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Characterization of methylene diphenyl diisocyanate-haptenated human serum albumin and hemoglobin

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ABSTRACT

Protein haptenation by polyurethane industrial intermediate 4,4′-methylene diphenyl diisocyanate (MDI) is thought to be an important step in the development of diisocyanate (dNCO)-specific allergic sensitization; however, MDI-haptenated albumins used to screen specific antibody are often poorly characterized. Recently, the need to develop standardized immunoassays using a consistent, well-characterized dNCO-haptenated protein to screen for the presence of MDI-specific IgE and IgG from workers' sera has been emphasized and recognized. This has been challenging to achieve due to the bivalent electrophilic nature of dNCOs, leading to the capability to produce multiple cross-linked protein species and polymeric additions to proteins. In the current study, MDI was reacted with human serum albumin (HSA) and hemoglobin (Hb) at molar ratios ranging from 1:1 to 40:1 MDI/protein. Adducts were characterized by (i) loss of available 2,4,6-trinitrobenzene sulfonic acid (TNBS) binding to primary amines, (ii) electrophoretic migration in polyacrylamide gels, (iii) quantification of methylene diphenyl diamine following acid hydrolysis, and (iv) immunoassay. Concentration-dependent changes in all of the above noted parameters were observed, demonstrating increases in both number and complexity of conjugates formed with increasing MDI concentrations. In conclusion, a series of bioanalytical assays should be performed to standardize MDI–antigen preparations across lots and laboratories for measurement of specific antibody in exposed workers that in total indicate degree of intra- and intermolecular cross-linking, number of dNCOs bound, number of different specific binding sites on the protein, and degree of immunoreactivity.
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Diisocyanates (dNCOs),[1] such as 4,4′-methylene diphenyl diisocyanate (MDI), are high-volume production chemicals used in the production of polyurethane foams, elastomers, paints, and other related products [1,2]. Exposure to dNCOs has most commonly been reported in the occupational setting [3,4]; however, concern about potential exposure through leaching of dNCOs from cured, semi-cured, or non-cured products has recently been raised in domestic settings [5].

dNCOs are low-molecular-weight compounds that must first react with (haptenated) autologous proteins to produce a functional antigen [6,7]. Apart from directly reacting with proteins at the site of exposure, diisocyanates can react with glutathione- or thiol-containing proteins, forming labile thiocarbamate adducts that may possibly be transported to sites distal to the site of exposure. It is not known whether the antigenicity of proteins adducted by isocyanates regenerated from thiocarbamates is different from that via direct haptenation. Synthetic methods have been reported for amino acid conjugation using thiocarbamates [8], which may possibly find utility in haptenation of whole proteins for specific antibody detection. The fate of the dNCO in the body and the ultimate protein adduct responsible for immunological sensitization currently remain unknown [9].

Diisocyanate asthma has been one of the most commonly reported causes of occupational asthma (OA); however, a downward trend in the number of reported cases has been noted during recent years [10–12]. This may be attributed to implementation of exposure limits and increased surveillance. Diagnosis of dNCO-induced OA remains confounded by methodological limitations [13].

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[1] *Abbreviations used:* dNCO, diisocyanate; MDI, 4,4′-methylene diphenyl diisocyanate; OA, occupational asthma; HSA, human serum albumin; Hb, h hemoglobin; TDI, toluene diisocyanate; PBS, phosphate-buffered saline; TMA, trimellitic anhydride; MWCO, molecular weight cutoff; RT, room temperature; MDA, methylenedianiline; HPLC, high-performance liquid chromatography; TNBS, 2,4,6-trinitrobenzene sulfonic acid; SDS, sodium dodecyl sulfate; MS/MS, tandem mass spectrometry; UPLC, ultra-performance liquid chromatography; qTOF, quadrupole time-of-flight; MS, mass spectrometry; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBST, PBS and Tween 20; SMPBST, skim milk/PBS–Tween 20; PAGE, polyacrylamide gel electrophoresis.

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Although dNCO asthma displays the pathological hallmarks of allergic asthma, including eosinophilic inflammation and increased airway reactivity, testing for dNCO-specific IgE for diagnosis of dNCO asthma is specific (96–98%) but not sensitive (18–27%) [14]. The low prevalence of detectable dNCO-specific IgE has been attributed to both assay limitations and a potential IgE-independent dNCO asthma mechanism(s) [15]. Ott and coworkers [14] reviewed issues related to dNCO antibody testing for use in diagnosis of dNCO asthma. Immunoassay standardization is critical for comparison of results across studies [16]. A number of factors that may confound results from these immunoassays include the dNCO used, the carrier protein employed, dNCO–protein reaction conditions, and postreaction processing of the haptenated protein. The variability of results obtained in these immunoassays may also be due in part to a lack of standardization in conjugate preparation and characterization.

dNCOs are reactive, bifunctional electrophilic compounds. When reacted to protein under aqueous conditions, dNCOs can covalently bind to protein amines and, reversibly, to thiols [17]. Such binding is in competition with hydrolysis of the dNCO to a diamine. Multiple species are formed with reaction of a dNCO to a protein, including (i) monomeric dNCO binding (1 N═C═O moiety reacts to the protein and the other hydrolyzes to an amine), (ii) polymeric binding onto a protein site (a second dNCO reacts to the amine formed from NCO hydrolysis), (iii) intramolecular cross-linking by the dNCO through two nucleophilic sites on a protein, or (iv) intermolecular cross-linking by the dNCO through nucleophilic sites on two different protein molecules. A single protein molecule such as human serum albumin (HSA) may also be haptenated at multiple nucleophilic sites by one or more ofthe species noted above [18].

