Isocyanate exposure in the workplace has been linked to asthma and allergic rhinitis. Recently, investigators have proposed that Th2 cytokine responses in lymph nodes draining the site of dermal application of chemicals including isocyanates may be used to identify sensitizers that cause asthma-like responses. The purpose of this study was to determine if the cytokine profile induced after dermal sensitization with isocyanates and serum IgE predict immediate (IHS) and methacholine-induced late (LHS) respiratory hypersensitivity responses after intranasal challenge. Dermal application of hexylmethane diisocyanate (HMDI), toluene diisocyanate (TDI), or methylene diisocyanate (MDI) significantly increased interleukin-4 (IL-4), IL-5, and IL-13 secretion in parotid lymph node cells. Isophorone diisocyanate (IPDI) increased IL-4 and IL-13, but not IL-5. Tolyl(mono)isocyanate (TMI), tetramethylene xylene diisocyanate (TMXDI), or the contact sensitizer dinitrochlorobenzene (DNCB), only induced minor increases in some of the Th2 cytokines. HMDI, TDI, MDI, and IPDI elicited greater increases in total serum IgE than DNCB, TMI, and TMXDI. All chemicals except TMXDI caused IHS after intranasal challenge of sensitized female BALB/c mice. Only HMDI-, TMI-, or TMXDI-sensitized and challenged mice had increases in LHS. All chemicals elicited epithelial cytotoxicity indicative of nasal airway irritation. The discordance between dermal cytokine profiles and respiratory responses suggests that dermal responses do not necessarily predict respiratory responses. Serum IgE also was not predictive of the respiratory responses to the isocyanates, suggesting that other unknown mechanisms may be involved.

Key Words: Isocyanates; airway hypersensitivity; Th2 cytokines; serum IgE; skin draining lymph nodes; intranasal instillation; dermal exposure; mice; dinitrochlorobenzene; hazard identification.

Th2 Cytokines in Skin Draining Lymph Nodes and Serum IgE Do Not Predict Airway Hypersensitivity to Intranasal Isocyanate Exposure in Mice

Aimen K. Farraj, Elizabeth Boykin, Najwa Haykal-Coates, Stephen H. Gavett, Donald Doerfler and MaryJane Selgrade1

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Isocyanate exposure in the workplace has been linked to asthma and allergic rhinitis. Recently, investigators have proposed that Th2 cytokine responses in lymph nodes draining the site of dermal application of chemicals including isocyanates may be used to identify sensitizers that cause asthma-like responses. The purpose of this study was to determine if the cytokine profile induced after dermal sensitization with isocyanates and serum IgE predict immediate (IHS) and methacholine-induced late (LHS) respiratory hypersensitivity responses after intranasal challenge. Dermal application of hexylmethane diisocyanate (HMDI), toluene diisocyanate (TDI), or methylene diisocyanate (MDI) significantly increased interleukin-4 (IL-4), IL-5, and IL-13 secretion in parotid lymph node cells. Isophorone diisocyanate (IPDI) increased IL-4 and IL-13, but not IL-5. Tolyl(mono)isocyanate (TMI), tetramethylene xylene diisocyanate (TMXDI), or the contact sensitizer dinitrochlorobenzene (DNCB), only induced minor increases in some of the Th2 cytokines. HMDI, TDI, MDI, and IPDI elicited greater increases in total serum IgE than DNCB, TMI, and TMXDI. All chemicals except TMXDI caused IHS after intranasal challenge of sensitized female BALB/c mice. Only HMDI-, TMI-, or TMXDI-sensitized and challenged mice had increases in LHS. All chemicals elicited epithelial cytotoxicity indicative of nasal airway irritation. The discordance between dermal cytokine profiles and respiratory responses suggests that dermal responses do not necessarily predict respiratory responses. Serum IgE also was not predictive of the respiratory responses to the isocyanates, suggesting that other unknown mechanisms may be involved.

Key Words: Isocyanates; airway hypersensitivity; Th2 cytokines; serum IgE; skin draining lymph nodes; intranasal instillation; dermal exposure; mice; dinitrochlorobenzene; hazard identification.

