

## **Abnormal Response Induced by Pesticides on Mammalian Immune System**

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**Abstract**—The environmental pollution induced by the chemical substances is regarded as a serious problem on the ecosystem. Recently, many people are interested in safety of foods. We focused on two pesticide chemicals methoxychlor and endosulfan presently used in agricultural farms in many countries. To investigate the influence of methoxychlor and endosulfan on immunoglobulin production, human hybridoma HB4C5 cells and mouse splenic lymphocytes were cultured in the medium containing these compounds at various concentrations. Human IgM production of HB4C5 cells slightly decreased in a dose-dependent manner. On the other hand, mouse splenic lymphocytes treated with each pesticide markedly decreased both IgA and IgG production levels but not the IgM. Moreover, to assess the influence of methoxychlor and endosulfan, we examined the effects of these compounds on mice by oral administration. The spleen was remarkably atrophied whereas the body weights were the same as in control group. These results suggest that these pesticides affect immune system as well as detoxification tissues. It can be suggested from our present findings that the exposure to these compounds via food may lead to the decline in immune activities of human body, resulting in diseases.

**Keywords:** pesticide, methoxychlor, endosulfan, oral administration, atrophy

### INTRODUCTION

The widespread environmental pollution caused by the chemical substances such as pesticides is a serious problem for creatures including human (McClure *et al.*, 2001; McKinlay *et al.*, 2008; Boobis *et al.*, 2008). Various chemical substances entering animal bodies are carried to the organs responsible for detoxification, such as liver and kidney, and excreted. On the other hand, some hydrophobic

substances among these chemicals are accumulated in our body (Somogyi and Beck, 1993).

In this study, we focused on the pesticidal chemicals used for the efficient cultivation of farm products. Many pesticides whose production and/or use are prohibited in Japan are still in use in other countries and those farm products produced by using pesticides and/or dusted with such chemicals are consumed and also exported. Therefore, human cannot avoid exposure to these pesticides that may occur through residues on cereals, vegetables and fruits harvested in other countries.

In some previous reports, influence of these pesticides on reproductive system has been studied (Welshons *et al.*, 1999; Bretveld *et al.*, 2007). However, the influence on immune system has not been well understood. We chose two pesticides for our investigation; methoxychlor possessing similar structure to DDT (Coats, 1983), and endosulfan (also referred as benzoepin) possessing similar characteristic property as endrin (Gegiou, 1974).

To examine the influence of these two pesticides on mammalian immune system, the effects on immunoglobulin production of human hybridoma cells and mouse lymphocytes prepared from spleen were evaluated. Human hybridoma cells and mouse splenic lymphocytes were cultured in the medium containing each pesticide at various concentrations. Moreover, for extensive investigation on the effect of pesticides, mice were orally administrated various concentrations of these two pesticides, individually for 2 weeks.

## MATERIALS AND METHODS

### *Reagents*

The pesticides, methoxychlor,  $\alpha$ -endosulfan and  $\beta$ -endosulfan were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Briefly, 10 mM of each pesticide was prepared in ethanol. For assays, each pesticide was diluted with ethanol to various concentrations.

Goat anti-human IgM, goat anti-human IgM-HRP (BioSource International, Inc., Camarillo, CA, USA), rabbit anti-mouse IgM, goat anti-mouse IgG, rabbit anti-mouse IgA, goat anti-mouse IgM-HRP, goat anti-mouse IgG-HRP conjugated and goat anti-IgA-HRP (Zymed, South San Francisco, CA, USA) were used for quantitative analysis by ELISA.

### *Cells*

Human-human hybridoma, HB4C5 cells producing monoclonal IgM were used for the assay of Ig production stimulating activity. HB4C5 cell line was a fusion product of a human B lymphocyte from a lung cancer patient and human fusion-partner NAT-30 cells (Murakami *et al.*, 1982a). Splenic lymphocytes were prepared from mice. Briefly, mice were killed by cervical dislocation, and spleens were excised from the cavity. Spleens were strained with 100 nm-pore nylon meshes, and collected cells were hemolyzed with hemolysis buffer (155

mM  $\text{NH}_4\text{Cl}$ , 15 mM  $\text{NaHCO}_3$ , 1 mM EDTA-2Na, pH 7.3). After washing twice with PBS, lymphocytes in spleen were obtained. HB4C5 cells and splenic lymphocytes were cultured in ERDF medium (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with 10  $\mu\text{g}/\text{ml}$  of insulin (Sigma, St. Louis, MO, USA), 20  $\mu\text{g}/\text{ml}$  of transferrin (Sigma), 20  $\mu\text{M}$  ethanolamine (Sigma), and 25 nM selenite (Sigma) (ITES-ERDF) (Murakami *et al.*, 1982b). HB4C5 cells and lymphocytes were incubated at 37°C under humidified 5%  $\text{CO}_2$ -95% air.

### *Animals*

Albino mice were purchased from the Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). Mice were housed in a room maintained at 24°C on a 12 h light/dark cycle in a specific pathogen-free facility, and provided tap water and diet ad libitum. Animal experiments using mice described herein were approved by the Ehime University Animal Care and Use Committee and were performed in accordance with applicable guidelines and regulations.

