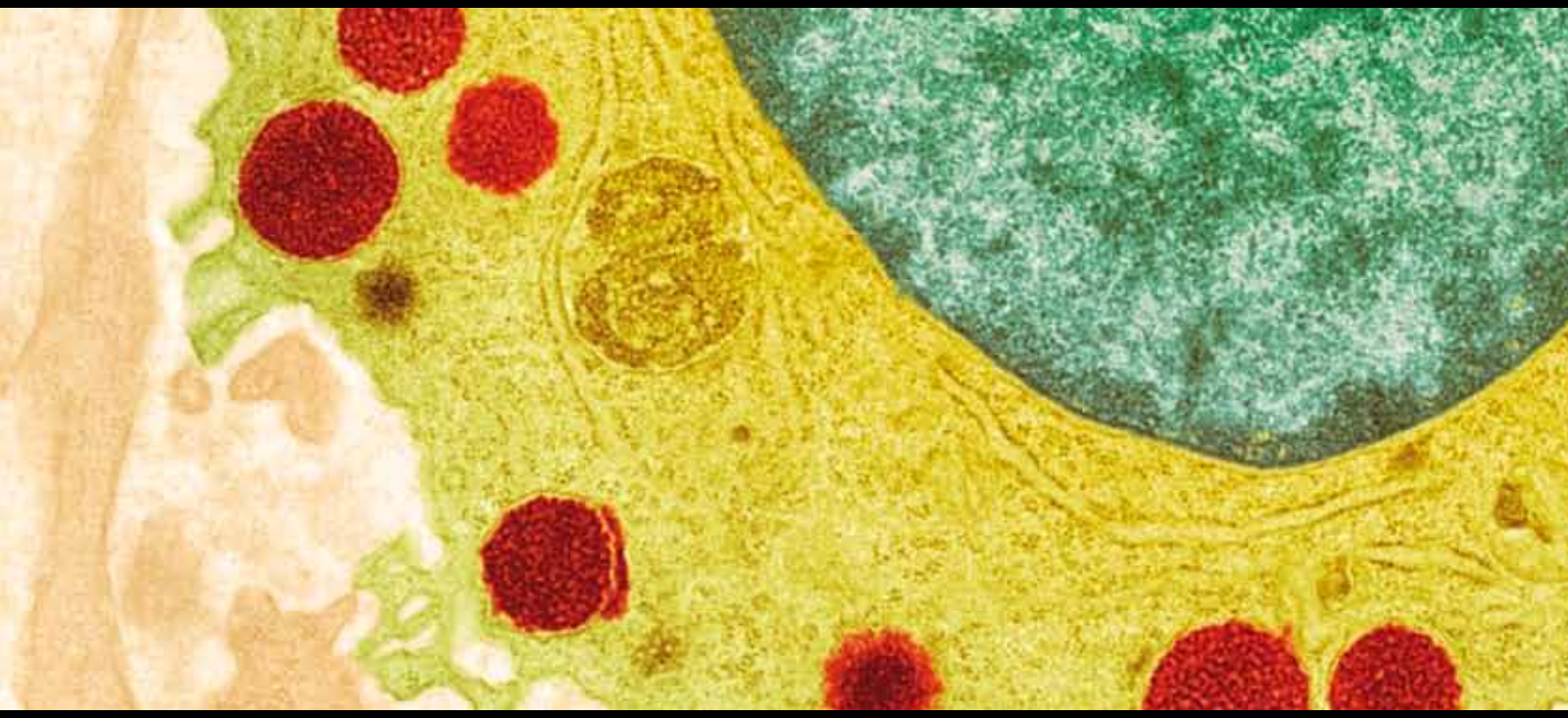
Journal of Allergy

Occupational Allergies

Guest Editors: Gordon L. Sussman, Donald H. Beezhold, and Arthur Sussman



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Editorial Board

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Editorial
Occupational Allergies

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Occupational immune diseases are new emerging illnesses that affect workers in industrialized societies. Occupational exposures to substances in the workplace environment can cause inflammation, allergy, or other potentially detrimental immune responses. Personal exposure to a variety of chemicals can exacerbate immune diseases such as contact dermatitis as well as respiratory diseases including rhinitis, asthma, and hypersensitivity pneumonitis.

Next to illnesses due to repeated traumatic injury, contact dermatitis is the second most commonly reported occupational illness. It can prevent individuals from performing job-related tasks or preclude working altogether. Occupationally related contact dermatitis is a significant public health burden with combined direct annual cost estimates of up to $1 billion in the USA for medical costs, workers compensation, and lost time from work.

