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Specific Antibodies to Diisocyanate and Work-related Respiratory Symptoms in Apprentice Car-Painters

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Abstract

Background. Isocyanates are the main cause of occupational asthma in most countries. Study of immunological markers of diisocyanate asthma may identify individuals at risk.

Objectives. 1) study changes in specific antibodies to hexamethylene diisocyanates (HDI); 2) describe the incidence of work-related respiratory symptoms in relation to changes in specific antibody levels.

Methods. Prospective study in 385 apprentice car-painters during their 18-month training. Participants were assessed upon entering and completing their training using questionnaires, methacholine challenges and measurements of HDI-specific immunoglobulin E (IgE), immunoglobulin G (IgG) and subclass 4 of IgG (IgG4) antibodies.

Results. Complete data are available for 298 subjects. Thirteen subjects (4.4%) reported ≥ 1 new work-related lower respiratory symptoms and 19 (6.4%), ≥ 1 new work-related nasal symptoms. Increases in levels of specific IgE and IgG above the 97th and 95th percentiles were significantly associated with duration of exposure. Increase in specific IgG was inversely related to incidence of work-related lower respiratory symptoms (OR=0.001, 95% CI 0.000-0.09) after adjusting for relevant covariates. The rise in specific IgG4 was significantly greater in those who did not develop work-related nasal symptoms (OR=0.09, 95% CI 0.01-0.7).

Conclusion. In this cohort of apprentice car-painters, a small proportion show increases in HDI-specific IgG and IgE after few months of exposure. Increases in specific IgG and IgG4 appear to have a protective effect on the incidence of work-related lower and upper respiratory symptoms, respectively. Assessment of specific antibodies to isocyanates may help identify subjects at risk of developing symptoms.

Keywords: HDI-specific antibodies, hexamethylene diisocyanates, work-related lower respiratory symptoms, work-related nasal symptoms.

Main messages

- In this cohort of apprentice car-painters, a small proportion show increases in HDI-specific IgG and IgE after few months of exposure.
- Increases in specific IgG and IgG4 appear to have a protective effect on the incidence of work-related lower and upper respiratory symptoms, respectively.
- Assessment of specific antibodies to isocyanates may help identify subjects at risk of developing symptoms.

Policy implication

Assessment of specific antibodies to isocyanates might be considered in surveillance programs of spray-painters starting exposure to isocyanates.

Introduction

Diisocyanates are now the most frequent cause of occupational asthma(OA) in most countries representing 14% to 25% of all cases(1)(2). The estimated prevalence of diisocyanate asthma is 10% among exposed workers(3). Since the 1970s, the annual incidence rate of OA due to toluene diisocyanates (TDI) has been estimated at below 1%, where TDI concentrations are controlled below 5 parts per billion (ppb)(4). Hexamethylene diisocyanate (HDI) is mainly used as a spray-paint-hardener in automobile body shops and in aircraft manufacturing.

Immune mechanisms may be implicated in the triggering process of OA due to diisocyanate.-Specific IgE and IgG antibodies can be detected in exposed workers(5). Elevated diisocyanate specific IgE antibodies exhibit poor sensitivity but high specificity for identifying workers with OA(6) whereas specific IgG levels may reflect either exposure or disease(7).

No prospective study has been conducted to determine the incidence of symptoms suggestive of OA and occupational rhinitis from the start of exposure to diisocyanates. We have previously completed prospective cohort studies of apprentices exposed to high-molecular-weight agents in order to study the natural history of probable OA(8)(9). The present study was performed using a similar approach. The aims were to: 1) longitudinally assess the changes in specific antibodies to HDI in young adults during the course of an apprenticeship in car-painting, 2) describe the occurrence of new work-related lower respiratory and rhinoconjunctivitis symptoms, and 3) examine the association between these work-related symptoms and changes in specific antibody levels as well as other possible risk factors.

Methods

Study design and population

This was a prospective cohort study of 385 car-painting apprentices recruited between 1999 and 2002 in the seven vocational schools in Montreal (Canada) and surroundings within 150 km from the *Hôpital du Sacré-Coeur de Montréal*, out of 12 schools offering the auto body training program in the Province of Quebec. The duration of this program is uniformly 1,590 hours with approximately 300 hours exclusively for painting. In 1998, prior to the study inception, 349 students graduated from this program in the whole Province. The cohort members were followed for a maximum of 19 months and the follow-up assessment was completed in 2004. The investigators met the students in their classroom to present the project and encourage their participation; at total of 486 apprentices were thus informed. Participants were assessed on two separate occasions, upon entry and at the end of the training program on the school premises. The only exclusion criteria were exposure to isocyanates during the two months preceding the commencement of the apprenticeship and exposure for a total of more than three months at anytime prior to the apprenticeship; this information was obtained through a questionnaire at the first visit.

