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Role of regulatory T cells in the induction of atopic dermatitis by immunosuppressive chemicals

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HIGHLIGHTS

- Prior exposure to immunosuppressive chemicals can aggravate atopic dermatitis.
- Regulatory T cells may be related to the induction of atopic dermatitis.

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ABSTRACT

Immunosuppressive environmental chemicals may exacerbate allergic diseases, including atopic dermatitis (AD). We examined the effects of the immunosuppressive environmental chemicals methoxychlor, parathion, piperonyl butoxide, dexamethasone, and cyclophosphamide on picryl-chloride-induced AD in NC/Nga mice. Mice were orally exposed (age, 5 weeks) to these chemicals; during their sensitization and challenge (age, 8–12 weeks) with picryl chloride, we measured ear thickness and scored skin dryness, erythema, edema, and wounding. After the challenge, we analyzed dermatitis severity and cytokine gene expression in the pinna, serum levels of IgE and IgG2a, T- and B-cell numbers and cytokine production in auricular lymph nodes, and counted splenic regulatory T cells. Exposure to environmental immunosuppressive chemicals markedly increased dermatitis severity and gene expression in the pinna; serum IgE and IgG2a levels; and numbers of helper T cells and IgE-positive B cells, production of Th1 and Th2 cytokines, and production of IgE in auricular lymph-node cells and markedly decreased the numbers of splenic regulatory T cells. Prior exposure to immunosuppressive environmental chemicals aggravates AD; a decrease in the numbers of regulatory T cells may influence this process.

1. Introduction

Current evidence suggests that environmental chemicals increase the potency of allergens and thereby play a role in the development of allergic diseases (Casillas et al., 1999; Peat and Li, 1999; Yanagisawa et al., 2008). Recently, we demonstrated that prior oral exposure to immunosuppressive environmental chemicals aggravates T-cell-mediated allergic reactions as measured by the local lymph node assay, a thymidine-uptake test used to screen chemicals for their potential to cause dermal hypersensitivity (Fukuyama et al., 2010a,b). Although the local lymph node assay is efficient and informative, it is not a good predictor of potential changes in the host's organ-specific functionality. Therefore, more detailed evaluations are needed to clarify the role of immunosuppressive environmental chemicals in the aggravation of allergic reactions. In the current study, we used a mouse model of atopic dermatitis (AD) to explore the mechanisms involved in the aggravation of allergic responses after oral exposure to several environmental chemicals.

AD is characterized by chronic and relapsing inflammatory dermatitis, immunologic disturbances, and pruritic and eczematous skin lesions (Jang et al., 2011; Tanaka and Matsuda, 2011). In recent years, AD has become one of the most common skin diseases: 10–20% of children worldwide are affected, and its incidence is increasing in industrial countries (Leung, 2000). The complex mechanisms of AD include increased numbers of activated circulating CD4+ and CD8+ T cells and marked infiltration of CD4+ T cells into the dermis. In particular, allergen-specific Th2-type T cells bearing the cutaneous lymphocyte antigen are recruited to the skin. The initial phase of AD is dominated by Th2-type T cells that produce IL-4, IL-5, and IL-13. During the subsequent chronic phase, the number of Th1 cells that produce IFN-γ increases (Anthoni et al., 2007; Tanaka and Matsuda, 2011).

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NC/Nga mice are the most extensively studied animal model of AD (Jang et al., 2011; Matsuda et al., 1997). These mice spontaneously develop AD-like eczematous skin lesions when kept in conventional housing but not when maintained under SPF conditions (Shiohara et al., 2004). However, even NC/Nga mice housed under SPF conditions develop AD-like skin lesions after repeated treatment with hapten (Mosmann and Coffman, 1989; Sampson and Albergo, 1984). In the current study, we used picryl chloride (1-chloro-2,4,6-trinitrobenzene), which causes overt dermatitis in 100% of NC/Nga mice (Choi et al., 2012; Shiohara et al., 2004). To explore the mechanisms of AD development in this model, we analyzed dermatitis severity and expression of inflammation-associated genes in the pinna, IgE and IgG_{2a} levels in serum, and T- and B-cell surface-antigen expression and local cytokine production in auricular lymph nodes. In addition, we hypothesized the relation between immunosuppressive environmental chemicals and autoreactive T or B cells, leading to abnormal hypersensitivity. Therefore, we measured the surface antigen expression of splenic regulatory T (Treg) cells.

