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Technical note

Immunochemical detection of the occupational allergen, methylene diphenyl diisocyanate (MDI), in situ

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ABSTRACT

Diisocyanate chemicals essential to polyurethane production are a well-recognized cause of occupational asthma. The pathogenesis of diisocyanate-induced asthma, including the pathways by which the chemical is taken up and its distribution in exposed tissue, especially the lung, remains unclear. We developed an antiserum with specificity for methylene diphenyl diisocyanate (MDI) the most abundantly produced and utilized diisocyanate worldwide, and established its ability to detect MDI in situ. Polyclonal MDI-specific IgG was induced by immunizing rabbits with MDI-conjugated to keyhole limpet hemocyanin (KLH) emulsified in complete Freund's adjuvant, followed by two booster injections with incomplete Freund's adjuvant. The antiserum contains IgG that recognize a variety of different MDI conjugated proteins, but not unconjugated or mock exposed proteins by dot blot analysis. The antiserum further demonstrates specificity for proteins conjugated with MDI, but not other commonly used diisocyanates. Immunochemical studies with cytospun airway cells and formalin-fixed paraffin embedded lung tissue sections from mice intranasally exposed to MDI (as reversibly reactive glutathione conjugates, e.g. GSH–MDI) demonstrated the antiserum's ability to detect MDI in tissue samples. The data demonstrate penetration of MDI into the lower airways, localized deposition in the epithelial region surrounding airways, and uptake by alveolar macrophages. The new immunochemical reagent should be useful for further studies delineating the uptake and tissue distribution of MDI, especially as it relates to adverse health effects from exposure.

1. Introduction

Diisocyanates, industrial chemicals with many important uses, are among the most commonly recognized causes of occupational asthma world-wide (Allport et al., 2003; Wisnewski et al., 2008). Methylene diphenyl diisocyanate (MDI) is the most abundantly produced and consumed diisocyanate, with applications in the generation of rigid foams and as a binding agent (Allport et al., 2003). Other diisocyanates, such as hexamethylene diisocyanate (HDI) and toluene diisocyanate (TDI), are less commonly used and have specialized applications as protective coatings and in the generation of "softer" flexible polyurethane foam (Allport et al., 2003; Wisnewski et al., 2008).

The pathogenesis of diisocyanate-induced asthma remains unclear, in part due to uncertainty regarding the reactivity of these chemicals with "self" molecules at the major sites of exposure. Animal studies with radio-isotope labeled diisocyanates suggest that a portion of inhaled chemical remains bound within lung tissue, however the distribution in different sub-anatomical locations remains unclear (Kennedy et al., 1994). Redlich et al. detected HDI along the airway epithelium of an exposed worker using polyclonal antiserum raised against HDI conjugated to ovalbumin (Redlich et al., 1997). Recent studies with TDI-specific monoclonal antibodies have demonstrated that TDI binds to proteins in the skin, which may be an important exposure route for inducing systemic immune sensitization (Nayak et al., 2014). Studies detecting MDI in situ, however, are lacking, as necessary reagents have yet to be generated and characterized.

In this report, we develop a rabbit antiserum with specificity for MDI and demonstrate its utility for detecting the chemical in lung tissue from exposed animals. Importantly, the polyclonal serum includes anti-MDI IgG antibodies whose epitopes are not destroyed by formalin fixation, allowing immunochemical detection of MDI in samples processed according to routine histological procedures (e.g. formalin-fixed paraffin-embedded). The potential applications of the new anti-MDI polyclonal serum toward understanding disease pathogenesis and as evidence of chemical exposure are discussed.

2. Methods

2.1. Chemicals and reagents

H_2O_2, the diisocyanates, 4,4′-diphenylmethane diisocyanate (MDI), 1,6 hexamethylene diisocyanate (HDI), and an 80:20 ratio mixture of

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2,4:2,6 toluene diisocyanate (TDI), albumins (from human, bovine and mouse), tropomyosin, keyhole-limpet hemocyanin (KLH), Tween 20, tetramethylbenzidine (TMB) liquid substrate solution, complete and incomplete Freund's adjuvant were from Sigma-Aldrich (St. Louis, MO). Biotin-labeled mouse monoclonal anti-rabbit IgG (6C1A8) with no cross-reactivity to IgG from other species was from ProSci Inc. (Poway, CA). Streptavidin–HRP and peroxidase conjugated anti-rabbit IgG were from BD Biosciences (San Jose, CA). Biotinylated goat anti-human albumin was from Bethyl (Montgomery, TX) and protein-A purified anti-IL-33 polyclonal rabbit IgG was from Enzo Life Sciences (Farmingdale, NY). Cyanogen bromide activated sepharose 4B was from Amersham Biosciences (Uppsala, Sweden) and protein A affinity columns were from BioRad (Hercules, CA). Biotin blocking solution was from Life Technologies (Eugene, OR). Diaminobenzidine (DAB) substrate, hematoxylin, and histomount were from a HistoMouse-MAX staining Kit (Invitrogen, Frederick, MD). Protein A sepharose 4B (Invitrogen). Normal rabbit and goat serum were from ThermoFisher Scientific (Waltham, MA). Amersham Hyperfilm MP from GE Healthcare Bio-Sciences (Pittsburgh, PA) was used in developing dot blots.