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responses and those that do not elicit asthma (Betts et al., 2002; Dearman et al., 1996, 1999, 2000, 2002; Hayashi et al., 2001; Plitnick et al., 2002; Vandebriel et al., 2000). The model, if validated, would minimize the duration of studies, reduce cost, and expedite the screening of chemicals for respiratory sensitization potential.

There is evidence, however, that the use of Th2 cytokine production or expression in the local draining lymph nodes to identify respiratory allergens may be problematic. In a recent study, investigators showed that the contact sensitizers, dinitrochlorobenzene (DNCB) and oxazolone (OXAZ), which have not been associated with asthma, elicited Th2 cytokine increases in draining lymph nodes of the skin that was comparable to that elicited by chemicals known to cause asthma (Traidl et al., 1999; Ulrich et al., 2001). This suggests that Th2 cytokine induction may not distinguish chemicals with potential to cause asthma.

Recently, we applied the cytokine profiling approach to assess the sensitization potential of six diisocyanates, some of which have been associated with asthma, elicited Th2 cytokine responses in local draining lymph nodes that were not predictive of total serum IgE levels or methacholine (Mch)-induced changes in ventilatory timing, a surrogate for late-onset airway hyperresponsiveness. In the present study, we test the hypothesis that cytokine profiling in draining lymph nodes and/or serum IgE is predictive of immediate and late airway hyperresponsiveness and nasal histopathology following intranasal challenge of dermally sensitized mice.

MATERIALS AND METHODS

Animals. Young adult (8-week-old) female inbred BALB/c mice were obtained from Charles River Laboratories (Raleigh, NC). Mice were housed in polycarbonate cages on beta-chip bedding in groups of six per cage, maintained on a 12-h light/dark cycle at approximately 22°C and 50% relative humidity in our Association for Assessment and Accreditation of Laboratory Animal Care approved facility, and held for a minimum of 5 days before treatment. Food (Prolab RMH 3000; PMI Nutrition International, St Louis, MO) and water were provided ad libitum. Six mice were assigned to each exposure group.

Experimental design. Figure 1 depicts the exposure regimen used for mouse exposure. Mice were exposed to one of the following seven chemicals: 1% tolylene-2,4-diisocyanate (TDI; Sigma Chemical, St Louis, MO), 2% 4,4′-methylenebis (phenyl isocyanate) (MDI; Sigma Chemical). 2% isophorone diisocyanate (IPDI; Aldrich Chemical, Milwaukee, WI), 2% dicyclohexylmethane-4,4′-diisocyanate (HMDI; Aldrich Chemical), 1% P-toly(lmono)isocyanate (TMI; courtesy of DuPont Haskell Laboratory, Newark, DE), meta-tetramethylene xylene diisocyanate (TMXDI; courtesy of DuPont Haskell Laboratory), or 1-chloro-2,4-di-tinortenzone (DNCB; Sigma Chemical). The concentrations for each of the chemicals were selected because they optimally enhanced cytokine responses in draining lymph node cells (Plitnick et al., 2002) and had equivalent potency in the LLNA as determined in a previous study (Selgrade et al., 2006). Mice were divided into three experimental groups: mice were treated dermally to chemical or vehicle and sacrificed at 14 days for cytokine profiling (Group A), six mice per chemical and a vehicle control were treated dermally with chemical throughout sensitization and challenged intranasally on Day 24 with the same chemical (Group B), and six animals per chemical received vehicle dermally throughout sensitization, but were challenged intranasally on Day 26 with chemical to control for nonspecific responses (Group C). On Days 0 and 5, mice were topically sensitized via dermal application of 100 μl of the test chemical dissolved in a 4:1 acetone and olive oil vehicle onto the shaved back. Vehicle groups of mice were similarly treated. On Days 10, 11, and 12, 25 μl of the chemical solution or vehicle was applied to each ear. On Day 14, Group A mice were sacrificed for the purpose of removing the parotid lymph nodes and to make single-cell suspensions that were analyzed for cytokine profiling. Blood was also collected from these mice. The remaining mice (Groups B and C) were again topically sensitized on the shaved back with 100 μl of the chemical solution or treated with vehicle on Day 19. All mice (both Group B sensitized and Group C nonsensitized) were then challenged via the airway on Day 24 by intranasal instillation with 60 ml of a 1% solution of one of the six chemicals in a 1:4 ethyl acetate and olive oil vehicle while lightly anesthetized using 3% isoflurane. Immediately after intranasal instillation of the chemicals, the mice were assessed for immediate airway hyperresponsiveness using whole-body plethysmography described below. On Day 26, respiratory responsiveness to increasing concentrations of aerosolized Mch was also measured using whole-body plethysmography. Thus, pulmonary physiology measurements on Day 24 tested specific responses to airway provocation with test chemicals, whereas measurements on Day 26 in the absence of chemical provocation described nonspecific airway reactivity.

