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Prior oral exposure to environmental immunosuppressive chemicals methoxychlor, parathion, or piperonyl butoxide aggravates allergic airway inflammation in NC/Nga mice

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ABSTRACT

Background: Immunosuppressive environmental chemicals may increase the potency of allergens and thereby play a role in the development of respiratory tract allergies, such as allergic rhinitis and asthma.
Objectives: We investigated the association between environmental immunosuppressive chemicals and the allergic airway inflammation development.
Methods: We used a mouse model of ovalbumin (OVA)-induced allergic airway inflammation. NC/Nga mice were exposed orally to pesticides parathion (an organophosphate compound) or methoxychlor (an organochlorine compound), or to an insecticide synergist piperonyl butoxide, prior to OVA intraperitoneal sensitization and inhalation challenge. We assessed serum IgE levels, B-cell counts, cytokine production, IgE production in hilar lymph nodes, eosinophil counts, chemokine levels in bronchoalveolar lavage fluid, and cytokine gene expression in the lung.
Results: Exposure to environmental immunosuppressive chemicals markedly increased serum IgE – IgE-positive B-cells, IgE and cytokines in lymph nodes – eosinophils and chemokines in BALF – *IL-10a* and *IL-17* in the lung.
Conclusions: Allergic airway inflammation can be aggravated by prior exposure to immunosuppressive environmental chemicals.

1. Introduction

Recent reports suggest that prior exposure to environmental immunosuppressive chemicals induces autoimmune reactions and allergic diseases in immature rodents (Bakker et al., 2000; Casillas et al., 1999; Mustafa et al., 2009; Sobel et al., 2005; Yanagisawa et al., 2008). We have previously demonstrated that prior exposure to immunosuppressive environmental chemicals aggravates T cell-mediated allergic reactions (Fukuyama et al., 2010), as well as mite- and chemical allergen-induced atopic dermatitis-like immunoreaction in immature mice (Fukuyama et al., 2011a,b, 2012). These data indicate that immunosuppression by environmental chemicals is closely related to the aggravation of allergic reactions. However, the reasons for the increasing prevalence of asthma are unclear, although environmental factors such as air pollutants have been implicated (Pearce et al., 2007).

Many environmental allergens, viral infections, and environmental irritants are known contributors to the development of asthma. Asthma is a chronic inflammatory disease of the airways characterized by reversible airway obstruction, airway hyperreactivity, and remodeling of the airways. Infiltration of eosinophils in the lungs is a fundamental trait of the inflammatory response in asthma and may be important in the pathogenesis of this disease (Lambrecht et al., 2000; Maestrelli et al., 2009; De Monchy et al., 1985; Whitehead et al., 2003).

OVA-sensitized and challenged mouse model is one of the most popular allergic airway inflammation models, in particular in the BALB/c strain (Lin et al., 2006). However, different susceptibilities to OVA may exist in different laboratory strains and with different antigen doses (Morokata et al., 1999, 2000). In the previous studies, more massive and prolonged allergic responses, such as eosinophilic infiltration and IgE production, were observed in NC/Nga strain mice relative to BALB/c strain mice. Therefore, it is suggested that NC/Nga strain is highly sensitive to several types of allergic diseases/reactions, such as atopic dermatitis and allergic airway inflammation (Iwasaki et al., 2001; Yamamoto et al., 2007). In addition, Iwasaki et al. (2001) demonstrated that both OVA-sensitized and challenged mice revealed severer airway inflammation than only OVA-challenged mice (there were almost no effect in only OVA-challenged group). Similarly, according to our unpublished data on strain-dependent differences in susceptibility to OVA-induced allergic airway inflammation, both OVA-sensitized

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and challenged protocol in NC/Nga mice are the most sensitive condition (only OVA-sensitized or -challenged group showed much the same patterns as intact group in allergic airway inflammation). Therefore, to detect the aggravation of allergic airway inflammation by environmental immunosuppressive chemicals, we selected the OVA-sensitized and challenged NC/Nga strain mice as a model for allergic airway inflammation. Moreover, to explore the mechanisms of allergic airway inflammation development, we analyzed leukocyte numbers, chemokine production, immunoglobulin (Ig) E levels in serum and B-cells, cytokine production in hilar lymph node (LN) cells, and cytokine gene expression in the lung.

