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Issues in Diisocyanate Antibody Testing

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Diisocyanates are used to produce a wide variety of polyurethane products; they are also recognized as an important cause of occupational asthma. Their chemical reactivity presents challenges to toxicologists and clinicians alike seeking to understand the mechanisms underlying diisocyanate asthma. In this article, we review the literature on immunoassay detection of IgE and IgG binding to diisocyanate–protein conjugates and assess the utility of such testing as a diagnostic tool and exposure indicator. Data from 29 studies of occupational exposure to diisocyanates revealed considerable variability in assay methodology and heterogeneity in the prevalence of positive antibody responses across laboratories. In studies that included both confirmed diisocyanate asthma subjects and exposed nonasthmatics, positive IgE responses identified cases with low sensitivity (18–27%), but high specificity (96–98%). Detection of IgG binding to diisocyanate conjugates is an indirect, qualitative indicator of disease status and past diisocyanate exposure. The utility of these assays is limited, however, due to a lack of (1) method standardization, (2) population norms to guide interpretation of results, and (3) demonstration that the assays improve either on disease prediction or on exposure confirmation beyond that of other indicators. Sources of assay heterogeneity are discussed and suggestions are offered for improving test performance and interpretability.

Keywords diisocyanate conjugate, ELISA methods, isocyanate biomarker, occupational asthma, specific IgE, specific IgG

INTRODUCTION AND BACKGROUND

Diisocyanates are monomers that react with polyols or similar compounds to generate a large variety of polyurethane products including flexible or rigid foams, elastomers, coatings, and adhesives. The chemical reactivity of diisocyanates (Brown, 1986; Kennedy and Brown, 1992), which contributes to their technical value, can be expected to play a key role in their general toxicity as well. Concerns about the risk of adverse effects due to diisocyanate exposure in the workplace relate mainly to respiratory health outcomes (Mapp et al., 1999), with the more severe outcomes including reactive airways dysfunction syndrome (RADS), asthma, and alveolitis (hypersensitivity pneumonitis).

The detection and characterization of diisocyanate-induced respiratory disease present many challenges to clinicians and researchers alike. Even after decades of investigation, the detailed mechanism(s) of diisocyanate-induced respiratory disease have yet to be elucidated (Karol, 1986; Redlich and Karol, 2002; Mapp et al., 1999; Raulf-Heimsoth and Baur, 1998). For some chemicals known to cause occupational asthma (OA), the presence of specific antibodies correlates well with clinical disease. This holds for high-molecular-weight (HMW) agents in which an IgE-mediated mechanism has been identified in most instances. It also applies to some low-molecular-weight (LMW) agents such as acid anhydrides where again specific IgE levels correlate well with disease state (Patterson et al., 1982; Cullinan, 2004). The presence of specific antibodies in the absence of disease, at least in the case of HMW antigens, has been viewed as

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evidence of subclinical sensitization and increased risk of subsequent disease development (Hagy and Settipane, 1971). With one LMW agent, trimellitic anhydride (TMA), the presence of specific antibody binding to TMA–protein conjugates was found to be predictive of who has or will develop immunologically mediated respiratory disease (Grammer et al., 1998).

With diisocyanates, there appears to be less concordance between the antigenic reactivity of diisocyanate–protein conjugates and the occurrence of disease compared with that seen with HMW and some LMW agents. In this regard it is worth noting that antigenicity, which has been defined as the ability of a peptide to react specifically with the functional binding site of a complementary antibody, is a purely chemical phenomenon, whereas immunogenicity, which is the ability of the peptide to induce an immune response in a competent host, depends on complex interactions with various elements of the host immune system (Van Regenmortel, 2001). Nevertheless, the search for immunologic markers of diisocyanate-induced respiratory disease, which may have been initiated to better understand the pathogenesis of the disease, has been pursued, in part, because of the desire to have an early and reliable indicator of diisocyanate-induced asthma. The importance of early diagnosis has been demonstrated in studies of disease outcome after removal from further exposure (Tarlo et al., 1997; Park and Nahm, 1997).

Clinical history alone has not been considered an accurate method of diagnosing OA, with positive predictive values varying from 30 to 46% (Malo et al., 1991). The most reliable method for diagnosing OA remains documentation of clinical and functional status changes during and after contact with the offending agent (Moscato et al., 2003). Ideally, the diagnosis would be confirmed by specific inhalation challenge (SIC) performed in a hospital laboratory, although use of peak expiratory flows to monitor work-related bronchoconstriction represents a more accessible option. SIC testing is not readily available due to its complexity, potential health risks, and expense (Bernstein and Jolly, 1999). Serial peak flow assessment requires rather extensive monitoring, expertise in its evaluation, and is problematic if the individual has been removed from exposure. Antibody testing is appealing due to its accessibility and relatively low cost, as well as the absence of potential side effects for the patient. For this reason, a number of studies have been conducted to investigate how well antibody tests perform in discriminating between exposed subjects with and without a confirmed diagnosis of OA due to diisocyanates.

Exposure to diisocyanates in the workplace has been traditionally assessed based on personal sampling of airborne concentrations. There is also an extensive literature on the use of biological markers of diisocyanate exposure based on measurement of protein adducts (Wisnewski et al., 2000; Sabbioni et al., 2001; Brown and Burkert, 2002) and on acid or alkaline hydrolysis of urine or plasma samples to assess metabolites consistent with diisocyanate exposure (Rosenberg and Savolainen, 1986; Skarping et al., 1991; Sennbro et al., 2005). The assessment of IgG binding to diisocyanate conjugates has also been investigated as a marker of past exposure in occupational and occasionally community settings (Orloff et al., 1998). This use could be appealing where prior measurement data are unavailable to confirm that diisocyanate exposure has occurred.

The objectives of this review are (1) to describe and critique the key methodologies employed in diisocyanate antibody testing, (2) to examine the utility of such tests as a diagnostic tool or marker of exposure, and (3) to offer suggestions for improving test performance and interpretation across studies. The review focuses on three diisocyanates, toluene diisocyanate (TDI), diphenylmethane diisocyanate (MDI), and hexamethylene diisocyanate (HDI), because of their wide usage and the extensive literature reporting immunoassay results using conjugates of monomeric forms of these diisocyanates.

PART I. LABORATORY METHODS IN DIISOCYANATE ANTIBODY TESTING

Various methodologies have been and continue to be used to generate diisocyanate conjugates and to assess antibody binding to these conjugates. Wisnewski and colleagues (2004) noted standardization issues in regard to these methodologies, particularly in reference to conjugate preparation, and suggested that this may have contributed to the heterogeneity of findings reported across studies. Key design and performance characteristics of these assays that could affect test outcome are identified and discussed in this section prior to examining the utility of these assays as markers of diisocyanate exposure or disease. The components discussed include the preparation of diisocyanate conjugates, the specific analytic methods for detecting serum antibodies bound to conjugate, and the criteria for defining a positive result of the assay.

Conjugate Preparation and Characterization

Although it would appear desirable to generate diisocyanate–protein conjugates under physiologic conditions that mimic those experienced during actual human exposure, this has been difficult to achieve in practice due to technical issues (Wisnewski et al., 2004). Conjugate preparation is a multistep process that involves deciding on: (1) the particular diisocyanates or commercial products to be conjugated, (2) the carrier protein, (3) reaction conditions (e.g., the concentrations of protein and diisocyanate to be used, mixing strategies, and reaction time and temperature), and (4) postreaction processing (i.e., use of quenching agents, and conjugate isolation). Various techniques have also been used to characterize the resulting conjugates (e.g., Gutman assay, 2,4,6-trinitrobenzenesulfonic acid [TNBS] analysis to determine free amino groups, mass spectrometry, and electrophoresis). The many variables (see Table 1) to be considered may have contributed to a lack of standardization in conjugate preparation and characterization methods across laboratories.