Respiratory morbidity is also a significant burden to public health leading to lost productivity. Prevalence rates for occupational rhinitis are significant, varying by occupation between 5% and 65% and costing an estimated $593/year/employee due to productivity losses. Conservative estimates made by the American Thoracic Society in 2003 estimate that 15% of chronic obstructive pulmonary disease and asthma cases were work related and cost approximately $7 billion in lost productivity in the USA With the changing work environment, new occupational hazards continue to emerge which require immunologic characterization. In order to reduce the morbidity and mortality associated with these illnesses, it is critical that we identify the allergens and understand the immunological mechanism by which they exacerbate immune-mediated respiratory and dermal diseases. Specific understanding of mechanism has direct implications in developing appropriate intervention and prevention strategies.

Occupational allergy can be stratified into high-molecular-weight-allergen and low-molecular-weight-allergen mediated responses. Different immunologic mechanisms mediate allergic reactivity to these occupational allergens as highlighted in this issue by Talini et al. High-molecular-weight (HMW) allergens (typically proteins) induce type I hypersensitivity responses or typical allergies by inducing IgE antibodies which lead to a continuum of symptoms including rhinitis (rhinosinusitis, conjunctivitis), hives, asthma, and life-threatening anaphylaxis. Patients with HMW-allergen-induced asthma show a greater frequency and severity of the early-phase response but are less likely to demonstrate a late-phase response. Occupational outbreaks of reactions to HMW allergens can occur episodically and can be severe and life altering for those affected. These allergies can affect large numbers of easily identified workers in specific industries which can reach epidemic proportions such as latex allergy and Baker's asthma. It can present in a less-well-defined population or as local occurrences such as agricultural or food processors exposed to soy, sea foods, pollens, molds, and so forth. Research areas include identification and characterization of high-molecular-weight occupational allergens. Using fungal enzymes as a prototypic HMW occupational allergen, Green et al. describe some of the characterized fungal enzyme allergens and discuss monitoring and avoidance strategies. Characterization of HMW allergens includes using proteomics, molecular techniques and generating recombinant allergens, and producing monoclonal antibodies for the development of immunoassays and improved detection of the allergens in the workplace.

Low-molecular-weight allergens (typically chemicals) induce type 4 hypersensitivity reactions by inducing allergen-specific T lymphocytes which can mediate contact dermatitis reactions as well as sensitizations that can lead to severe asthma such as isocyanates (auto painters) and trimellitic acid. Patients with LMW-allergen-induced asthma are more likely to demonstrate a late-phase airway response. The review by Anderson et al. describes the identification of low-molecular-weight allergens in the laboratory using the local lymph node assay to determine whether new chemicals being introduced can cause workplace sensitizations as well as testing various components to identify the specific sensitizer and potentially nonsensitizing replacements. They examined the effects of chemical exposure on immune function using selected assays from a comprehensive tiered approach. This can be used in detecting toxic effects following chemical exposure (in rodents) as adopted by the National Toxicology Program. The utility of analyzing potential replacement chemicals is highlighted by the study of Johnson et al. where the chemical *ortho*-phthalaldehyde (OPA) has been recommended as a substitute for glutaraldehyde as a sterilant in the healthcare industry. Their laboratory evidence suggested that the replacement of the chemical OPA is also a strong sensitizer. Characterization of the biochemical and immune mechanisms by which chemicals become allergens (haptenization) is described in a comprehensive review by Chipinda et al. Developing new methods for screening chemicals for potential sensitizers helps to build better models by which we predict whether chemicals are allergens. Yucesoy et al. describe new studies aimed at identifying occupationally sensitized individuals and understanding the genetic profile associated with sensitizing/anaphylactic agents.

It is important to improve our basic science knowledge and understanding of occupational allergies and their pathogenesis. If we are able to identify potential allergens, before clinical symptoms are observed, employers can take necessary precautions to minimize or eliminate their employee's exposure.

Acknowledgments

We dedicate this issue to one of our coeditors: Dr. Arthur Sussman, who passed away April 6 2011, just prior to our special issues' publication. Arthur Sussman was a pioneer in the field of allergy and immunology and witnessed its emergence first hand during his sixty years of medical practice. He contributed to this issue of the journal and it is fitting that this issue would highlight emerging issues in allergy and Immunology—occupational diseases. He will be sincerely missed.

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Gordon L. Sussman

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Clinical Study

Comparison between Airway Responses to High versus Low Molecular Weight Compounds in Occupational Asthma

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Occupational asthma (OA) is a heterogeneous disease, and the characteristics of the sensitizer responsible for OA may induce different clinical, functional, and biological manifestations. We examined the characteristics of 74 patients with OA induced by low molecular weight compounds (LMWC) or by high molecular weight compounds (HMWC) and diagnosed by specific inhalation challenge (SIC). Patients with OA induced by LMWC had a longer occupational exposure before the beginning of symptoms, a lower sputum eosinophilia, and a higher prevalence of late airway response (LAR), in comparison with patients with OA induced by HMWC. Pulmonary function tended to be poorer and atopy tended to be less frequent in LMWC-induced OA than in HMWC-induced OA. These data confirm and extend previous observations showing that the characteristics of the specific sensitizer inducing OA may determine different clinical, functional, and biological features, probably related to the difference pathogenetic mechanisms underlying these different types of OA.