### *Oral administration*

For oral administration, three mice per group were administered each pesticide or ethanol as control. The administration was performed against 8-week-old female mice. Mice were administered with 20  $\mu\text{L}$  (1 mL/kg) of pesticides solution containing 1 mM of pesticides everyday for 14 days (Breinholt *et al.*, 2000). After measurement of body weight, mice were killed by cervical dislocation, and spleens were excised at day 15. Following washing with PBS twice, the weight of spleen was measured. The mean tissue weight of the three control mice was calibrated as 100%, and relative weight loss rate was then calculated.

### *Assay for Ig production activity of pesticides*

The Ig production activity of each pesticide was examined by measuring the amount of IgM secreted by HB4C5 cells or of IgA, IgG and IgM secreted by splenic lymphocytes from mice into the culture medium. HB4C5 cells and splenic lymphocytes were inoculated into medium supplemented with each pesticide at various concentrations. The assay of the Ig production activity was performed in a 96-well culture plate, and HB4C5 cells and splenic lymphocytes were inoculated at a density of  $2 \times 10^5$  cells/ml and  $7.5 \times 10^5$  cells/ml, respectively. After cultivation for 24 h, the amount of Igs secreted into culture medium was determined by an enzyme-linked immunosorbent assay (ELISA).

### *Enzyme-linked immunosorbent assay (ELISA)*

ELISA was performed as previously described (Sugahara *et al.*, 2006; Nishimoto *et al.*, 2008). Briefly, 1.0  $\mu\text{g}/\text{ml}$  of an anti-human IgM and anti-mouse Igs antibody was added to a 96-well plate at 100  $\mu\text{l}/\text{well}$ , and incubated for 2 h at 37°C. After washing with 0.05% Tween20-PBS (T-PBS) three times, each well

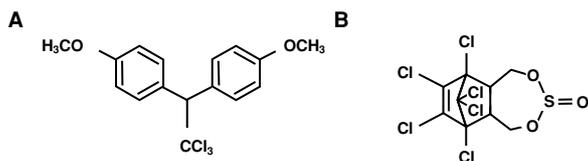


Fig. 1. Chemical structures of methoxychlor (A) and endosulfan (B). Endosulfan has two isomers,  $\alpha$  and  $\beta$ .

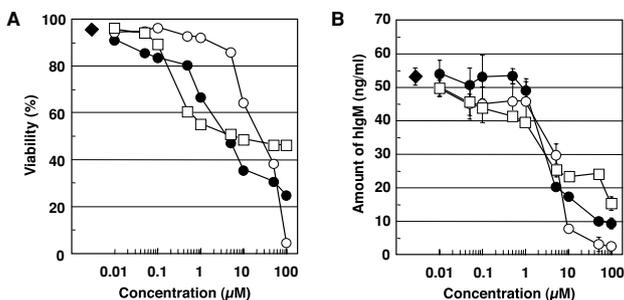


Fig. 2. Effect of pesticides on IgM production and viability of HB4C5 cells. (A) Cell viability was measured by trypan blue dye exclusion test. It is indicated that viability of the cells cultured with methoxychlor (●),  $\alpha$ - (○) and  $\beta$ -endosulfan (□) at various concentrations for 24 h. Control (◆) is ethanol. (B) HB4C5 cells were cultured with methoxychlor (●)  $\alpha$ - (○) and  $\beta$ -endosulfan (□) at various concentrations for 24 h. Control (◆) is ethanol. The amount of human IgM produced by HB4C5 cells in culture medium was measured by ELISA.

was blocked with 1.0% bovine serum albumin (BSA)-PBS solution for 2 h at 37°C. Following the blocking reaction, each well was treated with 50  $\mu$ l of culture supernatant for 1 h at 37°C. Then, 100  $\mu$ l/well of the HRP-conjugated anti-human IgM antibody or HRP-conjugated anti-mouse Igs that had been diluted 1000 times by 1.0% BSA-PBS was added and incubated for 1 h at 37°C. Following this, 0.6 mg/ml of 2,2'-azino-bis(ethylbenzothiazoline-6-sulfonic acid diammonium salt) (ABTS) dissolved in a 0.03% H<sub>2</sub>O<sub>2</sub>-0.05 M citrate buffer (pH 4.0) was added to the wells at 100  $\mu$ l/well, and the absorbance at 415 nm was measured after addition of 100  $\mu$ l/well of 1.5% oxalic acid to terminate the coloring reaction. The IgM concentration was quantitatively determined by a standard curve, using a standard solution for human IgM, mouse IgA, mouse IgG and mouse IgM, respectively.

## RESULTS

To examine the effect of pesticides on Ig production activity of immune cells in human body, we studied two pesticides, methoxychlor and endosulfan (Figs. 1A and B). Endosulfan has two different isomers,  $\alpha$  and  $\beta$ .

