Original Article

Comparison of cytokine profiles in bronchoalveolar lavage fluid of mice exposed to respiratory and contact sensitizers

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ABSTRACT — Respiratory sensitization to certain low molecular weight chemicals is a big concern for workers, but unfortunately there are no validated animal models to allow identification of sensitizing chemicals in the environment. In the present study, dermally sensitized and intratracheally challenged mice were used to investigate effective indicators of respiratory sensitizers. Changes in levels of total serum IgE and nine cytokines (G-CSF, IL-4, IL-5, IL-6, IL-12(p70), IL-13, IFN- γ , MCP-1 and TNF- α) in bronchoalveolar lavage fluid (BALF) were analyzed in BALB/c mice exposed to respiratory sensitizers (phthalic anhydride (PA); diphenylmethane-4,4'-diisocyanate (MDI); toluene diisocyanate (TDI); chloramine-T (CH); and piperazine (PI)) or contact sensitizers (2,4-dinitrochlorobenzene (DNCB); and oxazolone (OXA)). Non-sensitized mice were treated dermally with solvents and challenged intratracheally with the respective test chemicals as solvent controls. Increases in total serum IgE levels were observed in all treated mice, with apparent differences in cytokine profiles. PA caused statistically significant increases in Th2 cytokines, IL-4, IL-5 and IL-13, compared with the control. IL-5 was also found to be increased with CH. The other three respiratory sensitizers caused statistically significant increases in IL-13. In contrast, no change was apparent with contact sensitizers, DNCB and OXA, in these Th2 cytokines. Increases in the Th2 cytokines indicate that all five respiratory sensitizers induced immune responses in lungs. Interestingly, elevation of G-CSF levels in BALF appeared with all five respiratory sensitizers but not the two contact sensitizers. The findings suggest that G-CSF could be effective to identify respiratory sensitizers in animal models.

Key words: Cytokines, Bronchoalveolar lavage fluid, Respiratory sensitizer, Contact sensitizer, Dermal sensitization, Intratracheal challenge

INTRODUCTION

Repeated exposure to certain low molecular weight (LMW) chemicals causes allergic responses in the human airway. These chemicals include diisocyanates and acid anhydrides that are generally used to make resins and polymers, which is therefore a major concern for workers in many industries (Tarlo, 2003). The immune response features IgE production, cell infiltration and subsequent inflammation in airways, leading to respiratory troubles such as asthma (Mapp *et al.*, 2005). Epidemiological studies have shown that respiratory sensitizers induce complex symptoms, for example, diphenylmethane-4,4'-diisocyanate (MDI), a well-known example, which caus-

es allergic asthma with pathophysiological features such as eosinophilia and reactive airway dysfunction syndrome in the lungs (Leroyer *et al.*, 1998; Hur *et al.*, 2008). In addition, respiratory sensitizers may exhibit IgE-dependent or -independent responses (Kimber *et al.*, 1998) and mechanisms of LMW chemical-induced respiratory allergy remain to be defined.

At present, there are no validated methods to experimentally identify respiratory sensitizers and their classification is mainly based on epidemiological studies of human populations (Kimber *et al.*, 2007). However, various animal models have been proposed (Arts and Kuper, 2007; Boverhof *et al.*, 2008). Previous studies showed the effectiveness of dermal application as a route of sensi-

zation and cytokines as indicators (Dearman *et al.*, 2000; Van *et al.*, 2002). Respiratory allergy is generally considered to be based on Th2 immune responses and dermal application of several respiratory sensitizers was found to induce such responses in mice (Dearman and Kimber, 2001). In order to measure cytokines, draining lymph node cells and bronchoalveolar lavage fluid (BALF) may be used (Dearman *et al.*, 2000; Van *et al.*, 2002; Fukuyama *et al.*, 2008). For example, lymph node cells have been collected from chemical sensitized mice and cultured with or without concanavalin A, a stimulation factor of the production of IL-4, and shown by Dearman and co-workers to respond to several respiratory sensitizers by secreting Th2 cytokines (IL-4, IL-5 and IL-13) (Dearman *et al.*, 2000; Dearman and Kimber, 2001). Similarly, Th2 cytokines (IL-4, IL-5 and IL-13) are increased in BALF (Ban *et al.*, 2006). Therefore, Th2 cytokines are considered to be effective for identification of respiratory sensitizing potentials of chemicals. However, few systematic studies on various cytokine responses caused by chemical respiratory sensitizers in lungs were reported. In addition, most studies used a limited battery of test chemicals such as diisocyanates and acid anhydrides for analysis of BALF cytokines.