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TABLE 1
Factors in conjugate preparation and characterization

Parameter	Consideration
Reactants	
Compound	Use of monoisocyanates avoids cross-linking and produces more homogeneous antigens; use of commercial products rather than diisocyanate monomers may lead to more complex antigen structures
	Different solvents may be required for delivering diisocyanates to the reaction in a controlled manner
Carrier protein	Albumin and keratins have been identified as primary diisocyanate conjugates seen in human airways
Reaction conditions	
Mixing	Respective diisocyanate and protein concentrations
	Liquid, aerosol, or vapor phase addition of diisocyanates
Reaction pH and buffer	May impact formation of polyureas and other products
Reaction time and temperature	May impact intra- and inter-molecular cross-linking
Postreaction processing	Selection and use of quenching agents or dialysis to stop the reaction
	Filtration/centrifugation to remove insoluble polyureas and high-molecular-weight cross-reactants
Characterization	
Chemical substitution analysis	Gutman assay, TNBS, ultraviolet spectroscopy, high-performance liquid chromatography (HPLC)
Electrophoretic mobility	Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, isoelectric focusing
MALDI mass spectrometry on tryptic digests of HSA	For identifying potential sites of isocyanate conjugation

Choice of Diisocyanates to Be Conjugated to Protein

In principle, compound selection should be governed by the particular diisocyanates or polyisocyanates being processed in the targeted exposure environment. Despite the presence of the same isocyanate functional groups, these compounds differ dramatically in physical and chemical properties (Brown, 1986). Physical differences among the diisocyanates of primary interest suggest that reactions take place primarily in the vapor phase in the case of TDI and HDI and as an aerosol in the case of MDI. However, these are not necessarily the physical states of the diisocyanates used in commerce. For example, commercial MDI is typically sold as "polymeric" MDI and is a composite of 4,4′-MDI and several different polyisocyanate structures. The difference in vapor pressure between monomeric MDI and polymeric MDI is a factor of 50 (1.2×10^{-3} vs. 6×10^{-4} Pa at 25°, respectively) (Pemberton, 2001). The latter is a solid at room temperature while the former is a liquid. Similarly, due to the higher volatility of HDI, most commercial products are made of HDI prepolymers with small residues of monomer. The main prepolymers are HDI biuret and HDI isocyanurate.

In theory it would make sense to prepare conjugates using the commercially relevant starting materials (e.g., polymeric MDI or HDI prepolymer). However, due to the increased structural complexity of conjugates formed from heterogeneous commercial products, many investigators elect to prepare conjugates from HDI, MDI, or TDI monomer. There are exceptions, as several researchers specifically examined the performance of conjugates based on both monomer and commercial formulations. For example, with regard to HDI-based products, four investigators synthesized and tested conjugates from both the monomer and a prepolymer (Grammer et al., 1988; Welinder et al., 1988; Vandenplas et al., 1993; Redlich et al., 2001). In two studies, the correlations between antibody responses to conjugates based on the monomer and prepolymer were characterized as very good (Vandenplas et al., 1993; Redlich et al., 2001). Although there was a statistical correlation in the rank response scores for the HDI–HSA and HDI biuret–HSA conjugate in the Redlich study of 65 subjects, 34% of the responses were judged positive using the monomer versus 8% using the biuret conjugate. In a third study, a general lack of correlation was seen between exposure and antibody response for both monomer and prepolymer conjugates, and a single subject with symptoms compatible with work-related respiratory disease had no detectable IgE or IgG antibody to either the monomer or prepolymer conjugate (Grammer et al., 1988). In the remaining study, the conjugate based on a prepolymer could distinguish exposed from control subjects, whereas the difference was not significant when using

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the HDI monomer conjugate (Welinder et al., 1988). These data suggest that antibody binding to both monomer and commercial product-based conjugates does occur, but do not provide convincing evidence in favor of the utility of one over the other form of conjugates.

In earlier years, investigators also examined responses to conjugates formed from tolyl monoisocyanate (TMI) (Karol, 1980; Butcher et al., 1980; Baur et al., 1984; Tse et al., 1985; Wass and Belin, 1989; Kim et al., 1997). The use of monoisocyanates was advocated to avoid cross-linking of carrier protein and to produce more homogeneous antigen preparations that could be incorporated into routine diagnostic assays (Karol and Alarie, 1980; Karol, 1986). Wass and Belin (1989) reported that a TMI conjugate was less effective in eliciting a positive response than diisocyanate conjugates in three subjects with a diagnosis of OA, due to HDI in two cases and MDI in one case. As few studies have routinely included assays of conjugates derived from monoisocyanate molecules in recent years, we did not formally assess the prevalence of positive antibody responses to these conjugates.

Choice of Carrier Protein

Diisocyanates may react with a number of different proteins and peptides present in the airway fluids and tissues. In most studies, human serum albumin (HSA) has been the carrier protein of choice in preparing diisocyanate conjugates. A recent study found albumin to be the predominant soluble extracellular HDI-conjugated protein in airway lavage fluid of subjects exposed to an HDI aerosol, whereas keratin 18 was the predominant diisocyanate conjugate in human endobronchial biopsy samples (Wisnewski et al., 2000). Studies have also identified other molecules in the respiratory tract that are modified by isocyanates, including laminin (Kennedy, 1990), tubulin (Lange et al., 1999), and glutathione (Lantz et al., 2001).

Based on the reactivity of diisocyanates with many different biomolecules, Baur et al. (1994) suggested that the rather low sensitivity of diisocyanate–HSA conjugates might be partly explained by the existence of additional antigenic structures associated with other diisocyanate–protein conjugates. Tee and coworkers (1998) also proposed that antigens might be missed because of the choice of HSA as an almost exclusive carrier for the isocyanates in antibody testing. However, Wass and Belin (1989) had reported that HSA conjugates, but not conjugates to several other protein carriers, were able to bind to IgE in several patients with diisocyanate-induced OA. Recently, Wisnewski et al. (2004) also reported that HSA was an effective carrier protein in that HDI–HSA conjugates were recognized by IgG, but not HDI conjugates formed with two other proteins abundant in airway fluid, namely, transferrin and lactoferrin. It has also been shown that serum albumin is the major protein in plasma to which both MDI and TDI adducts are formed (Johannesson et al., 2004; Lind et al., 1997).

Varying Reaction Conditions

Even when reaction conditions favor the binding of diisocyanates to proteins, there are various further reactions that may have considerable influence on the conjugate structure (Kennedy and Brown, 1992). For example, only one isocyanate (NCO) group is needed for binding to a protein. The second NCO group may undergo either intramolecular or intermolecular cross-linking with dramatic changes in the overall protein structure of the resulting conjugate, as demonstrated for MDI by Jin and Karol (1988). The type of cross-linking favored can also be influenced by factors such as protein concentration and molar ratio of diisocyanate to protein (Jin and Karol, 1988).

A further contributing factor to the complexity of possible products is the time of reaction (Jin and Karol, 1988). Not all diisocyanates hydrolyze at the same rate and thus while one end of the diisocyanate may react with a site on a protein, the other end may "persist" as an isocyanate for variable time periods, depending on the isocyanate, and thus retain the potential for further reactions. Moreover, the second NCO group may hydrolyze to an aromatic amino group, giving the conjugate the image of a carrier of an amine. Such an amino group may even react with a second diisocyanate molecule, resulting in formation of a urea residue attached to the protein (Kennedy and Brown, 1992).

Postreaction Sample Handling

The use of quenching agents or dialysis to stop the reaction may also contribute to variability in the structure of the final antigen. For example, if the reaction is quenched by addition of a nucleophile in excess at a specific time, two major outcomes are expected. The first is the reaction of excess diisocyanate with the agent, thus stopping further incorporation into the protein. The second outcome is to "cap" any monofunctionally incorporated diisocyanate that retains a second reactive isocyanate group at the time of quenching, possibly yielding a unique antigenic determinant. Similarly, if dialysis is used to terminate the reaction, the final conjugate product may depend on the dialysate. For example, use of ammonium bicarbonate may result in the formation of an end product capped by urea.

Degree of Modification

The potential impact of variability in conjugate structure on the ability to detect specific antibody responses has been of concern to many investigators. Karol (1980) had suggested that differences in responses across research groups might be due in part to differences in antigen preparation. Comparisons of specific IgE responses to TDI–HSA conjugates prepared by two different methods revealed that conjugates with lower incorporation of TDI onto HSA (ratios of 21–26 mol TDI/mol HSA vs. 43–52 mol TDI/mol HSA) could better discriminate between a group of 9 TDI-exposed subjects with asthma symptoms (4 with proven OA by SIC) and 9 unexposed subjects (Dewair and Baur,

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1982). Additionally, in control subjects, radioallergosorbent test (RAST) values showed a higher correlation with total IgE in tests using the higher rather than lower substituted conjugates, suggesting nonspecific reactions of conjugate with antibody when using higher substituted conjugates. Conversely, a higher percent RAST inhibition was demonstrated in a sensitized subject using a lower vs. higher substituted conjugate. Other researchers also observed differences in the ability of RAST tests to detect IgE binding, depending on degree of diisocyanate incorporation in the conjugate (Spiazzi et al., 1991; Wass and Belin, 1989). These authors reported that radioactivity binding was maximized by conjugates having diisocyanate to HSA molar ratios between 3 and 15:1 and that molar ratios above 30:1 produced high nonspecificity. These conclusions were based on testing of a limited number of subjects with and without diisocyanate-induced asthma. The conjugates in these studies had been prepared by liquid-phase mixing of the diisocyanate with HSA, which was deemed to be the preferential carrier protein (Wass and Belin, 1989). The preferential antigenic site on the conjugate was also considered. Several authors have suggested that hydrophilic groups reacting with the isocyanate might lead to changes in the three-dimensional conformation of the HSA and formation of new antigenic determinants (Wass and Belin, 1989; Son et al., 1998).