2.2. Generation of diisocyanate-conjugated proteins

Diisocyanate conjugated proteins were prepared as previously described (Wisnewski et al., 2008, 2010). Briefly, proteins were dissolved in 20 mM phosphate buffered saline pH 7.4 at a concentration of 5 mg/ml. Diisocyanates were prediluted to 10% w/v in acetone, and 10 μl/ml of 10% diisocyanate was added to protein solutions to achieve a final concentration of 0.1% (w/v) diisocyanate. Diisocyanate–protein reactions were mixed vigorously and rotated end-over-end for 2 h at room temperature. Following centrifugation at 10,000 *g*, the supernatant was recovered and dialyzed against PBS to remove unreacted and/or hydrolyzed chemical as well as potential low molecular weight diisocyanate polymers. Following dialysis, samples were again centrifuged at 10,000 *g*, sterile filtered (0.2 μm), and stored at −80 °C. Diisocyanate conjugation to proteins was verified by changes in the different proteins' electrophoretic mobility and by mass spectrometry as previously described (Wisnewski et al., 2008, 2010). Control unconjugated proteins were "mock exposed" to 10 μl/ml of acetone without diisocyanate and identically processed.

2.3. Generation of polyclonal MDI-specific rabbit serum

To generate MDI-specific polyclonal rabbit serum, New Zealand White rabbits (Harlan Laboratories, Indianapolis, IN) were immunized subcutaneously with 500 μg of 4,4′-MDI-conjugated KLH emulsified in complete Freund's adjuvant, followed by two booster shots of 250 μg of MDI-KLH in incomplete Freund's adjuvant on days 14 and 28. Terminal bleed serum was collected on day 72 from 2 different rabbits, pooled, and depleted of anti-KLH immunoglobulins by affinity chromatography using KLH-coupled sepharose 4B. For immunochemistry studies, the IgG fraction was further purified by protein A affinity chromatography. All animal studies were conducted in accordance with institutional guidelines.

2.4. Dot blot analyses

One microliter of protein solutions (5 mg/ml, except for tropomyosin, 1 mg/ml) in PBS were spotted onto nitrocellulose membrane. Membranes were blotted with a 1:1000 dilution of anti-MDI polyclonal rabbit serum followed by a 1:2000 dilution of peroxidase conjugated anti-rabbit IgG. In studies with human albumin conjugated with different diisocyanates, dot blots were similarly probed with anti-MDI polyclonal serum, then subsequently stripped, and reprobed with biotinylated goat anti-human albumin, followed by peroxidase conjugated streptavidin. Replicate samples were also blotted with a 1:1000 dilution of pre-immune rabbit serum, normal rabbit serum and 2 μg/ml of polyclonal anti-IL-33 rabbit IgG. All bots were blocked with 3% dry milk in tris buffered saline (TBS) pH 7.4, washed with TBS containing 0.05% Tween 20, and developed with TMB liquid substrate, before exposure to film and processing. In some experiments, proteins were stained with 0.1% Ponceau S, and then washed before blotting with specific antibodies.

2.5. Immunochemistry

Studies were performed on replicate samples of cytospun airway cells obtained by bronchoalveolar lavage (BAL) and lung tissue sections of MDI exposed or control mice acquired during prior studies as previously reported (Wisnewski et al., 2015). Cytospun BAL cells fixed with −20 °C methanol and formalin-fixed paraffin-embedded tissue sections were blocked with 20% goat serum and stained with 2 μg/ml of KLH-depleted, protein-A affinity purified anti-MDI rabbit IgG, or 2 μg/ml of isotype control protein-A purified polyclonal rabbit IgG raised against IL-33 or 2 μg/ml protein-A purified normal rabbit IgG (not shown). Endogenous peroxidase was quenched before analysis using H_2O_2, and endogenous biotin was blocked before addition of biotin-labeled mouse monoclonal anti-rabbit IgG (with no cross-reactivity to other species IgG) using commercially available "biotin-blocking solution" (see Section 2.1 above). Slides were further developed with peroxidase-labeled streptavidin, DAB substrate, and hematoxylin counterstain. Micrographs were taken under a Nikon Eclipse NI-U model microscope (Tokyo, Japan) equipped with a Nikon DS-Ri2 image capture system and loaded into Nikon's NIS-Elements Basic Research software program for image analysis.

3. Results

3.1. MDI antisera's specificity for MDI conjugated vs. unconjugated proteins

The presence of MDI-specific IgG in the polyclonal rabbit serum was first assessed by dot blot analysis. As shown (Fig. 1A and B) the MDI antisera contained rabbit IgG that recognized MDI in a hapten-like manner; the antisera bound to a variety of different MDI conjugated proteins, regardless of the protein "carrier", but did not bind the corresponding unconjugated (mock reacted) proteins.

3.2. MDI antisera's specificity for MDI vs. other diisocyanates

We next tested the specificity of the rabbit polyclonal antisera for MDI vs. other diisocyanates commonly used industrially, namely TDI and HDI. As shown (Fig. 1C), the MDI antisera contained IgG that bound to albumin conjugated with MDI, but not HDI or TDI, or unconjugated (mock exposed) albumin preparations. In contrast, control biotinylated anti-human-albumin goat IgG recognizes albumin bound by all 3 diisocyanates; however binding is reduced following diisocyanate conjugation, likely due to chemical modification of the native albumin structure as previously described (Wisnewski et al., 2008, 2010).

3.3. Detection of MDI within airway cells from MDI–GSH exposed mice

We tested the ability of the MDI antisera to detect MDI in airway cells recovered by bronchoalveolar lavage (BAL). Immunochemical staining of cytospun BAL cells from mice sensitized and exposed to MDI, as reversibly reactive MDI–GSH conjugates (Wisnewski et al., 2015), identified MDI primarily within the cytoplasm of cells with alveolar macrophage or dendritic cell morphology, but not polymorphonuclear or lymphocyte morphology (Fig. 2). In contrast, no

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