1. Introduction

Occupational asthma with latency period can be induced by sensitization to either a specific allergen (high molecular weight compounds, HMWC) or chemical compounds (low molecular weight compounds, LMWC) present in the workplace [1]. The gold standard for the diagnosis of OA is represented by the Specific Inhalation Challenge (SIC) which is intended to demonstrate a direct relationship between exposure to a specific agent present in the workplace and an asthmatic response [2]. Few studies have analysed the variable patterns of response to HMWC and LMWC (early, dual, or late response) in limited numbers of patients, but how worker's characteristics may influence the pattern of response to the sensitising agents remains to be explored. Recent studies [3] have shown that there are significant differences in the type of airway changes induced by low and high molecular weight agents.

Sputum eosinophilia has been reported in a variable percentage of patients affected by occupational asthma, and some studies suggested that patients with asthma induced by LMWC may have a lower sputum eosinophil percentage than patients with asthma due to HMWC [4, 5]. Sputum eosinophils increase further after exposure to both HMWC and LMWC, showing the increase in allergic airway inflammation induced by these specific sensitizers [6]. Factors that influence the type of inflammatory responses are unclear but may include also the type of asthmatic reaction and the intensity of airway inflammation. In particular, it is not known if the type and/or the severity of airway inflammation may contribute to the determination of the pattern of airway response to the specific sensitizer.

We compared the clinical characteristics, the airway inflammatory pattern, and the model of specific airway response in patients with OA induced by HMWC or LMWC. The aim was to assess, in this specific model of asthma, whether the characteristics of the sensitizer and the different pathophysiologic mechanisms may be associated with a different asthma phenotype.

2. Materials and Methods

We studied 74 subjects with occupational asthma due to different sensitizers (diisocyanates, latex, hairdresser's products, wood, and flour dusts) observed consecutively in our asthma clinic: 48 were exposed to LMWC (isocyanates, persulfate salts, aziridine, and phenolic resins) agents, and 26 were exposed to HMWC (flour dusts, wood dusts, latex, detergents, and tobacco dusts). We selected only subjects in whom the diagnosis of occupational asthma had been performed by means of positive response to specific inhalation challenge (SIC). According to the international recommendations [2, 7], patients were all exposed to a known occupational sensitizer (Table 1) showed asthma deterioration at work and nonspecific bronchial hyperresponsiveness during a working period.

Bronchial hyperresponsiveness was determined by methacholine challenge test performed as previously reported [8]; a provocative dose of a 20% decrease in FEV1 from baseline (PD20FEV1) of less than 1000 mcg was considered as positive for bronchial hyperresponsiveness.

SIC was performed using different methods (Table 1): (a) for diisocyanates, subjects were exposed to vapours generated by blowing air on the surface of a small amount of toluenediisocyanate (TDI) or warming a small amount of methylenediphenyl diisocyanate (MDI) at 40°C, in a challenge chamber and monitoring isocyanate concentrations with a specific TDI/MDI detector (MDA model 7005 isocyanate detection equipment, MDA Scientific Inc., Glenview, IL); diluent was used as control exposure; the duration of the exposure was 30 min in a first test and 120 min in a second test (if the first resulted negative) [9]; (b) for dusts (flour, wood, persulfate, latex, and tobacco), subjects inhaled dusts by a mouthpiece connected to a small box where a suspension of the dust was obtained by blowing compressed air at 5 L/min through a bottle containing the dust; *lactose* powder was used as control test; the concentration of the dusts was measured by blowing air from the box through a cellulose nitrate filter of 0.8 μm porosity by means of a vacuum pump [10]; (c) in two cases (one exposed to phenolic resins and the other to detergents), a realistic way was employed in order to simulate in laboratory the exposure of the workplace (spreading the substance on a small surface); diluent was used as control test, and the duration of exposure was still 30 minutes. In all SIC, FEV1 was measured immediately before and 5, 15, 30, and 60 minutes after the exposure to the sensitizer, then hourly for 8 hours. A positive response was defined as a decrease in FEV1 from baseline of more than 15% during the first hour (immediate response) or between the second and the 8th hour (late response), and in absence of a more than 10% decrease in FEV1 during a control test performed in a different day with diluent (for diisocyanates or other simple chemicals) or with lactose dust (for other dust sensitizers).

One or two weeks before challenge, other measurements at diagnosis included skin prick tests to common allergens (to check for atopy), and collection of sputum induced by the inhalation of saline solution. The method for induction and processing has been previously described [11]. Total and differential counts of inflammatory cells (eosinophils, macrophages, neutrophils, and lymphocytes) were considered; we chose 2% as the upper limit of normal range for sputum eosinophils [12].

All patients gave their informed consent to the management of their personal data.