Recently, Wisnewski and colleagues conducted a study of auto body shop workers and reported that HDI–HSA conjugates formed by a vapor-phase exposure system performed better in distinguishing between high- and low-exposure jobs than conjugates formed by liquid addition of HDI to albumin (Wisnewski et al., 2004). In this study the vapor-phase conjugate had a molar ratio of HDI to HSA of <3:1, whereas the conjugates formed by liquid addition of HDI to albumin had molar ratios between 14 and 37:1. With the higher substitution ratio conjugates, these investigators were unable to differentiate statistically between painters and office workers in specific IgG binding. The vapor-phase method of conjugate preparation was advocated in part because it was thought to more closely parallel conjugates formed during actual workplace exposure.

Reaction Product Characterization

The complex nature of the products formed from the reaction of diisocyanates with proteins makes their characterization by traditional methods problematic. In the past, not all research groups even attempted to quantify the extent of modification. Most frequently, conjugates were characterized in terms of molar ratio of diisocyanate to protein on a chemical substitution basis. A much more detailed characterization of conjugates was made in a study by Wisnewski and colleagues (2004), in which a vapor-phase HDI-conjugate was even characterized in terms of the predominant conjugation sites on human albumin using mass spectroscopy methods.

While having a single well-characterized conjugate would appear to be beneficial in minimizing test variability across laboratories, it may also result in limiting the sensitivity and specificity of antibody detection. Just as the high reactivity of diisocyanates and changing laboratory conditions can lead to production of variable *in vitro* conjugates, *in vivo* exposure to diisocyanates may result in the production of variable antigenic epitopes depending on route, concentration, and duration of exposure, as well as on the form of the chemical or technical product used. Thus a panel of well-characterized conjugates prepared under different reaction conditions may be appropriate to enhance the sensitivity and specificity of detecting exposure or OA in exposed workers.

Immunoassay Methods to Detect Antibody

Given that total IgE levels in serum are many orders of magnitude lower than total IgG levels, greater sensitivity can be expected to be required in detecting IgE compared to IgG binding. Two main classes of immunoassay, RAST and enzyme-linked immunosorbent assay (ELISA), have been employed in assessing the antigenicity of diisocyanate–protein conjugates. Generally, an indirect noncompetitive sandwich format has been utilized with conjugate fixed to a solid phase for immobilization. Following incubation and washing, bound antibody is determined by assessing ^{125}I-labeled anti-human IgE or IgG in RAST or enzyme-labeled anti-human IgE or IgG in ELISA. In RAST, radioactive emissions from the ^{125}I are read in counts per minute by a gamma scintillation counter, whereas in ELISA, the optical density (OD) is read by spectrophotometer after catalytic enzyme reaction using chromogenic substrates. An issue specific to some ELISA assays for IgE is that the use of secondary reagents to amplify the signal (e.g., using rabbit anti-goat IgG to detect goat anti-human IgE bound to human IgE) may be problematic because of cross-reactivity with human IgG. The use of a biotin–streptavidin ELISA has been suggested to circumvent this issue (Bernstein et al., 2006). Supplemental inhibition assays have also been carried out on positive sera by preincubating sera with increasing concentrations of antigen to examine the specificity of antibody binding or assess potential cross-reactivity to other antigens.

Beyond variability associated with the different assay detection systems, test performance may be impacted by differences in how conjugates are processed in these screening assays. Covalent attachment of the antigen to the cellulose disc in the RAST or physical adsorption of the antigen to the microtiter plates in the ELISA could, for example, alter antigenic determinants or make them inaccessible (Peterman et al., 1988; Butler et al., 1993). Length of incubation may also impact test performance (Spiazzi et al., 1991). In ELISA, binding of the conjugate could be influenced by the chemical makeup of the solid phase, with the degree of modification impacting the hydrophobicity of the conjugate. Inhibition assays help to confirm the specificity of serum reactions and rule out reactions due to altered antigenic determinants formed during attachment or adsorption to the solid phase.

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Commercial assays have been used in some, but not the majority of, diisocyanate antigenicity studies. Generally, these assays have not provided a description or detailed characterization of the diisocyanate conjugates used in the assay. Commercial assays that have been used in published studies include a Phadebas RAST kit (Pharmacia and Upjohn Ltd., Uppsala, Sweden) and an automated Pharmacia CAP System assay, one version based on radioisotope detection and a second using fluorescent enzyme immunoassay (FEIA) to assess antibody response. A significant correlation between the performance of Phadebas RAST and CAP FEIA assays was reported in parallel tests of these two assays (Mazur and Pethran, 1993). In another study, performance of the CAP FEIA was compared with that of a Pharmacia enzyme-linked assay using the same set of HDI, MDI, and TDI conjugates (Baur et al., 1996). This study was undertaken in a large group of symptomatic workers showing prior sensitization to diisocyanates. Correlation coefficients for a quantitative response measure ranged from 0.76 to 0.84 depending on conjugate. The overall prevalence of positive responses was also very similar between the two assays.

Criteria Used in Defining and Interpreting Test Outcomes

Additional considerations potentially impacting test performance or interpretation of results include differences in scoring assay responses, choice of serum dilution levels, confirming positive responses by repeat or inhibition testing, size and composition of referent populations, cross-reactivity, and the timing of blood drawing relative to date of last exposure.

Defining a Positive Test Result

Among the studies reviewed, various parameters were used to quantify assay results and categorize responses as positive or negative. Depending on assay and investigator, both absolute (e.g., minimum required signal for a positive result) and relative criteria have been employed in categorizing test outcomes. Additionally, different procedural requirements may have been incorporated into the categorization process, such as a requirement to confirm positives through repeat testing or by demonstration of a certain percent inhibition. Some investigators, but not all, have reported the underlying quantitative measurements as well as the categorized results, and a few have reported results according to other classification algorithms such as the end titer at which a signal is detected. Reporting of quantitative measurements can be important where there is a signal that is measurable on a statistical basis, even though below the threshold used to define a positive response.

RAST assay results have typically been presented as a ratio of measured radioisotope binding on the diisocyanate conjugate disk divided by that of the corresponding HSA-only control disk. However, subtracting HSA binding from conjugate binding before calculating the ratio may yield results more consistent with RAST inhibition results (Karol et al., 1995). Tee et al. (1998) also reported that subtracting HSA binding from conjugate binding improved test performance in discriminating among OA cases and noncases. For classification of positive responses, a RAST ratio of 2 or higher has been used most often (Keskinen et al., 1988; Cvitanovic et al., 1989; Park and Nahm, 1996, Deschamps et al., 1998, Nosko et al., 1998), but a ratio of 3+ (Wass and Belin, 1989) has also been employed, as well as a RAST ratio greater than the highest of 22 nonexposed controls (Welinder et al., 1988). Few studies have examined the consequences of using different definitions for a positive response on test performance. In one study that did, increasing the RAST ratio cutoff from 2 to 3 decreased the sensitivity of the test in detecting proven OA cases from 28% to 19%, but increased the test specificity from 91% to 98% (Tee et al., 1998). The percent positive responses among non-OA cases decreased from 9% to 2%. It should be noted that all subjects were tested with TDI–HSA, MDI–HSA, and HDI–HSA conjugates, and a positive result with any conjugate was taken as a positive response. These data demonstrate the expected result that less stringent definitions will lead to higher prevalence rates of positive responses in both OA cases and noncases.

Investigators utilizing commercial assays generally classified findings as positive based on recommended threshold values provided by the manufacturer. Minimum threshold values have been incorporated into the decision criteria by other investigators as well (Baur et al., 1984; Skarping et al., 1996; Jakobsson et al., 1997). In specific IgE testing, Baur and colleagues (1984) additionally required that readings exceeded a control mean plus 2 standard deviations after regression adjustment for total IgE. Dewair and Baur (1982) had noted a correlation between specific IgE and total IgE in unexposed subjects, and Spiazzi et al. (1991) observed an effect of high total IgE on specific IgE results, when employing higher substitution ratio diisocyanate conjugates. A method for defining a positive response by incorporating regression adjustment for total IgE was developed and recommended by Karol et al. (1995) based on good agreement between this method and the RAST inhibition assay.

With ELISA assays, the cutoff point for a positive test has typically been set based on the distribution of OD readings among unexposed referents or established negative laboratory controls. While, in general, 2–3 standard deviations above the control mean has been used in defining a positive response (Cartier et al., 1989; Bernstein et al., 1993; Lushniak et al., 1998; Park et al., 1999; Daftarian et al., 2002), some investigators have utilized OD ratios of 2+ or 3+ calculated in a similar fashion as RAST ratios (Patterson et al., 1987; Grammer et al., 1988; Liss et al., 1988; Vandenplas et al., 1993), and others have used the highest observed control value (Welinder et al., 1988; Skarping et al., 1996; Jakobsson et al., 1997). Available data comparing different criteria for classifying responses as positive or negative are limited, but do indicate that some of the heterogeneity in the prevalence of positive responses to antibody tests across laboratories could be due to differences in classification algorithms. Comparability of data would be improved by reaching a consensus on one or two algorithms for defining a positive response.

