INCONSISTENCIES BETWEEN CYTOKINE PROFILES, ANTIBODY RESPONSES, AND RESPIRATORY HYPERRESPONSIVENESS FOLLOWING DERMAL EXPOSURE TO ISOCYANATES

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Abstract

Cytokine profiling of local lymph node responses has been proposed as a simple test to identify chemicals, such as low molecular weight diisocyanates, that pose a significant risk of occupational asthma. Previously, we reported cytokine mRNA profiles for dinitrochlorobenzene (DNCB) and six isocyanates: toluene diisocyanate (TDI), diphenylmethane-4,4’-diisocyanate (MDI), dicyclohexylmethane-4,4’diisocyanate (HMDI), isophorone diisocyanate (IPDI), p-tolyl(mono)isocyanate (TMI), and meta-tetramethylene xylene diisocyanate (TMXDI). The present study was conducted to test the hypothesis that relative differences in the cytokine profile are predictive of relative differences in total serum IgE and respiratory responses to methacholine (Mch) following dermal exposure to the chemicals. After a preliminary experiment to determine an exposure regimen sufficient to achieve responses to Mch following dermal diisocyanate exposure, BALB/c mice received 9 dermal exposures over a period of 28 days to one of six isocyanates, DNCB, or vehicle. Mice were then challenged with increasing doses of Mch and responsiveness was assessed using whole body plethysmography. Serum antibody responses and cytokine mRNA profiles in the draining lymph node were also assessed. In separate experiments, cytokine protein assays were performed after 5 dermal exposures over a 14 day period. The response pattern for IL-4, IL-10, and IL-13 for the different isocyanates was highly reproducible as determined by RNAsese protection assay, RT-PCR or cytokine protein levels. However, the relative differences in Th2 cytokine profiles were not predictive of relative differences in either total serum IgE or respiratory responses to Mch following dermal exposure. The data suggest that the cytokine profiling approach needs to be further developed and refined before adoption and that
other approaches to hazard identification should be pursued as well. Based on the weight of evidence from all the assays performed, it appears that all 6 isocyanates tested have some potential to cause respiratory hypersensitivity following dermal exposure.
Introduction

Work-related asthma has become the most frequently diagnosed occupational respiratory illness (Petsonk 2002). Low molecular weight allergens, including diisocyanates, acid anhydrides, some reactive dyes, and platinum salts, cause about 40% of the cases of occupational asthma (Bernstein 2003). These compounds (or a metabolite of the compound) must be reactive enough to haptenate a larger molecule, usually a host protein, before they can induce immune responses typical of allergic asthma, such as T-helper (Th2) cell activation and IgE antibody. Low molecular weight sensitizers are typically electrophiles, or proelectrophiles, capable of reacting with hydroxyl, amino, and thiol functionalities on proteins (Karol et al. 2001). However, these characteristics are not sufficient to identify chemicals with the potential to cause asthma. Whole body plethysmography has been used in animal models to assess both early and late phase airway responses to chemical allergens in guinea pigs, rats, and mice (Griffiths-Johnson and Karol 1991; Matheson et al. 2001; Vanoirbeek et al. 2004; Zhang et al. 2004), but this approach does not generally lend itself to routine toxicity testing due to cost and technical complexity. Hence, both industry and regulatory agencies continue to seek tests that can be used to screen chemicals for this potential.

A tiered approach to hazard identification of chemicals with the potential to induce asthma has been proposed based on the notion that low molecular weight chemicals that cause occupational asthma are a subset of a larger group of chemical sensitizers that yield positive results in animal tests for allergic contact dermatitis (ACD) (Dearman et al. 2003). The first step in such an approach would be a positive response in the local lymph node assay (LLNA) (Dearman et al. 2003). The credibility of this
approach has been improved by animal studies demonstrating that dermal exposure to chemicals can lead to hypersensitivity of the respiratory tract (Arts et al. 1998; Ban et al. 2006; Herrick et al. 2002; Klink and Meade 2003; Matheson et al. 2001; Pauluhn 2005; Sailstad et al. 2003; Scheerens et al. 1999). It has been suggested that immune responses after dermal exposure might be used to predict whether IgE or Th2 responses would likely result from exposure to a chemical by other routes and hence predict the potential to induce allergic asthma. Several approaches that build upon the LLNA design have been suggested for the second tier of testing to distinguish the subset of chemicals with the potential to induce asthma from other ACD positive chemicals. Elevation of total serum IgE levels following dermal exposure to a chemical (the mouse IgE test) has been suggested as a basis for the identification of respiratory sensitization potential (Dearman 1998; Hilton et al. 1996). Differential production of IgE can also be assessed locally in draining lymph nodes following dermal chemical exposure by quantifying IgE bound to CD23 on B cells using flow cytometry (Manetz and Meade 1999). Cytokine profiling bases hazard identification on the induction of high levels of Th2 cytokines and low levels of Th1 cytokines in the draining lymph node (Dearman et al. 2003; Plitnick et al. 2005) relative to responses induced by appropriate negative and vehicle controls. None of these assays have been effectively validated due to variable results amongst different laboratories, and to date these assays have not been used in any official hazard identification capacity.