Characteristics of subjects (age, sex, smoking habit, atopy, duration of symptoms and exposure, latency, type of response, sputum eosinophilia, and functional data) were compared between two groups with asthma induced by HMWC or LMWC.

Descriptive analysis for data collected at diagnosis was performed, with data expressed as mean (±standard deviation, SD) or median (range) for normally and nonnormally distributed data, respectively. PD20FEV1 methacholine was

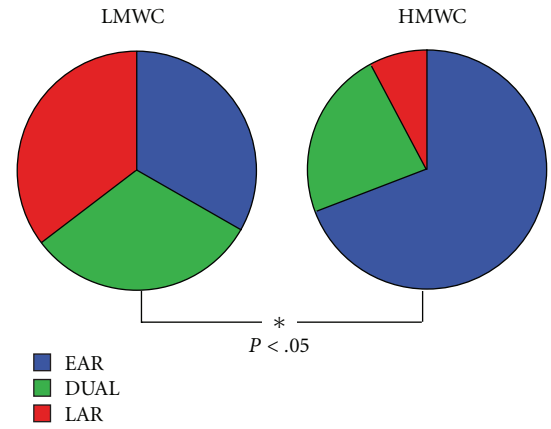

Figure 1: Type of response to SIC in subjects sensitized to high and low molecular weight agents. (EAR: early response, LAR: late response).

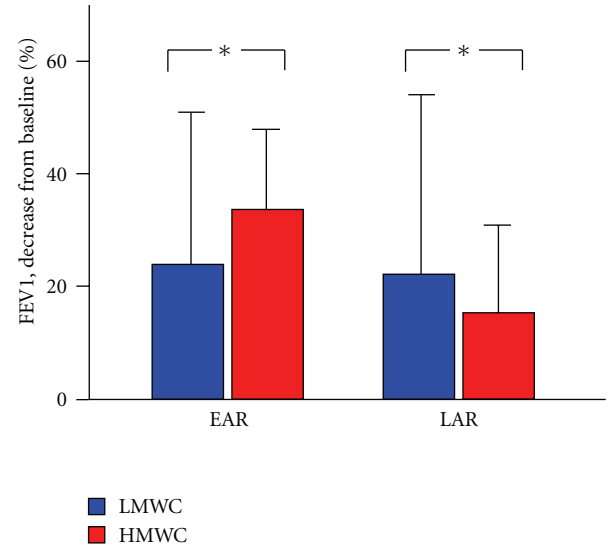

Figure 2: Magnitude of the early (EAR) and late (LAR) responses (expressed as percent decrease in FEV1 from baseline) during SIC to low (LMWC) or high (HMWC) molecular weight compounds, $^*P < .05$.

Table 1: Characteristics of the compounds used for performing specific inhalation tests (SIC), concentrations used during SIC and duration of the exposure.

Agent	Control	Number of subjects	Challenge concentration	Time exposure
TDI vapours	Diluent	37	0.002–0.003 ppm	30–120′
Flour dust	Lactose dust	20	0.3–0.5 mg/m^3	30′
Wood dusts	Lactose dust	3	0.3–0.5 mg/m^3	30′
Persulfate salts	Lactose dust	6	0.05–0.1 mg/m^3	30′
Aziridine	Lactose dust	2	0.03–0.05 mg/m^3	30′
Latex solution	Normal saline	3	<0.0001 mg/m^3	30′
Tobacco dusts	Lactose dust	1	0.3–0.5 mg/m^3	30′
Phenolic resins and Detergents vapours	Diluent	2	Not measured*	30′

* Subjects simulated the job activity in laboratory.

Table 2: Characteristics of the patients examined, according to the type of the occupational sensitizer.

	LMWC	HMWC
Patients	48	26
Age, years	43.8 ± 12.0	38.9 ± 10.8
Gender,		
Male	34 (70.8)	20 (76.9)
Female	14 (29.2)	6 (23.0)
Atopy	12 (25)	12 (46.1)
Smoking habit		
Nonsmokers, *N* (%)	23 (47.9)	14 (53.8)
Smokers, *N* (%)	4 (8.3)	2 (7.7)
Ex-smokers, *N* (%)	21 (43.8)	10 (38.4)
Therapy *N*/*Y*	29/19	14/12
Duration of symptoms, yrs	6.1 ± 6.9	6.4 ± 5.7
Duration of exposure, yrs	20.1 ± 13.1	15.2 ± 7.7*
Latency period, yrs	13.9 ± 12.7	8.7 ± 5.7*
Baseline FEV1		
L	3.01 ± 0.67	3.39 ± 0.68*
% pred	89.2 ± 16.0	93.5 ± 14.8
Baseline PD20FEV1 (mg)	0.22 (0.01–5.00)	0.18 (0.02–5.00)
Sputum eosinophilia		
Eosinophils, %	0.95 (0–32)	6.8 (0–43.1)*

Data are presented as *n* (%), M ± SD or GM (range; PD20FEV1) or median (range; eosinophils, %).
* $P < .05$.
LMWC: low molecular weight compound; HMWC: high molecular weight compound.

reported as geometric mean and log-transformed for statistical analysis. Comparison among groups was performed by appropriate parametric (analysis of variance, Chi-square test, and unpaired *t*-test) and nonparametric tests (Mann-Whitney *U* test). A *P* value lower than 5% was considered as significant, and a *P* value between 0.1 and .05 was considered as expression of a trend.