Draining lymph node cell suspensions and cytokine responses. The parotid lymph nodes from Group A mice were excised and then placed in RPMI-1640-supplemented with 10% fetal calf serum (FCS) (GIBCO, Carlsbad, CA), 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO). Single-cell suspensions were then prepared by homogenizing the lymph nodes and then resuspending in RPMI-1640 with 10% FCS. The suspensions were spun down and resuspended in 1 ml of the RPMI. The cells were counted, and the viability was then determined using the trypan blue dye exclusion method. The cell numbers were adjusted to 1 × 10^7 viable cells/ml. Wells were seeded at 10^6 cells per well in a 24-well microtiter plate. The cells were cultured in the presence of 2 mg/ml concanavalin A (ConA; Sigma Chemical) for 24 h at 37°C in a humidified atmosphere of 5% CO₂. The supernatants were collected and centrifuged at 150 × g for 10 min. Cytokine proteins in cell supernatants were detected using the Luminex 100 (Luminex Corporation, Austin, TX) and LINCOplex kits (LINCO Research, Inc., St Charles, MO) for simultaneous
measurement and detection of the following cytokines: IL-4, 5, 13, and interferon (IFN)-γ.

In vivo airway responses. Antigen-specific airway responsiveness was measured on Day 24 immediately after exposure to each chemical in unrestrained mice, using whole-body plethysmography (Buxco Electronics, Troy, NY). Pressure signals were analyzed using BioSystem XA software (SFT3812, version 2.0.2.48, Buxco Electronics) to derive whole-body flow parameters that were used to calculate enhanced pause (Penh). Enhanced pause (Penh) was used as an index of airway hyperresponsiveness as previously described (Gavett et al., 2003; Hamelmann et al., 1997). Baseline Penh measurements for each animal were recorded for 10 min and averaged. The mice were then intranasally instilled with one of the chemicals and placed back in the chambers within 2 min of dosing. Penh readings were then monitored and averaged over a 30-min postinstillation period.

Non-specific airway responsiveness to increasing concentrations of aerosolized Mch in anesthetized, unrestrained mice in a 12-chamber whole-body plethysmograph system was measured 48 h after intranasal challenge on Day 26. After measuring baseline parameters for 7 min, an aerosol of saline or Mch in increasing concentrations (10, 32, and 100 mg/ml) was nebulized through an inlet of the chamber. The response to saline or Mch was measured over the aerosolization period (1 min), an aerosol drying step (2 min), and an additional 4-min period.

Bronchoalveolar lavage. Group B mice were deeply anesthetized with ip injection of sodium pentobarbital (0.5 ml of 5 mg/ml). Blood samples were collected by cardiac puncture and serum samples were collected and stored at −80°C. The abdominal aorta and renal artery were then severed to exanguinate the mice. After exsanguination, the trachea was canulated and the lungs were lavaged with two 1-ml aliquots of Ca²⁺, Mg²⁺, and phenol red-free Hank’s balanced salt solution (HBSS, 35 ml/kg; Life Technologies, Bethesda, MD). Approximately 85% of the total instilled volume was recovered in all treatment groups. The BAL fluid was maintained on ice and centrifuged at 360 × g for 10 min at 4°C. BAL cells were resuspended in 1 ml HBSS and counted (Z1; Coulter, Hialeah, FL). Cytospin preparations of BAL cell samples were prepared and stained with Wright-Giemsa Stain Pack (Fisher Diagnostics, Middleton, VA) using an automated slide stainer (Hematek 2000; Miles Inc., Elkhart, IN). Cell differentials and percentages were determined by counting 200 cells per slide.