The protocol was approved by the IRB of *Hôpital du Sacré-Coeur de Montréal*. Signed informed consent was obtained from each participant.

Questionnaire and immunological investigations

At the first visit, the health assessment included a respiratory questionnaire derived and translated from the standardized questionnaire of the International Union Against Tuberculosis and Lung Diseases(10), skin-prick tests, measures of serum antibodies, spirometry and a bronchial challenge test. At the second visit, except for the skin-prick tests, the same investigations were performed, and a questionnaire on exposure characteristics and symptoms at work was administered.

Allergy skin-prick tests included eleven common aeroallergens, namely mixed trees, mixed grass and ragweed pollens; *Alternaria*, *Aspergillus*, and *Hormodendrum*, feathers, *Dermatophagoides farinae* and *pteronyssinus*; and cat and dog dander (Omega, Montreal, Canada). Histamine phosphate (5mg/ml) was used as a positive control and the diluent (glycerine 50%) as the negative control. The largest wheal diameter was assessed 10 to 15 minute after introduction of the antigen. A positive reaction was defined as a diameter ≥ 3 mm in the presence of a positive reaction to histamine and without a reaction to the diluent. Atopy was defined as a reaction to ≥ 2 aeroallergens.

Spirometry testing was performed with a Collins spirometer according to standards(11). Bronchial challenge tests were done with a Wright nebulizer, with concentrations of methacholine from 0.03 to 32 mg/ml according to a modified protocol(12).The provocative concentration of methacholine causing a 20% decrease in forced expiratory volume one second(FEV1) (PC20) was interpolated from individual dose-response curves. Reference values for FEV1 and forced vital capacity (FVC) were those from Knudson and co-workers(13). A PC20 ≤ 16 mg/mL represented bronchial hyperresponsiveness (BHR)(14).

For each subject, serum HDI-specific IgE, IgG and IgG4 antibodies were measured at the Imperial College School of Medicine.

Conjugate preparation

A previously optimized method for the conjugation of hexamethylene diisocyanate – human serum albumin (HDI-HSA) was used for the study(15). Briefly, equal volumes of 0.002 M hexamethylene diisocyanate (HDI) (Sigma Chemical Company, Dorset UK) and 5 mg/ml human serum albumin (HSA) (Sigma Chemical Company, Dorset UK) are mixed and incubated overnight at 4°C on a rocker and roller. The diisocyanate conjugate was dialysed against 8 litres distilled water at 4°C, changed hourly for the first 2 hours, then twice over 48 hours, to remove any unbound diisocyanate. HDI:HSA conjugate was coupled to cyanogen bromide activated discs. The HDI:HSA conjugate revealed a distinct 67 kDa band on SDS PAGE gel.

Measurement of specific IgE using a radioallergosorbent assay (RAST)

Specific IgE were measured using a radioallergosorbent test (RAST). Serum samples (50 µl) were added in duplicate to HDI:HSA coupled RAST discs and incubated overnight at room temperature. Discs were washed three times with 2.5ml RAST wash solution (0.9% w/v sodium chloride with 0.2% v/v Tween 20 and 0.05% sodium azide) and aspirated to dryness. Bound IgE was determined by adding 50µl ¹²⁵I anti-human IgE (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) per disc, incubating overnight at room temperature and washing three times with RAST wash and aspirated to dryness. Radioactivity was measured using a Cobra II Auto Gamma Counter (Packard Instrument Company, Meriden, CT, USA). The positive cut off point for RAST assay was considered to be >2% binding, the % binding being the % of the counts per minute of the sample, divided by the total counts added to the assay. The specificity of IgE anti-HDI:HSA was determined (see online supplement).