3. Results

Patients' characteristics are summarized in Table 2. These characteristics were similar in both groups with asthma induced by HMWC and LMWC, except for duration of exposure, latency period, and sputum eosinophilia. Duration of exposure and latency periods were higher in subjects with asthma due to LMWC, who had also a lower sputum eosinophilia. FEV1 was lower in absolute value, but not in percentage of predicted, in patients with LMWC- than in patients with HMWC-induced asthma. Atopy was more frequently observed in patients with HMWC- than in patients with LMWC-induced asthma, but the difference was not statistically significant. Atopic subjects had a higher FEV1 ($P = .02$) and a higher percentage of eosinophils ($P = .005$).

The comparison among groups of subjects with asthma due to different sensitizers was strongly affected by the low number of subjects included in the different groups, except for patients sensitized to diisocyanates ($N = 37$) and to flour dust ($N = 20$) who were different for age, atopy, duration of exposure, and sputum eosinophil percentage, in the same way as the difference between LMWC and HMWC. There was also a difference in the gender, related to the specific jobs (e.g., female in the subjects exposed to persulfate, or male in subjects exposed to diisocyanates).

Patterns of response following SIC were different for HMWC and LMWC. Subdividing subjects by type of response, immediate responses (early + dual response) were common in subjects exposed to HMWC (Figure 1). Also, in subjects with higher sputum eosinophilia, immediate responses were higher (61.1% versus 94.1%, $P = .02$). Considering all types of responses, magnitude of the early (EAR) responses was higher during SIC to LMWC while the magnitude of the late (LAR) responses was higher during SIC to HMWC (Figure 2).

4. Discussion

The present study shows that few clinical characteristics may differentiate patients with occupational asthma induced by LMWC from those with asthma induced by HMWC. In particular, duration of exposure before the beginning of asthma symptoms and the severity of the eosinophilic airway inflammation were the only findings which differentiated two groups of patients. In the same way, the pattern or airway response was consistently different, with patients with LMWC-induced asthma showing a higher frequency and

a greater severity of the isolated LAR than patients with HMWC-induced asthma who showed, on the contrary, a greater frequency and severity of the early response. These data confirm and extend previous observations confirming that some clinical and functional characteristics are different between subjects with occupational asthma induced by LMWC or by HMWC.

Our observations concern a wide range of sensitizing agents and a consistent number of subjects, as in other previous few studies [3, 13]. Differently from other previous papers which have considered only a subset of baseline measurements, our data include several clinical and functional findings which may be measured in these patients, including also the evaluation of the level of eosinophilic airway inflammation and the pattern of response to SIC. In effect, previous studies have shown that patients with LMWC-induced asthma had a greater duration of exposure to the specific sensitizer before the beginning of asthma symptoms than patients with HMCW-induced asthma [14, 15], or that LMWC asthma showed less frequent sputum eosinophilia [5] or a lower frequency of immediate airway response to SIC [3, 16]. All these data have been confirmed in our study which included a large set of clinical, functional, and biological findings of the disease at the baseline assessment.

However, other studies did not show relevant difference in terms of type and level of airway inflammation, level of asthma severity at the diagnosis, or rate of recovery from asthma after work cessation, between asthma induced by HMWC or LMWC [17–20]. However, some of these studies included small number of subjects, with several confounding factors (like the persistence or removal from exposure, or the severity of the disease).

This difference in clinical, functional, and biological features is probably related to the different pathophysiologic mechanisms underlying HMWC and LMWC occupational asthma [21]. Despite the large heterogeneity of the pathogenetic mechanisms underlying this disease, OA induced by HMWC is sustained by an IgE-mediated mechanism, which is well know to induce immediate airway reaction, due to mastcell activation which initiates the inflammatory cascade leading to a late reaction and the recruitment of eosinophils in the airways. Differently, LMWC elicit a lymphocyte-specific sensitization, with predominant isolated late response and lower eosinophilic inflammation. These features, in addition to the longer duration of exposure before the beginning of symptoms and the lower pulmonary function, make LMWC-induced asthma as a good model of late-onset asthma, different from HMWC-induced asthma which has many findings more typical of early-onset asthma. The hypothesis of different characteristics and outcome between early- and late-onset asthma has been suggested by other authors [22] and might be further supported by the different outcome of asthma after removal from work exposure. In effect, independently from the specific characteristics of the occupational agent, patients who at diagnosis had higher levels of sputum eosinophila reported a better outcome in the followup than patients without sputum eosinophilia, probably because a better response to the corticosteroid treatment [23].