2. Materials and methods

2.1. Reagents

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, dexamethasone ($C_{22}H_{29}FO_5$, 98–102% pure), 0.5% methylcellulose solution, and olive oil were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cyclophosphamide monohydrate ($C_7H_{15}C_{l2}N_2O_2P{\cdot}H_2O$, 100.6% pure) was purchased from Sigma–Aldrich Japan (Tokyo, Japan). Picryl chloride (wetted with ca. 15% water; $C_6H_2ClN_3O_6$; 100.2% pure) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). Methoxychlor, parathion, and piperonyl butoxide were diluted in corn oil and dexamethasone and cyclophosphamide were diluted in 0.5% methylcellulose solution. After recrystallization with ethanol, picryl chloride was dissolved in acetone–olive oil (v/v, 4:1) solution to 0.5% or 1%.

All antibodies for flow cytometry were purchased from BD Pharmingen (Tokyo, Japan).

2.2. Animals

Female NC/NgaTnd mice (age, 3 weeks) were purchased from Charles River Japan Laboratories (Atsugi, Kanagawa, Japan) and housed individually under controlled lighting (lights on, 07:00 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 Code of Ethics for Animal Experimentation of the Institute of Environmental Toxicology.

2.3. Experimental protocol

After a 1-week acclimation period, NC/Nga mice (age, 4 weeks) were allocated into 5 or 6 groups (n = 8 mice per group) for each chemical (methoxychlor, parathion, piperonyl butoxide, dexamethasone, and cyclophosphamide): intact group (no treatment), vehicle-only control group (oral administration with corn oil or 0.5% methylcellulose solution, followed by sensitization and challenge with picryl chloride), low- and high-dose groups (oral administration of chemical, followed by sensitization and challenge with picryl chloride), and Treg analysis group (oral administration with high-dose chemical only). Chemical doses were: methoxychlor, 30 and 300 mg kg^{-1} day^{-1}; parathion, 0.15 and 1.5 mg kg^{-1} day^{-1}; piperonyl butoxide, 30 and 300 mg kg^{-1} day^{-1}; dexamethasone, 1 mg kg^{-1} day^{-1}; and cyclophosphamide, 10 mg kg^{-1} day^{-1}. 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, excretion, etc.). On each of days 1–5, mice were given an oral dose of the test solution (methoxychlor, parathion, piperonyl butoxide, dexamethasone or cyclophosphamide) or vehicle. Four weeks after the last oral administration (day 29), 100 μL of 1% picryl chloride was applied to each mouse's abdomen, which had been clipped free of fur 24 h previously, for sensitization. For challenge, a 25-μL aliquot of 0.5% picryl chloride was applied to the dorsum of each ear of each mouse on days 29, 32, 36, 39, 43, 46, 50, 53, 56, and 59. On days 30, 37, 44, 51, and 58, we measured ear thickness by using a gauge and determined clinical scores for skin dryness, erythema, edema, and wounding. Scores were assigned according to the following system: 0, no symptoms; 1, mild; 2, moderate; and 3, severe (Takano et al., 2006). On day 60 (the day after the last challenge), all mice were anesthetized and then euthanized by pentobarbital injection. Blood was collected from the inferior vena cava and serum samples assayed for substance P and total IgE and IgG_{2a} levels. The right pinna was removed from each mouse, pooled, and stored in RNAlater (Applied Biosystems, Tokyo, Japan) until used for RNA analysis. Auricular lymph nodes (LN) and spleens were removed, and pooled by tissue type in RPMI 1640 (Gibco, Tokyo, Japan). Single-cell suspensions from LNs and spleens were prepared by passage of the tissues through sterile 70-μm nylon cell strainers into 1 mL or 10 mL RPMI 1640 supplemented with 5% fetal calf serum (FCS, Gibco), respectively. The cell counts of the resulting suspensions were determined on an automated cell counter (model Z2, Beckman Coulter, Tokyo, Japan).