Measurement of specific IgG and IgG₄ antibodies

Specific IgG and IgG₄ were measured using an Enzyme Linked ImmunoSorbent Assay (ELISA) Half a 96 well microtiter plate (Immulon 2HB, Thermolabsystems, Massachusetts, USA) was coated with the pre-determined optimal coating concentration (100 µl of 1:400 diluted HDI:HSA conjugate for IgG and 200 µl of 1:400 diluted HDI:HSA conjugate for IgG₄); and the other half with 100 µl (IgG) or 200 µl (IgG₄) of 1 mg/ml human serum albumin (Sigma, Dorset, UK) in phosphate buffered saline (PBS) and incubated overnight at 4°C. Plates were washed twice with PBS/0.05% Tween 20 (VWR International Ltd., Poole, England) and then blocked with 200 µl PBS/1% HSA for two hours at 4°C. Following four washes, 100 µl of serum (diluted 1:10) for IgG and 200 µl serum (diluted 1:10) for IgG₄ were added to both HDI:HSA conjugate and HSA coated wells and the plates were incubated for two hours at room temperature. Tests were done in duplicate. After a further four washes, biotinylated anti-human IgG/IgG₄ (BD Biosciences Pharminagen, Oxford, UK) (100 µl for IgG/ 200 µl for IgG₄ diluted 1:1000 in PBS/1%HSA) was added to all wells and incubated for one hour at room temperature. After six washes streptavidin-peroxidase (Sigma, Steinheim, Germany) (100 µl for IgG/ 200µl for IgG₄ diluted 1:1000 in PBS/1%HSA) was then added to each well and the plates were incubated for 30 minutes at room temperature. After eight washes substrate (TMB, Sigma, Dorset, UK) (100 µl for IgG/ 200µl for IgG₄) was added to each well. The reaction was stopped after 10 minutes with 50 µl of 1.9M sulphuric acid and absorbance read on a plate reader (Dynatech Lab Ltd., West Sussex, UK) at 450 nm to produce an optical density. We subtracted the binding of specific antibodies to HSA from the HDI:HSA conjugate binding to give a final HDI:HSA specific binding(16). A positive, negative and background control were run on each plate and results were

only accepted if the coefficient of variation was below 0.25%. The specificity of IgG anti-HDI:HSA was determined (see online supplement).

Assessment of isocyanate exposure during training

Hygiene surveys were conducted in five of the participating schools. The industrial hygienist responsible for one school was on leave of absence during the hygiene survey period; no arrangements could be made to set up the survey in the seventh which was the smallest school with . Personal breathing zone (PBZ) air sampling (n=51) was performed during regular and specific activities such as mixing, spraying with High Volume Low Pressure guns, cleaning spray guns, and area air sampling (n=41) in the spray cabins and the workplace background using standardized sampling techniques for periods varying from 5 to 120 minutes. Diisocyanate (monomers and oligomers of HDI) levels were measured with high pressure liquid chromatography (HPLC) in the accredited laboratories of the *Institut de recherche Robert-Sauvé en santé et en sécurité du travail* (IRSST)(17).

Statistical analyses

Threshold values were determined for high levels of specific IgE, IgG and IgG4 (97th percentile of the distribution). New work-related symptoms, lower respiratory (hereafter referred to as respiratory) or nasal and/or ocular (rhinoconjunctivitis) symptoms, were defined as at least one respiratory or rhinoconjunctivitis symptom occurring at work and improving off work (weekends, holidays) as reported at the end-of-apprenticeship assessment.

Student's *t* or Mann-Whitney tests were used to study the relationship between specific antibody values and incident work-related symptoms. Chi-squared analyses were performed to determine the association between dichotomized variables (high specific antibodies, host characteristics and incident work-related symptoms). Linear regression models were used to determine the association between mean changes in specific antibody levels with the duration of exposure and other potential risk factors: atopy and BHR at baseline.

Quantile regressions were performed instead of traditional least squares because it was hypothesized that in a population of non-diseased subjects, as in this study, exposure would affect only a minority of susceptible subjects in a dose-response manner. In the least squares model, the majority of the observations presenting no relationship will only add noise to the data. By focusing the analysis in the highest percentiles of the population, it was intended to isolate the susceptible apprentices and estimate the relationship without the noise contributed by the majority of non-reactive subjects. Therefore, instead of using traditional linear regression models, quantile (percentile) regression models were used (see online supplement) to study changes in specific antibody levels according to independent variables – duration of exposure, atopy and BHR at baseline – specifically in the most reactive individuals following exposure to HDI, (i.e., in those with increases in specific antibody levels above the last percentiles (93rd, 95th and 97th) (18)(19). In this analysis, only linear model were used in the absence of firm scientific reasons to postulate a given non-linear model. Thus, it is possible that for the extremes of the range of the independent variable the quantile regression lines are not in their proper ordering since each p-level is estimated independently.

Multivariate logistic regression analyses were carried out to assess the effect of changes in antibody levels on the incidence of work-related symptoms taking into account other potential risk factors such as duration of exposure, baseline host characteristics (atopy, BHR, non-work-related rhinoconjunctivitis symptoms, non-work-related lower respiratory symptoms, physician-

diagnosed asthma) and smoking) irrespective of their level of significance in the univariate analysis. Changes in levels of specific antibodies were determined in regards to duration of exposure within the overall populations of apprentices in car-painting that was otherwise quite homogeneous in terms of demographic and exposure characteristics; no external comparison group was thus studied.

Analyses were performed using SPSS for Windows (version 11.0, Chicago, Il, USA) and R (R Development Core team, Vienna, Austria R development(19) software packages. A two-sided p value ≤ 0.05 was considered statistically significant.