5. Conclusions

In summary, we may speculate that the chemical characteristics of the specific sensitizer responsible for OA are responsible for a different pathophysiologic mechanism, which may determine different clinical, functional, and biological manifestation of the disease, including the pattern of specific airway response. In this way, occupational asthma may represent a good model for studying the heterogeneity of asthma and the difference between early- and late-onset asthma.

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Review Article

Industrial Fungal Enzymes: An Occupational Allergen Perspective

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Occupational exposure to high-molecular-weight allergens is a risk factor for the development and pathogenesis of IgE-mediated respiratory disease. In some occupational environments, workers are at an increased risk of exposure to fungal enzymes used in industrial production. Fungal enzymes have been associated with adverse health effects in the work place, in particular in baking occupations. Exposure-response relationships have been demonstrated, and atopic workers directly handling fungal enzymes are at an increased risk for IgE-mediated disease and occupational asthma. The utilization of new and emerging fungal enzymes in industrial production will present new occupational exposures. The production of antibody-based immunoassays is necessary for the assessment of occupational exposure and the development of threshold limit values. Allergen avoidance strategies including personal protective equipment, engineering controls, protein encapsulation, and reduction of airborne enzyme concentrations are required to mitigate occupational exposure to fungal enzymes.

1. Introduction

In the United States, the 2010 civilian workforce accounted for 139 million people [1] who spend up to a quarter of their lifetime and half of their waking lives at work [2]. With changes in the global market, particularly with the rise of biotechnology, new occupational hazards have emerged. Approximately, 200 biotic (organisms or particles of viral, prokaryote, or eukaryote origin) and an even greater number of abiotic (physical and chemical) agents have been associated with adverse health outcomes. In certain occupational settings, particularly those engaged in handling purified microbial proteins in baking and manufacturing sectors, workers are at increased risk of becoming sensitized and developing respiratory disease.

Occupational asthma (OA) is the most common respiratory disease reported in the workplace [3–8]. OA has been defined as either irritant induced or immune mediated [6, 7]. Immunologically mediated OA accounts for approximately 90% of cases [9], but the severity of disease is dependent on the concentration, route, agent of exposure, and the latency period [6, 7]. Both high- and low-molecular-weight antigens can induce OA, but the immunological mechanisms are distinctly different. High-molecular-weight allergens are generally proteins that are greater than 5 kDa, and production of immunoglobulin E (IgE) results in the release of mediators from mast cells and eosinophils [6, 7].

More than 250 high-molecular-weight allergens that induce OA have been identified [4, 6, 7]. Many are derived from animals or plants, and exposure usually involves mixtures of many proteins [4, 6]. Occupations where high-molecular-weight allergens have been characterized include seafood processing (tropomysin), dairy, poultry, citrus, greenhouse, baking, healthcare (latex), pharmaceutical (drugs), and detergent manufacturing (fungal enzymes) [6]. Some of the best examples of high-molecular-weight occupational allergens are the fungal enzymes. They are particularly suited for study because they are often used as purified preparations in baking, food, detergent, textile, and pharmaceutical industries [6, 10]. In this paper, we will focus on the fungal enzymes as model high-molecular-weight allergens in industrial settings and describe the main enzymes that have been associated with occupational sensitization and asthma. Identification of emerging fungal enzymes in manufacturing and biotechnology industries is discussed as well as new methods to detect and quantify

Table 1: Fungal enzymes utilized in different industries and associations with occupational sensitization.

Industry	Fungal Enzyme	
	Characterized occupational allergen	Uncharacterized occupational allergen
Agriculture	Protease, lipase	
Animal feed	α-amylase, cellulase, lipase, phytase, protease, and xylanase	Beta-glucanase*, endo-xylanase
Pulp and paper production	Cellulase, hemicellulase, lipase, and xylanase	Esterase*, laccase, lignin peroxidase, manganese peroxidase, pectinase*, and mannose
Waste management	Lipase	Esterase*, cytochrome P450, laccase, lignin peroxidase, manganese peroxidase, and monooxygenase
Biotechnology	α-amylase, cellulase, glucoamylase, hemicellulase, and protease	Cytochrome P-450 monooxygenase, glucose oxidase, glutathione-transferase*, lignin peroxidase, and manganese peroxidase,
Detergent	α-amylase, cellulase, lipase, and protease	
Food processing	α-amylase, cellulase, glucoamylase, lactase, lipase, protease, and xylanase	Glucose isomerase, invertase, and pectinase*
Biofuels	α-amylase, cellulase, glucoamylase, protease, and xylanase	
Bakery	α-amylase, cellulase, glucoamylase, hemicellulase, lipase, protease, and xylanase	Glucose oxidase, lipoxygenase
Brewing and wine production	Cellulase, glucosidase, protease, and xylanase	Alpha-acetolactate decarboxylase, beta-glucanase*, and pectinase*
Pharmaceutical	Lactase, lipase, and protease	Alpha-galactosidase*, catalase*, cytochrome P450 oxygenase, and glutathione transferase*
Textile	α-amylase, cellulase, lipase, protease, and xylanase	Catalase*
Leather processing	Lipase, protease	
Hygiene products	Glucoamylase, protease	Catalase, *glucose oxidase

Adapted from Baur [10] and from the Concordia University Genomics Project website: https://fungalgenomics.concordia.ca/home/indappl.php.
*Denotes fungal enzymes associated with allergic sensitization in other fungal bioaerosols or environmental allergens.

fungal enzymes in the occupational environment. Methods to avoid fungal allergens in the workplace are additionally covered.

