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Diisocyanate conjugate and immunoassay characteristics influence detection of specific antibodies in HDI-exposed workers

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Summary

Background The structural characteristics of diisocyanate chemical protein antigens vary depending upon the methods of production, and may influence diisocyanate antigen immunoassays. The impact of different antigen preparation methods on immunoassay sensitivity, specificity, and predictive value for identifying workers with diisocyanate asthma (DA) has not been systematically evaluated.

Objective Evaluate the influence of preparation methodology of hexamethylene diisocyanate human serum albumin (HDI-HSA) conjugates on the performance of specific antibody assays for identifying workers with confirmed HDI asthma.

Methods Asthmatic reactions to HDI exposure were assessed in 80 autobody shop workers by specific inhalation challenge (SIC). HDI-specific IgE and IgG in serum were measured by RAST and ELISA with seven different HDI-HSA conjugates prepared in liquid phase with monomeric or polymeric HDI, or vapour-phase monomeric HDI. The HDI : HSA substitution ratios were determined by mass spectrometry.

Results DA was confirmed by SIC in 23 subjects. The maximal sensitivity for detecting specific IgE among workers with positive SIC results was higher with RAST and with polymeric vs. monomeric HDI-albumin conjugates (21.7% vs. 8.7%) with a generally high specificity ($\geq 95\%$). HDI-HSA specific IgG antibody was also detected in 22–43% of HDI asthmatics depending upon the conjugate used. The specificity of specific IgG varied from 88% to 96%, and it was higher for monomeric (vs. polymeric) HDI-albumin conjugates with low (vs. high) substitution ratios.

Conclusion The test performance of specific IgE and IgG immunoassays for identifying a positive SIC response varied with different HDI-HSA conjugates. Standard test antigens and common immunoassays must be used to minimize inter-laboratory variability.

Keywords conjugates, diisocyanate asthma, ELISA, HDI, IgE, IgG, RAST

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Introduction

Serologic evaluation of workers in occupations that use chemicals known to cause asthma may help identify workers with disease. In many industrialized countries, diisocyanate agents are the most common causes of occupational asthma (OA) [1]. These low-molecular-weight reactive chemicals are used in a variety of industrial applications including the manufacture of rigid and flexible urethane foam products and as hardeners in spray paints.

Numerous investigators have assayed serum-specific antibodies reactive with antigens prepared by using con-

jugated human serum albumin (HSA) with various diisocyanate chemicals [2–6]. Diisocyanate-specific IgE (reactive with diisocyanate-HSA) is frequently undetectable in workers with diisocyanate asthma (DA) [2, 7] despite the clinical resemblance of the disease to a type 1-mediated hypersensitivity response. Diisocyanate-specific IgG responses are more commonly found in workers with DA, but may also be detected in asymptomatic workers and have been suggested to be markers of exposure [8, 9]. On the other hand, one independent laboratory demonstrated that the presence of toluene diisocyanate (TDI)-specific IgG antibodies is closely related to the results of specific inhalation

challenge (SIC) with TDI [5]. Such findings question the clinical utility of serologic antibody data in DA, and whether or not DA may involve non-humoral pathogenic mechanisms. However, numerous technical issues might also contribute to the outcome of serologic assays for diisocyanate-specific antibodies including the methods used for generating the diisocyanate-HSA conjugate (used as the 'antigen'), as well as the type of immunoassay (RAST vs. ELISA).

Ideally, the 'antigen' used in diisocyanate serology studies should mimic those that form *in vivo* under occupational exposure conditions. However, the antigenic form of diisocyanates *in vivo* remains poorly understood. It has recently been suggested that HSA conjugates produced by exposure to hexamethylene diisocyanate (HDI) vapours (rather than liquid) may yield more biologically relevant conjugates [10]. In addition, it has been speculated that conjugates prepared with pre-polymeric forms of diisocyanate (vs. monomers) may be more relevant, given that pre-polymeric diisocyanates have supplanted monomeric forms in most industries [11]. However, few studies have compared the effects of different methods of conjugate preparation with the outcome of serologic tests.