Results

We successfully recruited 422 apprentices (86.8%) among a total of 486 contacted at the information session. The number of apprentices registered in each school varied according to its size; we enrolled proportionally 8, 18, 51, 64, 67, 71 and 143 subjects in the respective schools. Complete data were available for 385 subjects. The group of 298 out of 385 (78.5%) apprentice car-painters who remained in the study (varying between 63.1% to 87.9% between schools) consisted mainly of young men (91%) with a mean age of less than 25 years (Table 1), and slightly more than 50% of them were current smokers.

Table 1. Baseline characteristics of apprentices recruited in the cohort

	Participants at follow-up (n=298, 78.5%)	Subjects lost to follow-up (n=87, 21.5%)
Sex n, F/ M	28/270	10/77
Age, mean ($\pm$ SD)	23.49 ($\pm$ 7.7)	22.80 ($\pm$ 7.1)
Smoking, (S + Ex-S vs NS), n (%)	157 (52.7)	54 (62.1)
Atopy †, n (%)	104 (34.9)	26 (30.0)
Asthma diagnosed by a physician, n (%)	56 (18.8)	16 (18.4)
Probable asthma ‡, n (%)	22 (7.4)	8 (10.9)
Past exposure §, n (%)	14 (4.6)	2 (1.7)
Non-work-related respiratory symptoms, n (%)	49 (16.4)	18 (20.5)
Non-work-related rhinoconjunctivitis symptoms, n (%)	101 (33.9)	33 (38.3)
PC20 $\leq$ 16 mg/ml, n (%)	57 (19.1)	22 (25.2)
FEV1 $\leq$ 80% predicted, n (%)	20 (6.7)	6 (6.9)
School		
A	11 (3.7)	5
B	102 (34.2)	29
C	41 (13.8)	24
D	53 (17.8)	8
E	51 (17.1)	7
F	5 (1.7)	2
G	35 (11.7)	12

Legend: S: smoker; Ex-S: ex-smoker; NS: non-smoker; †: Atopy: a positive reaction to at least two skin-prick tests from a battery of 11 common aeroallergens;

‡: Probable asthma: $\geq$ 2 symptoms suggestive of asthma and a PC20 $\leq$ 16 mg/ml;

§: Past exposure = contact $\leq$ 3 months with isocyanates and without contact in the last 2 months (not meeting the exclusion criteria).

Close to 20% reported having received a diagnosis of asthma, while less than 10% had probable asthma according to the epidemiological definition where both at least two respiratory symptoms suggestive of asthma and a PC20 $\leq$ 16 mg/ml are requested criteria(20). One fifth of these apprentices demonstrated BHR (PC20 $\leq$ 16 mg/ml) at baseline. A minority of participants (<5%) reported a past exposure to isocyanates of less than three months. Table 1 presents the baseline characteristics of both the subjects who participated in the follow-up at the end of their apprenticeship (maximum duration of 19 months, median 9 months; duration of effective exposure to HDI: maximum: 7 months, median: 3 months, duration <1 considered as 0), and those lost to follow-up. The majority of those in the latter group had dropped out from the training program (72.3 %), 23.5% were absent at the time of the reassessment visit, 3.4% declined to participate and one was deceased. Although higher proportions of subjects lost to

follow-up reported non-work-related respiratory and rhinoconjunctivitis symptoms, had a lower PC20, and were smokers compared to those assessed at follow-up, the two groups did not differ significantly at baseline.

Specific antibody levels at the beginning and at the end of the training period and associated baseline host factors

Detectable levels of specific IgG, IgG4, and IgE antibodies at baseline and at the end of the apprenticeship were found at both visits. Threshold values (97th percentile) for IgE level at visit 1 and visit 2 were 0.80 and 0.94 (% binding), respectively; those for IgG level were 0.40 and 0.41 (OD), and those for IgG4 level: 1.04 and 0.94 (OD) at visit 1 and visit 2, respectively. For the increase in antibody levels the cut-off values at the 97th percentile were 0.34 % binding for delta IgE, 0.25 OD for delta IgG and 0.29 for delta IgG4. Specific IgE antibody levels were associated with atopy; specific IgE levels being higher in atopic subjects both at the first visit ($p<0.001$) and the second visit ($p<0.001$) (in Mann-Whitney tests) (results not shown).

Development of work-related symptoms and associated baseline host factors

Thirteen subjects developed work-related respiratory symptoms while nineteen developed work-related symptoms of rhinoconjunctivitis. Only one subject developed both rhinoconjunctivitis and respiratory symptoms in the workplace. For the 13 subjects who developed work-related respiratory symptoms, Table 2 presents the associations with baseline host factors.