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Calibration With Laboratory Controls

Negative and positive laboratory controls are often used to define positive responses in an ELISA assay and to simultaneously control for plate-to-plate variability in the assay. This approach may indirectly contribute to bias in estimates of specific antibody prevalence across studies if the laboratory controls have been selected specifically because of low OD readings obtained with these samples. Such controls may not be representative of the broader general population of non-occupationally exposed individuals. In a study of blood donors without known occupational or hobby exposure to diisocyanates, the OD cutoff point for a positive test was set as the mean + 3 standard deviations of 8 negative laboratory controls. In random sampling from the same target population, only about 0.2% of the observations would be expected to exceed the OD cutoff point by chance. However, positive specific IgG responses to HDI–HSA, MDI–HSA, and TDI–HSA conjugates were observed for 13%, 0%, and 5% of the blood donors, respectively (Bernstein et al., 2006). The percentages for two of the conjugates are clearly statistically higher than would be expected if the blood donors and negative laboratory controls were drawn from the same target population. No clear explanations for these differences were found. However, if antibody screening is to be useful in interpreting individual responses, it needs to be understood in terms of normal population responses (Brown et al., 2002). To date, there has only been limited assessment of the performance of these assays in general population samples and limited evaluation of possible host-related determinants of variability in OD readings.

Inhibition Assays

Inhibition assays have been utilized on a limited basis both to confirm the specificity of antibody response and to assess cross-reactivity with other antigens. The test can be time-consuming and is generally performed only where the initial immunoassay yields a positive reading well above the limit of quantification for the assay. In several studies, use of an inhibition assay was formally included in the definition of a positive assay response. Selden et al. (1989) utilized a threshold value plus ELISA inhibition of greater than 50% to define a positive specific antibody response, and Redlich et al. (2001) required at least 25% inhibition to confirm a positive response. Other investigators have reported inhibition results for selected subjects or performed inhibition assays using multiple diisocyanate conjugates (Pezzini et al., 1984; Tse et al., 1985; Liss et al., 1988; Keskinen et al., 1988; Wass and Belin, 1989). Because there has not been consistent reporting of inhibition assays, the reduction in percent positive responses through inclusion of this criterion is not well established. The lack of inhibition testing would be most apt to have an impact on assay outcome where OD readings are marginally positive. An ELISA validation study by Lynn et al. (1996) demonstrated diminished precision and interlaboratory agreement for reference samples having low specific antibody levels. Demonstration of positive inhibition rules out the formation of neoantigens during attachment of conjugate to the solid phase as an explanation for a positive response in the initial assay, but may not establish the specificity of the antibody binding.

In the blood donor population already discussed, inhibition testing was performed on 19 samples that showed binding of IgE or IgG to any of 3 diisocyanate–HSA conjugates (Bernstein et al., 2006). Inhibition assays were performed for samples in which OD readings were $\geq$0.2 and were elevated relative to 8 negative laboratory controls. Nevertheless, the OD readings of the examined samples were considerably below the positive control result. Among the 19 inhibition tests run using homologous conjugates, 9 (47%) showed at least 50% inhibition without concomitant inhibition of HSA alone. Because not much is known about the availability of antigenic sites in solution versus those bound to the microtiter plate, further work is needed to understand this methodology with complex antigens.

Confirmation of Positives by Repeat Testing

Another factor potentially impacting prevalence estimates across studies is whether or not a repeat test was required to confirm positive sera. Among 29 studies we reviewed in detail, explicit mention of repeat tests to confirm positive responses was found in only 7 articles (Karol, 1980; Patterson et al., 1987; Grammer et al., 1988; Cartier et al., 1989; Park et al., 1996; Tee et al., 1998; Redlich et al., 2001). Typically, tests were repeated twice, but several investigators reported undertaking additional confirmatory tests where duplicate testing yielded discrepant results. It was not always indicated whether the duplicate runs were made on the same or different days. With ELISA assays, duplicate tests should be carried out on different days since variability between plates and days may be much larger than intraplate variability (Nielsen, 2002). In assessing OD readings for IgG binding to HDI–HSA and TDI–HSA conjugates using samples from the blood donor population, mean intra-assay coefficients of variation (CVs) were 7 and 11% and interassay CVs were about 40% for the two conjugates, respectively (Bernstein et al., 2006). In this study samples were run in triplicate on the same plate and were repeated three times on separate days.

Effect of Serum Dilution Level

Differences in choice of serum dilution levels used in ELISA procedures could also impact the reported prevalence of positive ELISA responses, particularly where absolute thresholds are used in defining a positive response. The commercial and RAST assays appear to have been run mostly with undiluted serum. Serum dilution levels reported in ELISAs have ranged from 4 to 500; only a few studies have presented results run at multiple dilution levels. In a study of IgG responses to diisocyanate conjugates that included 455 occupationally exposed, asymptomatic individuals, Selden et al. (1989) reported a decrease in the percent positive responses from 10 to 5% based

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on applying a less strict criterion of a positive test at 1:20 dilution with 50+% inhibition versus one additionally requiring a positive response at a 1:100 dilution. In each case, a 0.5 absorbance value was used to define a positive result. Redlich and colleagues (2001) examined IgG binding to an HDI–HSA conjugate in 65 auto body shop workers (11% with work-related respiratory symptoms) exposed to HDI and HDI-biuret. Results were presented by the highest serum dilution (testing performed at multiple dilution levels) at which the ELISA OD exceeded 2 times that of negative controls. In their analyses, these investigators reported the prevalence of specific IgG responses to HDI–HSA to be 34%, but also indicated that only 14% and 5% of the specimens were positive at dilution levels of $\geq$1:16 and $\geq$1:128 times, respectively. These findings are consistent with Selden et al. (1989) and suggest that at least some of the heterogeneity in results across studies may be due to use of different dilution levels in defining positive responses.

Cross-Reactivity

Evaluation of cross-reactivity has been carried out with analogous mono-, di-, and polyisocyanate conjugates using either standard or inhibition immunoassays. Inhibition assays would be expected to provide stronger evidence than standard assays of partial or complete cross-reactivity. Some studies also compared responses using different protein carrier conjugates. No studies were found that examined cross-reactivity to conjugates formed with more distantly related chemicals such as isothiocyanates.

Baur (1983) examined cross-reactivity in six patients with proven histories of asthmatic reactions to diisocyanates. One subject, who had been exposed to HDI, MDI, and TDI, showed positive RAST responses to HSA conjugates of the three monomers as well as to the corresponding monoisocyanate conjugates, but did not react to a TDI conjugate formed with ovalbumin (OA). The substitution ratios of the conjugates were later characterized as varying between 10 and 26 moles of isocyanate per mole of HSA (Baur et al., 1984). In inhibition assays, each of the heterologous conjugates inhibited the response regardless of which conjugate was used for the solid phase. In this subject both a pure 2,4-TDI–HSA and a pure 2,6-TDI–HSA conjugate inhibited a mixed monomer TDI solid phase antigen as well. For other subjects, varying degrees of cross-reactivity were demonstrated, although one subject exposed to TDI and MDI demonstrated specific IgE responses only to TDI conjugates. In a later paper where standard assay results were presented for 36 subjects with a positive RAST response to at least one conjugate, apparent cross-reactivity to multiple conjugates was again demonstrated (Baur et al., 1984).

Keskinen and colleagues (1988) conducted RAST inhibition tests in seven individuals with diisocyanate asthma and positive specific IgE. All seven cases had immediate reactions in SIC tests; five had been exposed to MDI and two to HDI. The inhibition to the homologous conjugate ranged from 94 to 100%. Inhibition was regarded as partial for conjugates of heterologous diisocyanates, ranging from 39 to 93%. Total IgE was reported to be higher in these subjects than in other subjects without positive RASTs. Using inhibition studies, Liss et al. (1988) demonstrated partial cross-reactivity in one MDI-exposed employee with asthma symptoms, whose IgE was inhibited best by MDI–HSA and less by TDI–, HDI–, and *p*-tolyl-monoisocyanate–HSA, but not at all by a TDI–transferrin conjugate. Similar findings of partial inhibition have been reported by other researchers (Wass and Belin, 1989; Grammer et al., 1990). Wass and Belin (1989) concluded that it was important to prepare conjugates based on the same diisocyanate as present in the workplace. In the absence of a positive response with that conjugate, demonstration of a positive response to conjugates prepared from diisocyanates not known to be present in the workplace should be regarded with suspicion.

Study Design Considerations

Several aspects of study design may also be important in interpreting the findings of individual studies. These include the extent to which study participants have been accurately categorized in regard to exposure intensity and OA status, the timing of sample collection relative to date of most recent exposure, and the selection of referents appropriate for addressing the targeted research questions.