Here, we report the results of the first study comparing serologic results using diisocyanate-HSA conjugates prepared with vapour and liquid monomeric HDI, as well as polymeric HDI. In addition, we compare RAST- and ELISA-based methods for detecting specific IgE. The study was conducted using a large group of HDI-exposed workers, in whom a diagnosis of OA was confirmed or excluded by specific inhalation testing. The implications of the data, for performing and interpreting diisocyanate serology assays, are discussed.

Methods

Subjects

Serum was collected from 80 spray painting workers with well-documented work exposure to HDI during evaluations conducted at two occupational pulmonary disease clinics in Québec, Canada: Hôpital du Sacré Coeur in Montreal (Drs Cartier and Malo) and Hôpital Laval in Sainte-Foy (Dr Boulet), to which subjects were referred for the purpose of confirming or excluding DA (DA⁺ or DA⁻, respectively). All subjects were evaluated by clinical and occupational history, spirometry and skin prick testing to common aeroallergens. Before conducting SIC tests, airway hyperresponsiveness was assessed by methacholine testing [12] (see Table 1). A diagnosis of DA was confirmed or excluded in all patients based on the results of SIC testing according to previously described methods [13]. Serum was obtained from all workers for measurement of specific antibodies. All patients gave prior in-

Table 1. Hexamethylene-diisocyanate (HDI)-exposed worker characteristics ($n = 80$), grouped according to the outcomes of specific inhalation challenge; i.e., challenge positive with diisocyanate asthma (DA⁺) and challenge negative with no diisocyanate asthma (DA⁻)

	DA ⁺ ($n = 23$)	DA ⁻ ($n = 57$)
Mean age (years) (range)	43.2 (21–64)	38.9 (22–63)
Gender (M/F)	23/0	55/2
PC ₂₀ (geometric mean)	1.1 mg/mL	3.9 mg/mL
Atopy (%)	48%	47%
Smoking status	5% current 55% never 41% ex-smoker	30% current 33% never 37% ex-smoker
Mean months of exposure	232.6	190.9
Mean months from last exposure	8.2	3.01
SIC reaction type	Early 48% Late 35% Dual 17%	Negative

Atopy was defined as two or more positive skin prick test to common aeroallergens.

M/F, male/female; SIC, specific inhalation challenge; PC₂₀, provocative concentration that results in a 20% decline of forced expiratory volume in 1 s (FEV₁).

formed consent, and an ethics review committee at each participating institution approved the study protocol.

Hexamethylene diisocyanate-human serum albumin conjugate preparation and characterization

Seven chemical protein conjugates were prepared in two laboratories, following different methods. As shown in Table 2, diisocyanate-HSA molar ratios for all conjugates are represented as a subscript number (e.g.: HDI₁₆). Four antigen conjugates were prepared in the laboratory of Dr Adam Wisnewski (Yale School of Medicine, New Haven, CT, USA), and three antigens were prepared in the University of Cincinnati Allergy Laboratory according to previously published methods [10, 14]. For the HDI vapour-exposed albumin conjugate, HDI vapour concentrations ranging from 20 to 200 parts per billion (p.p.b.) were generated in a close-circuit system and monitored with an Autostep monitor (GMD, Pittsburg, PA, USA). HSA at 0.5% w/v was exposed to HDI vapour in open 60 mm Petri dishes. In addition, the Yale Laboratory prepared HDI-modified HSA conjugates in liquid phase. Monomeric conjugate (HDI₃₃) was prepared by reacting liquid HDI at a final concentration of 0.1% (Sigma Chemicals, St Louis, MO, USA) with HSA 0.5% in a buffered borate solution (pH 9.5) for 2 h at 37 °C in PBS, pH 7.2 (Gibco BRL; Grand Rapids, MI, USA) dialysed against PBS four times and 0.2 µm filtered. The same protocol was followed for preparation of pre-polymeric HDI₂ (N-100 biuret) and pre-polymeric HDI_{0.3} (N-3300 isocyanurate) (both from Bayer, Pittsburg, PA, USA), but the conjugates were pre-

Table 2. Antigen characteristics (i.e. molar ratios) determined by mass spectrometry (MALDI)