In this paper, we investigate the association between environmental immunosuppressive chemicals and allergic airway inflammation development following exposure to several types of environmental immunosuppressive chemicals: organochlorine pesticides (OC) such as methoxychlor, organophosphorus pesticides (OP) such as parathion, and an agricultural insecticide synergist used mainly with OP or pyrethroids such as piperonyl butoxide. These three chemicals were chosen on the basis of previous studies. Methoxychlor exposure has been demonstrated to result in atrophy of CD4+CD8+ double-positive T-cells in the thymus (Takeuchi et al., 2002a,b). Parathion markedly inhibits antigen-specific IgM production (Casale et al., 1984), and piperonyl butoxide administration depletes T-cells in the spleen and thymus, induces hypoplasia of the bone marrow, and inhibits T-cells proliferation in lymphoid tissues (Mitsumori et al., 1996; Diel et al., 1999; Battaglia et al., 2010). In addition, we have previously shown that methoxychlor, parathion and piperonyl butoxide exposure results in an increase in thymocyte apoptosis in vitro, and markedly inhibits sheep red blood cells (SRBC)-specific IgM production in mice (Fukuyama et al., 2012). However, although these chemicals contribute to suppressing immune functions, we have obtained contradictory results from studies in which T-lymphocyte-mediated allergic reactions and atopic dermatitis were exacerbated by prior oral exposure to methoxychlor, parathion, or piperonyl butoxide (Fukuyama et al., 2010, 2011a,b, 2012). To establish these paradoxes, we undertook the current study using a typical animal model of allergic airway inflammation.

2. Materials and methods

2.1. Animals

Female inbred NC/Nga mice (3-week old) were purchased from Charles River Japan (Atsugi, Kanagawa, Japan) and acclimatized for 6 days before the start of the experiment. Mice were housed individually under controlled lighting (lights on from 7:00 to 19:00 h), temperature (22 ± 3 °C), humidity (50 ± 20%), and ventilation (at least 10 complete fresh-air changes hourly). Food (Certified Pellet Diet MF, Oriental Yeast, Tokyo, Japan) and water were available ad libitum. The current study was conducted in accordance with the Animal Care and Use Program of the Institute of Environmental Toxicology (IET IACUC Approval No. AC12008).

2.2. Chemicals

Methoxychlor standard ($C_{16}H_{15}Cl_3O_2$, >97% pure), parathion standard ($C_{10}H_{14}NO_5PS$, 99.5% pure), piperonyl butoxide ($C_{19}H_{30}O_5$, >98% pure) standard, and 0.5% methylcellulose solution were purchased from Wako Pure Chemical Industries (Osaka, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). Albumin from chicken egg white (ovalbumin, OVA) was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan) and Sigma–Aldrich Japan K.K. (Tokyo, Japan). Aluminum hydroxide hydrate gel suspension (ALUM) was purchased from Cosmo Bio Co., Ltd. Methoxychlor, parathion and piperonyl butoxide were diluted in corn oil to concentrations (w/v) described below. For the intraperitoneal (i.p.) sensitization, OVA (10 μg) was dissolved in PBS (0.2 mL/animal) containing ALUM (2 mg). For the inhalation challenge, OVA was dissolved in PBS to 0.1% (w/v). To confirm the effect of the methoxychlor, parathion or piperonyl butoxide, we adopted OVA concentrations that were no adverse effect to allergic airway inflammation only sensitization/challenge.