Table 2. Presence of baseline host characteristics and their association with incident work-related lower respiratory symptoms and work-related rhinoconjunctivitis symptoms.

Factor n (%)	Study cohort at inception	Subjects with incident work-related lower respiratory symptoms	$P\ddagger$ value	Subjects with incident work-related symptoms of rhinoconjunctivitis	$P\ddagger$ value
N	298	13		19	
Atopy	104 (34.9)	6 (46.2)	0.38	8 (42.1)	0.49
Physician-diagnosed asthma	56 (18.8)	6 (46.2)	0.01	2 (10.5)	0.34
BHR	57 (19.2)	6 (46.2)	0.01	2 (10.5)	0.37
PC20 $\leq$ 16 mg/ml†					
Non-work-related respiratory symptoms	49 (16.4)	4 (30.8)	0.15	1 (5.3)	0.17
Non-work-related symptoms of RC	101 (33.9)	7 (53.9)	0.12	4 (21.1)	0.22

Legend: †: n=297 for methacholine bronchial challenge test; ‡: p value in chi-squared analysis comparing cases (with incident symptoms) with referents within the cohort; RC: rhinoconjunctivitis.

Reporting a diagnosis of asthma and a positive methacholine challenge test (PC20 $\leq$ 16 mg/ml) were significantly associated with the incidence of work-related respiratory symptoms. The development of work-related rhinoconjunctivitis symptoms was not associated with any of the same host factors assessed at the beginning of the study (Table 2). Ten of the thirteen (76.9%) subjects with work-related respiratory symptoms presented baseline characteristics suggestive of respiratory status impairment (physician-diagnosed asthma, BHR and/or non-work related symptoms) whereas only six of the 19 (31.6%) subjects with work-related rhinoconjunctivitis did (results not shown).

Airborne HDI concentrations in the training workshops

During spray painting, measurable levels of HDI monomer in PBZ were systematically found in four schools out of five, but the levels were all low (0.0006 to 0.006 mg/m³) compared to the permissible level in the Province of Quebec (threshold limit value-time weighted average: TLV-TWA: 0.034 mg/m³) (Table 3).

Table 3. HDI monomer and oligomer concentrations by task in five vocational schools

Sampling site	HDI type	No. of personal samples	Concentration (ug/m ³)		LOD(%)
			median	maximum	
Spraying	monomer	29	0.001	0.006	41.4
	oligomer	29	0.283	0.916	27.9
Mixing	monomer	13	0.0003	0.0003	92.3
	oligomer	13	0.4365	0.6890	69.2
Brush cleaning	monomer	5	-	-	100
	oligomer	5	0.079	0.079	80

LOD(%): percentage of samples below the detection limit

However measured levels of HDI oligomers were high, ranging from 0.033 to 0.916 mg/m³ for PBZ, when referring to the standard values for monomers in the absence of standard values for HDI oligomers. Levels were lower in other activities, i.e. mixing and cleaning spray guns. Airborne HDI concentrations from area air sampling were lower than from PBZ. When spraying, students were wearing masks but not always those recommended; also, the masks were often removed inappropriately for inspecting the work. The quantitative results and work practices were similar between schools.

Relationship between specific antibody levels and symptoms developed in the workplace

Specific IgG antibody levels at the first and second visits were not associated with neither work-related respiratory symptoms nor with symptoms of rhinoconjunctivitis, as shown in Table 4.

Table 4. Association between incident work-related symptoms and specific antibody levels at baseline, and changes (delta) in levels of antibodies between visits

		Lower respiratory symptoms			Rhinoconjunctivitis symptoms		
		Yes	No	P value	Yes	No	p value
No. of subjects		13	285		19	279	
<i>Levels of antibody at baseline (Visit 1)†</i>							
IgG	Median	0 (173)	0 (148)	0.19	0 (156)	0 (149)	0.61
IgG4	Median	0.149 (163)	0.069 (149)	0.55	0.126 (187)	0.069 (147)	0.04
IgE	Median	0.30 (179)	0.249 (148)	0.2	0.23 (151)	0.24 (149)	0.9
<i>Changes in levels of antibodies between visits‡</i>							
Delta IgG	Mean ± SD	-0.064±0.12	0.013±0.123	0.02	0.002±0.126	0.01±0.124	0.77
Delta IgG4	Mean ± SD	-0.003±0.106	-0.001±0.174	0.8	-0.108±0.2	-0.008±0.168	0.01
Delta IgE	Mean ± SD	0.120±0.536	-0.001±0.143	0.41	-0.03±0.066	0.006±0.184	0.38

Legend: †: comparisons with Mann-Whitney test; ‡: comparisons with Student *t* test.