Conjugate	Diisocyanate used	HDI (%) / HSA (%)	Hapten-protein ratio (HPR)
Vapour HDI ₆ -exposed albumin*	Monomeric HDI	20–200 p.p.b./0.5	6
Pre-polymeric HDI _{0.3} *	Desmodur [®] N-3300 (isocyanurate)	0.1/0.5	0.3
Pre-polymeric HDI ₂ *	Desmodur [®] N-100 (biuret)	0.1/0.5	2
Monomeric HDI ₃ [†]	Monomeric HDI	0.2/1.0	3
Monomeric HDI ₁₆ [†]	Monomeric HDI	0.2/1.0	16
Monomeric HDI ₂₃ [†]	Monomeric HDI	2/1.0	23
Monomeric HDI ₃₃ *	Monomeric HDI	0.1/0.5	33

Numbers in subscript represent the HDI–HSA hapten–protein substitution ratio (HPR). Concentration of HDI and HSA are expressed as percent (w/v).

*Prepared in Yale Laboratory.

[†]Prepared in Cincinnati Laboratory.

HDI, hexamethylene diisocyanate; HSA, human serum albumin.

diluted in acetone, and made at a final HDI concentration of 0.1%.

Three monomeric HDI antigens were prepared at the University of Cincinnati following the methods described by Tse and Pesce [15]. Conjugates were prepared with HDI concentrations of 0.2 (HDI₃ and HDI₁₆) and 2% (HDI₂₃) w/v, respectively. Pharmaceutical-grade HSA was obtained from Baxter Healthcare Corp., Glendale, CA, USA, as a 20% (w/v) solution. A 1% w/v HSA solution in PBS (0.01 M sodium phosphate, 0.14 M NaCl, pH 7.4) was stirred in an ice bath with HDI (Eastman Kodak Co., Rochester, NY, USA) or no HDI (untreated HSA). Aliquots were removed at various incubation points. The reaction was stopped by addition of an equal volume of ice-cold 2 M NH₄CO₃. Each solution was centrifuged at 10 000 × g, 20 min, and the supernatant was dialysed at 4 °C against three changes of 40 volumes of NH₄CO₃ and then four changes of 40 volumes of PBS. The conjugates were sterilized by 0.2 µm membrane filtration, and the protein concentration was determined by the BioRad procedure.

Native gel analysis

Proteins were mixed with 10% glycerol in gel running buffer and then electrophoresed on 10% native polyacrylamide gels, pH 8.3, as described previously [10, 16] and stained with GelCode Blue from Pierce (Rockford, IL, USA).

Characterization of conjugates by mass spectrometry

Conjugates were characterized to determine hapten : protein molar ratios by mass spectrometry, at the University of Cincinnati Mass Spectrometry Facility. The instrument used was Bruker Reflex IV MALDI Time-of-Flight (Billerica, MA, USA). Sinapinic acid was used as the matrix. HDI–HSA conjugates were compared with untreated HSA for determination of the increased mass attributable to HDI substitution, and the HDI : HSA molar ratios were

calculated according to the molecular weights of diisocyanates and HSA.

Enzyme-linked immunosorbent assay immunoassays

Specific IgE and IgG antibodies were assayed by ELISA in triplicate sera samples. High-binding 96-well microplates (Corning Inc., Corning, NY, USA) were coated with 100 µL/well (IgG) or 200 µL/well (IgE) of a 10 µg/mL solution of HDI–HSA or HSA-carrier protein diluted in 0.15 M NaCl by incubation for 2 h at 37 °C, and overnight at room temperature. Positive reference serum containing high levels of anti-HDI–HSA-specific IgG and IgE antibodies was obtained from an HDI-exposed worker. Eight negative control sera were obtained from volunteers with no known exposure to diisocyanates. For both ELISA-specific IgE and IgG, positive and negative control sera (1 : 10 dilution) were included on each plate, and plates were read when the positive reference serum (1 : 10 dilution) reached 0.6 mean optical density (OD_{405 nm}).