A number of studies have demonstrated different cytokine profiles for chemicals associated with asthma when simultaneously compared to ACD positive chemicals thought not to be associated with asthma (Dearman et al. 2003; Plitnick et al. 2002; Van
Och et al. 2002). However, few studies have tried to use this approach to categorize chemicals with unknown potential for inducing asthma. Using an RNase protection assay, Plitnick et al. (2005) studied cytokine expression for 6 isocyanate species, including some well-documented asthmagens based on human and animal studies, but also some chemicals for which there was little or no data to support or refute a role in the induction of asthma. Based on Th2 mRNA cytokine profiles of interleukin (IL)-4, -10, and -13, high and low responders were identifiable, but without a phenotypic anchor to respiratory responses it was not possible to relate these profiles to differences in the potential to induce asthma. The present study was conducted to 1) demonstrate the reproducibility of high and low Th2 cytokine responses to the different isocyanates using both mRNA expression (assessed by real time PCR) in the lymph node and production of cytokines in lymph node cell cultures and 2) test the hypothesis that relative differences in the cytokine profile are predictive of relative differences in total serum IgE and respiratory hyperresponsiveness to Mch following dermal exposure to the chemicals.

Materials and Methods

Chemicals. Toluene-2,4-diisocyanate (TDI) and 1-chloro-2,4-dinitrobenzene (DNCB) were purchased from Sigma Chemical Co. (St. Louis, MO). 4,4'-Methylenebis phenyl isocyanate (MDI), isophorone diisocyanate (IPDI) and dicyclohexylmethane-4,4'-diisocyanate (HMDI) were purchased from Aldrich Chemical Co. (Milwaukee, WI). P-tolyl (mono) isocyanate (TMI) and meta-tetramethylene xylene diisocyanate (TMXDI) were provided by DuPont Haskell Laboratory. All chemicals were solubilized in
acetone:olive oil (AOO) (4:1 v/v). Acetone, 99+%, HPLC grade was purchased from Sigma Chemical Co. Olive oil was purchased from Sigma Chemical or in the case of the LLNA from a local retailer.

Animals. Female BALB/c mice (8-12 weeks old) were obtained from Charles River Breeding Laboratories (Raleigh, NC or (for LLNA) Kingston, NY). Mice were housed in plastic shoebox cages containing beta chips, or in individual wire-bottom cages (LLNA studies), and were fed a standard diet of commercial rodent chow (Purina chow, St. Louis, MO) and water ad libitum in rooms maintained on 12- h light/dark cycles. Additional mice from each facility were (routinely) monitored serologically and were found to be free of Sendai, mouse pneumonia, mouse hepatitis, and other murine viruses, as well as mycoplasma. Mice were also monitored for, and found to be free of, ectoparasites and endoparasites. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committees of NHEERL, U.S. EPA or The Dow Chemical Company (LLNA studies).

Experimental Design. Four separate experiments were conducted. 1) A preliminary study (Fig. 1, experiment 1) was done to determine whether respiratory hyperresponsiveness to Mch could be detected following dermal exposure to a subset of the diisocyanate chemicals as well as DNCB (an ACD positive chemical not associated with asthma). The 14 day protocol mimicked those reported by Plitnick et al. (2005) and Dearman et al. (2002). Because studies showing hyperresponsiveness to Mch following dermal exposure to other chemicals employed longer sensitization regimens (Klink and Meade 2003), the 14-day protocol was extended to include an additional round of
sensitizations, the 28 day protocol (Fig.1). For this preliminary experiment, mice (12 per group) were exposed to 2% HMDI, 2% MDI, or 1% DNCB based upon doses reported by Plitnick et al. (2005). At time 0 and 5 days mice were exposed dermally on the shaved back with 100 μl of chemical. On Days 10, 11, and 12, 12.5 μl of chemical was applied to each side of both ears. On day 14 animals were assessed for Mch responsiveness and 6 mice/group were sacrificed in order to collect bronchoalveolar lavage fluid (BALF) and serum. The remaining 6 mice/group were subsequently treated with 100 μl of chemical on the shaved back on day 19 and then on days 24, 25, and 26 were treated on both sides of the ears with 12.5 μl of chemical. On day 28 mice were assessed for Mch responsiveness and BALF and serum were collected. Serum was assessed for total IgE, and BALF was assessed for total and differential cell counts, total protein and lactate dehydrogenase (LDH) activity. 2) Based on results of the preliminary study, the 28 day protocol was used to expose mice in the following treatment groups (6 mice/group): 1% TDI, 2% MDI, 2% HMDI, 2%IPDI, 1%TMI, 1% TMXDI, 1% DNCB, and vehicle (experiment 2). Doses used were the same as those used by Plitnick et al. (2005). At 28 days mice were assessed for responsiveness to Mch, total serum antibody IgE and IgG1, total and differential cell counts in BALF and cytokine message in the “auricular” lymph nodes (IL-4, IL-10, IL-13, and interferon γ (IFNγ). For the location of the “auricular” lymph node used in this study see the illustration in (Dean et al. 2001). 3) In a separate experiment 6 mice/group were exposed in the same manner to the same chemicals under 2) above. In addition groups exposed to 1% MDI, HMDI, and IDPI were added to the experiment. On day 14 “auricular” lymph nodes were removed and cells were cultured and assessed for production of cytokine proteins (experiment 3). 4) Also, LLNA studies
were performed as previously described (Woolhiser et al. 1999) in order to obtain dose response curves for the various chemical, and evaluate immunologic potency of the different test materials.