Levels of specific IgG4 detected at the first visit were found to be associated with work-related rhinoconjunctivitis symptoms, the group with higher levels of IgG4 antibodies at the first visit being more likely to develop rhinoconjunctivitis symptoms during the apprenticeship. There was no association between these symptoms and the levels of specific IgG4 antibodies detected at the second visit (results not shown). Levels of specific IgG4 antibodies were not associated with work-related respiratory symptoms developing during the apprenticeship. No significant difference in median levels of specific IgE antibodies detected either at the first or second visit was seen between subjects with incident work-related symptoms and those without.

Variation in levels of specific antibodies in the course of the apprenticeship

1. Linear regression analysis

Linear regression analyses did not show any significant relationship between changes in levels of the specific antibodies and the duration of exposure to isocyanates after adjusting for atopy and BHR at baseline.

2. Percentile regression analyses

The percentile regression analyses presented below explore the relationship between the high increases in specific antibody levels with duration of exposure adjusting for atopy and BHR a baseline.

A. IgG. The percentile regression model for the variations in IgG level revealed ascending slopes as a function of the duration of exposure at the 95th percentile ($B=0.025$, 95%CI: -0.017 - 0.069), and was significant ($B=0.035$, 95%CI: 0.012 - 0.056) at the 97th percentile of the distribution of the detected level of specific IgG antibodies (Figure 1). Nine subjects increased their IgG levels above the cut-off of the 97th percentile (value of 0.25 OD). No other variable was associated with the increase of high levels of IgG antibodies.

B. IgG4. There was no increase in the highest percentiles of specific IgG4 levels neither with time, nor with the other parameters.

C. IgE. Percentile regressions for the variations in levels of specific IgE antibodies revealed ascending slopes as a function of the length of exposure and were significant at the 95th percentile ($B=0.03$, 95% CI 0.004 - 0.047), and the 97th percentile ($B=0.04$, 95% CI 0.003 - 0.056). Nine subjects increased their IgE levels above the cut-off of the 97th percentile (value of 0.34 OD). An association was also shown between changes in levels of specific IgE antibodies and atopy status at the 97th percentile ($B=0.32$, 95% IC: 0.190 - 0.611) and the 95th percentile ($B=0.233$, 95% IC: 0.018 - 0.429).

Variations in levels of specific antibodies and their association with symptoms in the workplace

Univariate analyses

The increase in levels of IgG antibodies was lower in subjects who developed work-related respiratory symptoms (Table 4). The variations in levels of specific IgG in the group without work-related respiratory symptoms compared to the group with such complaints indicated an increase in the levels of specific IgG antibodies between the two visits. The increase in levels of IgG4 antibodies was lower in subjects who developed rhinoconjunctivitis symptoms in the

workplace (Table 4). Higher levels of specific IgG4 antibodies were detected in subjects who remained asymptomatic. The variation in levels of specific IgE antibodies was not associated with new symptoms in the workplace. Anecdotally, two out of the 13 subjects who developed work-related respiratory symptoms also had a significant increase in bronchial responsiveness (i.e., a 3.2-fold decrease in PC20 from the first to the second visit), thus considered as having probable OA(21). One of these two subjects demonstrated a small increase in IgG levels (84th percentile of the distribution), the other a decrease (-0.37) next to the maximum decrease measured in this population of apprentices (-0.39).

Multivariate regression analysis

The effect of variation in levels of specific antibodies (delta IgG, delta IgG4 and delta IgE) on the development of work-related symptoms was estimated through multivariate logistic regression analyses to take into account other potential risk factors (duration of exposure, baseline host characteristics and smoking). Table 5 shows that new work-related respiratory symptoms were determined by delta IgG (a significant negative association) after adjusting for the other covariates.

Table 5. Effect of changes in antibody levels on the incidence of work-related symptoms taking into account duration of exposure, host characteristics and smoking.

	OR	95% CI
Determinants of work-related lower respiratory symptoms† (n=13)		
Delta IgG‡	0.001	0.000-0.09
Determinants of work-related rhinoconjunctivitis symptoms† (n=19)		
Delta IgG4‡	0.09	0.01-0.55
Smoking	0.34	0.13-0.90

Legend: †: multivariate logistic regression of delta IgE, delta IgG, delta IgG4, adjusting for duration of exposure, baseline host characteristics (atopy, BHR, non-work-related rhinoconjunctivitis symptoms, non-work-related lower respiratory symptoms, physician-diagnosed asthma and smoking); OR: odds ratio; 95% CI: 95% confidence intervals. ‡ : the OR values represent the change of risk by unit of change in antibody levels (expressed in OD).