Enzyme-linked immunosorbent assay-specific immunoglobulin E. Plates were blocked for 1 h with blocking buffer [2% BSA in TBS (0.1 M Tris HCl 0.15 M NaCl, pH 7.6), 0.01% Tween 20] at 37 °C. Samples and reagents were diluted in blocking buffer and plates were incubated with the serum (200 µL/well) at 37 °C for 1 h. Plates were washed three times at each step with TBS-Tween 0.05%, pH 7.6. Specific IgE was detected using biotinylated (ε-chain specific) affinity-purified goat anti-human IgE antibody (1 : 2000 dilution), followed by alkaline phosphatase-labelled Streptavidin at a 1 : 2000 dilution [17] (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The substrate consisted of 1 mg/mL *p*-nitrophenyl phosphate in 10% (w/v) diethanolamine, 0.5 mM MgCl₂, pH 9.8.

Enzyme-linked immunosorbent assay-specific immunoglobulin G. Plates were blocked for 1 h with blocking buffer (5% BSA in PBS, 0.05% Tween). Samples and

reagents were diluted in blocking buffer and plates were incubated with the serum (100 µL/well) at room temperature for 1 h. Plates were washed three times at each step with PBS-Tween 0.05%. Serum-specific IgG antibodies were using goat anti-human IgG (γ-chain specific) alkaline phosphatase conjugate (Sigma Chemicals product # A-3187, St Louis, MO, USA) according to a previously described method [17]. The substrate consisted of 1 mg/mL *p*-nitrophenyl phosphate in 10% (w/v) diethanolamine, 0.5 mM MgCl, pH 9.8.

Radioallergosorbent test

A RAST assay was performed in Yale Laboratory as described previously [10]. Briefly, HDI-HSA antigens were conjugated to CNBr-activated Whatman paper discs prepared inhouse. A disc was placed in 75 µL of serum and incubated for 16 h at room temperature, and washed afterwards. HDI antigen-specific IgE bound to these coated discs was detected by using 75 µL of ¹²⁵I-labelled goat anti-human IgE tracer (HyCor Biomedicals, Garden Grove, CA, USA). The results were expressed as the ratio of binding (cpm) to HDI-HSA vs. untreated HSA-coated paper discs.

Enzyme-linked immunosorbent assay inhibition

ELISA inhibition assays were performed in four workers' sera that exhibited high specific IgG binding. Serum samples were mixed with diluent, 1, 10, 100 or 1000 µg/mL of each HDI-HSA antigen and untreated HSA, and then incubated overnight. All the steps were done at room temperature. Pre-incubated sera were then tested for HDI-HSA-specific IgG as described above. Percent inhibition was calculated as follows:

% Inhibition =

$$100 \times \frac{\text{mean OD non-inhibited well} - \text{OD inhibited well}}{\text{mean OD non-inhibited well}}$$

The I_{50} (inhibitor concentration required for 50% inhibition of antibody binding) was determined by linear regression.

Data analysis

Specific IgE results of the ELISA and RAST assays were expressed in terms of sensitivity, specificity, positive and negative predictive values (PPV, NPV) as well as the likelihood ratio of a positive test (⁺LR) [18]. Although not considered to be a valid diagnostic test, we analysed specific IgG for test sensitivity and specificity merely as a means to further compare our panel of antigens. ELISA results were considered to be positive when the mean OD_{405 nm} (1 : 10 dilution) was at least 0.1 and ≥ 3SD above

the mean of eight negative control sera, and no observed binding to HSA alone. RAST ratios > 2 were considered to be positive [8, 19]. Subject characteristics were compared using the Mann-Whitney rank sum test and Fisher's test.

Results

Hexamethylene diisocyanate-exposed workers' characteristics

The demographic characteristics of the subjects are listed in Table 1. As shown, there were 23 HDI-exposed workers in whom DA was confirmed and 57 in whom DA was excluded by SIC testing. All workers were Caucasian and no significant differences existed between the two groups regarding age, gender and atopy. The mean active occupational exposure to HDI as well as duration since workers were last exposed to HDI were higher in the DA⁺ group. Current smoking was more prevalent in the DA⁺ group compared with the DA⁻ workers ($P = 0.02$). The geometric mean methacholine PC₂₀ was lower among workers with confirmed DA. Asthmatic reactions after SIC with diisocyanates included 48% of workers having early responses, and 35% with late or dual (17%) responses.