HSA is the most common carrier protein used for dNCO antibody immunoassays [15] due to its prevalence in plasma [19] to form MDI adducts. Other molecules, such as keratin 18 [19], tubulin [20], and the peptide glutathione [21], have been found to be modified by dNCO exposure. Hemoglobin (Hb)–MDI haptenation occurs in vivo following MDI exposure. Sabbioni and coworkers reported MDI bound to the N-terminal valine of Hb in MDI-exposed rats and proposed Hb–MDI as a biological marker of MDI exposure [22]. The same authors also found the N-terminal adduct with valine in globin of a TDI-exposed worker and in two women with polyurethane-covered breastimplants [23]. The immunogenicity of adducted proteins that have been identified other than albumin has not been tested; however, the fact that haptenated keratin and tubulin were identified immunochemically suggests that multiple haptenated protein species formed following exposure may be antigens.

Due to the lack of characterization of protein–dNCO adducts, antibody reactivity toward other endogenous haptenated proteins may be overlooked using conventional detection approaches; however, other proteins assessed as carrier proteins for dNCOs have not been as effective at detecting dNCO-specific antibody in the blood of exposed workers. It is not known whether this is due to varying degrees of dNCO adduction to different proteins or antibody recognition. Characterization of different dNCO–protein conjugates, therefore, is important to further our understanding of dNCO haptenation and adduction.

Our previous research has been directed toward delineating the concentration-dependent increase in specific dNCOs such as toluene diisocyanate (TDI) binding sites on HSA [18]. In these studies, the predominant TDI binding sites on HSA were lysine residues, although binding to the N-terminal arginine and to glutamine were also observed. In another study performed in our laboratory [24], MDI bound to the same sites as TDI, but overall the reactivity was reduced. The extent of conjugation was also influenced by buffers, with conjugation being greater in phosphate buffer compared with ammonia carbonate buffer as a result of faster kinetics of the competing hydrolysis of the dNCOs in ammonium carbonate buffer compared with phosphate-buffered saline (PBS).

In the current study, we further characterize MDI–HSA conjugation/adduction with the aim of developing a standardized approach for screening IgE- and IgG-specific dNCO-haptenated HSA. MDI reactivity toward Hb was also examined and compared with HSA because dNCO-adducted hemoglobin has been measured from the blood of exposed workers and used as a biological marker of dNCO exposure [25]. Although diisocyanate-adducted hemoglobin in vivo immunogenicity or antigenicity has not yet been reported in the literature, Wong and coworkers [26] reported that acrylonitrile-adducted Hb was antigenic. In another study, Pien and coworkers [27] found that rats exposed to the respiratory allergen and inducer of late respiratory systemic syndrome, trimellitic anhydride (TMA), by inhalation produced IgG that recognized bothTMA-haptenated albumin and Hb. They demonstrated, through cross-inhibition studies, that TMA–albumin and TMA–Hb share antigenic determinants. Collectively, these studies suggest that haptenated hemoglobin can be antigenic.

Materials and methods

Chemicals

Unless otherwise specified, all reagents were acquired from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification. Ethyl acetate (reagent grade) was purchased from J.T. Baker/Avantor Performance Materials (Center Valley, PA, USA). Sodium tetra borate, sodium hydroxide, hydrochloric acid, dialysis membranes (molecular weight cutoff [MWCO] of 12,000–14,000), 98% sulfuric acid, and *N*-acetyl glycine were purchased from Fischer Scientific (Fair Lawn, NJ, USA).

Preparation of MDI–protein adducts

MDI–protein adducts were prepared as described previously [24]. Briefly, protein solutions were prepared in 0.1 M PBS (pH 7.4) at 0.5 mg/ml. MDI was dissolved in dry acetone at 1.8, 9, 18, and 72 μg/ml for HSA conjugation and at 1.84, 9.2, 18.4, and 73.6 μg/ml for Hb conjugation immediately before use. Each MDI solution was added at 34.5 μl to 5 ml of 0.5 mg/ml protein with mixing, resulting in MDI/protein molar ratios of 1:1, 5:1, 10:1, and 40:1. Samples were then incubated at room temperature (RT) for 1 h. Following incubation, samples were dialyzed for 18 h against 4 L of distilled deionized water using 12,000 to 14,000 MWCO dialysis tubing (Sigma–Aldrich). The samples were stored at −20 °C until analysis.

Analysis of number of moles of MDI bound per mole protein

MDI-conjugated proteins (2 ml of 0.5 mg/ml HSA–MDI or Hb–MDI) were hydrolyzed by incubating with 1 ml of 3 M H_2SO_4 at 100 °C for 16 h. Methylenedianiline (MDA)-spiked protein standards (1–16,000 ng/ml) were run in parallel. Following hydrolysis, samples and standards were cooled to RT. Then 5 ml of saturated sodium hydroxide was added, vortexed, and put in an ice bath to cool for 10 min. The resulting MDA from samples and standards was extracted into 6 ml of ethyl acetate and subsequently evaporated at 40 °C under N_2 to 1 ml. The ethyl acetate extracts were then back-extracted into 500 μl of 0.5% H_2SO_4. Then 250 μl of saturated borate buffer (pH 8.5) and 450 μl of acetonitrile were added to 250 μl of each H_2SO_4 extract and vortexed for 1 min, and then 50 μl of 14.4 mg/ml fluorescamine in acetonitrile was added. This was vortexed for 1 min, and 100 μl was injected onto a Supelco