The negative association between new work-related symptoms of rhinoconjunctivitis and delta IgG4 remained significant after adjusting for the same factors. A negative association, though of borderline significance, was found between smoking and new work-related rhinoconjunctivitis symptoms. No interactions were seen between covariates in both these models.

Discussion

Specific antibodies, IgG, IgG4 and IgE to HDI were detected in subjects entering a training program in car-painting without any known prior exposure to diisocyanates. The presence of measurable levels of specific antibodies to HDI and even of high titers of specific antibodies can be explained by a high reactivity to exposure to isocyanates either in the general environment or to accidental exposure(s) occurring prior to the commencement of the car-painting program. It is not excluded that some apprentices may have performed isolated spray-painting activities before entering a formal training program. Also, cross-reactivity with antibodies developed subsequent to exposures to substances having a structure close to that of HDI is a possibility. Immunologic cross-reactivity has been described earlier between different albumin-bound isocyanates(22), for TDI(6) and between monomeric and polymeric diphenylmethane diisocyanate conjugates(23). The significant association between specific IgE antibodies and atopy at the beginning of the study denotes a predisposition in atopic subjects to develop specific antibodies following exposure to isocyanates, which is surprising since isocyanate-induced OA is not associated with atopy(5).

No longitudinal study has prospectively assessed the changes in specific HDI antibody levels from the first exposure to diisocyanate in a healthy population and there is, as yet, no definition for antibody values considered as positive in that context. In our inception cohort, the duration of exposure was short. This can explain the differences observed in specific antibody levels between the first and second visits that were not significant. However, the maximum values, the 95th and the 97th percentile values of the IgG and IgE distributions are higher at the second visit compared to the first, which indicates a modification limited to the highest values (a more reactive subpopulation of individuals) subsequent to the exposure.

Respiratory symptoms in the workplace are significantly associated with host factors present at the beginning of the study – i.e., asthma diagnosis and/or bronchial HBR – and to a lesser extent with the duration of exposure. The first two parameters (diagnosed non-occupational asthma and BHR) reflect the presence, even prior to the beginning of exposure, of a deteriorated respiratory status. The development of work-related rhinoconjunctivitis symptoms, weakly associated with baseline personal risk factors (Table 2), appears to be mostly attributable to exposure to HDI in the school (Table 5). Therefore, at this initial stage of exposure to HDI, we observe two groups of subjects: those who develop rhinoconjunctivitis symptoms mostly attributable to exposure and not to baseline host factors (Table 2), and those who develop work-related respiratory symptoms mainly related to a pre-exposure personal history of asthma and BHR. Rhinoconjunctivitis symptoms seem to be the first functional manifestation related to the duration of exposure. Occupational rhinitis can precede OA, more so for proteinaceous agents(24) and is a strong determinant for the development of asthma(25). In the current study, a group of apprentices was identified with incident work-related respiratory symptoms without any prior evidence of work-related rhinoconjunctivitis symptoms.

In those who developed rhinoconjunctivitis symptoms, pre-exposure levels of IgG4 were significantly higher. A similar finding was observed in laboratory animal workers exposed to rat urinary allergens(26).

The percentile regressions show that the highest changes in IgG antibody level are positively associated with the duration of exposure revealing the existence of a more reactive subpopulation (3%). The highest changes in IgE antibody level were also positively associated

with the duration of exposure identifying a more prone subpopulation (5%). The increase in levels of specific IgE antibodies also shows an association with atopy, which is an unexpected finding in the case of diisocyanate(5).

The increase in specific IgG levels was lower in the group reporting work-related respiratory symptoms compared to those without such symptoms, which suggests a protective effect of specific IgG. The incidence of work-related rhinoconjunctivitis symptoms was associated with a lower reactivity of specific IgG4, also suggesting a protective role of this class of antibodies(27)(16). Smoking was associated with a lower incidence of work-related rhinoconjunctivitis symptoms, although only with borderline significance ($p=0.07$). This may suggest that smoking exerts a real protective effect or that smokers tend to underreport their symptoms(28).