**In vivo airway responsiveness.** Respiratory responsiveness to increasing concentrations of aerosolized Mch were assessed in unanesthetized, unrestrained mice in a 12-chamber whole-body plethysmograph system (Buxco Electronics, Sharon, CT) 48 h after diisocyanate exposure on days 14 (preliminary experiment) or 28. Enhanced pause (Penh) was used as an index of airflow obstruction as previously described (Gavett et al. 2003; Hamelmann et al. 1997). After measuring baseline parameters for 7 min, an aerosol of saline or Mch in increasing concentrations (10-100 mg/mL) was nebulized through an inlet into 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 minute period.

**Serum collection and bronchoalveolar lavage.** Mice were anesthetized by i.p. injection with 0.5 ml of 5 mg/ml sodium pentobarbital, and blood samples were collected by cardiac puncture. The blood was placed in serum separator tubes and kept at room temperature for 1-2 h prior to centrifugation. Serum was collected and stored at -70°C. Following collection of the blood, the abdominal aorta and diaphragm were severed. The rib cage was carefully opened to expose the lungs. An incision was made in the ventral neck area to expose the trachea. A blunted 24 gauge needle (modified to approximately 15 mm) was inserted into the trachea and tied in place with surgical silk. The lungs were
lavaged twice with a single aliquot of 1 ml of HBSS (Hanks Balanced Salt Solution). BALF samples were stored on ice until centrifuged at 100 x g for 15 min at 4 °C. The supernatant was removed and stored at -70°C and subsequently assessed for total protein and lactate dehydrogenase (LDH). The cell pellet was resuspended in 1 ml of Hanks balanced salt solution (HBSS) and total cell counts were obtained using a Coulter Counter (Coulter Corp., Miami, FL) An additional 150-200 μl of the resuspended cells were centrifuged onto glass slides using a Shandon Cytospin (Pittsburgh, PA) at 750 rpm for 7 minutes. The slides were air dried and stained (Modified Wright-Giemsa Stain Pak, Fisher Scientific, Pittsburgh, PA and the Hema-Tek Slide Stainer, Miles Inc., Elkhart, IN) for differential cell counts. Differential cell counts were performed by counting 200 cells per slide (one slide per animal). BALF supernatants were assayed for total protein using Pierce Coomassie Plus Protein Assay Reagent (Pierce Biotechnology, Inc, Rockford, IL). Concentrations were determined from a standard curve using bovine serum albumin (BSA) standards obtained from Sigma Chemical Co. (St. Louis, MO). Supernatants were also assayed for LDH activity using a commercially prepared kit and controls from Sigma Chemical Co. Both assays were modified for use on a KONELAB 30 clinical chemistry spectrophotometer analyzer (Thermo Clinical Labsystems, Espoo, Finland).

**Total IgE and IgG 1 assays.** Rat anti-mouse IgE (Pharmingen, San Diego, CA) or rat anti-mouse IgG\(_1\) (Pharmingen) in phosphate buffered saline (PBS, pH 7.3) was placed in 96-well microtiter plates, sealed, and incubated overnight at 4°C. Following blocking with PBS plus 1% BSA, test sera or mouse standards for IgE (purified mouse IgE, κ monoclonal anti-trinitrophenol; Pharmingen.) or IgG\(_1\) (purified mouse IgG\(_1\), κ, Clone
MOPC-31C, Pharmingen) were diluted in blocking buffer and applied in 2-fold dilutions to the plates. The biotinylated detection antibody (rat anti-mouse IgE or rat anti-mouse IgG₁ (Pharmingen) was diluted in blocking buffer. Streptavidin horseradish peroxidase (Zymed, San Francisco, CA) was added and detection was accomplished with 3,3’,5,5’ tetramethylbenzidine (TMB) (DAKO Corp., Carpinteria, CA). Optical density was read on a Spectramax 340PC® Plate Reader (Molecular Devices Corp., Menlo Park, CA) at a wavelength of 650 nm. Softmax Pro® version 2.6.1 (Molecular Devices Corp.). Software was used for data collection and conversion from optical density to antibody concentrations. The lower limits of quantification were 3.12 ng/ml (IgG₁) and 6.25 ng/ml (IgE).

