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NOTE

Toxicology

Effect of prenatal exposure to combined immunosuppressive agrochemicals in a mouse model of allergic airway inflammation

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ABSTRACT. The aim of this study is to identify the combined effect of multiple chemicals to the development of allergy. In this study, the effect of prenatal exposure to an organochlorine agent methoxychlor (MXC) and/or an organophosphate agent parathion (PARA) on trimellitic anhydride-induced allergic airway inflammation was examined in mice. Eosinophil infiltration in the bronchoalveolar lavage fluid (BALF) was significantly enhanced by MXC + PARA exposure compared to that of the control, MXC, and PARA groups. In the hilar lymph node, only slight increases in B-cell infiltration, as well as IL-6 and IL-9 secretions were observed in MXC + PARA group, and no effect was observed in the individual treatment groups. Our findings imply that prenatal exposure to some combinations of multiple chemicals may exacerbate the allergic inflammatory responses including eosinophils and cytokine production.

KEY WORDS: allergic airway inflammation, combined toxicity, methoxychlor, parathion, prenatal exposure

Millions of chemicals are currently released into the environment such as atmosphere, soil, and underwater, which are hazardous to both human and wildlife populations worldwide. Chemical risk assessment is thus required by each government, community (e.g. OECD), and regulation (e.g. Registration, Evaluation, Authorization and Restriction of Chemicals) for every chemical company. Despite the requirement of individual risk assessment for each chemical by the current registrations, most chemicals in the environment or food product are present in combinations. At present, information about combined effect of multiple chemicals is limited.

The effect of short-term oral exposure to a combination of environmental immunotoxic chemicals on the adaptive immune system and allergic airway inflammation has been recently investigated by our group [7, 11]. Nishino *et al*. [11] focused on three immunosuppressive agrochemicals, namely methoxychlor (MXC), parathion (PARA), and piperonyl butoxide (PBO), which are an organochlorine compound, an organophosphate compound, and an agricultural insecticide synergist, respectively. They showed that exposure to a combination of MXC + PARA induced higher immunotoxic responses in murine subjects, such as antigen-specific IgM and B-cell counts, than exposure to a single chemical. Fukuyama *et al*. [7] further examined the effect using a mouse model of allergic airway inflammation. MXC + PARA and PBO + MXC combinations induced a significant increase in allergic airway inflammation compared to that induced by exposure to the individual chemicals, as indicated by the IgE responses, eosinophil counts, and levels of pro-inflammatory chemokines and cytokines. This evidence suggests that exposure to a combination of chemicals induced more severe immunotoxic responses than those induced by the exposure to a single chemical.

However, the combined toxicity of multiple environmental chemicals against immature immune system has not been fully understood yet, although immature immune system is higher sensitive than mature immune system. Therefore, this study aimed to provide more information on combined toxicity of chemicals against immature immune response and therefore investigated the effect of prenatal exposure to a combination of MXC and PARA on allergic airway inflammation in mice. MXC and PARA were used as representative environmental chemicals which have toxic effect on immune function by different pathway. While humans are at low risk of exposure to MXC or PARA nowadays, the information acquired in this study will provide the basis to assess the risk to combined toxicity of multiple environmental chemicals.

