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Use of long term dermal sensitization followed by intratracheal challenge method to identify low-dose chemical-induced respiratory allergic responses in mice

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ABSTRACT

The inhalation of many types of chemicals, including pesticides, perfumes, and other low-molecular weight chemicals, is a leading cause of allergic respiratory diseases. We attempted to develop a new test protocol to detect environmental chemical-related respiratory hypersensitivity at low and weakly immunogenic doses. We used long-term dermal sensitization followed by a low-dose intratracheal challenge to evaluate sensitization by the well-known respiratory sensitizers trimellitic anhydride (TMA) and toluene diisocyanate (TDI) and the contact sensitizer 2,4-dinitrochlorobenzene (DNCB). After topically sensitizing BALB/c mice (9 times in 3 weeks) and challenging them intratracheally with TMA, TDI, or DNCB, we assayed differential cell counts and chemokine levels in bronchoalveolar lavage fluid (BALF); lymphocyte counts, surface antigen expression of B cells, and local cytokine production in lung-associated lymph nodes (LNs); and antigen-specific IgE levels in serum and BALF. TMA induced marked increases in antigen-specific IgE levels in both serum and BALF, proliferation of eosinophils and chemokines (MCP-1, eotaxin, and MIP-1β) in BALF, and proliferation of Th2 cytokines (interleukin (IL)-4, IL-10, and IL-13) in restimulated LN cells. TDI induced marked increases in levels of cytokines (IL-4, IL-10, IL-13, and IFN-γ) produced by restimulated LN cells. In contrast, DNCB treatment yielded, at most, small, nonsignificant increases in all parameters. Our protocol thus detected respiratory allergic responses to low-molecular weight chemicals and may be useful for detecting environmental chemical-related respiratory allergy.

1. Introduction

The inhalation of many types of chemicals, including pesticides, perfumes, and other low-molecular weight chemicals, is a leading cause of allergic respiratory diseases (Brown and Jason, 2007; Jeebhay and Quirce, 2007; Kreutzer et al., 1999). Environmental and lifestyle factors and increasing use of such chemicals have often been suggested as contributors to such diseases (Ban and Hettich, 2005; Peden, 2000). These diseases are characterized by episodic obstruction, inflammation, and nonspecific hyperresponsiveness of the airway to a variety of stimuli, including histaminergic and cholinergic agents (Subcommittee on 'Occupational Allergy' of the European Academy of Allergology and Clinical Immunology, 1992). Development of these diseases requires sensitization, which is thought to be triggered by dermal or respiratory exposure to the sensitizing chemical, binding of the chemical to self-proteins, and subsequent presentation to chemical-specific lymphocytes. In susceptible individuals, this may result in the production of specific IgE antibodies, airway inflammation characterized by activated CD4+ T cells, eosinophils, and mast cells, and increased levels of interleukin (IL)-4 and IL-5 (Ban et al., 2006; Maestrelli et al., 1997; Mapp et al., 1999; Wisnewski and Redlich, 2001).

Several animal models have been used to identify chemical respiratory allergies (Arts et al., 2003; Ban et al., 2006). However, although environmental chemical allergens tend to have weak, minimal immunogenicity, these methods have focused on the detection of strong allergic reactions. Therefore, protocols are needed for the detection and treatment of weakly immunogenic and low-dose respiratory allergies. In a previous study, we developed a new detection method of environmental chemical-related hypersensitivity using typical T helper (Th) 1 (2,4-dinitrochlorobenzene, DNCB) and Th2 (trimellitic anhydride, TMA; and toluene diisocyanate, TDI) sensitizers by a long-term dermal sensitization protocol (Fukuyama et al., 2008). The study showed that, unlike low-dose 2,4-dinitrochlorobenzene (DNCB)

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treatment, treatment with low-dose TMA or TDI induced a typical Th2 response such as increase in antigen-specific serum IgE levels and IL-4 production in local lymph nodes (LNs). In contrast, secretion of Th1 cytokines in LNs was induced by treatment with the same low dose of the Th1 type allergen DNCB, with the production of high levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-2; Th1 cytokine production was not induced by the same concentrations of TMA or TDI (Fukuyama et al., 2008). These results demonstrated that our method is able to detect allergic reactions caused by chemicals occurring at weakly immunogenic and low doses in the environment. However, this test method is not fully capable of assessment of respiratory sensitizers because it lacks an airway challenge phase. In this issue, we attempt to develop our detection method of environmental chemical-related hypersensitivity moreover using another challenge route (respiratory tract).