2.3. Experimental protocol

The experimental protocol used in this study is depicted in Fig. 1. Following a 6 days acclimatization period, NC/Nga mice (4-week old) were allocated randomly to groups ($n = 8$ mice per group) for dosing, vehicle control, or no treatment (intact) group. According to our preliminary study, only OVA sensitization or challenge group showed much the same patterns as intact group in allergic airway inflammation. Therefore, data of only OVA sensitization or challenge are not shown in this issue. Doses of the chemicals were as follows: methoxychlor, 30 or 300 mg/kg day; parathion, 0.15 or 1.5 mg/kg day; piperonyl butoxide, 30 or 300 mg/kg day. Dosing groups and vehicle control group were given an oral dose of the test solution or vehicle (corn oil) only on days 1–5, respectively. Based on the EPA Immunotoxicity Guidelines established in 1998, the highest dose level used in a host should "not produce significant stress, malnutrition, or fatalities". Accordingly, in this study, the maximum doses used were selected to be <1/3 of the LD50 (dose at which ≥50% of animals would be expected to die) and concurrently to avoid the induction of clear systemic toxicity (i.e., changes in appearance, posture, behavior, respiration, consciousness, neurologic status, body temperature and excretion) (Fukuyama et al., 2012, in press). Actually, there were no abnormal signs during examination period. In body weight measurement, treated groups were comparable with vehicle control and intact groups (data not shown). In addition, the objective in this study was to investigate the relationship between immunosuppressive chemicals and allergic responses using confirmed environmental immunosuppressive chemicals using a mice model. The examination of relation to human exposures is now underway. For sensitization, OVA/ALUM was injected intraperitoneally into each mouse on days 29, 36, and 42. For challenge, OVA/PBS was administered by inhalation on days 43, 45, 47, 50, and 52. One day after the last challenge (day 53), all animals were anesthetized and sacrificed by injecting pentobarbital sodium (75 mg/kg, i.p.). Blood samples were taken from the inferior vena cava, and serum samples were assayed for IgE levels. Bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea and lavaging the lungs 3 times with 1 mL PBS supplemented with 1% heat-inactivated fetal calf serum (FCS; Life Technologies Co., Ltd., Tokyo, Japan). The first BALF fraction from each animal was centrifuged at 350 × *g* for 5 min, then supernatants were pooled, and chemokine levels were measured. The cell pellets of all three fractions were resuspended, pooled and centrifuged at 350 × *g* for 5 min. The supernatants were removed, and the cell pellets were used for differential cell counts. Hilar lymph nodes (LN) removed from each mouse were pooled in RPMI 1640 medium (Life Technologies Co., Ltd., USA). Single-cell suspensions were prepared from LNs by passage through a sterile 70-μm nylon cell strainer in 1 mL RPMI 1640 supplemented with 5% FCS. Single-cell suspensions were used to analyze the IgE-positive B-cells and cytokine production. Each animal's accessory lobe was removed, submerged in RNAlater Solution (Life Technologies Co., Ltd., Tokyo, Japan) overnight, and then used to extract total RNA.

2.4. Inhalation exposure

For the inhalation challenge, animals were exposed to 0.1% OVA/PBS mist continuously for 30 min/day. The animals were individually held in animal holders (Tokiwa Kagakukikai Co., Ltd., Tokyo, Japan) attached to a nose-only exposure chamber (total volume 31.2 L, Tokiwa Kagakukikai Co., Ltd.) so that only their noses were exposed to the chemical mist. The mist was generated by an atomizer (Ikeuchi Co., Ltd., Tokyo, Japan) with compressed air (ES4AD-5, Kobelco, Tokyo, Japan), supplied to the exposure chamber through an air filter (F3000-10-Y, CKD Corporation, Aichi, Japan). Airflow to the chamber was controlled by an area flowmeter (NSPO-4, Nippon Flow Cell, Tokyo, Japan) at a rate of 20 L/min. The chamber air was exhausted through an air filter system consisting of a glass wool filter, a mist trap, and an activated charcoal filter (Tokiwa Kagakukikai Co., Ltd.), and was emitted to the atmosphere by using a blower (TFO-K4P, Hitachi, Ltd., Tokyo, Japan). The actual concentration, mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were monitored by gravimetric analysis by using an air sampler (MF-200, Oct Science Co., Ltd., Osaka, Japan) and an Andersen type personal sampler (Model 1312S, Kanomax Japan, Inc., Osaka, Japan). The mean values of actual concentration of active ingredient, MMAD and GSD were kept at approximately 5 mg/m^3, 4.0 μm and 2.0, respectively, throughout the inhalation exposure.

2.5. Assay for total IgE

Total IgE levels in serum were measured by using enzyme-linked immunosorbent assay (BD OptEIA Mouse IgE ELISA Set, BD Pharmingen, Tokyo, Japan) according to the manufacturer's protocol.

2.6. Flow cytometry of BALF and hilar LN cells

The following antibodies used for flow cytometric analysis were purchased from BD Pharmingen: fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgE (clone R35-72), phycoerythrin (PE)-conjugated hamster anti-mouse CD11c (HL3), PE-conjugated anti-mouse Gr-1 (RB6-8C5), peridinin chlorophyll protein-conjugated anti-mouse CD3 (145-2C11), PE-cyanin-7-conjugated anti-mouse CD4 (RM-4-5), allo-phycocyanin-conjugated anti-mouse CD45R/B220 (RA3-6B2), and allo-phycocyanin-cyanin-7-conjugated anti-mouse CD8 (53-6.7). To avoid