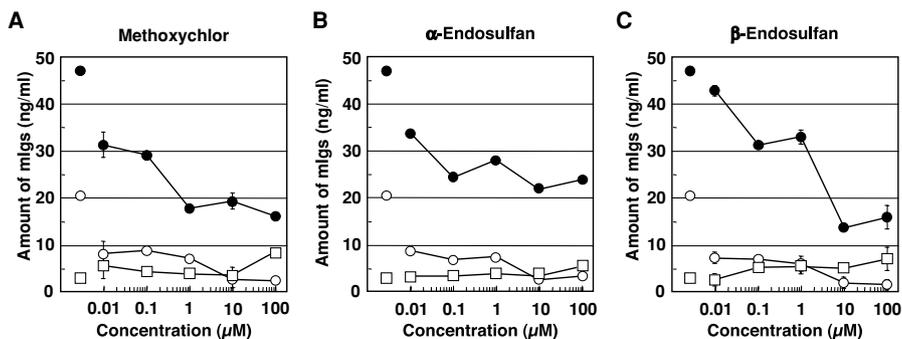


Fig. 3. Ex vivo effect of pesticides on Igs production of mouse splenic lymphocytes. It is indicated that the amount of IgA (●), IgG (○) and IgM (□) production on splenic lymphocytes cultured with methoxychlor (A),  $\alpha$ - (B) and  $\beta$ -endosulfan (C) at various concentrations for 24 h. The amount of each Ig in culture medium was measured by ELISA. Control was indicated at the left side as a single plot in each figure.

Table 1. Relative weight rate of spleen on pesticides administrated mice.

	Control	Methoxychlor	$\alpha$ -Endosulfan	$\beta$ -Endosulfan
7 days	$0.50 \pm 0.01$	$0.33 \pm 0.07$	$0.35 \pm 0.06$	$0.37 \pm 0.01$
14 days	$0.53 \pm 0.01$	$0.30 \pm 0.04$	$0.30 \pm 0.02$	$0.28 \pm 0.02$

The values (%) were calculated by spleen weight per body weight.

To assess the effect of these pesticides on human IgM production, HB4C5 cells were cultured with methoxychlor and endosulfan at various concentrations for 24 h. As shown in Fig. 2A, methoxychlor and  $\beta$ -endosulfan indicated cytotoxicity at low concentration (between 0.01 and 1  $\mu$ M) against HB4C5 cells. On the other hand, only  $\alpha$ -endosulfan had almost no toxicity at same concentration range. In Fig. 2B, the amount of IgM produced by hybridoma cells treated with  $\alpha$ - and  $\beta$ -endosulfan at same dose range was slightly lower in the culture medium. We found from this result that methoxychlor and endosulfan at low concentration range had no obvious affect on HB4C5 cells. In case of treatment of pesticides at more than 1  $\mu$ M, cell viability and IgM production levels decreased in a dose dependent manner.

Next, we investigated whether mouse immune response effect of methoxychlor and endosulfan are similar in HB4C5 cells. Splenic lymphocytes were prepared from young female mouse as shown above. Even though splenic lymphocytes treated with both pesticides were alive, the IgA and IgG production was down-regulated in a dose-dependent manner (Fig. 3). On the other hand, IgM production level slightly increased and/or constant. These results suggest that each pesticide regulate Igs production except for IgM and/or inhibit Igs secretion.

To observe the histological effect, each pesticide at 1 mM concentration was administrated to mice for 14 days. The administrated concentration was determined from the value of LD<sub>50</sub> (Gupta *et al.*, 1981). Methoxychlor and endosulfan was used at concentration levels of one/two thousand and one/forty of LD<sub>50</sub>, respectively. We did not observe any body weight loss of mice during the experimental period. As shown in Table 1, spleen was enucleated in the high concentration pesticides-administrated mice and became atrophied. Both pesticides induced weight loss of spleen within 7 and 14 days; the rate (spleen weight/body weight) being approximately 60% against control.

### CONCLUSIONS

In human hybridoma cells, methoxychlor,  $\alpha$ - and  $\beta$ -endosulfan slightly decreased the amount of IgM in culture medium and cell viability at low dose. The intensive toxic effects of these pesticides were not observed between 0.01 to 1  $\mu$ M. However, the toxicity increased dose-dependently at higher concentrations.

In mouse splenic lymphocytes, each pesticide markedly decreased IgA and IgG production levels except IgM. Splenic lymphocytes treated with each pesticide at low concentration were reduced in IgA and IgG production, whereas they were alive. These pesticides may have suppression effect on secretion mechanism or specific response activity of Ig class. In addition, we found that the atrophy of spleen induced by each pesticide-administration. These results suggest that pesticides not only reduce IgA and IgG level but also atrophy the immune tissue if the animals consume the pesticides orally. IgG plays an important role in infectious diseases against viruses or bacterium (Insel *et al.*, 1994; Nimmerjahn and Ravetch, 2008). Hence, animals may suffer from opportunistic infectious diseases by decline of immune response when methoxychlor or endosulfan was chronically taken and accumulated in our body.

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