2.4. Enzyme immunoassay for serum substance P

Serum levels of substance P were measured by using an enzyme immunoassay (Substance P EIA Kit, Cosmo Bio, Tokyo, Japan) according to the manufacturer's protocol. The optical density at 405 nm was read by using a microplate reader (SpectraMax 190, Molecular Devices, Tokyo, Japan).

2.5. Enzyme-linked immunosorbent assay for total serum immunoglobulin

Total IgE and IgG_{2a} levels in serum were measured by using enzyme-linked immunosorbent assays (OptEIA Mouse Kit, BD Pharmingen, San Diego, CA, USA) in accordance with the manufacturer's protocol. The optical density at 405 nm was read by using a microplate reader.

2.6. IgE production by B cells in auricular LNs

B cells were isolated from auricular LNs by a magnetic cell-sorting system (autoMACS Separator, Miltenyi Biotec, Tokyo, Japan) and B220 microbeads (Miltenyi Biotec). To stimulate IgE production, we cultured B cells (1×10^6 cells/well) for 8 days with anti-CD40 ligand antibodies (150 ng/mL; R&D Systems, Tokyo, Japan) and recombinant IL-4 (150 ng/mL; R&D Systems) in 24-well plates at 37 °C in 5% CO_2. Total IgE concentrations in supernatants were measured by using enzyme-linked immunosorbent assays (BD Pharmingen).

2.7. Flow cytometry of auricular LNs

Auricular LNs were stained with fluorescein-isothiocyanate (FITC)-conjugated rat anti-mouse IgE (clone R35-72), FITC-conjugated hamster anti-mouse CD3 (clone 145-2C11), phycoerythrin (PE)–Cy5-conjugated rat anti-mouse CD45R/B220 (clone RA3-6B2), and phycoerythrin–Cy5-conjugated rat anti-mouse CD4 (clone RM4-5; all from BD Pharmingen). To avoid nonspecific binding, 1×10^6 cells were incubated with 20% normal goat serum for 10 min at 4 °C, followed by incubation with FITC- and PE–Cy5-conjugated monoclonal antibodies for 30 min at 4 °C in the dark. Cells were washed twice with 5% fetal calf serum in PBS, resuspended at 1×10^6 cells per tube in 1 mL PBS, and then analyzed on a FACSCaliber flow cytometer (BD Pharmingen) using Cell Quest software (BD Pharmingen). For each sample, 20,000 events were collected and analyzed for expression of antigens.

2.8. Cytokine production from T cells in auricular LNs

To stimulate T-cell receptor signaling, we cultured single-cell suspensions obtained from LNs (1×10^6 cells/well) with either anti-CD3 (2 μg/mL; BD Pharmingen) or anti-CD28 (2 μg/mL; BD Pharmingen) or both antibodies for 24 h or 96 h in 24-well plates at 37 °C in 5% CO_2. The concentrations of IL-4, IL-5, IL-6, IL-13, IL-17A, and interferon gamma (IFN-γ) in the supernatants were assayed by using the BD Cytometric Bead Array (BD Pharmingen) in accordance with the manufacturer's protocol.

IFN-γ in the supernatants was quantified after culture for 24 h in the presence of anti-CD3. IL-6 levels in the supernatants were quantified after culture for 24 h in the combined presence of anti-CD3 and anti-CD28. Amounts of IL-4, IL-5, IL-13, and IL-17A in supernatants were quantified after culture for 96 h in the combined presence of anti-CD3 and anti-CD28.

2.9. Cytokine gene expression in pinnae

Total RNA was extracted from pinnae by using NucleoSpin RNA II (Takara Bio, Tokyo, Japan) according to the manufacturer's protocol. The PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio) was used to reverse-transcribe RNA into cDNA, in accordance with the manufacturer's protocol. PCR primers (Table 1) for genes encoding IL-4 (*Il4*), IL-5 (*Il5*), IL-12/IL23P40 (*Il12b*), IL-13 (*Il13*), IL-17A (*Il17a*), IFN-γ (*Ifng*), and β actin (*Actb*) were purchased from Takara Bio. Resulting cDNAs were amplified by quantitative real-time polymerase chain reaction (PCR) analysis by using the Thermal Cycler Dice system (Takara Bio). The data acquired for each sample were normalized to the expression levels recorded for the housekeeping gene *Actb*.