Characterization of hexamethylene diisocyanate-protein antigens

Changes in albumin after exposure to different forms of HDI were assessed initially by means of native gel analysis (see Fig. 1). The conjugates with higher substitution ratios showed more marked changes (lanes 7 and 8) compared with low substituted conjugates (lanes 1, 3, 4 and 6) in both liquid and vapourized forms. Hapten : protein molar ratios of HDI-HSA conjugates were assessed by mass spectrometry. Figure 2 illustrates an example of the mass spectrometry-MALDI determination of the mass of two

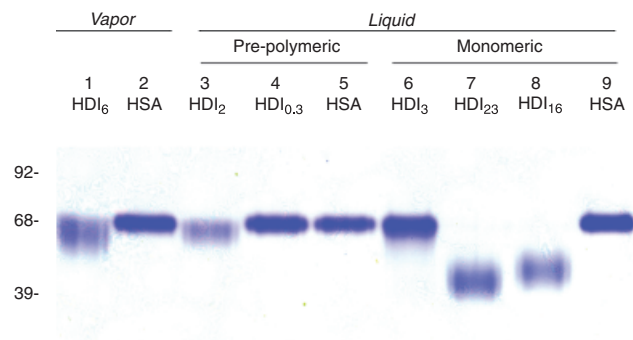


Fig. 1. Changes in albumin after exposure to hexamethylene diisocyanate (HDI) (native gel analysis): lane 1, vapourized HDI (HDI₆); lane 3, pre-polymeric HDI₂; lane 4, pre-polymeric HDI_{0.3}; lane 6, monomeric HDI₃; lane 7, monomeric HDI₂₃; lane 8, monomeric HDI₁₆. Lanes 2, 5 and 9 represent untreated human serum albumin (HSA).

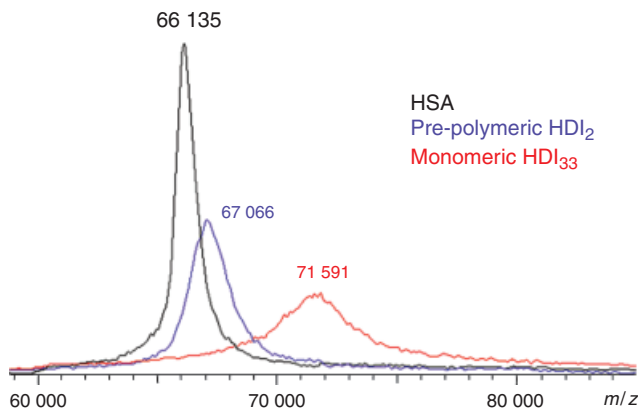


Fig. 2. Representation of two hexamethylene diisocyanate-human serum albumin (HDI-HSA) conjugates (pre-polymeric HDI₂ and monomeric HDI₃₃) and one non-conjugated (untreated) HSA value determined by mass spectrometry.

different conjugates (pre-polymeric HDI₂ and monomeric HDI₃₃) compared with untreated HSA. The increased mass of the conjugated albumins is attributable to HDI substitution.

The characteristics of HDI antigens are shown in Table 2. As shown, the vapour-modified HDI₆-HSA conjugate had a relatively low substitution ratio (i.e. 6) compared with HDI monomeric conjugates (HDI₃₃, HDI₁₆ and HDI₂₃), except for monomeric HDI₃. Conjugates prepared with pre-polymeric HDI reagents had lower molar ratios (2 and 0.3, respectively).

Significant and specific mean inhibition by homologous HDI-HSA antigens was demonstrated with the I₅₀ (inhibitor concentration required for 50% inhibition of antibody binding) for HDI-HSA conjugates ranging 1.7–5.8 µg protein/mL (data not shown). Non-specific binding to HSA was not detected (<20%).

Antigen-specific immunoglobulin G and immunoglobulin E antibodies

Specific antibodies were assayed with seven HDI conjugates, one vapour-exposed albumin, two pre-polymeric and four monomeric HDI conjugates. Specific IgG was measured by means of ELISA and specific IgE was measured with both ELISA and RAST techniques. Because adequate serum volume was not available, specific IgE data could not be performed for pre-polymeric HDI_{0.3} and monomeric HDI₃₃ conjugates.