Ideally, controls should be representative of the study population except for the factor of interest. In practice, referent populations have varied considerably in size and often appear to have been selected for convenience. If one is investigating antibody testing as a means of discriminating between OA cases and noncases, the referent population should consist of subjects with suspected OA but who were determined not to have diisocyanate-induced OA through workplace or laboratory challenge tests. If investigating antibody testing as a marker of past exposure, the referent population should consist of individuals determined not to have had past exposure to the agent of interest.

Timing of sample collection relative to date of last exposure may also impact the interpretation of findings. After high acute exposures, antibody responses may be detected within 3 to 4 weeks following exposure (Karol, 1986). The *in vivo* half-life of circulating IgE is only several days (Prussin and Metcalfe, 2006) and the *in vivo* half-life of specific IgE antibody responses after removal from further exposure was estimated to be 5 to 7 months for diisocyanate–HSA conjugates (Tee et al., 1998). In another study, RAST values were reported to return to normal levels 4 to 6 months after exposure in nonatopic individuals and after a longer period of time in atopic individuals (Karol, 1986). These results are similar to those seen after removal from further occupational exposure to acid anhydrides (Taylor et al., 1987). Thus, the prevalence of positive responses would be expected to decline in relation to interval since last exposure. Specific IgE is not thought of as a potential marker of exposure, as positive tests are relatively uncommon even among known occupationally exposed individuals in the absence of work-related symptoms.

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The *in vivo* half-life of circulating IgG is longer than that for IgE, being 18–23 days (McPherson and Massey, 2006), and the in vivo half-life of specific IgG responses after removal from further exposure appears to be longer than for the corresponding IgE responses. The absorbance values of specific IgG responses to diisocyanate conjugates declined with a half-life varying from 1.1 to 6.4 years in one study of OA cases (Malo et al., 2006). In a second study of 5 subjects with TDI-induced OA, the mean time for absorbance values to decline by one-half was estimated to be 4.5 years (Park et al., 2002).

Treatment of the serum once collected is also important due to concerns of immunoglobulin stability, particularly when repeated freeze–thaw cycles occur, as for laboratory negative and positive controls. Thus descriptions are needed not only of exposure history in regard to study and referent subjects, but also of the postcollection handling of sera samples.

PART II. PREVALENCE ESTIMATES OF SPECIFIC IgE AND IgG BINDING TO DIISOCYANATE CONJUGATES BY DISEASE AND EXPOSURE STATUS

Methods

Literature Search

For the prevalence assessment, candidate English-language articles indexed in MEDLINE (1966 through 2006) were identified using the following combinations of search terms: "diisocyanates and immunology," "diisocyanates and IgE," and "diisocyanates and IgG." These articles were cross-referenced against an existing library of articles maintained by the authors on the broader topic of diisocyanate antibody testing and were individually screened to identify those potentially relevant to the prevalence review.

Inclusion Criteria

Studies were selected for inclusion based on testing sera for the presence of IgE or IgG antibody binding to diisocyanate conjugates (positive or negative response) in a minimum of 10 subjects occupationally exposed to diisocyanates and characterized relative to respiratory health status. The assessment was restricted to results obtained using conjugates of three diisocyanates: TDI, MDI, and HDI. These monomers were singled out because of their extensive use in commerce and the extent of publications reporting immunoassay results for these conjugates. Findings related to the use of other mono- or polyisocyanate conjugates were discussed earlier in Part I of this article. Results of several studies pertaining to environmental or general populations are described later, but were not included in the initial prevalence analyses.

Exclusions

Aside from exclusions related to sample size and the use of other conjugates, one study was excluded because it used a novel criterion to define a positive test: the 25th percentile of all subjects tested (Petsonk et al., 2000). Such a criterion is valid for internal comparisons, but is not readily adaptable to external comparisons. Another study (Grammer et al., 1990) was excluded because of substantial overlap of subjects with a prior reported study (Cartier et al., 1989).

Data Synthesis

Given the absence of interlaboratory cross-validation studies for antigenicity testing with diisocyanate conjugates, we first evaluated the heterogeneity of prevalence estimates across studies controlling for subject condition (that is, occupationally exposed persons with or without an established OA diagnosis). Individuals without an OA diagnosis could include asymptomatic as well as symptomatic individuals whose respiratory symptoms were judged not to be due to OA in the original study reports. This evaluation was undertaken separately for each combination of antibody class, exposure agent (HDI, MDI, TDI, or Mixed), and conjugate for which comparable data were available in two or more studies. Heterogeneity was assessed by a chi-square test for homogeneity using SAS Version 8.2 software (SAS Institute, Cary, NC). Lack of homogeneity could be due to a variety of factors including differences in laboratory procedures/performance, differences in exposure conditions, and differences in the timing of testing versus date of last exposure.

Next, antibody prevalence trends were examined by subject condition (e.g., OA vs. no OA), initially comparing crude rates calculated by pooling results across all relevant studies. A statistical analysis controlling for laboratory- or study-specific differences by stratification was undertaken where prevalence data were available for levels of the factor of interest within multiple studies. This analysis was based on a Mantel–Haenszel test statistic and cumulated evidence of an effect only from within-study contrasts. For factors, such as hapten–protein ratio of the conjugates, control for heterogeneity was only feasible for studies that internally compared results for different conjugates (for example, see Wisnewski et al., 2004).

Results

Selected characteristics of the 29 studies (28 with assessment of specific IgE and 19 with assessment of specific IgG meeting the inclusion criteria) are summarized in Table 2. The publication dates spanned a 26-year period. All except the two earliest studies of specific IgG assessments used ELISA methods. Seven of the 28 studies examining specific IgE employed commercial assays, either a Phadebas RAST kit or a Pharmacia CAP System. Where results were reported using multiple definitions for a positive result, the definition yielding the highest prevalence rate was generally used in this evaluation (see Table 2 for the definition of a positive response as used in each study). Characterization of the conjugates was relatively limited. For example, the molar ratio of TDI to HSA was indicated in only 8

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TABLE 2
Summary information on 29 studies included in assessment of diisocyanate antibody responses among occupationally exposed individuals

Author	Year	Study ID	Number of subjects	Exposure agent	Diagnosis method	IgE Assay	IgG Assay	HDI	MDI	TDI	Exposure timing	Definition of positive response
Karol	1980	1	37	TDI	SIC/ Clinical	RAST	–	–	–	35	<3 Wks since last exp.	Net cpm > M + 2SD (geometric) nonsensitive workers ($n = 25$)
Zammit-Tabona	1983	2	11	MDI	SIC	RAST	RAST	–	NR	–	Variable	RAST ratio > 2 (pooled control)
Baur	1984	3	621	Mixed	Clinical	RAST	–	22	15	26	<3 Wks since last exp.	RAST > 0.35 U/ml + >CM + 2SD ($n = 50$)
Pezzini	1984	4	28	Mixed	SIC	RAST	–	–	6	5	4–30 days since exp.	RAST > CM + 2SD ($n = 50$)
Tse	1985	5	76	MDI	Clinical	RAST	RAST	–	NR	–	Current	RAST ratio > 2 (pooled control)
Patterson	1987	6	23	TDI	Clinical	ELISA	ELISA	–	–	11	Not stated	OD > 2 × control value ($n = 5$) at 1:2 dil. IgE; 1:10 dil. IgG
Grammer	1988	7	150	HDI	SIC	ELISA	ELISA	NR	–	–	Low level current exp.	OD > 2 × CM ($n = 3$) at 1:5 dil.
Keskinen	1988	8	121	Mixed	SIC	PRAST	–	NR	NR	NR	Not stated	RAST ratio > 2
Liss	1988	9	46	MDI	Clinical	RAST	ELISA	–	NR	–	Current exp.	RAST%bind>2 × CM%bind; OD > 3 × CM ($n = 14$)
Welinder	1988	10	30	HDI	Clinical	RAST	ELISA	8	–	–	Current exp.	RAST ratio > 1.8; OD > max control value ($n = 22$)
Cartier	1989	11	62	Mixed	SIC	ELISA	ELISA	NR	NR	NR	Not stated	OD > 2 × CM
Selden[a]	1989	12	622	Mixed	Clinical	-	ELISA	8	6	10	Not stated	OD > 0.5 A units for 1:20 dil. + 50% inhibition
Wass[a]	1989	13	269	Mixed	Clinical	RAST	–	15	8	15	Not stated	RAST ratio > 3
Bernstein	1993	14	243	MDI	Clinical	ELISA	ELISA	–	3	–	Current exp.	OD > CM + 3SD at 1:10 dil. ($n = 10$)
Mazur	1993	15	94	Mixed	NA	PRAST, CAP-FEIA	–	NR	NR	NR	Current exp.	RAST ratio > 2 CAP > 0.35 U/ml
Vandenplas	1993	16	20	HDI	Clinical	ELISA	ELISA	NR	–	–	Not stated	ELISA index > 2 at 1:100 dil.
Karol	1994	17	63	TDI	SIC	RAST	ELISA	–	–	34	>1 Wk since last exp.	RAST%bind > CM + 2SD ($n = 94$) >25% inhibition ELISA OD>2 × CM
Park	1996	18	43	TDI	SIC	PRAST	–	NR	NR	NR	Current exp.	RAST ratio > 2
Skarping	1996	19	174	Mixed	NA	RAST	ELISA	32	21	–	Current exp.	RAST spec binding > 0.3%; ELISA OD > max control value ($n = 20$)
Jakobsson	1997	20	163	MDI	NA	RAST	ELISA	–	NR	–	Current exp.	RAST = spec binding>0.3%; ELISA OD > max cont. value $n = 49$
Deschamps	1998	21	68	TDI	Clinical	CAP-RAST	–	–	–	NR	Current exp.	CAP > 0.35 U/ml
Lushniak	1998	22	18	MDI	Clinical	RAST	ELISA	NR	3	NR	Current exp.	%bind > CM + 3SD $n = 9$ OD > CM +3 SD $n = 9$
Nosko	1998	23	26	MDI	Clinical	RAST	–	–	NR	–	Current exp.	RAST ratio > 2
Tee	1998	24	101	Mixed	SIC/ Clinical	PRAST	–	NR	NR	NR	< 1 year	RAST ratio > 2
Park	1999	25	83	TDI	SIC	ELISA	ELISA	–	–	5	Not stated	OD > CM + 2SD $n = 20$
Redlich	2001	26	65	HDI	Clinical	RAST	ELISA	49	–	–	Current exp.	RAST: >25% inhibition OD > 2 × CM + >25% inhibition
Daftarian	2002	27	100	TDI	NA	CAP-FEIA	ELISA	–	–	NR	Current exp.	CAP > 0.35 U/ml; OD > CM + 3SD $n = 6$
Wisnewski	2004	28	214	HDI	SIC	RAST	ELISA	3	–	–	Not stated	RAST ratio > 2 mean end titers
Ye	2006	29	410	TDI	SIC	ELISA	ELISA	–	–	12	Not stated	OD > CM + 2SD $n = 80$