Assessment of cytokine mRNA message

Lymph nodes were removed under RNase free conditions, placed immediately into RNA later and stored at -70°C. RNA extraction was done by removing the lymph node from the RNA later, placing it into the QIAsol lysis buffer, homogenizing, and then following the protocol supplied with the RNeasy lipid tissue mini kit (Qiagen, Valencia, CA). The extracted RNA was eluted into 60μl of RNase free water, then aliquoted into 20 μl volumes and stored at -70°C. The quantity of RNA was determined by the GeneQuant Spectrophotometer (Pharmacia, Cambridge, England) and the integrity was determined by using an RNA 600 Nano Assay run on the Agilent 2100 Bioanaylyzer (Agilent, Palo Alto, CA). Complementary DNA (cDNA) was synthesized using the TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA) and conditions described in Applied Biosystem’s protocol for reverse transcription. The PCR reactions each required 20X Assay-on-Demand primers and probes and the TaqMan Universal PCR Master Mix.
GenBank mRNA sequences for IL-4, IL-10, IL-13, and IFN\(\gamma\) primers were X03532, M37897, M23504, and K00083, respectively. Each 30 \(\mu l\) reaction contained: 2\(\mu l\) RNase free water, 15 \(\mu l\) master mix, 1.5 \(\mu l\) target primer, 1.5 \(\mu l\) control primer (control primer was 18s diluted 1:3) and 10 \(\mu l\) of the unknown cDNA (cDNA diluted 1:10). Reactions were run in triplicate. The reactions were placed in 96-well plates and amplified in the ABI Prism 7900 using standard cycling parameters: 2 minutes at 50\(^0\)C, 10 minutes at 95\(^0\)C, 40 cycles of 15 seconds at 95\(^0\)C and 1 minute at 60\(^0\)C. Expression changes were calculated using the comparative Ct method (User Bulletin #2, Applied Biosystems). The housekeeping gene 18s ribosomal RNA was used as an endogenous reference to normalize target gene C\(T\) values. Gene transcription is expressed as an n-fold difference relative to the control. These numbers were averaged for each group and expressed as percent of control.

**Detection of cytokine proteins.** Methods for cytokine profiling were adopted from Dearman et al. (2002) and from Van Och et al. (2002). Fourteen days after the initial exposure, the draining auricular nodes from both sides were excised. The nodes for individual mice were weighed and placed in RPMI 1640-supplemented with 10% fetal calf serum (FCS, GIBCO), 100 \(\mu g\) penicillin/ml, and 100 \(\mu g\) streptomycin/ml. A single-cell suspension was prepared under aseptic technique by disrupting the node with a homogenizer, and cells were resuspended in RPMI-1640 with 10% FCS. Cell suspensions were centrifuged (180 x g, 10min) and the cell pellet suspended in 1 ml of the media. Total cell counts were obtained using a Coulter Counter (Coulter Corp, Miami, FL). Cells were diluted and seeded in 24-well microtiter plates. For each cell
suspension one well was seeded with $10^6$ and one with $10^7$ cells. Concanavalin A (ConA) (2 µg) was added to cultures with $10^6$ cells and cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 24 hours. Culture supernatants were then collected by centrifugation at 150 x g for 10 minutes, frozen on dry ice, and stored at –80°C. The remaining wells ($10^7$ cells without conA) were incubated for 120 hours before collecting supernatants and storing as described above. Cytokine proteins in cell supernatants were assessed using the Luminex 100 (Luminex Corporation, Austin, TX) and LINCOplex kits (Linco Research, Inc., St. Charles, MO) for simultaneous detection and measurement of 8 cytokines (IL-2,4,5,6,10,12,13, IFNγ and a monocyte chemotactic protein (MCP)-1).

**Local Lymph Node Assay (LLNA).** Following topical application to the ears, an intravenous (IV) injection of 20 µCi $^3$H thymidine ($^3$H-TdR, Amersham) in 0.25 ml PBS (Sigma) was delivered to each mouse via the lateral tail vein on the morning of test day 5. Approximately 5 hours later nodes were collected, cell suspensions were prepared, and $^3$H thymidine incorporation was measured as previously described (Woolhiser et al. 2000). EC3 were determined, as described by Basketter et al. (1999).