Positive results for specific IgE, IgG or both were obtained in 24 subjects out of 80 HDI-exposed workers tested. The detailed results for both specific IgE (by ELISA and RAST) and IgG are shown in Table 3. Subjects were grouped based on the response to SIC (DA⁺ and DA⁻). Comparative results of specific IgE assays are also summarized in Table 4. In general, RAST results achieved a higher sensitivity (8.7–21.7%) than did ELISA-specific IgE

(0–8.7%). Maximal sensitivity (22%) was achieved by measuring RAST binding to pre-polymeric HDI₂. The test specificity of both IgE assays was ≥95% for the entire antigen panel, except for the monomeric HDI₁₆ (89.5%), with only one serum sample (subject # 16) giving consistent false-positive results (see Table 3). The NPV for specific IgE with either immunoassay was suboptimal, ranging between 71% and 76% for the HDI antigen panel. However, it is noteworthy that the positive predictive value (83%) of a positive RAST test was optimized with the use of pre-polymeric HDI₂. Furthermore, the ⁺LR of RAST was 12.1 with the aforementioned conjugate, far exceeding all other antigens tested (0–7.2) (Table 4).

Specific IgG ELISA results with the same panel of antigens are listed in Table 5. As mentioned in 'Methods', evaluation of test performance characteristics relative to the outcomes of SIC tests was used as a means to compare immune reactivity to various HDI-HSA antigens. As opposed to specific IgE, specific IgG responses are not generally considered to be valid markers of clinical sensitization. Diisocyanate-specific IgG antibodies were detected by ELISA in 22–43% of the DA workers, with the highest percentage being achieved with monomeric HDI₂₃. The specificity of specific IgG varied from 88% to 96%, and it was higher for monomeric (vs. polymeric) HDI-albumin conjugates with low (vs. high) substitution ratios. In this case, immune reactivity to specific IgG was not enhanced with the use of pre-polymeric conjugates. The HDI vapour-modified HSA conjugate (vapour HDI₆-HSA) possessed the lowest sensitivity, the highest specificity, the highest ⁺LR as well as the highest PPV (71%).

Discussion

In this study, we compared the performance of two different specific IgE immunoassays (RAST and ELISA) as well as an ELISA for specific IgG using a diverse panel of HDI-HSA conjugates. The present study focused on sera obtained in HDI-exposed spray painters, in that these comprised the vast majority of diisocyanate cases presenting to participating clinics. The study population was unique in that DA diagnoses were rigorously confirmed by SIC testing (the current diagnostic gold standard), which were conducted by experienced personnel in specialized laboratory facilities [20].

First, we evaluated whether the use of pre-polymeric vs. monomeric HDI-HSA antigens affected the test performance of immunoassays. For the ELISA-specific IgG assay (Table 5), optimal test sensitivity was achieved with monomeric conjugates possessing ligand-HSA substitution ratios ranging between 16 and 33 compared with the monomeric HDI-HSA ratio of 3. Interestingly, although the sensitivity was lower, the specificity was optimized (95%) for low-substituted monomeric HDI₃ relative to the highly substituted monomeric conjugates, as was the

Table 3. Positive results obtained for specific IgG and specific IgE (ELISA and RAST techniques) in 24 out of 80 HDI-exposed workers