Histopathology. After removal of the right lung lobes of Group B mice, the left lung lobe was intratracheally perfused with 4% paraformaldehyde at a constant intra-airway pressure of 30 cm of fixative. After 1 h, the trachea was ligated, and the inflated left lung lobe was immersed in a large volume of the same fixative for 4 h. The lungs were then placed in 70% ethanol at 4°C. After fixation, the left lung lobe was microdissected along the axial airways, and sections were then excised at the level of the fifth airway generation as described previously (Farraj et al., 2003). Fixed lungs were processed to paraffin blocks, sectioned at a thickness of 5 μm, placed on glass slides, and stained with hematoxylin and eosin.

The head of each mouse was excised from the carcass, and the eyes, skin, skeletal muscle, and lower jaw were removed. The heads were immersed in 4% paraformaldehyde for 4 h. After fixation, the heads were decalcified in 13% formic acid for 7 days and then rinsed in tap water for at least 4 h. The nasal cavity of each mouse was transversely sectioned at three specific anatomic locations according to a modified method of Young (1981). The most proximal nasal section was taken immediately posterior to the upper incisor teeth (proximal, T1); the middle section was taken at the level of the incisive papilla of the hard palate (middle, T2); the most distal nasal section was taken at the level of the second palatal ridge (distal, T3). These tissue blocks were embedded in paraffin, sectioned at a thickness of 5 μm, and then stained with hematoxylin and eosin for light microscopic examination.

Total serum IgE. Total serum IgE was measured in Group B mice 48 h after intranasal challenge with the chemicals using a 96-well Nunc ELISA plate (Nalge Nunc International, Rochester, NY) coated with 100 ml per well of Anti-

Mouse IgE antibody (Pharmingen, San Diego, CA) in coating buffer and incubated overnight at 4°C. After washing, the plates were incubated with 1% bovine serum albumin at room temperature for 1 h (Calbiochem, La Jolla, CA). Serum samples at 1:10 dilution or anti-mouse IgE standards (Serotec, Oxford, UK) were then added followed by incubation at room temperature for 1 h. After washing, biotinylated anti-mouse IgE (Pharmingen) was then added and allowed to incubate at room temperature for 1 h. After washing, 15 μg/ml of streptavidin peroxidase (Pharmingen) was added followed by incubation at 25°C for 1 h. After washing, tetramethylbenzidine substrate (Alpha Diagnostic International, San Antonio, TX) was added to produce a color reaction. After color development, the reaction was terminated by the addition of H₂SO₄ (Alpha Diagnostic International). Optical density was determined at 450 nm using a SpectraMax 340pc plate reader (Molecular Devices, Sunnyvale, CA). The mean concentrations were determined using Softmax Pro software (Molecular Devices).

Statistics. For the analysis of the immediate hypersensitivity Penh responses cytokine protein levels, and serum IgE, a two-factor ANOVA was used (SAS Institute Inc., Cary, NC). The two factors were chemical exposure and sensitization. Pairwise comparisons were performed to find significant differences (p < 0.05). For the analysis of Mch-induced Penh responses, a multivariate ANOVA was used.

RESULTS

Cytokine Responses in Draining Lymph Nodes (Group A Mice)

Relative to vehicle control there were significant increases in secreted IL-4 protein from parotid lymph node cells stimulated ex vivo with ConA in mice that were topically exposed to HMDI (181-fold increase over vehicle; p < 0.01), TDI (157-fold; p < 0.01), MDI (72-fold; p < 0.01), IPDI (58-fold; p < 0.01), TMI (8-fold; p < 0.01), and TMXDI (3-fold; p < 0.05) relative to mice exposed to the ethyl acetate/olive oil vehicle (Fig. 2A). The HMDI- and TDI-induced increases were also significantly greater than all other groups (p < 0.01; p < 0.05 vs. MDI). Both MDI and IPDI induced increases that were significantly greater than TMI, and TMXDI (p < 0.05), which induced responses significantly different from DNCB. There were no significant differences between mice that were exposed to DNCB and mice that were exposed to vehicle.