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MXC, PARA, and trimellitic anhydride (TMA, $C_9H_4O_5$, >97% purity) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Dynabeads mouse T-Activator CD3/CD28 was purchased from Thermo Fisher Scientific, Inc. (Yokohama, Japan). Mouse BD Fc Block, APC-conjugated anti-mouse CD3, PE-Cy7-conjugated anti-mouse CD4, PerCP-conjugated anti-mouse CD45R/B220, APC-Cy7-conjugated anti-mouse CD11c, PerCP-Cy5.5-conjugated anti-mouse Gr-1, FITC-conjugated anti-mouse CD23, PE-Cy7-conjugated anti-mouse CD117, APC-conjugated anti-mouse CD49b, PE-conjugated anti-mouse CD49d, and BD CBA Mouse Flex Set (cytometric bead array, IL-4, IL-6, IL-9, IL-13, IL-17A, INF-γ, KC, and TNFα) were purchased from BD Pharmingen (Tokyo, Japan). Pregnant female BALB/c mice were purchased from Charles River Japan Laboratories (Atsugi, Japan) and kept under the following conditions: temperature of 22 ± 2°C, humidity of 50 ± 20%, and 12 hr light/dark cycle. The mice were provided with certified pellet diet and water *ad libitum*. The study protocol was approved by the Animal Care and Use Committee of the Institute of Environmental Toxicology, which is fully accredited by the AAALAC international (IACUC Protocol No. AC15006). Starting on the thirteenth day of pregnancy, female BALB/c mice (F0) were orally administered with MXC (10 mg/kg), PARA (0.15 mg/kg), MXC+PARA (10+0.15 mg/kg), or vehicle (corn oil) (control) for 5 days (6–8 animals/group). MXC and PARA at those doses did not induce severe toxicity and immunosuppressive effect in our previous study [6]. Following prenatal MXC and/or PARA treatment, 8-week-old F1 pups were repeatedly treated with TMA to induce allergic airway inflammation according to a previous study [12]. In brief, each neonatal mouse was topically treated with 0.15 mg TMA dissolved in acetone-olive oil (4:1, v/v) 3 times a week for 3 consecutive weeks (total 9 times). Two weeks after the final treatment, the mice were inhaled with 0.5 mg TMA using a snout-only inhalation exposure system. Twenty-four hr afterwards, the mice were euthanized using isoflurane anesthesia. Bronchoalveolar lavage fluid (BALF) samples were collected by cannulating the trachea and lavaging the lungs three times with 1 m*l* PBS supplemented with 1% FBS. After centrifugation of BALF sample (350 × g, 5 min), the cell pellets were resuspended, pooled, and used for differential cell counts using fluorescence-activated cell sorting (FACS) flow cytometry (FACSVerse cytometer, BD Pharmingen). Hilar lymph nodes (LNs) were also isolated from each mouse and single-cell suspensions were prepared by passage through a sterile 70-μm nylon cell strainer in 1 m*l* of RPMI 1640 supplemented with 5% FBS. Single-cell suspensions were used in FACS and cytokine evaluation. For FACS analysis, 1×10^6 cells were first incubated with 1 μg of Mouse BD Fc Block, followed by incubation with monoclonal antibodies for T cells and B cells. Cells were washed and analyzed on a FACSVerse flow cytometer. For cytokine determination, single-cell suspensions of LNs (5×10^5 cells/well) were incubated with Dynabeads mouse T-Activator CD3/CD28 for 24 hr. IFNγ, IL-4, -6, -9 and -17A secretions were in the supernatant were evaluated by ELISA [13]. Data are expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used to evaluate the significance of difference between groups and followed by the Tukey-Kramer *post-hoc* test. Statistical significance was determined at $P<0.05$ and $P<0.01$.

Exposure to MXC+PARA combination induced a significant increase in eosinophil infiltration compared to those in the other groups, whereas eosinophil infiltrations caused by either MXC or PARA exposure were not significantly different from that of the control group (Fig. 1A). The MXC+PARA-treated group also showed a significantly higher mast cell count than those of the control and PARA groups, whereas no changes were observed between vehicle and PARA treatment groups (Fig. 1B). Mast cell count of the MXC treatment group was almost doubled compared to the vehicle control group, although significant difference was not observed. Significant up-regulation of basophils infiltration was noted in the