Subject #		Vapour HDI ₆			Pre-polymeric				Monomeric									
					HDI _{0.3}	HDI ₂			HDI ₃			HDI ₁₆			HDI ₂₃			HDI ₃₃
		G	E ELISA	E RAST		G	E ELISA	E RAST	G	E ELISA	E RAST	G	E ELISA	E RAST	G	E ELISA	E RAST	
DA ⁺	1	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	3	+	—	—	+	+	—	+	+	—	—	+	—	—	+	—	—	+
	4	+	—	+	+	+	+	+	+	—	+	+	—	+	+	—	+	+
	5	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—
	6	+	—	+	+	+	—	+	+	—	+	+	—	+	+	—	+	+
	7	—	—	—	—	+	—	+	+	—	—	+	—	+	+	—	+	+
	8	—	—	—	—	—	—	—	—	—	—	+	—	—	+	—	—	—
	9	+	—	—	+	+	—	—	+	—	—	+	—	—	+	—	—	+
	10	—	—	—	—	+	—	—	—	—	—	+	—	—	+	—	—	+
	11	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—
	12	—	—	+	—	—	+	+	—	—	—	—	+	+	+	+	+	—
	13	+	—	—	+	+	—	—	+	—	—	+	—	—	+	—	—	+
DA [−]	14	—	—	—	+	+	—	—	+	—	—	+	—	—	+	—	—	+
	15	—	—	—	—	+	—	—	—	—	—	+	—	—	+	—	—	+
	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	17	+	—	—	+	+	—	—	+	—	—	+	—	+	+	—	—	+
	18	—	—	—	+	+	—	—	—	—	—	+	—	—	+	—	—	+
	19	—	—	—	—	—	—	—	—	—	—	+	—	—	+	—	—	+
	20	—	—	—	—	+	—	—	—	—	—	+	—	—	+	—	—	+
	21	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	+	—
	22	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
	23	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
	24	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—

Subjects were grouped by responses to SIC (DA⁺ [#1–#13] and DA[−] [#14–#24]).

Specific IgE data for pre-polymeric HDI_{0.3} and monomeric HDI₃₃ could not be obtained because adequate serum volume was not available.

HDI, hexamethylene diisocyanate; DA, diisocyanate asthma.

Table 4. Specific IgE antibody results in 80 HDI-exposed workers tested with five different conjugates and two different techniques (ELISA and RAST)

Conjugates	Assay	Sensitivity (%) (TP/TP+FN)	Specificity (%) (TN/TN+FP)	PPV (%)	NPV (%)	⁺ LR
Vapour HDI ₆ -exposed albumin*	RAST	13 (3/23)	98.2 (56/57)	75	73.7	7.2
	ELISA	0 (0/23)	98.2 (56/57)	0	70.9	0
Pre-polymeric HDI ₂ *	RAST	21.7 (5/23)	98.2 (56/57)	83.3	75.7	12.1
	ELISA	8.7 (2/23)	98.2 (56/57)	66.7	72.7	5
Monomeric HDI ₃ [†]	RAST	8.7 (2/23)	98.2 (56/57)	66.7	72.7	5
	ELISA	0 (0/23)	98.2 (56/57)	0	70.9	0
Monomeric HDI ₁₆ [†]	RAST	17.4 (4/23)	89.5 (51/57)	40	72.9	1.7
	ELISA	4.3 (1/23)	98.2 (56/57)	50	71.8	2.4
Monomeric HDI ₂₃ [†]	RAST	17.4 (4/23)	96.5 (55/57)	66.7	74.3	5
	ELISA	4.3 (1/23)	98.2 (56/57)	50	71.8	2.4

Data for pre-polymeric HDI_{0.3} and monomeric HDI₃₃ could not be obtained because adequate serum volume was not available.

PPV (%) = TP/TP + FP; NPV (%) = TN/TN + FN; PPV, positive predictive value; NPV, negative predictive value; TP, true positives, TN, true negatives, FP, false positives, FN, false negatives.

⁺LR = sensitivity/1 – specificity; LR, likelihood ratio.

*Prepared in Yale Laboratory.

[†]Prepared in Cincinnati Laboratory.

HDI, hexamethylene diisocyanate.

Table 5. Specific IgG antibody results in 80 HDI-exposed workers tested with seven different conjugates (ELISA)

Conjugates	Sensitivity (%) (TP/TP+FN)	Specificity (%) (TN/TN+FP)	PPV (%)	NPV (%)	⁺ LR
Vapour HDI ₆ -exposed albumin*	22 (5/23)	96 (55/57)	71	75	5.5
Pre-polymeric HDI _{0.3} *	22 (5/23)	93 (53/57)	56	75	3.1
Pre-polymeric HDI ₂ *	39 (9/23)	89 (51/57)	60	78	3.6
Monomeric HDI ₃ [†]	26 (6/23)	95 (54/57)	67	76	5.2
Monomeric HDI ₁₆ [†]	35 (8/23)	88 (50/57)	53	77	2.9
Monomeric HDI ₂₃ [†]	43 (10/23)	88 (50/57)	59	79	3.6
Monomeric HDI ₃₃ *	30 (7/23)	88 (50/57)	50	76	2.5

PPV (%) = TP/TP+FP; NPV (%) = TN/TN+FN; PPV, positive predictive value; NPV, negative predictive value; TP, true positives, TN, true negatives, FP, false positives, FN, false negatives.