There were significant increases in secreted IL-5 protein in lymph nodes stimulated ex vivo with ConA in mice that were topically exposed to HMDI (11-fold; p < 0.05), MDI (27-fold; p < 0.01), and TDI (29-fold; p < 0.01) relative to mice exposed to the ethyl acetate/olive oil vehicle (Fig. 2B). Both TDI and MDI induced increases that were significantly greater than all other groups (p < 0.01). There were no significant differences between mice that were exposed to IPDI, TMI, TMXDI, or DNCB and mice that were exposed to vehicle.

There were significant increases in secreted IL-13 protein in lymph nodes stimulated ex vivo with ConA in mice that were topically exposed to HMDI (55-fold; p < 0.01), TDI (82-fold; p < 0.01), MDI (63-fold; p < 0.01), IPDI (26-fold; p < 0.01), TMI (7-fold; p < 0.01), TMXDI (5-fold; p < 0.01), and DNCB (3-fold; p < 0.05) relative to mice exposed to the ethyl acetate/olive oil vehicle (Fig. 2C). HMDI, TDI, and MDI each induced responses that were significantly greater than all other groups.
There were significant increases in secreted IFN-γ protein in lymph nodes stimulated ex vivo with ConA in mice that were topically exposed to HMDI (43-fold; *p* < 0.01), TDI (88-fold; *p* < 0.01), MDI (340-fold; *p* < 0.01), IPDI (50-fold; *p* < 0.01), TMI (12-fold; *p* < 0.01), TMXDI (6-fold; *p* < 0.01), and DNCB (8-fold; *p* < 0.01) relative to mice exposed to the ethyl acetate/olive oil vehicle (Fig. 2D). The MDI-induced increase was significantly greater than all other groups (*p* < 0.01). HMDI, TDI, and IPDI each induced increases that were significantly greater than all other groups except MDI (*p* < 0.01).

Airway Physiology (Group B and C Mice)

Increases in expiratory time and peak expiratory flow and decreases in inspiratory time and peak inspiratory flow contribute to increases in Penh. These changes in ventilatory timing may coincide with airflow obstruction or increased airway resistance at the level of the nasal, tracheobronchial, or pulmonary regions (Hamelmann et al., 1997). All chemicals except TMXDI elicited significant increases in average Penh immediately after intranasal chemical challenge of sensitized mice relative to both vehicle-exposed and unsensitized mice challenged with chemical, the control for nonspecific responses (Fig. 3). Sensitization and challenge with TMI induced a Penh response that was significantly greater than that induced by DNCB, IPDI, MDI, and TMXDI (*p* < 0.01). Sensitization and challenge with HMDI induced an immediate Penh response that was significantly greater than that induced by DNCB, IPDI, MDI, and TMXDI (*p* < 0.01).

There were very small differences between the chemical treatment groups in response to MCH at baseline (not shown) and Mch concentrations of 10 and 32 mg/ml (Figs. 4A and 4B). At 100 mg/ml Mch, HMDI sensitization and challenge caused...
a 1.8-fold increase in Penh relative to vehicle-exposed mice (p < 0.01; Fig. 4C). TMI sensitization and challenge caused a 1.9-fold increase in Penh relative to vehicle-challenged mice (p < 0.01). The responses to HMDI or TMI were significantly greater than all other groups (p < 0.01). TMXDI sensitization and challenge caused a significant increase in Penh relative to vehicle-exposed mice and unsensitized mice challenged with TMXDI (p < 0.01). Dermal sensitization and intranasal challenge with IPDI, MDI, TDI, or DNCB did not result in any significant differences relative to the vehicle controls, nor were there differences from vehicle in any of the unsensitized groups challenged with chemicals intranasally.