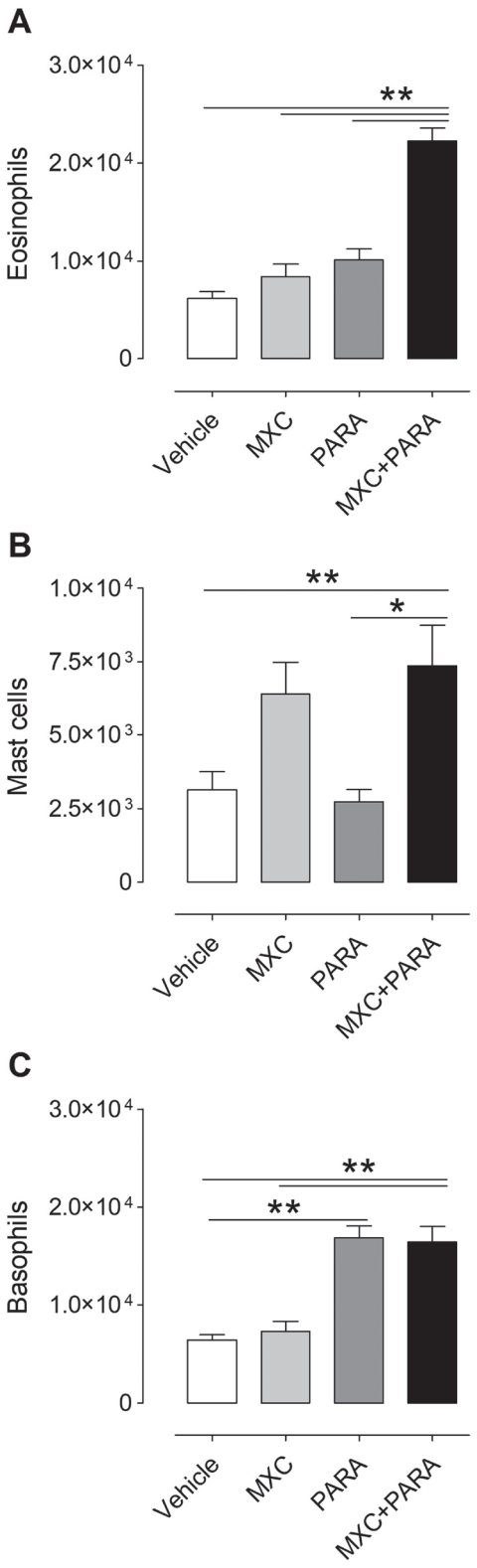

Fig. 1. Cell counts in BALF. (A) Eosinophil, (B) Mast cell and (C) Basophil counts in BALF from mouse models of allergic airway inflammation after prenatal exposure to MXC and/or PARA. Each value is presented as the mean ± SEM. n=6–8 per group. **P*<0.05, ***P*<0.01 (Tukey-Kramer *post-hoc* test).

Table 1. Helper T cell and B cell infiltrations, and cytokine productions in hilar lymph node from mouse models of allergic airway inflammation after prenatal exposure to MXC and/or PARA

	Control	MXC	PARA	MXC + PARA
Helper T cells ($\times 10^6$)	1.01 ± 0.45	1.26 ± 0.44	1.25 ± 0.33	1.33 ± 0.43
B cells ($\times 10^6$)	1.94 ± 0.93	2.66 ± 0.85	2.78 ± 0.94	3.03 ± 0.69[a)]
IL-4 (*p*g/m*l*)	128 ± 176	227 ± 159	296 ± 196	236 ± 245
IL-5 (*p*g/m*l*)	108 ± 138	171 ± 101	235 ± 205	241 ± 242
IL-6 (*p*g/m*l*)	16.7 ± 19.3	30.6 ± 13.8	53.8 ± 37.5	63.8 ± 56.4[a)]
IL-9 (*p*g/m*l*)	54 ± 54	64 ± 43	128 ± 72	155 ± 106[a,b)]
IL-13 (*p*g/m*l*)	83 ± 141	131 ± 108	198 ± 141	163 ± 187
IL-17A (*p*g/m*l*)	6.2 ± 9.1	17.5 ± 14.0	19.3 ± 19.1	16.1 ± 21.0

Results are expressed as mean ± SEM. n=6–8 per group. a) $P<0.05$ vs. control group, b) $P<0.05$ vs. MXC treatment group.

MXC+PARA group compared to the vehicle control and MXC treatment groups; however, the PARA treatment group exhibited similar induction as the MXC+PARA treatment group (Fig. 1C). Table 1 shows the responses of LNs to prenatal administrations of MXC, PARA, and MXC+PARA. Even though all groups showed no effect on helper T cells infiltration, the MXC+PARA group showed a significant increase in B cell infiltration compared to that of the control group. There were no differences in B cell infiltration among the MXC+PARA, MXC, and PARA groups. Prenatal exposure to MXC and/or PARA increased IL-4, -5, -6, -9, -13, and -17A secretions, as compared to vehicle treatment. However, significant increases were only observed in IL-6 and IL-9 secretion. In particular, IL-9 secretion in the MXC+PARA group was significantly higher than that in the MXC group.