⁺LR = sensitivity/1 – specificity; LR, likelihood ratio.

*Prepared in Yale Laboratory.

[†]Prepared in Cincinnati Laboratory.

HDI, hexamethylene diisocyanate.

positive likelihood ratio (5.2). It is noteworthy that HDI vapour-modified HSA (HDI₆) performed in a manner similar to monomeric HDI₃ with low test sensitivity (22%), high specificity (96%) and high ⁺LR (5.5). The pre-polymeric HDI conjugates used in this study both had low HDI-albumin ratios (i.e. 0.3 and 2). Interestingly, the pre-polymeric antigen (HDI_{0.3}) with the lowest ratio had a sensitivity and specificity that were similar to monomeric HDI₃ and the vapour HDI₆ conjugate (22% and 93%, respectively). Therefore, the ELISA-specific IgG test was optimized by using HDI-HSA conjugates with relatively low substitution ratios.

The RAST assay was more sensitive than ELISA-specific IgE for all five HDI-HSA antigens tested. Pre-polymeric HDI₂ yielded optimal sensitivity for both ELISA and RAST IgE assays. The superiority of RAST could be related to higher binding capacities of the paper disc-bound conjugates for low-affinity IgE antibodies. The overall specificity was excellent for both assays and for all antigens tested regardless of HDI substitution ratios. However, the RAST when performed with pre-polymeric HDI₂ was clearly superior, exhibiting optimal sensitivity, specificity, PPV (83%) and a positive likelihood ratio (12.1). These findings suggest that pre-polymeric HDI chemicals, which are the predominant moieties used in spray paint hardeners, are more suitable antigens for detection of specific IgE. In workers exposed predominantly to pre-polymeric diisocyanates, Vandenplas et al. [21] reported that specific asthmatic reactions occurred after inhalation of pre-polymeric TDI but not with monomeric TDI, suggesting that pre-polymeric diisocyanates are the principal inducers of OA. These findings provide evidence that would support the routine use of pre-polymeric HDI-HSA antigens in future assessments of serum-specific IgE antibodies.

Second, we evaluated the impact of using vapour vs. liquid phase-prepared HDI-HSA conjugates on test performance. As mentioned, specific IgG with vapourized

HDI-HSA yielded optimal specificity and a positive likelihood ratio, and the lowest sensitivity when compared with all other antigens. In a recent study, Wisniewski et al. assessed sera of 203 healthy autobody shop workers and 11 workers with HDI asthma (confirmed by SIC) for the presence of specific IgE assayed by RAST. The vapourized HDI-HSA and HDI-HSA prepared in the liquid phase were compared. The sensitivity of RAST was 55% for the vapour HDI-exposed albumin vs. 18% for the liquid phase-prepared conjugate in the asthmatic group (*n* = 11) [10]. In contrast, our data exhibited a lower sensitivity (13%) of RAST with the vapour-modified conjugate, which may be explained by intrinsic differences between the study populations.

Our results agree with previous studies in which, regarding the conjugate and the technique used, diisocyanate-specific IgE is detected in a low number of exposed workers. It is possible that the influence of the conjugates or technique is relatively minor. A recent publication by Jones et al. evaluated bronchial specimens of workers after specific challenge with diisocyanates. They found an absence of Cε and mRNA IL-4, suggesting that DA might be a non-IgE-mediated disease at least in patients in whom specific IgE is not detected [22].

In summary, the utilization of various forms of HDI-HSA conjugates introduced variability into the results of specific antibody immunoassays. In addition to selection of test antigens, subtle differences in assay methodology are likely to introduce additional variability. Thus, this study demonstrates that meaningful comparisons of test results between different laboratories will require future use of standardized antigens and common assay procedures.

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