In the initial months of exposure, the changes in levels of specific IgG4 antibodies appear to have a protective effect against the development of rhinoconjunctivitis symptoms, whereas the changes in levels of the other specific antibodies (IgG and IgE) studied are not significant. Recent studies in immunotherapy have shown that the blocking antibody activity is found in the IgG4 fraction as opposed to the IgG4 depleted fraction of IgG(29). As work-related rhinoconjunctivitis symptoms were mostly reported in the absence of work-related respiratory symptoms and as nasal symptoms have been reported to appear first or at the same time as respiratory symptoms suggestive of OA, the association observed between variations in levels of specific IgG4 and rhinoconjunctivitis symptoms during this stage of exposure suggests that this class of specific antibodies would be one of the first detectable manifestations of the immunologic response to protect individuals exposed to HDI. On the other hand, the presence of larger levels of specific IgG4 antibodies in a number of subjects, probably due to personal factors and/or previous environmental exposure to HDI, seems to be a risk factor leading to the subsequent development of work-related rhinoconjunctivitis symptoms. This apparently contradictory phenomenon has already been observed in immunotherapy studies, which on the one hand have shown a bad prognostic relative to symptoms in cases of early predominance of specific IgG4(30) and on the other hand, a good correlation between the improvement of symptoms and an increase in specific IgG4(30)(31). The dynamics of this type of antibody response and the interactions with the other types of antibodies are still poorly known. Lee and colleagues(27) showed that laboratory workers with positive IgE had higher specific IgG4 titers when comparing asymptomatic to symptomatic subjects. Moreover, IgG4 values are higher for subjects who do not have measurable IgE, which suggests that specific IgG4 are also a factor blocking the increase in IgE antibodies. The cross-sectional study performed by Jeal and colleagues(16) shows larger values for the ratio of specific IgG4/IgE in asymptomatic compared to symptomatic subjects.

In conclusion, our study intended to identify early markers of immunological sensitization that can be related to the onset of symptoms or that can exert a protective effect. Small changes in antibody levels were documented in our study, which might have been expected in subjects exposed for a relatively short period (maximum of 19 months) and to low concentrations of isocyanates. However, we were able to show that increases in specific IgG may exert a protective effect on the development of work-related symptoms, which is relevant to public health interventions. Confirmation of the significant roles of specific IgG and personal characteristics

described in our study in the development of work-related respiratory symptoms is warranted in a longer follow-up study.

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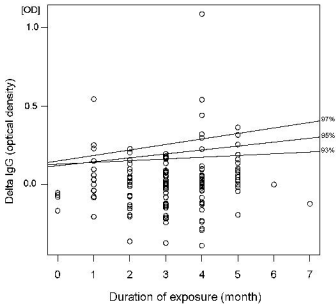
Figure 1. Variation of the estimated changes in levels of specific IgG antibodies (delta IgG) between the second and first visits at the 93rd, 95th and 97th percentiles of the distribution of delta IgG according to the duration of exposure (n=298).

Legend: The lines represent the estimated percentile regression lines at the 93rd, 95th and 97th percentiles of delta IgG as a function of time.

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Specific Antibodies to Diisocyanate and Work-related Respiratory Symptoms in Apprentice Car-Painters

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Web-only appendix

Methods

Immunological investigations

Determining the specificity of IgE anti-HDI:HSA RAST

Competitive RAST inhibition assays were conducted to determine the self inhibition of binding of IgE to HDI:HSA. The specificity of IgG anti-HDI:HSA ELISA was determined by ELISA inhibition. The inhibition was carried out by incubating equal volumes of serum and HDI:HSA conjugate (range of dilution from neat to 1:1000) overnight at room temperature and then 16 hr with the HDI:HSA coated RAST disc. The disc was washed and processed as above in the RAST assay. Inhibition of IgE binding to HDI:HSA coated RAST discs by prior incubation of serum samples with HDI:HSA conjugate demonstrated 76% inhibition for the neat with no inhibition for the 1:1,000 dilution.

Determining the specificity of IgG anti-HDI:HSA Enzyme Linked ImmunoSorbent Assay (ELISA)

The specificity of the IgG ELISA was determined by ELISA inhibition. Briefly equal volume of serum and a range of HDI:HSA dilution (neat to 1000) was incubated overnight at room temperature. The sera were added in the ELISA as above. Inhibition of IgG binding to HDI:HSA by prior incubation of serum samples demonstrated 60% inhibition for the neat with no inhibition for the 1:1,000 conjugate dilution. For each sample in the IgG and IgG₄ ELISA the background binding to HSA only was subtracted from the HDI:HSA conjugate, thus avoiding any non specific binding.

Statistical Analyses – Quantile regressions

A ‘p’ quantile is the value that divides a data set into two parts at the ‘p’ percentage point. This point can be easily identified after sorting the data. By contrast, in a regression context this is not so easily estimated. The least squares classical linear regression is defined as: “find b such that $\sum (y - x*b)^2$ is a minimum”. If we alternatively define a function R such that: $R(x) = p * x$ if $x > 0$ or $= (p-1) * x$ if $x < 0$, then the p-quantile regression is defined as: “Find b such that $\sum (R(y - x * b))$ is a minimum”. This problem can be solved by the simplex method of linear programming.

Thus, all the observations are taken into account to estimate the quantile regression line. We used the statistical program R with the package ‘quantreg’ with the methods ‘br’ i.e. the default Barrowdale-Roberts l-p simplex algorithm to estimate our models(1, 2).

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