The respiratory tract is routinely exposed to a variety of environmental allergens. The presentation of their derivative epitopes to T lymphocytes, a key event in the adaptive immune response, takes place in the lung-associated LNs (Ban et al., 2006; Banchereau and Steinman, 1998; Lambrecht et al., 2000). In previous mouse studies, treatment with TMA and TDI provoked a strong Th2 immune response associated with increases in total serum concentrations of IgE and IgE-positive B cells in the respiratory tract (Ban and Hettich, 2001; Matheson et al., 2005). However, little is known about the mechanism of the effect of very low doses of environmental chemicals.

In light of these previous results, the aim of this study was to improve our methods for detecting environmental chemical-related respiratory hypersensitivity at low and weakly immunogenic doses. To that end, we used long-term dermal sensitization followed by intratracheal challenge.

2. Materials and methods

2.1. Chemicals

For dermal sensitization, each test chemical was dissolved in 4:1 acetone:olive oil (AOO). For intratracheal challenge, each test chemical was dissolved in a sterile physiological saline solution. TMA and DNCB were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). TDI (95% 2,4 isomer), acetone, and olive oil were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DNCB is a contact allergen, whereas the others are respiratory allergens. The concentrations used are presented in Table 1. These concentrations of TMA, TDI, and DNCB were selected to avoid systemic toxicity and/or excessive local sensitization (particularly in the preliminary test) while still permitting comparisons of the sensitizing potencies of the chemicals. In addition, we focused on the EC3 values (the concentration of a chemical required to elicit a threshold positive response) in local lymph node assay (LLNA) and LLNA was used to select concentrations of TMA, TDI, and DNCB.

2.2. Animals

Female BALB/c mice (age, 7 weeks) purchased from Charles River Japan Laboratories (Atsugi, Kanagawa, Japan) 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.

Table 1
Chemical concentrations used

Groups	Sensitization	Challenge
TMA	0.1%	Low: 0.001% High: 0.01%
TDI	0.01%	Low: 0.0001% High: 0.001%
DNCB	0.03%	Low: 0.0003% High: 0.003%

TMA, trimellitic anhydride; TDI, toluene diisocyanate; DNCB, 2,4-dinitrochlorobenzene.

This study was conducted in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science (Japanese Association for Laboratory Animal Science, 1987).

2.3. Test and control groups

The mice were divided into five groups for each chemical (TMA, TDI, and DNCB): Group −/− (both sensitization and challenge with solvent only), Group −/+ (sensitized with solvent only and challenged with high-dose test solution), Group +/− (sensitized with high-dose test solution and challenged with solvent only), Group $+/+^{low}$ (sensitized with test solution and challenged with low-dose test solution), and Group $+/+^{high}$ (sensitized with test solution and challenged with high-dose test solution).

2.4. Sensitization and challenge protocol

After a 1-week acclimatization period, mice were allocated randomly to dose and control groups ($n = 8$ per group). On days 1–3, 8–10, and 15–17, a 25-μL aliquot of the test solution or solvent alone was applied to the dorsum of each ear of each mouse for dermal sensitization. Two weeks after the last sensitization (day 31), mice were anesthetized by intraperitoneal (i.p.) injection of pentobarbital sodium (50 mg/kg), and a challenge with a 50-μL aliquot of test solution or solvent alone was injected into intratracheal using a 29-G needle. On the day after the challenge (day 32), all mice were anesthetized with pentobarbital sodium (75 mg/kg) and sacrificed, and blood samples, BALF, and LNs were taken.