Note. CAP = Pharmacia CAP System, FEIA = fluorescent enzyme immunoassay, PRAST = Phadebas RAST, SIC = specific inhalation challenge, NA = not applicable, CM = control mean.

[a]These two studies appear to summarize data drawn from a common pool of subjects; one study reports on IgE-specific and the other on IgG-specific findings.

of 16 studies reporting IgE and 5 of 8 studies reporting IgG testing with TDI–HSA conjugates. Among the 29 studies, 14 were conducted in an actively working population, and the interval between last exposure and blood draw date was <30 days in 3 other studies. In 10 studies, the relationship between last date of exposure and draw date was not clearly indicated; in one other study it was reported as >1 week and in another as up to 1 year.

In four studies, respiratory status was assessed based on questionnaire, but no clinical or SIC determination of OA status was made.

Heterogeneity Across Studies

The heterogeneity test assesses the extent to which differences in prevalence estimates across studies could be due to

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TABLE 3
Heterogeneity in the prevalence of IgE-specific antibodies across studies

Subject condition and exposure agent	Conjugate	Total subjects positive/tested	Study IDs	*p* Value
Exposed with OA diagnosis				
HDI	HDI–HSA	18/52 = 35%	8,11,16,28	.074
MDI	MDI–HSA	15/60 = 25%	2,4,5,8,9,11	.026
TDI	TDI–HSA	81/240 = 34%	1,4,6,8,11,17,18,25,29	<.001
Exposed without OA diagnosis				
HDI	HDI–HSA	19/754 = 3%	3,7,8,10,11,16,19,26,28	.153
MDI	MDI–HSA	15/864 = 2%	2,3,5,8,9,11,13,14,19,20,22,23	.001
Mixed	HDI–HSA	36/642 = 6%	3,13,15,24	<.001
Mixed	MDI–HSA	29/642 = 5%	3,13,15,24	<.001
Mixed	TDI–HSA	33/642 = 5%	3,13,15,24	.016
TDI	TDI–HSA	18/653 = 3%	1,3,6,11,13,17,21,25,27,29	.063

chance alone. When significant, it provides evidence for possibly important unaccounted sources of variability among the studies and renders combined estimates of prevalence across studies somewhat problematical. Heterogeneity test results are summarized in Table 3 for specific IgE and in Table 4 for specific IgG assays. These tests were run separately by subject condition, namely among documented OA cases and occupationally exposed individuals without an OA diagnosis. With regard to specific IgE, significant heterogeneity was observed with respect to MDI and TDI conjugates, both among documented OA cases and noncases. Significant heterogeneity was not observed with HDI conjugates. With respect to specific IgG, evidence of heterogeneity was detected for all three diisocyanate conjugates among exposed individuals without an OA diagnosis and for the TDI–HSA conjugate for subjects with an OA diagnosis (see Table 4).

Prevalence of Specific IgE and IgG by Subject Condition

The prevalence of specific IgE and IgG to diisocyanate conjugates is summarized in Table 5 by OA case status among employees exposed to any diisocyanate. The differences between cases and noncases were statistically significant across all diisocyanate–HSA conjugates and both antibody classes based on those studies providing data on both OA cases and noncases. Of the 13 studies providing data for one or more conjugates and antibody types, 9 defined cases based on SIC and the remaining 4 defined cases based on clinical assessment only. For specific IgE, the prevalence of positive responses ranged from 20 to 28% among OA cases and from 3 to 4% among noncases irrespective of conjugate. Very similar prevalence estimates were obtained when comparing only results where exposure agent matched conjugate. Considering only studies that included both cases and noncases, specific IgE identified cases with a sensitivity ranging from 18 to 27% and a specificity of 96–98% depending on the conjugate tested (HDI–HSA, MDI–HSA, or TDI–HSA). Prevalence rates were higher for specific IgG, both among OA cases and noncases, and the differences between OA cases and non-cases remained statistically elevated. There was a modest trend toward a higher prevalence of specific IgG for HDI–HSA both among cases and noncases compared to that for the MDI and TDI conjugates.

TABLE 4
Heterogeneity in the prevalence of IgG specific antibodies across studies

Subject condition and exposure agent	Conjugate	Total subjects positive/tested	Study IDs	*p* Value
Exposed with OA diagnosis				
HDI	HDI–HSA	17/25 = 68%	11,16	.116
MDI	MDI–HSA	16/37 = 43%	2,5,9,11	.050
TDI	TDI–HSA	52/161 = 32%	6,11,17,25,29	.003
Exposed without OA diagnosis				
HDI	HDI–HSA	70/453 = 15%	7,10,11,16,19,26	<.001
MDI	MDI–HSA	53/688 = 8%	2,5,9,11,15,19,20,22	<.001
TDI	TDI–HSA	25/323 = 8%	6,11,17,25,27,29	.002

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TABLE 5
Prevalence of specific IgE and IgG antibodies by conjugate and condition

	Occupational exposure to any diisocyanate				
	No OA diagnosis		With OA diagnosis		
Antibody class and conjugate	Positive/tested	Number of studies	Positive/tested	Number of studies	p Value[a] (number of studies)
IgE					
HDI	64/1,760 = 4%	14	45/182 = 25%	7	<.001 (6)
MDI	46/1,787 = 3%	14	36/179 = 20%	8	<.001 (6)
TDI	56/1,520 = 4%	15	97/341 = 28%	11	<.001 (9)
IgG					
HDI	75/462 = 16%	7	17/25 = 68%	2	.001 (2)
MDI	53/688 = 8%	8	16/37 = 43%	4	.002 (4)
TDI	31/332 = 9%	6	52/161 = 32%	5	<.001 (5)

[a]For p value based on stratified analysis of studies reporting results for both conditions.

Limited prevalence data were also available regarding symptomatic versus asymptomatic occupational exposure among persons without an OA diagnosis (see Table 6). These results were suggestive of a difference between symptomatic and asymptomatic individuals with regard to specific IgE. Results for specific IgG were based on too few observations and studies to be meaningful.