**Statistics.** The data were analyzed using analysis of variance (ANOVA) models (SAS, Version 8.02, SAS Inc, Cary, NC). With the exception of the 2 & 4 week IgE data, the analysis involved a one-way ANOVA with exposure as the independent variable. In cases where the distributional and homoscedastic assumptions appeared to be violated, either a transformation of the data was performed in order to satisfy these assumptions or
a Kruskal-Wallis test was used. A cluster analysis was performed to examine groupings of exposures with similar responses. Serum IgE data for 2 & 4 week data were analyzed using a two-way ANOVA with exposure and time (week) as the two independent variables. In this analysis, when the assumptions were violated, a transformation of the data was performed and then the analysis proceeded. Subsequent to the ANOVA or alternative analysis, pairwise comparisons were performed. The significance levels associated with these comparisons were not adjusted for multiple comparisons. The level of significance for evaluation of factors or pairwise comparisons was set at P< 0.05.

Results

Comparison of 14 and 28 day protocols

A preliminary experiment was done using diisocyanates (2%MDI and 2% HMDI) and 1% DNCB to evaluate the feasibility of increasing respiratory hyperresponsiveness as a result of exclusively dermal exposures to diisocyanates. Because the dermal sensitization studies noted in the Introduction employed exposure regimens considerably longer than the standard 14 day cytokine profile protocol (Dearman et al. 2002; Plitnick et al. 2005), exposure protocols for 28 as well as 14 day protocols were used (Figure 1). Figure 2 shows that after both the 14 and 28 day protocols, total serum IgE titers for the diisocyanate-treated groups were significantly increased (p<0.01) compared to DNCB and vehicle. Titers in the DNCB treated group were significantly increased (p<0.01) over vehicle only at 28, but not 14 days. Responses to diisocyanates and DNCB at 28 days were significantly greater (p<0.01) than responses at 14 days. There were no differences in lavage fluid protein, LDH, or differential cell counts among the different treatment
groups at either time point (data not shown). Figure 3 shows that there were no significant differences in responsiveness to Mch following the 14 day exposure protocol, but hyperresponsiveness to Mch was observed in animals treated with HMDI in comparison with the other treatment groups at 28 days (p<0.05). Based on these results, the 28 day protocol was used to assess mice treated with the 6 diisocyanates for hyperresponsiveness.

**Comparison of 6 Diisocyanates and DNCB using 28 day protocol**

Cytokine mRNA expression in lymph nodes removed at 28 days produced an expression pattern similar to that observed by Plitnick et al. (2005) at 14 days in that the diisocyanates could be grouped into high (TDI, MDI, and HMDI) and low (IPDI, TMI, and TMXDI) responders based on cluster analysis (Fig. 4). However, when DNCB was added to the analysis, all of the diisocyanates except TDI clustered with DNCB. In the Plitnick study the 6 diisocyanates were compared in one experiment and selected diisocyanates were compared to DNCB in a separate experiment, hence clustering without DNCB is more analogous to the Plitnick design. Figure 5 shows the total serum IgE and IgG\(_1\) responses for the same mice. IgE responses for HMDI, IPDI, and TMI groups were statistically greater than those for TDI, MDI and TMXDI groups. All were significantly greater than vehicle and DNCB groups, which were not significantly different from each other. HMDI-treated mice had IgG\(_1\) titers significantly higher than IPDI, TMXDI and DNCB groups, which were significantly greater than TDI and MDI and TMI groups. IgG\(_1\) titers for all treatment groups were different from vehicle. Based on serum antibody responses, the treatment groups did not cluster in the same groups
seen with cytokine mRNA expression. Figure 6 shows that airway responsiveness to Mch for groups treated with TMI, HMDI, and TMXDI was significantly greater than that for mice treated with vehicle alone, whereas groups treated with MDI, TDI, IPDI, or DNCB had slightly greater PENH but were not statistically more responsive to Mch than the vehicle-treated group. As with the serum antibodies, treatment groups did not cluster (based on Mch responsiveness) in the same manner seen with cytokine mRNA expression. Again, no differences were seen in differential cell counts, lavage fluid protein, or LDH (data not shown). Table 1 summarizes the result from different endpoints assessed in the 28-day protocol.