The objectives of this study were to identify effective cytokines as markers for detecting respiratory sensitizing potentials of chemicals. In the study, we analyzed nine BALF cytokines (G-CSF, IL-4, IL-5, IL-6, IL-12(p70), IL-13, IFN- γ , MCP-1 and TNF- α) and serum total IgE of dermally sensitized and intratracheally challenged mice using five known respiratory sensitizers and two contact sensitizers systematically. Our results provide useful information on the identification of respiratory sensitizers using BALF cytokines in animal models.

MATERIALS AND METHODS

Animals

Female BALB/c mice (6-7 weeks old) were purchased from Charles River (Kanagawa, Japan) and individually identified by marking with ink. They were housed in plastic cages containing wood-chips, and allowed food and water *ad libitum*. Before treatment, the mice were quarantined for 1 week and acclimatized in the room maintained on a 12-hr light-dark cycle. All of the animal experiments were conducted in accordance with current Japanese laws and the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan) and were approved by the Environmental Health Science laboratory IACUC of Sumitomo Chemical Co., Ltd.

Chemicals

Toluene diisocyanate (TDI) and acetone were purchased from Kanto Chemical (Tokyo, Japan); phthalic anhydride (PA), diphenylmethane-4,4'-diisocyanate (MDI), chloramine-T (CH), piperazine (PI), 2,4-dinitrochlorobenzene (DNCB), olive oil and diethyl ether from Nakalai Tesque (Kyoto, Japan); dimethyl sulfoxide (DMSO) from Dojindo Laboratories (Kumamoto, Japan); oxazolone (OXA) from Sigma-Aldrich (Tokyo, Japan); midazolam from Astellas Pharma (Tokyo, Japan); and medetomidine and butorphanol from Meiji Seika Kaisha (Tokyo, Japan). PA, MDI, TDI, DNCB and OXA were dissolved in 4:1 acetone:olive oil (AOO) for dermal application. PA was heated to aid solution in AOO using a water bath before application. CH and PI were dissolved in DMSO for dermal application. For intratracheal instillation, PA, CH, PI and OXA were dissolved in DMSO and mixed with olive oil, while MDI, TDI and DNCB were mixed with olive oil.

Dermal application and intratracheal instillation

For dermal application, 25% (w/v) PA, 2% (w/v) MDI, 1% (w/v) TDI, 10% (w/v) CH, 5% (w/v) PI, 1% (w/v) DNCB, 1% (w/v) OXA or vehicle (AOO or DMSO) were used (Table 1). Fifty μ l of chemical was applied on each shaved flank on days 0, 1, 6 and 7. On days 12, 13 and 14, 25 μ l aliquots were applied on the dorsum of both ears. Non-sensitized mice were similarly treated dermally with solvents. On day 27, 50 μ l of 0.05% (w/v) PA, 0.0001% (w/v) MDI, 0.001% (w/v) TDI, 0.00001% (w/v) CH, 0.00001% (w/v) PI, 0.01% (w/v) DNCB or 0.001% (w/v) OXA were intratracheally instilled into both sensitized and non-sensitized mice (Table 1). Briefly, mice were anesthetized with a subcutaneously injection of midazolam (4 mg/kg), medetomidine (0.1 mg/kg) and butorphanol (5 mg/kg) in saline into the scruff of the neck. The trachea was then exposed surgically on the ventral side of the neck and intrapulmonary aerosolizer (PennCentury, PA, USA) was instilled via the mouth with monitoring of the exposed trachea for accurate instillation of chemicals. There were 8-10 mice per experimental group. The doses of dermal application were determined based on the previous studies (Dearman *et al.*, 1998; Plitnick *et al.*, 2003, 2005; Farraj *et al.*, 2007) and our unpublished data, which showed that a topical application of chemicals had little effect on mice skin. In brief, chemicals at various concentrations were applied on mice skin, and then the skin was observed 24 hr after application. The doses of intratracheal instillation were determined based on our pilot studies that chemicals at various concentrations were applied into lungs, and then the symptoms of mice were observed 24

Table 1. Doses for dermal application and intratracheal instillation of chemicals

Chemical	Dermal application (Sensitization)		Intratracheal instillation (Challenge)	
	Concentration (%)	Solvent	Concentration (%)	Solvent
PA	25	AOO	0.05	DMSO/Olive oil
MDI	2	AOO	0.0001	Olive oil
TDI	1	AOO	0.001	Olive oil
CH	10	DMSO	0.00001	DMSO/Olive oil
PI	5	DMSO	0.00001	DMSO/Olive oil
DNCB	1	AOO	0.01	Olive oil
OXA	1	AOO	0.001	DMSO/Olive oil

For dermal application, PA, MDI, TDI, DNCB and OXA were dissolved in 4:1 acetone:olive oil (AOO) and CH and PI were dissolved in DMSO. For intratracheal instillation, PA, CH, PI and OXA were dissolved in DMSO and mixed with olive oil and MDI, TDI and DNCB were mixed with olive oil. n = 8-10 mice/group.

hr after application. The doses with little effect on mice symptoms were selected for intratracheal instillation of chemicals.