Prevalence of Specific IgG by Exposure Intensity and Duration

Several studies have reported that assays to detect specific IgG responses to diisocyanate–HSA conjugates may be useful indicators of prior diisocyanate exposure (Selden et al., 1989; Skarping et al., 1996; Lushniak et al., 1998; Redlich et al., 2001; Wisnewski et al., 2004). Among studies reporting specific IgG responses relative to known occupational exposure, we identified one that correlated a quantitative biomarker of diisocyanate exposure (hydrolyzable amine metabolites in plasma and urine) with an assessment of specific IgG responses in the same study population (Skarping et al., 1996). This study was conducted in a facility that utilized heated HDI- and MDI-based glues as adhesives. Urine and plasma was collected from 174 employees, and the amine analogues of the corresponding diisocyanates were measured after acid hydrolysis of the urine and plasma samples. These results were then correlated with specific IgG responses to HDI–HSA and MDI–HSA conjugates, with the hapten–protein molar ratios being 32 and 21, respectively. A modest statistical association was observed between ELISA OD values and both plasma and urinary MDI metabolites. These results were taken to

TABLE 6
Prevalence of specific IgE and IgG antibodies by conjugate and symptom status

	Occupational exposure to any diisocyanate excluding subjects with an OA diagnosis[a]					
	Exposed no symptoms		Exposed		Exposed with symptoms	
Antibody class and conjugate	Positive/tested	Number of studies	Positive/tested	Number of studies	Positive/tested	Number of studies
IgE						
HDI	0/163 = 0%	3	56/1,356 = 4%	9	8/241 = 3%	3
MDI	0/189 = 0%	4	35/1,330 = 3%	7	11/268 = 4%	5
TDI	8/393 = 2%	7	36/824 = 4%	4	12/303 = 4%	7
IgG						
HDI	–	–	68/438 = 16%	6	7/24 = 29%	1
MDI	4/48 = 8%	2	45/589 = 8%	4	4/51 = 8%	3
TDI	18/173 = 10%	2	8/109 = 7%	2	5/50 = 10%	4

[a]Insufficient data to perform stratified analysis of exposure subgroups controlling for laboratory.

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support the view that specific IgG is an index of diisocyanate exposure, but the association was characterized as weak (Skarping et al., 1996). A weak association may not be unexpected since a positive specific IgG response, if reflective of a true immune response, requires immune recognition of a diisocyanate–protein conjugate as an antigen at some time in the past coupled with continuing generation of specific IgG antibodies in response to relatively recent exposure.

In a study of 75 auto body shop employees without clinically apparent asthma, Redlich et al. (2001) showed a higher prevalence of specific IgG responses to an HDI–HSA conjugate among spray painters and technicians (39%) compared to office employees (14%). In the same target population, Wisnewski and colleagues (2004) showed higher mean end-titer responses to HDI–HSA conjugates prepared by vapor-phase mixing among painters and technicians versus office employees. Similarly, in a small study of 18 employees of a facility producing MDI-based polyisocyanate foam products, 6 of 9 exposed employees without a diagnosis of OA demonstrated specific IgG binding compared to 0 of 9 control subjects (Lushniak et al., 1998). Selden et al. (1989) compared specific IgG responses among 256 asymptomatic employees assigned to foaming operations characterized as using "modern" ($n = 186$) or "old" ($n = 39$) equipment or involving a closed process ($n = 31$). Percent positive IgG responses decreased from 33% to 5% to 0% among personnel assigned to "old" equipment, "new" equipment, and closed process, respectively.

Prevalence of Specific Antibodies in Populations Without Known Occupational Exposure

Aside from the 29 studies discussed earlier, we reviewed 3 studies using immunoassays as possible markers of past environmental exposure or disease in community settings (Patterson et al., 1985; Nuorteva et al., 1987; Orloff et al., 1998) and a study of specific antibody responses in a blood donor population (Bernstein et al., 2006). One of the community studies involved an outbreak of illness in school children (Patterson et al., 1985). It was hypothesized that the illnesses may have been triggered by MDI contamination related to the use of polyurethane insulation in the schools. Sera were collected from 59 children and staff members of 3 affected schools and from 9 control children. Samples were then submitted for ELISA analysis of specific antibody responses to an MDI–HSA conjugate. In the 59 samples, 1 specific IgG and 5 specific IgE responses were detected to an MDI–HSA conjugate; however, there were also 2 specific IgE responses detected in the 9 control children. The authors concluded that there was no difference in the proportion of positive responses between the two groups and no association between antibody responses and symptoms compatible with MDI-related health effects.

Two other studies were conducted among residents living in the vicinity of flexible foam production facilities (Nuorteva et al., 1987; Orloff et al., 1998). In the earlier Finnish study, sera from 62 residents, who had been identified by questionnaire as asthmatic, were submitted for analysis of specific IgE to TDI, HDI, and MDI conjugates (Nuorteva et al., 1987). A positive response to all three conjugates was observed in one subject. This individual had, however, reported prior occupational exposure to diisocyanates. It was also noted that the prevalence of asthma was actually lower among those residents living closer to the facility and the study concluded that the facility had not had a noticeable impact on the prevalence of asthma in its surroundings.

In the U.S. study, sera from 113 residents living near another flexible foam production facility were submitted for ELISA analysis of IgE and IgG binding to TDI–HSA, MDI–HSA, and HDI–HSA conjugates (Orloff et al., 1998). Similar to the prior study, this study lacked an internal comparison group of residents not residing in the vicinity of flexible foam facilities. A total of 10 residents (9%), including 1 with known occupational exposure to diisocyanates, had positive responses to 1 or more of the 3 conjugates tested at 1:10 and 1:100 dilutions. No inhibition testing was performed in this study and the adequacy of prior air sampling for diisocyanates during the period of operation has been criticized (Levine et al., 2001). A study of 139 blood donors with no known occupational or hobby exposure to diisocyanates was conducted later by the same laboratory and used the same ELISA procedures (Bernstein et al., 2006). Among the 139 subjects, positive IgG responses to the HDI–HSA (13%) and TDI–HSA (5%), but not the MDI–HSA (0%), conjugates were detected. Histograms of the OD readings showed a greater spread of OD values for the more highly substituted conjugates. Responses classified as positive had only marginally elevated OD values relative to negative laboratory controls and values $<1/2$ of the OD of the positive control. Further analyses demonstrated no statistical differences in response percentages by occupation, gender, or age of participant. These results suggest that variability even within a given laboratory can occur over time and emphasize the importance of including internal referent groups when conducting such studies.

Discussion of Prevalence Findings

Antibodies as Markers of Disease

For HMW allergens, antigen-specific IgE has been consistently detected in subjects with immediate-type hypersensitivity reactions to those allergens. Evidence of a relationship between detection of specific IgE and immediate-type respiratory sensitization is also convincing for some LMW compounds such as trimellitic anhydride (Grammer et al., 1998), but this has not been the case for diisocyanates. Even with trimellitic anhydride, there are some immunologic syndromes (e.g., late respiratory systemic syndrome) associated with detection of antigen-specific IgG rather than IgE (Grammer et al., 1998). With respect to diisocyanates there is also general agreement that IgE binding to diisocyanate conjugates, when detected, is a specific marker for OA (Tee et al., 1998). This is supported in our review by the

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generally low prevalence (3–4%) of specific IgE binding to diisocyanate conjugates in exposed subjects without a diagnosis of diisocyanate asthma and even lower prevalence among exposed individuals with no symptoms. Unfortunately, up to now, a positive IgE response is seen, on average, in less than 30% of the diisocyanate asthma cases confirmed by SIC. Thus, its utility as a diagnostic tool is limited in that a negative test result does not rule out a positive diagnosis. Similar to trimellitic anhydride, there are indications that at least some cases of diisocyanate asthma occur in the absence of any evidence of IgE involvement in the response to SIC challenge (Jones et al., 2006). In patients with diisocyanate-induced asthma without detectable specific IgE antibodies in their serum these investigators found no evidence of local bronchial IgE production after diisocyanate exposure sufficient to provoke an asthmatic response and postulated that two separate forms of asthma are induced by diisocyanates, one at least partly mediated through an IgE mechanism and another form totally independent of IgE involvement.

The role of IgG as a risk marker of diisocyanate asthma is also not well understood. In a review of studies examining antigen-specific IgG for a variety of HMW and LMW allergens, it was noted that although specific IgG responses correlated with intensity of exposure to these allergens, these antibodies might be present as bystanders due to their apparent lack of association with respiratory symptoms (Cullinan, 1998). In regard to diisocyanates, a few studies have considered IgG to be at least a useful marker of diisocyanate-induced asthma (Cartier et al., 1989; Park et al., 1999). Isocyanate-specific IgG responses have been reported in a number of case studies of allergic alveolitis (Malo et al., 1983; Bascom et al., 1985; Walker et al., 1989), and, in two larger studies, positive IgG responses were reported in 8 of 8 and 10 of 14 cases, respectively (Vandenplas et al., 1993; Baur, 1995). Because specific-IgG responses were also observed in healthy employees, Baur (1995) concluded that their presence was not, in itself, a reliable indicator of disease status. With regard to LMW haptens in general, the detection of specific IgG may provide corroborative evidence of the diagnosis (Grammer, 1999).

In the present review, we found specific IgG responses to diisocyanate conjugates to be more prevalent than IgE responses among proven cases of diisocyanate asthma, but the reported prevalence of specific IgG was also higher in subjects without proven diisocyanate asthma. The relative risk of a positive test among known cases versus non-cases was greater for specific IgE than for specific IgG across all conjugates and in both stratified and non-stratified analyses. These findings detract from the usefulness of IgG antigenicity testing as a specific diagnostic tool for diisocyanate asthma.