2. Fungal Enzymes in Occupational Disease

The industrial utility of fungi has been well known since antiquity. In addition to the role of fungi as saprophytes in the environment, many species have commercial use, for example, mushrooms as food sources, ingredients in food preparation (cheese flavoring *Penicillium roqueforti*), alcoholic fermentation, and the conversion of sugars in bread dough to carbon dioxide (*Saccharomyces cerevisiae*). In Asia, *Aspergillus oryzae* is an essential ingredient for the production of soy sauce and the fermented drink, sake. *Rhizopus* spp. secrete a wide variety of enzymes including cellulolytic, proteolytic, lipolytic, and pectinolytic enzymes that are used in the production of various foods such as Tempe from Indonesia [46]. *Rhizopus oryzae* has also been identified as a biocatalyst for biodiesel fuel production [47]. Other fungi such as *Yarrowia lipolytica* have more recent applications in the biodegradation of industrial products [48]. Advances in industrial enzymology following World War II have enabled researchers to identify and utilize various enzymes and proteases that fungi produce to break down carbohydrate and lignin containing plant material in the environment [49]. To date, close to 200 fungal enzymes have been purified from fungal cultures and the biochemical and catalytic properties characterized [10, 50]. These enzymes have great utility in pharmaceutical, agricultural, food, paper, detergent, textile, waste treatment, and the petroleum industries.

Industrial fungal enzymes are high-molecular-weight proteins that are catalysts [10, 49]. A description of the common enzymes used in various industries is presented in Table 1. The most widely used enzymes of occupational importance are derived from the genus *Aspergillus* and include α-amylase, xylanase, and cellulase. A summary of the proteomic and immunologic properties of these enzymes is presented in Table 2. Other enzymes are also utilized from rhizosphere fungal species belonging to the genera *Rhizopus* and *Humicola* (Table 2). These enzymes usually have intracellular or other functional roles associated with apical hyphal growth. It is uncommon for individuals in the general population to be exposed and sensitized to these antigens. In fact, in the general population, the prevalence of sensitization to fungal enzymes has been reported to be as low as 1% and as high as 15% [22, 51]. However, in the occupational environment, workers that handle purified fungal enzymes are at an increased risk of becoming sensitized to enzymes [10, 23, 24, 42, 52–56]. This is especially the case for workers whose occupation requires debagging, sieving, weighing, dispensing, and mixing enzymes [24, 53–56]. Eight-hour time-weighted average exposures demonstrate

Table 2: Fungal enzymes associated with occupational sensitization and asthma in occupational environments.

Fungal Order	Fungal species	Allergen	Putative function	Calculated size (kDa)	Accession number	Patient reactivity	Occupational environment	Reference
Ascomycota								
Diaporthales	*Cryphonectria parasitica*	Cry p AP	Aspartic protease	43	X63351	5.7%†	Food processing	[11]
Eurotiales	*Aspergillus niger*	Asp n 14*	Beta-xylosidase	105	AF108944	4–11%††	Bakers, textile, detergent, animal feed	[12–15]
		Asp n 25*	3-phytase B (phosphatase)	84	L20567	37%††	Animal feed	[16, 17]
		Asp n glucoamylase	Glucoamylase	68	X00548, AM270061	5–19%† 63%††	Bakers	[10, 13, 18]
		Asp n hemicellulase	Hemicellulase	22.6	A19535	8–43%† 100††	Bakers	[19–21]
	Aspergillus oryzae	Asp o 21*	TAKA-amylase A	53	X12725, X12727, M33218, D00434	0.9–35%† 1–32%††	Bakers, pharmaceutical	[12, 20–37]
		Asp o lactase	Lactase		AP007164	29%–31†	Pharmaceutical	[38–40]
		Asp o lipase	Lipase			SR	Detergent	[41]
	Thermomyces lanuginosus (Humicola lanuginosa)	The l lipase	Lipase	32	EU370914, AF054513	3%††	Detergent, food processing	[42]
Hypocreales	*Trichoderma viride*	Tri v cellulase	Cellulase	42	EF602036	35%††		
	Trichoderma reesei	Tri rs cellulase	Cellulase	48	AY928809	13%†	Biotechnology	[43]
Sordariales	*Humicola insolens*	Hum in cellulase	Cellulase	45	P56680	SR	Detergent	[41]
Zygomycota								
Mucorales	*Rhizomucor miehei*	Rhi m AP	Aspartic protease	46	M18411	28.6%†	Food processing	[11]
	Rhizopus oryzae	Rhi o lipase	Lipase	42	M38352, AB433531, AF229435	SR	Pharmaceutical	[44]

