12.6 \times 10^5$ cells, approximately 130% that of the control group (Figures 4 and 5C). In the MXC 400 mg/kg treatment group, the number of annexin V-positive thymocytes was $100.60 \pm 29.8 \times 10^5$ cells, approximately 325% that of the control group (Figures 4 and 5D). The number of annexin V-positive thymocytes in the DEX treatment group ($170.4 \pm 41.3 \times 10^5$ cells) was approximately 550% that of the control group (Figures 4 and 5E) and this difference was highly significant ($P < 0.01$).

DNA fragmentation analysis

We used agarose gel electrophoresis to assess the degradation pattern of nuclear DNA in MXC-induced cell death because it has been observed that oligonucleosomal cleavage accompanies apoptosis in most systems.[25–27] In the 300 and 400 mg/kg MXC treatment groups, we observed increase in fragmented DNA of 200 bp (Figure 6C and 6D), whereas essentially no fragmented DNA was detected in the control and MXC 150 mg/kg treatment groups (Figure 6A and 6B). In the positive control DEX treatment group, marked DNA fragmentation was noted (Figure 6E).

Discussion

Our results indicate that MXC at doses of 400 mg/kg administered three times in 8 h by oral gavage modulated the immune response in young female Balb/c mice. The indicators of apoptosis affected were thymus weight, number of thymocytes, caspase activity (3/7, 8, and 9), number of annexin V-positive thymocytes and DNA fragmentation in thymocytes (see Figures 3–6). This study is the first to demonstrate these particular effects of *in vivo* environmental chemical-induced thymocyte apoptosis in short time.

Detection of *in vivo* chemical-induced apoptosis in short time is difficult because of the rapid clearance of apoptotic cells by phagocytic cells.[8–12] To overcome this problem, we used a protocol in which mice were killed 8 h after receiving the last of three oral doses of MXC. In addition, we focused on several endpoints such as movement of PS, DNA fragmentation, and caspase activity. By using this procedure we detected more apoptosis in MXC-treated mice than in controls.

A relatively early marker of apoptosis is movement of PS residues to the external leaflet of the plasma membrane.[28] This process is thought to facilitate macrophage recognition, engulfment, and removal of dying cells.[29,30] Our results indicate that MXC increased the

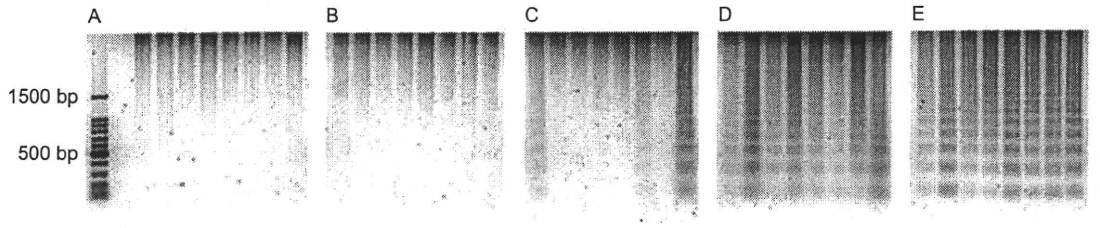

Figure 6. Agarose gel electrophoresis of DNA extracted from the thymus (A: control; B: MXC 150 mg/kg; C: MXC 300 mg/kg; D: MXC 400 mg/kg; E: DEX) of mice treated with test solution. The lanes correspond to each mouse in the experimental group. Molecular weight markers are indicated to the left.

number of PS-exposed cells in a dose-dependent manner (see Figures 4 and 5). This upregulation of PS movement thus seems to be a useful endpoint for identifying chemical-related thymocyte apoptosis.

A later-stage marker of apoptosis is DNA fragmentation. Apoptosis has been characterized biochemically by the production of internucleosomal DNA fragments of 180–200 bp resulting from the activation of an endonuclease. We observed prominent increases in the DNA fragmentation in the 300 and 400 mg/kg MXC treatment groups (see Figure 6). This upregulation of DNA fragmentation also seems to be a useful endpoint for identifying chemical-related thymocyte apoptosis.