Airway Pathology (Group B and C Mice)

The principal morphologic alteration in mice that were dermally sensitized and intranasally challenged with TDI, MDI, HMDI, IPDI, TMI, TMXDI, or DNCB was moderate sloughing of the airway epithelium lining the septum, naso- and maxilloturbinates and lateral walls in the T1, T2, and T3 regions of the nasal cavity. The olfactory epithelium lining of the dorsal meatus in sections T1, T2, and T3 and ethmoid turbinates in sections T2 and T3 was the site of individual cell necrosis (Fig. 5). This epithelial change was accompanied by an inflammatory influx that consisted of macrophages and neutrophils. Table 1 lists the prevalence of this olfactory epithelial lesion in sensitized and unsensitized mice after airway challenge with each of these chemicals. Interestingly, MDI, TDI, and TMI caused these lesions with the greatest prevalence, suggesting greater cytotoxic potential. These lesions were likely not an immune-mediated response, but rather an irritant effect of the chemicals because mice sensitized with vehicle and challenged with TDI, MDI, HMDI, IPDI, TMI, TMXDI, or DNCB had similar airway lesions (Table 1). There was no evidence of any lesions in the nasal airways of any vehicle-treated mice.

No exposure-related alterations were microscopically evident in the lungs of any of these mice (data not shown).

Total Serum IgE (Group A, B, and C Mice)

There were significant increases in total serum IgE in Group A mice with IPDI (p < 0.0001), HMDI (p < 0.0001), MDI (p < 0.0001), TDI (p < 0.0001), TMI (p < 0.0001), TMXDI (p < 0.0001), and DNCB (p < 0.0001) relative to the vehicle control (Fig. 6A). The response to IPDI was significantly greater than all other groups. The response to HMDI or MDI was significantly greater than all other groups except IPDI (p < 0.01).

There were also significant increases in total serum IgE in Groups B and C after dermal sensitization and intranasal challenge with each of the chemicals relative to vehicle control: HMDI (59-fold; p < 0.0001), MDI (49-fold; p < 0.0001), IPDI (33.4-fold; p < 0.0001), TDI (24.4-fold; p < 0.0001), DNCB (15-fold; p < 0.0001), TMI (8.5-fold; p < 0.0001), and TMXDI (p < 0.0001).
HMDI, MDI, IPDI, and TDI each elicited responses that were significantly greater than the responses to DNCB, TMI, and TMXDI ($p < 0.0001$). The response elicited by DNCB was significantly greater than that elicited by TMI or TMXDI ($p < 0.05$). These findings are similar to our findings in a previous study (Selgrade et al., 2006).

**DISCUSSION**

In the present study, all the chemicals tested had the capacity to induce immediate airway hypersensitivity responses and/or enhanced responses to Mch challenge. However, only TDI, MDI, HMDI, and IPDI elicited significantly larger increases in Th2 cytokines in the parotid lymph nodes and total serum IgE levels relative to the other chemicals tested (Table 2). This is the first report to demonstrate that dermal sensitization followed by intra-airway challenge results in disparate immediate and late airway reactivity responses that do not correspond with elevated Th2 cytokine responses in lymph nodes draining the site of dermal application or serum IgE levels.

Some of the results presented here are unexpected based on our current understanding of hypersensitivity responses to allergen challenge. In general, the asthmatic response after allergen elicitation may be subdivided into an immediate, short-lived hypersensitivity response and a later response that is longer in duration. The immediate response is thought to be due to antigen cross-linking of IgE antibodies on mast cells resulting in release of preformed mediators that cause increased vascular permeability and airway smooth muscle contraction (Janeway et al., 2005). The IgG to IgE class switch is mediated by IL-4 produced by Th2 cells. Airway inflammation is not an essential component of this response (Lambert et al., 1998). The late response is characterized by edema, a second phase of smooth muscle contraction, and airway hyperresponsiveness to nonspecific stimuli (e.g., MCH) and is mediated by Th2 cytokines (Janeway et al., 2005). In the present study, DNCB and all isocyanates except TMXDI elicited an immediate hypersensitivity response as indicated by elevated Penh after intranasal challenge. As expected, this corresponded with strong increases in Th2 cytokines and serum IgE responses in mice exposed to HMDI, MDI, TDI, and IPDI. In contrast, TMI and DNCB elicited only mild increases in IL-4, IL-5, and/or IL-13, and serum IgE while still causing immediate hyperresponsiveness of similar magnitude to the high Th2 cytokine-eliciting chemicals. DNCB was previously not known to induce respiratory responses. The divergent cytokine responses and similar immediate hypersensitivity responses were elicited despite the fact that the doses of each hapten used for dermal application elicited relatively similar immunogenic responses as determined by the LLNA (Selgrade et al., 2006). Thus, the *ex vivo* cytokine and serum IgE end points do not necessarily predict immediate antigen-specific airway reactivity.