Serum total IgE and BALF cytokines

Mice were anesthetized with inhalation of diethyl ether 24 hr after intratracheal instillation. Blood samples were collected from the abdominal aorta, and sera were stored frozen at -20°C until measurement of total IgE levels. Mice were sacrificed after the collection of blood samples and the trachea was exposed surgically on the ventral side of the neck. An incision was made on the upper trachea and a needle was inserted to allow BALF to be obtained using 1.0 ml of PBS (pH 7.3) containing 0.05 mM ethylenediaminetetraacetic acid (Dojindo Laboratories, Kumamoto, Japan) and 1 mg/ml bovine serum albumin (Wako Pure Chemical Industry, Osaka, Japan). BALF samples were centrifuged (800 × g, 10 min, 4°C) and the supernatants were frozen at -80°C until cytokine measurement. Serum total IgE and BALF cytokines (G-CSF, IL-4, IL-5, IL-6, IL-12(p70), IL-13, IFN-γ, MCP-1 and TNF-α) were measured by ELISA (Yamasa, Tokyo, Japan) and Bio-Plex (Bio-Rad, Tokyo, Japan), respectively, according to manufacturer's instructions. One-sided Student's T test was performed to assess whether the measurement values of sensitized groups were significantly greater than those of each control.

RESULTS

Serum total IgE

Mice were treated with chemicals as shown in Fig. 1. Non-sensitized mice were treated dermally with solvents

and challenged intratracheally with the respective test chemicals as solvent controls. Remarkable differences in symptoms were not observed between sensitized and non-sensitized mice 24 hr after intratracheal challenge. Sera were collected from dermally treated mice 24 hr after intratracheal challenge. Respiratory sensitizers, PA, MDI, TDI, CH and PI, showed statistically significant increases in total serum IgE compared with each control (Fig. 2). As with these respiratory sensitizers, contact sensitizers DNCB and OXA caused increases in serum total IgE (Fig. 2).

BALF cytokines

BALF was obtained 24 hr after intratracheal challenge (Fig. 1). Nine cytokines (G-CSF, IL-4, IL-5, IL-6, IL-12(p70), IL-13, IFN-γ, MCP-1 and TNF-α) in BALF were measured by multiplex bead assays. Production of cytokines differed among chemicals, as shown in Fig. 3. The reproducibility of the results was confirmed by additional studies for each chemical (data not shown).

The level of IL-4 showed a statistically significant increase in PA-sensitized mice compared with the control. However, the other chemicals did not cause any increase of IL-4 in sensitized mice. Intratracheal challenge with CH also resulted in elevated IL-4 in BALF, but was observed in both CH-sensitized and control mice. Thus, it was considered that the responses were not based on immune reactions. PA and CH, but not the other chemicals used in this study, caused statistically significant increases in the levels of IL-5 compared with controls. PA, TDI, CH, PI and DNCB induced statistically significant increases in the levels of IL-6. Elevation of IL-12(p70) levels was statistically significant with PA, MDI and TDI. Similarly

Fig. 1. Experimental design. Fifty μ l aliquots of chemicals were applied on each shaved flank on days 0, 1, 6 and 7. On days 12, 13 and 14, 25 μ l of the same chemical was applied on the dorsum of both ears. On day 27, 50 μ l of the chemical was intratracheally instilled and serum collection and bronchoalveolar lavage were performed 24 hr thereafter. n = 8-10 mice/group.

Fig. 2. Serum total IgE, measured by ELISA. *P < 0.05, **p < 0.01, ***p < 0.001 compared with each respective control group. n = 8-10 mice/group.

Fig. 3. BALF cytokines. IL-4, IL-5, IL-6, IL-12(p70), IL-13, IFN- γ , MCP-1, G-CSF and TNF- α were measured using a multiple cytokine assay system (Bio-Plex). *P < 0.05, **p < 0.01, ***p < 0.001 compared with each respective control group. n = 8-10 mice/group.

Cytokine profiles in BALF of mice exposed to chemical sensitizers

IL-13 was found to be increased with PA, MDI, TDI and PI, and IFN- γ with PA, MDI, TDI and CH. Only PA and TDI caused statistically significant increase in MCP-1. All respiratory sensitizers tested in this study (PA, MDI, TDI, CH and PI) induced statistically significant increases in the levels of G-CSF compared with controls. In contrast, no such change was evident with contact sensitizers (DNCB and OXA). PA, MDI and TDI caused statistically significant increase in TNF- α .