**Cytokine protein profiles at 14 days**

An additional 14 day study was conducted to determine whether the inconsistency between cytokine profile, antibody response, and hyperresponsiveness was due to assessment of message rather than protein or length of exposure (14 vs 28 days). The lymph nodes were removed to evaluate *ex vivo* cytokine production using the approach of Dearman *et al.* (2002) (IL-4 measured after 24 hr culture with ConA and all other cytokines measured after 120 hrs without Con A) or an adaptation of the van Och *et al.* (2002) approach assessing all cytokines after 24 hours with ConA. Figure 7 shows results for Th2 cytokines IL-4, IL-10 and IL-13 following 24 hrs in culture with ConA. Several observations can be made from these data: First, the treatment groups cluster in a manner similar to that for message shown in Figure 4 and as reported by in the Plitnick *et al.* (2005) study. Second, the cytokine responses for animals treated with 1% MDI, HMDI, and IPDI were not significantly different from the responses for animals treated
with 2% of the same chemical. Third, the IFNγ response in the 24 hour ConA cultures did not produce the expected result in that this cytokine was not detected in cultures from DNCB treated mice and was detected in cultures from mice treated with 1% and 2% MDI (Fig 7D). IL-2 and IL-12(P70) were elevated to a similar degree in all cultures; IL-5 was absent in all except TDI and MDI cultures, and MCP-1 was undetectable in all cultures (data not shown). Figure 8 demonstrates that the IFNγ response detected after culture of cells for 120 hours did not differ among the different exposure groups again indicating that this Th1 cytokine does not distinguish between DNCB and the diisocyanates. In this particular experiment we did not detect IL-4,-5,-10 or -13 in the 120 hour supernatants. IL-2 was elevated to a similar degree in all cultures (data not shown). Working with fewer treatment groups we have attempted the 120 hour assay several times with variable results with respect to IL-10 and 13 (data not shown). This variability occurred with both ELISA and Luminex assays and led us to conclude that the 120 hour supernatants did not provide a reproducible assay. MCP-1 was detected at 120 hrs (Fig 8b) and with the exception of TMXDI responses clustered into the same high and low responder groups seen with the 24 hr cultures and mRNA assays.

**Local lymph node assessment of biologic equivalency.**

To address the biologic equivalency of the doses used, LLNA dose responses for the six diisocyanates and DNCB were determined (Fig 9). The EC₃, typically determined when assessing ACD, varied among the different chemicals by a factor of 4. More relevant to the current study, the stimulation index for the doses administered in previous experiments, 1%TDI, 2% MDI, 2% HMDI, 2%IPDI, 1%TMI 1%TMXDI, and 1%
DNCB, were 25, 45, 38, 50, 23, 28, and 32 respectively. The data indicate that the doses administered varied in their LLNA response by a factor of 2 in some cases. However, as noted in figure 2 the cytokine response to MDI, IPDI, and HMDI at the 2% dose did not differ significantly from that at 1%.

**Discussion**

These data support the findings of Plitnick *et al.* (2005), which reported TDI, MDI, HMDI, IPDI, TMI, and TMXDI can be divided into high and low responders based on Th2 cytokine profiles. The pattern of responses for IL-4, IL-10, and IL-13 were very similar when message was assessed by RNAse protection assay (Plitnick *et al.*, 2005), or RT-PCR, or when cytokine protein was measured *ex vivo*. However, the relative differences in the Th2 cytokine profiles were not predictive of relative differences in either total serum IgE or respiratory responses to Mch (our phenotypic anchor) following dermal exposure to the chemicals. In addition, total serum IgE itself was not an accurate predictor of Mch hyperresponsiveness. These results suggest that the use of cytokine profiling for hazard identification may be premature (Dearman *et al.* 2003; Holsapple *et al.* 2006) and that other approaches to hazard identification should be pursued.

In addition to our inability to link cytokine profiles with the anticipated respiratory phenotypes, we found it difficult to replicate some of the results that have been previously reported for cytokine profiling (Dearman *et al.* 2003; Van Och *et al.* 2002). In particular, we were unable to demonstrate differences in Th1 cytokine (primarily IFNγ) expression between DNCB and the isocyanates despite using several different methods to assess cytokine profiles. With respect to the Th2 cytokines, assessing message by either RNAse protection or RT-PCR worked well. Given current
microarray technologies, it might be useful to examine message expression for a wider range of proteins in hopes of developing a more predictive profile. We used two approaches to assess production of cytokines in cultures of lymph node cells (Dearman et al. 2003; Van Och et al. 2002). We found the first approach (IL-4 assessed after 24 hrs in culture with ConA and other cytokines assessed without ConA at 120 hours) to be challenging and not reproducible with respect to the 120 hour cultures. Assessing all of the cytokines after 24 hours with ConA was more reproducible and more readily lends itself to multiplexing assays such as the Luminex assay. In order to demonstrate the utility and reproducibility of cytokine profiling, whatever method is chosen needs to be validated across several laboratories.

With respect to multiplexing, we added MCP-1 to our multiplex assay because, following antigen stimulation in vitro, production of MCP-1 is enhanced in peripheral blood mononuclear cells of workers with diisocyanate induced occupational asthma (Lummus et al. 1998). This was the only cytokine that produced a response at 120 hour. Although MCP-1 was not more accurate than the other cytokine responses in predicting enhanced hyperreactivity, it does illustrate the possibility of adding assays for other proteins to the multiplex assay.