HMDI, TMI, and TMXDI elicited increases in nonspecific airway hyperresponsiveness. Only HMDI, however, caused

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Olfactory epithelial lesion in dorsal meatus (prevalence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Unsensitized MDI</td>
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<td>Unsensitized IPDI</td>
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</tr>
<tr>
<td>Sensitized DNCB</td>
<td>3 of 6</td>
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FIG. 5. Light photomicrographs of right dorsal meatus from proximal nasal airways (T1 section) from rats dermally sensitized and intranasally challenged with (A) vehicle, (B) TDI, (C) MDI, (D) HMDI, (E) IPDI, (F) TMI, (G) TMXDI, or (H) DNCB. O, surface olfactory epithelium; S, submucosa; B, bone; NP, right nasal passage; arrows, sight of cell necrosis; bars, 50 μm.
a strong Th2 cytokine response in the parotid lymph node. These findings are similar to those in our previous study where, in the absence of any respiratory challenge, dermal exposure alone with HMDI, TMI, or TMXDI, but not the other isocyanates or DNCB, increased nonspecific airway hyper-responsiveness (Selgrade et al., 2006). Our findings with TMI and TMXDI are in contrast to the clinical findings that workplace exposure to TMI or TMXDI was not associated with specific IgG antibodies or asthma symptoms (Grammer et al., 1993). However, it is difficult to draw conclusions about risk based on one negative human study. Additional work is needed to assess the potential of these chemicals to induce asthma.

The data suggest that more than one immunologic mechanism can result in the respiratory responses of interest, and therefore, Th2 cytokine secretion from skin draining lymph nodes is an unreliable predictor of airway hypersensitivity in this model. All chemicals tested elicited increases in the Th1 cytokine IFN-\(\gamma\), suggesting a mixed T-helper response as opposed to a response skewed toward the activation of Th2 cells. These findings are consistent with the findings of other investigators who demonstrated that low molecular weight chemicals linked to asthma displayed a mixed Th1/Th2 cytokine response in the lungs of mice (Tarkowski et al., 2007; Vanoirbeek et al., 2004, 2006). Recently, it was demonstrated that dinitrofluorobenzene (DNFB), a compound that is similar in activity to DNCB and known to cause contact hypersensitivity, elicited hypersensitivity reactions in the pulmonary airways of mice (Kraneveld et al., 2002; van Houweligen et al., 2002). These responses to DNFB, however, were not mediated by Th2 cytokines or IgE, but were in fact more classic (Th1) delayed-type hypersensitivity responses. However, a critical role for mast cells and mast cell–derived mediators was described in this model of nonatopic asthma.

Similarly, dermal sensitization and intranasal challenge with picryl chloride causes Th1 cell–mediated delayed-type hypersensitivity in the lungs of mice and increased airway hyper-responsiveness (Garssen et al., 1994). It is entirely possible that the immunologic mechanisms underlying HMDI, TMI, and TMXDI increases in the Mch response were not identical. Further characterization of the T-cell phenotypes in each of the isocyanate treatment groups might more specifically reveal the nature of these mechanisms and identify appropriate biomarkers for chemical-induced allergy.

The respiratory responses we observed could not be correlated with differences in nasal or pulmonary airway pathology. Dermal sensitization followed by intranasal challenge with any of the chemicals used did not elicit any pulmonary airway lesions characteristic of occupational allergic airways disease. These findings are in contrast to reports that TDI increased inflammatory cells including neutrophils as well as ventilatory responses 24 h after challenge (Furusho et al., 2006; Tarkowski et al., 2007; Vanoirbeek et al., 2004, 2006). The inflammatory cell infiltrates into the airways in these studies, however, were mild and cannot be directly compared to our results which were obtained 48 h after exposure. The absence of an inflammatory response in the lungs of mice in the present study may be due to the lack of sufficient distribution to the lower airways due to the ethyl