Caspase activation is a step in apoptosis, and measurement of caspase 3/7, 8, and 9 activity is a reliable indicator of caspase-dependent apoptosis.[31] Caspases 8 and 9 are thought to be initiator caspases, and caspases 3 and 7 effector caspases. Caspase 3, the first caspase linked to apoptosis that is upstream of DNA fragmentation, is activated by caspases 8 and 9. Caspase 3 is required for DNA degradation and chromatin condensation. Thus the dose-dependent increase in caspase 3 (see Figure 3A) indicated an increase in MXC-induced thymocyte apoptosis. Caspase 8 is the apical caspase in the tumor necrosis factor–family death-receptor or extrinsic pathway. Caspase 8 activation initiates Fas (Apo-1/CD95), tumor necrosis factor receptor and death receptor-3.[32–36] Active caspase 8 cleaves and activates the downstream effector caspase 3, which cleaves cellular targets and induces apoptosis. Furthermore, caspase 8 cleaves the BH3-only protein Bid and generates a small fragment of Bid, which translocates to the mitochondrial outer membrane and inhibits the function of anti-apoptotic proteins Bcl-2 and Bcl-x_L. This step leads to the activation of the intrinsic death pathway.[7] Caspase 9 is the apical caspase in the intrinsic or mitochondrial pathway. Active caspase 9 further activates downstream effector caspases, including caspases 3 and 7, which in turn cleave hundreds of cellular components and result in irreversible cell death. In the present study, caspase 8 and 9 activity in thymocytes treated with MXC increased significantly. This result suggests that MXC-induced thymocyte apoptosis affects both intrinsic and extrinsic apoptosis pathways.[7]

To detect *in vivo* MXC-induced thymocyte apoptosis in short time, we used our original protocol and MXC at rather high doses (about one fourth of the LD50). In recent years, several reports have suggested that oral MXC exposure suppresses immune function by inducing thymic atrophy.[13,14] Whether this thymic atrophy occurs because MXC triggers apoptosis is not clear, however. Our study is the first to demonstrate severe thymic atrophy with cellular depletion and increased thymocyte apoptosis in MXC-treated mice, findings indicating that MXC induces thymocyte apoptosis. Takeuchi et al.[21] demonstrated that morphometrical analysis revealed a significant decrease in the size of the thymic cortical area in MXC-treated rat pups. In addition, phenotyping of thymic lymphocytes revealed a significant decrease in the DP immature T cells ($CD3^{int}CD4^{+}CD8^{+}$) located in the cortex area. These reports and our results indicate that MXC may cause thymic cortical atrophy via a thymocytes apoptotic process. In general, DP cells represent the majority of thymic lymphocytes located in the cortex area and have low expression of Bcl-2, which is associated with resistance to apoptosis.[37,38] DP cells are sensitive when exposed to chemicals, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin and organotins, and tend to show apoptotic cell death in the *in vitro* and *in vivo* studies.[39–43] This suggests that immunotoxic chemicals that have the potential to cause thymic atrophy may affect DP thymocytes and result in apoptosis, as was observed with MXC.

Numerous environmental toxic chemicals such as pesticides, perfumes, organotins, and heavy metals play roles in the dysregulation of immune functions, including thymocyte apoptosis.[44–52] Such immunosuppression may predispose the highly sensitive immune system to loss of tolerance to self-antigens and subsequent increased risk for autoimmune disease and allergies. Alternatively, altered repertoires of T cells responsive to foreign antigens may cause the neonate to become more susceptible to infections.[44–46,53] From this point of view, we attempted to develop a method for detection of *in vivo* chemical-induced thymocyte apoptosis and we obtain positive reactions using MXC as a typical chemical. Whether the same events occur after administration of other chemicals

that appear in many places in the environment remains uncertain, however. Experiments are currently under investigation in our laboratories.

In summary, we detected *in vivo* MXC-induced thymocyte apoptosis in short time by measuring thymus weight, number of thymocytes, caspase activity (3/7, 8, and 9), number of annexin V-positive thymocytes and DNA fragmentation in thymocytes.

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Declaration of interest

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