No studies were identified that specifically assessed the contribution of antibody testing to discriminating between diisocyanate asthma cases and noncases taking into account serial peak expiratory flow testing and methacholine challenge. Using SIC as the gold standard, Baur et al. (1998) reported a sensitivity of 62% and specificity of 56% for methacholine challenge alone in identifying diisocyanate asthma cases. Combining clinical history and methacholine challenge, the sensitivity declined to 52% but the specificity increased to 80% as some of the SIC-positive cases did not report a history of work-related asthmatic symptoms. When adequate serial peak expiratory flow records have been utilized, Anees et al. (2004) reported a sensitivity of 78% and specificity of 92% with respect to diagnosed cases of OA due to a variety of causes. Currently, there is a lack of data demonstrating that immunoassay detection of IgE and IgG antibody binding to diisocyanate–protein conjugates improves or does not improve on disease prediction beyond that of other indicators such as detailed symptom history relative to exposure, serial peak flow monitoring, and methacholine challenge.

In recent years, other competing immunoassays have been proposed for discriminating between SIC positive and negative individuals. These include lymphocyte proliferation assays (Wisnewski et al., 1999) as well as *in vitro* assays of the ability of diisocyanate conjugates to stimulate monocyte chemoattractant protein-1 (MCP-1) production (Bernstein et al., 2002). Because of the limited experience with MCP-1 testing, it remains to be seen if the early promise will be fulfilled in practice (Hendrick, 2002). Other assays may yet emerge that are better able to distinguish between SIC positive and negative individuals as more becomes known regarding the mechanisms underlying OA due to diisocyanates.

Antibodies as Indicators of Exposure in Occupational Cohorts

A higher prevalence of specific IgG compared to specific IgE binding to diisocyanate conjugates was apparent in occupationally exposed individuals with and without diisocyanate asthma. Among all subjects classified as occupationally exposed to diisocyanates, but without a diagnosis of OA, the prevalence of specific IgG ranged between 8 and 15% depending on diisocyanate but did not vary depending on whether or not the subjects reported symptoms. Given the relatively low percentages of positive responses seen in occupationally exposed populations, measurement of specific IgG binding cannot be regarded as a sensitive indicator of individual exposure. On a group basis, several investigators have reported a statistically higher prevalence of specific IgG binding or higher end titers when comparing employees subdivided into exposed versus unexposed or high versus low exposure categories (Selden et al., 1989; Lushniak et al., 1998; Redlich et al., 2001; Wisnewski et al., 2004). Only one study (Skarping et al., 1996) correlated specific IgG binding with more traditional indicators of exposure. In this study the correlations were characterized as rather weak and the authors expressed a preference for measurement of diisocyanate metabolites in plasma following acidic hydrolysis as an indicator of relatively recent exposure. In work settings, where symptomatic exposure is reported to have occurred during nonroutine work activities or after upset conditions, or where skin contact exposures are suspected, antigenicity tests taken at an appropriate

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time interval after exposure incidents could be of value from a clinical and exposure assessment perspective (Karol, 1986). The presence of specific IgG binding to diisocyanate conjugates is an indirect, qualitative indicator of past exposure that would not be expected to be as useful as other exposure assessment tools in monitoring and controlling routine exposures in the workplace.

Antibodies as Markers of Diisocyanate Exposure in Community Settings

Given the heterogeneity in prevalence rates for specific IgE and IgG across laboratories and the general lack of standardization of these assays, their use to assess potential low-level environmental exposure to diisocyanates is of questionable value. Findings based on results from a single laboratory would be very difficult to interpret in the absence of an appropriate control group run in parallel with study subjects and without detailed diisocyanate exposure histories. Furthermore, the low prevalence rates of specific IgE and IgG responses seen in occupationally exposed subjects without a diagnosis of OA do not support their use as a means of documenting past low-level environmental exposure.

SUMMARY AND RECOMMENDATIONS

Our analyses indicate that there has been considerable heterogeneity in the prevalence of specific IgE and IgG binding to diisocyanate conjugates across studies. To our knowledge, this is the first review to evaluate the consistency of diisocyanate immunoassay results across laboratories while controlling for subject condition (occupationally exposed subjects with and without a diagnosis of OA) and to examine systematically the utility of these assays in (1) discriminating among OA cases and noncases both within and among laboratories and (2) assessing past exposure in occupational and environmental settings.

Potential explanations for the observed heterogeneity are evident in the differences in assay methodology as well as the criteria used to define positive findings—differences that have been noted previously (Bernstein et al., 2002; Wisnewski et al., 2004). While progress has been made in understanding the important parameters that impact conjugate formation, to date, there has been no agreement reached on a set of conjugates that optimizes specificity and sensitivity in the detection of OA due to diisocyanates, and in fact, due to the complexity of exposure to isocyanate-containing products, identifying such a set of conjugates may not be realistic. There does seem to be some consensus that the use of conjugates with low molar substitution ratios increases the specificity of the conjugate as a disease or exposure marker and that albumin is the preferred carrier protein. However, a greater effort at interlaboratory cross-validation is needed to verify these conclusions.

With respect to antigenicity testing, factors ranging from the choice of serum dilutions to be run in the assay to criteria to be used in classifying results as negative or positive will affect the sensitivity and specificity of the test in regard to its proposed uses. Concern over how differences in defining the cutoff point for a positive response impact prevalence estimates across studies is not unique to diisocyanate antibody testing; the same issue has been raised in regard to immunoassay tests for other antibodies (Martin et al., 2000). Currently, there is no consensus on the need for confirming positive results through repeat testing (either on the same or separate days in the case of ELISA assays). There are also issues related to cross-inhibition due to heterologous diisocyanates or to related compounds such as isothiocyanates. In light of these considerations, additional data are needed for establishing population norms, as has been required for other clinical laboratory tests (Martin et al., 2000). A recent study by Bernstein and colleagues (2006) demonstrated that the background prevalence of IgE or IgG binding to diisocyanate conjugates in a general population can be quite high, depending on the assay methodology and criteria used to define a positive result. Consequently, until consensus test methodology is in place, the availability of appropriate within-laboratory control data will be critical to interpreting test results.

Despite the many limitations, there remains a real need for a reliable laboratory test to help identify individuals who have or will develop diisocyanate-induced respiratory disease. To this end, some steps in the direction of building a consensus among investigators and clinicians for common diagnostic assays and interpretation criteria would be helpful. Practice parameters for allergy diagnostic testing have been developed that discuss standardization of antibody testing in general (Bernstein and Storms, 1995). Efforts at interdisciplinary consensus building regarding the early diagnosis of occupational asthma in general have been organized in the past bringing together clinicians, toxicologists, immunologists, epidemiologists, and biochemists. Most recently, these efforts led to a report outlining the major unanswered questions and research needs in occupational asthma (Tarlo and Malo, 2006). A Workshop on Low Molecular Weight Occupational Allergens sponsored by the American Thoracic Society in 2005 is expected to result in the recommendation of new approaches to address these challenging issues. Taking the theoretical frameworks being discussed and translating them into practice remains a key challenge.

In the interim, the authors have summarized in Table 7 suggestions for improving antibody test performance and reliability based on our assessment of the literature. These suggestions relate to conjugate preparation, assay standardization, and study design. Ambiguities in interpretation of findings can also be avoided where measurement data are reported quantitatively (e.g., optical density readings) as well as qualitatively (positive/negative results), conjugate and serum samples are aliquoted into small volumes, thereby avoiding repeated freeze and thaw cycles, and quality control measures are reported, such as coefficients of variability both within and between ELISA runs.

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TABLE 7
Improving diisocyanate antibody test performance and reliability

Factor	Suggestion
Conjugate preparation	
Diisocyanate selection	Selecting diisocyanates to match those present in the exposure environment helps avoid ambiguities in interpretation
Hapten–protein molar ratio	Low molar ratios < 15:1 appear less subject to background binding than more highly substituted conjugates
Carrier protein	HSA is most commonly used and performs well when tested against other carrier proteins
Characterization	At a minimum, conjugates should be characterized in terms of molar ratios and migration during gel electrophoresis
Assay standardization	
Positive/negative controls	Include positive and negative controls on each ELISA plate for quality assurance purposes
Dilution level	Run sera samples at multiple dilutions to address possible matrix interferences at lower sera dilutions
HSA effect	Subtracting HSA binding from conjugate binding before calculating RAST ratios may improve performance
Defining positive responses	At least 50 samples from normal nonexposed subjects are needed to set the cutoff point for a positive test
Adjustment for total IgE	Adjust percent binding for total IgE to improve agreement with inhibition test findings
Study design	
Study population	Describe both in terms of exposure and disease status
Referent population	Choice depends on issue being addressed (e.g., in assessing diagnosis utility, referents should be exposed noncases)
Timing of sera collection	Reporting intervals between last exposure and blood sampling reduces ambiguity in interpreting results
Confirmation of positives	Confirm positive responses by duplicate testing on separate days and by inhibition assay where possible

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