### TABLE 2

**Summary of Findings**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Th2 cytokine response in draining node</th>
<th>Serum IgE (26 days)</th>
<th>Immediate airway response</th>
<th>Nonspecific airway response</th>
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<td>TMXDI</td>
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<td>Weak</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>DNCB</td>
<td>Weak IL-13</td>
<td>Weak</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

FIG. 6. Total serum IgE levels in mice sacrificed (A) on Day 14 after only dermal sensitization or (B) on Day 26 after sensitization and intranasal challenge. Bars represent average amounts of IgE (\(\mu\)g/ml serum) ± SEM (n = 6 per group). *Significantly greater than all groups (\(p < 0.0001\)). + Significantly greater than all groups except IPDI (\(p < 0.0001\)). \# Significantly greater than vehicle (\(p < 0.0001\)). & Significantly greater than all chemicals except MDI (\(p < 0.0001\)). • Significantly greater than all groups except HMDI and IPDI sensitized and challenged groups (\(p < 0.0001\)). † Significantly greater than TMI, TMXDI, DNCB, and all vehicle groups (\(p < 0.0001\)). § Significantly greater than TMI, TMXDI, and all vehicle groups (\(p < 0.0001\)). || Significantly greater than all vehicle groups (\(p < 0.0001\)).
acetate and olive oil vehicle used (Farraj et al., 2004). In addition, this model used only a single antigen provocation which is different from the daily and/or chronic occupational exposures that often result in significant airway remodeling. Pulmonary allergic inflammation is not a necessary prerequisite for airway hypersensitivity as measured by increased responsiveness to Mch. Klink and Meade (2003) showed that dermal exposure to 3-amino-5-mercapt-1,2,4-triazole induced sensitization and increased responsiveness to Mch in mice in the absence of significant airway inflammation. Similarly, we demonstrated that repeated dermal exposure to isocyanates without airway challenge causes increased responsiveness to Mch in mice (Selgrade et al., 2006), in the absence of airway inflammation although inflammation occurred in the skin. Also several investigators in elegant studies have modulated the inflammatory responses without affecting the airway response or the airway response without affecting the inflammatory response. (Blumer et al., 2007; Card et al., 2006; Carey et al., 2007).

An easily measurable biomarker of exposure that universally predicts the respiratory sensitization potential of all chemicals remains elusive. Th2 cytokines and IgE are key mediators of asthma for many protein allergens and chemical haptens. The realization, however, that low molecular weight chemicals may elicit hypersensitivity by more than one mechanism strongly suggests a different approach to hazard identification; one that moves away from the search for a single mechanism of action. The gene expression profile to one that focuses on improving, without airway challenge causes increased responsiveness to Mch in mice (Selgrade et al., 2006), in the absence of airway inflammation although inflammation occurred in the skin. Also several investigators in elegant studies have modulated the inflammatory responses without affecting the airway response or the airway response without affecting the inflammatory response. (Blumer et al., 2007; Card et al., 2006; Carey et al., 2007).

An easily measurable biomarker of exposure that universally predicts the respiratory sensitization potential of all chemicals remains elusive. Th2 cytokines and IgE are key mediators of asthma for many protein allergens and chemical haptens. The data in this study and others suggest, however, that Th2 cytokines in the skin and serum IgE do not portend the respiratory hypersensitivity effects of all low molecular weight chemicals (Bernstein et al., 2002; Deschamps et al., 1998; Weissman and Lewis, 2000). Recently, genes other than Th2 cytokines and serum IgE have garnered interest in the field of isocyanate-induced asthma and potentially may more reliably predict isocyanate sensitization potential. These include glutathione S-transferases (Pirilla et al., 2001), HLA class II genes (Bignon et al., 1994), matrix metalloproteinases (Lee et al., 2001), and monocyte chemoattractant protein (Lummus et al., 1996). The realization, however, that low molecular weight chemicals may elicit hypersensitivity by more than one mechanism strongly suggests a different approach to hazard identification; one that moves away from the search for a single gene expression profile to one that focuses on improving, streamlining, and expediting the measurement of chemical-induced respiratory hyperresponsiveness in animals. The improvement of noninvasive techniques for measuring airway function with subsequent wide-spread acceptance and validation may facilitate hazard identification of asthma-causing chemicals.

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