2.5. Antigen-specific serum IgE

TMA–ovalbumin (OVA) conjugate was prepared by Imject Immunogen EDC Kit in accordance with the manufacturer's protocol (Pierce Biotechnology, Inc. Rockford, MD, US). TDI–MSA conjugate (mouse serum albumin, Sigma–Aldrich Chemical Company Inc., Tokyo, Japan) and DNCB–MSA conjugate were prepared as described, by a modified version of the method of Ye et al. (2006). Briefly, TDI or DNCB was added to 1% HSA in PBS with constant stirring. Aliquots were taken 5, 10, 20, 30, and 40 min after the beginning of the reaction. Excess ammonium carbonate (2M, Sigma–Aldrich Chemical Company Inc., Tokyo, Japan) was added to each aliquot to terminate the reactions. All reactive samples were centrifuged at 3000 × *g* for 40 min to remove unreacted TDI and DNCB. The samples were then extensively dialyzed for 3 days against 0.1 mol/L ammonium carbonate and precipitated with an equal volume of 20% trichloroacetic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Samples were then redissolved in 1 mol/L sodium hydroxide (Sigma Aldrich) and dialyzed with deionized water for 24 h. The protein content of each conjugated sample was determined by the Lowry method (Lowry et al., 1951).

Specific serum IgE was measured by ELISA. Briefly, each flat-bottomed microplate well (Nalge Nunc International K.K., Tokyo, Japan) was coated with chemical–protein conjugates (100 μL, 0.1 mg/mL) in coating buffer (BD Pharmingen, San Diego, CA, USA) and incubated overnight at 4 °C. The content of each well was removed and the plate was washed fifth with wash buffer (BD Pharmingen) following each addition–incubation procedure. Nonspecific binding was blocked by incubation with 200 μL 10% heat-inactivated goat serum (Sigma–Aldrich Chemical Company Inc., Tokyo, Japan) in PBS for 1 h at room temperature. Mouse serum was diluted in PBS (from 1:4 to 1:4096). The dilute serum (100 μL) was added to each well and incubated for 2 h at room temperature. Biotin-conjugated rat anti-mouse IgE monoclonal antibody (BD Pharmingen, 2 μg/mL, 100 μL) was added to each well and incubated for 1 h at room temperature. Streptavidin–horseradish peroxidase (SAv–HRP) conjugate (BD Pharmingen, dilution 1:1000, 100 μL) was added to each well and incubated for 30 min at room temperature. The plate was developed with tetramethylbenzidine (TMB) (100 μL/well) in the dark, at room temperature, for 30 min. Optical density (OD) was read at a wavelength of 450 nm with an Immuno-Mini reader (NJ-2300, Nippon Intermed, Tokyo, Japan).

2.6. BALF analysis

BALF was collected by cannulating the trachea and lavaging the lung three times with 1 mL phosphate-buffered saline (PBS, Gibco, Tokyo, Japan) supplemented with 1% heat-inactivated fetal calf serum (FCS; Gibco, Tokyo, Japan) warmed to 37 °C. The first BALF fraction from each animal was then centrifuged at 1300 rpm for 5 min, and then the all the supernatants from all mice in a group were pooled. The supernatant was assayed for chemokine and cytokine levels (IL-6, MCP-1, eotaxin, and MIP-1β). The cell pellets of the first fraction and another two fractions were pooled and centrifuged at 1300 rpm for 5 min. The supernatant was removed and the cell pellet was resuspended in 1 mL PBS supplemented with 5% FCS for cell counts and differentials.

The number of cells was determined with a Coulter counter Z2 (Beckman Coulter Co., Tokyo, Japan). For differential cell counts, half of the cells were used for Cytospins (Shandon Inc., Pittsburgh, PA) and the remaining cells were used for flow cytometric analysis (de Haar et al., 2005). The Cytospin preparations were stained with Giemsa stain (Wako Pure Chemical Industries, Ltd. Osaka, Japan), and 200 cells in each sample were examined to differentiate the numbers of macrophages, eosinophils, and neutrophils present (according to standard leukocyte typing).