Our data show that respiratory hyperresponsiveness can occur as a result of dermal exposure to certain isocyanates, including TMI, and TMXDI, both chemicals for which there are little or no data to support or refute a role in the induction of asthma. Under the dermal exposure conditions reported here, HMDI, TMI and TMXDI demonstrated an overall greater potential for respiratory effects than TDI, MDI and IPDI. Neither MDI nor TDI, both of which are known asthmagens, produced a statistically
significant increase in hyperresponsiveness to Mch when applied dermally over a 26 day period. Hence, the present dermal exposure regimen is not sufficient (using Mch hyperresponsiveness as an endpoint) to identify all chemicals which might pose a risk via the respiratory route. Because there is a trend toward increased responsiveness in groups dermally exposed to TDI, MDI, IPDI, and DNCB, a more prolonged exposure protocol might be needed in order to demonstrate Mch hyperresponsiveness. Klink and Meade (2003) demonstrated increased airway responsiveness to Mch following dermal exposure to a pesticide (3-amino-5-mercaptop-1,2,4-triazole) 7 days per week for 35 days. Scheerens et al. (1999) demonstrated increased airway reactivity in mice sensitized with TDI weekly by the dermal route for 7 weeks; however hyperreactivity occurred only after a respiratory challenge. Also, hyperresponsiveness to Mch was demonstrated in a recently described mouse model for TDI asthma that involves 6 weeks of sensitization via the respiratory route (Matheson et al. 2005), and an earlier model by the same group demonstrated hyperresponsiveness following 3 sub-cutaneous injections of neat TDI followed by 3 inhalation exposures over a period of 24 days (Matheson et al. 2001). Hence, increasing the sensitization period and or adding a respiratory challenge following dermal sensitization could perhaps result in a protocol that would make it possible to use dermal sensitization and airway hyperreactivity to identify asthmagens. Dermal exposure is worth pursuing because it is more amenable for routine screening purposes than inhalation exposures.

Total serum IgE for all isocyanate treated mice was elevated over DNCB suggesting that this also might be a useful biomarker. The fact that elevated IgE did not always reflect increased hyperreactivity to Mch is not surprising. Using adoptive transfer
and a mouse model of house dust mite allergy, Lambert et al. (1998) demonstrated that airway hyperresponsiveness to Mch could be transferred with lymphocytes, but not serum, and that immediate hyperresponsiveness to antigen could be transferred with serum (presumably IgE), but not cells. The airway hyperresponsiveness was therefore presumed to be cell-mediated rather than IgE mediated. DNCB also increased total IgE, but not to the level observed in the diisocyanate treated mice. It has been difficult to consistently demonstrate TDI specific antibodies in humans suffering from TDI asthma (Liu and Wisnewski 2003), and humans in some cases exhibit late phase but not immediate responses. The uniform genetic background of the BALB/c mice used in this study as compared to the human population may be one reason for this discrepancy.

Regardless of the end points chosen for screening chemicals, more work is needed to identify appropriate positive and negative controls and criteria for determining whether an unknown is more like the negative or more like the positive control. As has been noted for cytokine profiling the differences in Th1 and Th2 cytokine expression are relative, not absolute. The same appears to be true for IgE and could be true for other endpoints as well. DNCB is frequently used as the negative control; however, total serum IgE was increased in mice treated with DNCB, and IgG₁ was significantly elevated. IgG₁, which in mice is coregulated with IgE via the IL-4/Th2 pathway (Purkerson and Isakson 1992), is capable of sensitizing mast cells (Daeron et al. 1980; Vaz et al. 1970), and in some cases has been shown to be the fore runner of IgE (Robinson et al. 1996). Also, the expression of IL-4 mRNA following DNCB treatment was not the result we expected from a negative control. Although we might assume that the assay is not appropriately predictive, perhaps the real problem is that our negative control is not entirely negative.
In this regard DNCB-induced hypersensitivity responses in mouse lung have been demonstrated (Kraneveld et al. 2002). It is likely that there is a distribution of chemicals (possibly along a bell or some other shaped curve) with respect to capacity to produce respiratory sensitization with some very strongly biased in a Th1 or Th2 direction and others falling in between. Positive and negative controls need to be at the extreme ends of that curve. A variety of potential negative controls should be tested and compared across several laboratories.