DISCUSSION

In this study, five respiratory sensitizers (PA, MDI, TDI, CH and PI) induced statistically significant increases in total serum IgE (Fig. 2), but so did both contact sensitizers, DNCB and OXA, similarly tested (Fig. 2). Previous reports also showed that dermal application of not only respiratory sensitizers but additionally a contact sensitizer DNCB increased serum total IgE in mice (Ban and Hettich, 2001; Selgrade *et al.*, 2006). While serum IgE is considered to be one of the triggers of chemical-induced asthma (Holsapple *et al.*, 2006; Arts and Kuper, 2007), epidemiological studies have suggested that IgE inde-

pendent responses may also be involved (Kimber *et al.*, 1998). The available data thus suggest that increase in total serum IgE is related to immune reactions, but itself can not be used as an indicator to discriminate between respiratory and contact sensitizers, at least in dermally sensitized mice.

Cytokine profiling of dermally sensitized mice consistently demonstrated their effectiveness as indicators of respiratory sensitizers (Dearman *et al.*, 2000; Dearman and Kimber, 2001). Dermal application of PA, MDI, TDI, CH and PI caused Th2 immune responses in mice (Dearman *et al.*, 1998; Plitnick *et al.*, 2003, 2005; Farraj *et al.*, 2007). We therefore used these chemicals to analyze immune responses in lungs. The present study showed that all five induced statistically significant increases in BALF cytokines as a result of dermal sensitization and intratracheal challenge (Fig. 3). However, differences in BALF cytokine profiles were observed, PA, MDI, TDI and PI for example causing statistically significant increases in IL-13, whereas no change was apparent with CH. Earlier work indicated skin exposures of respiratory sensitizers such as PA, MDI, TDI, CH and PI to induce Th2 responses featuring increased levels of IL-4, IL-5 and

Fig. 3. (Continued.)

Table 2. Summary of results

Chemical	PA	MDI	TDI	CH	PI	DNCB	OXA
Serum total IgE	+++	+++	+++	+++	+	++	+++
BALF cytokine							
IL-4	+	–	–	–	–	–	–
IL-5	++	–	–	+	–	–	–
IL-6	++	–	+	+	+	+	–
IL-12 (p70)	++	+	+	–	–	–	–
IL-13	+++	+	+	–	++	–	–
IFN- γ	++	+	+	++	–	–	–
MCP-1	+	–	+	–	–	–	–
G-CSF	+++	+	++	+	++	–	–
TNF- α	++	+	+	–	–	–	–

The measurement results of chemical-sensitized mice were compared with those of each control. + indicates a statistically significant increase (+ $P < 0.05$, ++ $P < 0.01$ and +++ $P < 0.001$); – indicates no change. $n = 8$ –10 mice/group.

IL-13 in lymph nodes. The elevation of IL-4 levels was due to increases in IL-4 producing T cells in lymph nodes (Dearman *et al.*, 2000; Dearman and Kimber, 2001). However, our results showed that IL-5 and IL-13 were increased in BALF by two and four examples, respectively, whereas IL-4 showed a statistically significant increase only in BALF of PA sensitized mice (Fig. 3). These results imply that IL-4 producing T cells were increased in lymph nodes but not in lungs of mice sensitized with MDI, TDI, CH or PI. In addition to Th2 cytokines, production of Th1 cytokines like IL-12(p70) and IFN- γ was elevated by several of our respiratory sensitizers (Fig. 3). It seems that the doses and frequencies of dermal application influence the Th1/Th2 balance and induce complex immune responses with Th1 and Th2 cytokines. Our results indicate that we may need to analyze a panel of several cytokines to assess immune responses.

Respiratory sensitizers increased Th2 cytokines in BALF (Fig. 3), which showed that immune responses were caused in lungs. The present study showed that all five respiratory sensitizers induced statistically significant increase in G-CSF, but not DNCB and OXA (Fig. 3). G-CSF functions as a stimulatory factor for neutrophils, which are reported to be involved in chemical-induced asthma in man (Park *et al.*, 1999). Increase in neutrophils was also observed in animal models with dermal sensitization (Matheson *et al.*, 2005; Vanoirbeek *et al.*, 2004). Influx of eosinophils observed in human and animal models was also appears related to allergic asthma. However, our study showed that IL-5, which is closely related to the influx of eosinophils in lungs, was only increased by

PA and CH (Fig. 3). The animal model used in this study featured intratracheal challenge only once and BALF was collected 24 hr after the challenge, to detect immune responses at an early stage. A more sustained challenge may be required to generally induce influx of eosinophils. Thus, it was considered that neutrophils, rather than eosinophils, may be effective indicators in this topically intratracheal challenge model. Notably, G-CSF was increased by all respiratory sensitizers used in this study (Table. 2). The findings thus suggest that G-CSF and related cytokines could be effective to identify respiratory sensitizers in animal models.

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