This study aimed to provide information about the developmental effect of oral exposure to a combination of multiple chemicals. We showed in a recent study that oral exposure to a combination of the organochlorine agent MXC and organophosphorus agent PARA synergistically suppresses the adaptive immune system [11] and aggravates allergic airway inflammation in mice [7]. However, immune responses are strongly affected by the age of the animal, and MXC and PARA are also easily transported from mother to juvenile through the placenta and breast milk. Therefore, in this study, we further examined the effect of prenatal exposure to MXC and PARA on allergic airway inflammation in mice to provide more information on combined toxicity of chemicals. MXC is an organochlorine pesticide developed as an alternative to dichlorodiphenyltrichloroethane owing to its shorter half-life, lower toxicity in mammals, and greater biodegradability [2, 4, 9]. MXC is an endocrine disruptor and previous studies reported that MXC affects the immune and reproductive systems by binding to estrogen receptors [5, 8]. In contrast, PARA is an organophosphorus pesticide developed to replace organochlorine pesticide due to its lower persistence in humans and animals. The pesticide activity of PARA is attributed to its ability to inhibit acetylcholinesterase, and recent studies showed that PARA plays a role in the dysregulation of immune system through a completely different mechanism than that of MXC [6]. Chemicals are basically existing in combination in real environment, therefore, the effect of combined exposure to multiple chemicals should be ensured. However, synergic or additive effects of these different types of agrochemicals on immune function has not been examined yet. We thus investigated in the present study a possible interaction between two different types of agrochemicals on the immune function *in vivo*. PARA and MXC were used as representative environmental chemicals which potentially have different impact on the immune function. In this study, TMA-induced airway inflammation model was used to examine the effect of prenatal exposure to MXC and PARA combination on the immune system. MXC+PARA combined exposure showed significant increases in eosinophils in the BALF compared to those of the individual treatment and control groups (see Fig. 1). Significant increase in eosinophils is observed in asthma patients [3], and eosinophil-deficient mice showed a decreased airway hyperresponsiveness, resulting in decreases in airway regeneration and mucus production [10]. Since eosinophils are considered to play an essential role in the activation of allergic airway inflammation [1] we concluded that in the present study, MXC+PARA combination aggravated part of the allergic airway pro-inflammatory response via eosinophils infiltration. Mast cell infiltration was also significantly increased by the exposure to MXC+PARA compared to those of the vehicle control group and PARA group. However, response of MXC+PARA group was comparable with that of MXC group, which indicates that MXC may strongly contribute to the increase of mast cell in MXC+PARA group. On the contrary, basophil infiltration was significantly increased by the exposure to MXC+PARA compared to those of the vehicle control group and MXC group, thus PARA play an important role to induce the basophil infiltration. To examine the contribution of T cells, B cells, and related cytokines to the effect of prenatal exposure to MXC and PARA combination, we analyzed the mouse hilar LNs 24 hr after TMA induction (see Table 1). Helper T cells, which play a central role in developing respiratory allergy, were not affected by the prenatal exposure to MXC, PARA, or MXC+PARA. However, significant B cells infiltration was observed in MXC+PARA group compared to that of the vehicle control, MXC and PARA groups showed little to no difference in B cells infiltration. The production of several cytokines, such as IL-6 and IL-9, were significantly upregulated by MXC+PARA exposure compared to those of the control group. However, similar tendency was also observed in MXC and PARA groups, suggesting a limited additive effect between MXC and PARA on T cells and B cells stimulation. In conclusion, our findings indicated that prenatal combined exposure to MXC and PARA, which have different impact on immune function, significantly induced eosinophil infiltration in a mouse model of allergic airway inflammation compared to

that of vehicle control and single chemical exposure groups. However, the interaction between MXC and PARA in immature mice was additive and quite limited, causing no significant increase in mast cell, basophil, helper T cell, B cell, and related cytokine levels. These findings suggest that combination of MXC and PARA may not induce the synergic toxic effect on immune system. While humans are at low risk of exposure to MXC or PARA nowadays, the information acquired in this study will provide the basis to assess the risk to combined toxicity of multiple environmental chemicals. Follow-up study will be performed to find the possible pairs of chemicals which may have synergic impact on immune system. It is also important to note that the transient exposure to MXC and/or PARA at early stages in life may exacerbate allergic airway inflammation in the later stages of life. Therefore, since the events between administration and outcome remain quite uncertain, future study has to take into consideration the key mechanisms underlying our findings.

CONFLICTS OF INTEREST. The authors state no conflict of interest.

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