Finally, it has been suggested that comparisons between unknowns and positive and negative controls be done under conditions of exposure that lead to equivalent immunogenicity with respect to lymphocyte proliferation in the draining lymph node. Although a 2-fold difference in the LLNA stimulation index was observed between the lowest responding and highest responding chemical treatment used in this study (Fig. 9d), a good degree of overlap for stimulation index values can be observed across dose groups suggesting these doses would be within the general range of error for assay reproducibility. At a concentration of 2% MDI, HMDI, and IDPI all yielded stimulation indexes somewhat higher than DNCB. However, lowering the dose of each of these to 1% had no effect on the cytokine response (Fig 7). We previously demonstrated a lack of dose response for cytokine message in response to trimellitic anhydride and DNCB (Plitnick et al. 2002). It is also worth noting that two of the three isocyanates which caused a significant increase in Mch hyperresponsiveness had average stimulation indexes slightly below that for DNCB, whereas 2 (TMI and TMXDI) of the 3 isocyanates that did not cause increased hyperresponsiveness had stimulation indexes slightly higher than DNCB. This suggests that these cytokine profiles can be attributed to the
chemical’s inherent potential and is not a function of immunogenicity resulting from the doses used in this study.

In summary, all of the isocyanates tested here appear to have some potential to induce respiratory hypersensitivity based on the endpoints measured in these studies. All induced an increase in total serum IgE relative to DNCB. HMDI, TMI, and TMXDI treatment resulted in the highest hyperresponsiveness to Mch. TDI, MDI and HMDI enhanced Th2 cytokine responses in the draining lymph node and are known to induce asthma in humans (see Plitnick et al 2005 for review of human and animal studies for all of the diisocyanates tested here). Using the dermal route of exposure to screen chemicals for the capacity to cause respiratory hypersensitivity has promise. However, additional work is needed to optimize the exposure protocol and select the most appropriate endpoints to assess. In this regard hyperresponsiveness to Mch has some appeal because it assesses a respiratory endpoint and can be done in the same animal used to assess immune endpoints.

Acknowledgments

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References


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+ describes magnitude of significant response; ± indicates trend but not significant; ( ) results from preliminary experiment

**Figure Legends**

**Figure 1:** Experimental protocols for 14 and 28 day studies.

**Figure 2:** A comparison of total serum IgE responses at 14 and 28 days. Data points represent the mean and standard error of the mean (SEM) for 6 mice. a Significantly increased over DNCB; b Significantly increased over vehicle control (p<0.01).
**Figure 3:** Airway responsiveness to methacholine (Mch) aerosol at 14 and 28 days. Data represent means and SEM of enhanced pause (Penh) responses to saline and increasing concentrations of Mch aerosols for 12 mice per group (14 days, A) or 6 mice per group (28 days, B), following subtraction of baseline values. * $P < 0.05$, HMDI group vs. DNCB- and vehicle-treated groups. † $P < 0.05$, HMDI group vs. all other groups.

**Figure 4:** Expression of cytokine message in lymph nodes extracted at 28 days as measured by real time PCR. Data represents the mean and SEM for 6 mice.

**Figure 5:** Total serum IgE and IgG1, 28 day protocol as measured by ELISA. Data points represent the mean and SEM for 6 mice. Like letters are not significantly different from each other.

**Figure 6:** Airway responsiveness to Mch, 28 day protocol. A. Values shown are means and SEM of Penh responses to increasing concentrations of Mch aerosols, after subtraction of responses to saline aerosol ($n = 6$/group). B. Regression lines were calculated for plots of Penh (- saline response) vs. log [Mch (mg/ml)]. Values shown are means and SEM of slopes of the regression lines. * Slopes significantly different ($P < 0.05$) compared with slope of the regression line for vehicle-treated group.

**Figure 7:** Cytokine protein profile produced after culture of lymph nodes for 24 hr with ConA. Data points represent the mean and SEM for 6 mice. Like letters are not significantly different from each other.
**Figure 8:** Cytokine protein produced after culture of lymph nodes for 120 hours without ConA. Data points represent mean and standard error for 6 mice. A. There were no differences in IFN$\gamma$ response. B. MCP-1 was detected at 120 hrs and with the exception of TMXDI responses clustered into the same high and low responder groups seen with the 24 hr cultures and mRNA assays. Like letters are not significantly different from each other.

**Figure 9:** LLNA responses. Data points represent individual mice treated with test chemical (4-5 mice per group). Bars represent the mean. Potency estimates (EC3 values) were calculated for each chemical using a linear regression model as described in Basketter et al, 1999.
Figure 1

Experiment 1:
- Serum IgE
- Hyperresponsiveness
- BALF

Experiment 3:
- Cytokine protein profiling

Figure 2

Experiment 2:
- Hyperresponsiveness
- Cytokine m-RNA Serum
- Serum IgE, IgG1
- BALF

Bar chart showing total serum IgE levels across different treatments and protocols.
Figure 3

14 Day Protocol

28 Day Protocol
Figure 6

A

Effect of Mch (mg/ml) on Perh (saltine response) showing different responses for various compounds.

B

Graph showing the slope of Perh vs. log(Mch) for different treatments: Vehicle, DNCB, IPDi, TDI, MDI, TAMDI, TMI, and HMOI. Asterisks indicate significant differences.
Figure 7

Figure 8
Figure 9