Adapted from the IUIS Allergen Nomenclature Subcommittee, Allergome (http://www.allergome.org/), and [45].
*Denotes allergens deposited to the IUIS Allergen Nomenclature Subcommittee.
SR: Patients with a positive SPT or specific IgE.
—†Denotes that patients were tested with SPT.
—††Denotes that patients were serologically screened using Pharmacia UniCap, Rast, or Immunoblot.

that occupations weighing the enzyme preparations have the lowest average exposure compared to those workers that sieve [24]. These workers are often exposed to levels of dust that exceed 4 mg m^{-3}, the threshold limit value (TLV) for inhalable dust [57]. For other industrial environments that use lipase and cellulose in production, occupational exposure is highest in production areas and laboratories [42].

Adverse health effects associated with enzyme exposure are well characterized in the baking industry. In some countries, bakery exposures to enzymes are one of the leading causes of occupational allergy [58]. Fungal enzymes are commonly used as baking additives to improve the dough, increase shelf life, and decrease production time [19, 49, 59]. Airborne concentrations ranging from 5.3 ng m^{-3} to 200 ng m^{-3} have been reported in occupational environments [12, 59, 60]. Occupational sensitization to fungal enzymes was first reported by Flindt [61]. Later, Baur et al. [62] demonstrated IgE sensitization in workers handling these products. Since the original study, fungal enzymes have been identified as potent allergens in the occupational environment [25, 26]. Prevalence of sensitization to *Aspergillus* enzymes ranges from 8% for glucoamylase [13], 11% for xylanase [13], 13% for cellulase [13], and up to 34% for α-amylase [19, 27]. Sensitization to α-amylase in bakery workers results in decreased peak expiratory flow [63–66] and OA [20, 28, 67]. In one report, workers exposed to fungal enzymes induced an immediate bronchospastic reaction [49]. In the United States, the prevalence of work-related wheeze, runny nose, frequent sneezing, and specific IgE to fungal enzymes was significantly higher among highly exposed workers [68]. However, other irritant-induced mechanisms associated with high total dust levels have also been reported in a cohort of British bakers [29, 56]. To date, atopy has been hypothesized to be an important risk factor for OA to fungal enzymes.

Occupational exposure to enzymes has been demonstrated in other industries including manufacturing [41, 53, 69], pharmaceutical [25, 38], food processing [70], animal feed, and biotechnology [43]. Like in baking environments, workers handling or in direct contact with fungal enzymes and with a history of atopy are at increased risk of becoming sensitized [10, 23, 24, 42, 52–56]. Sensitization to proteolytic enzymes has also been demonstrated in the manufacture of detergents [53, 71]. In the future, additional uses for fungal enzymes in industrial environments will be identified. Recent examples include the use of α-amylase and glucoamylase for the production of ethanol in the biofuel industry [72, 73]. If proper methods of exposure prevention are not followed and exposure is not monitored in these industries, it is possible that new groups of workers will suffer adverse health outcomes and become sensitized to enzymes. In the following sections we describe the major fungal enzymes, prevalence of sensitization, and occupational environment that they are most likely to be encountered.

3. Fungal Enzyme Allergens

3.1. α-Amylase. Fungal amylase is the most well-characterized fungal enzyme used in the occupational environment. Originally discovered by Takamine in 1884 [49], bakers have used α-amylase as a supplement to cereal flour to improve carbohydrate fermentation by yeasts and ultimately the quality of the bread [49]. α-amylase cleaves long-chain carbohydrates into simpler sugars including maltose [49]. Derived from *A. oryzae*, α-amylase is a 478 amino acid glycoprotein with a molecular weight of 53 kDa (Table 2; [6]). Occupational sensitization to α-amylase was first reported by Flindt [61] and has subsequently been identified as an allergen in baking [74], pharmaceutical [25], animal feed [12], and biotechnology industries [43]. The allergen was originally designated Asp o 2 by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Subcommittee but now has been redesignated Asp o 21 [27]. Since this preliminary work, α-amylase has been identified as one of the principle sensitizers in large-scale bakeries [24, 56]. The prevalence of sensitization among bakers is variable and ranges from 0.9% to 34% [13, 18, 19, 21, 23, 24, 27–35, 54, 66, 67, 75]. Concentrations as high as 40 ng m^{-3} have been reported in baking environments [60]; however, α-amylase concentrations in the low ng m^{-3} range have been associated with an increased frequency of sensitization [58].

The most common tasks associated with α-amylase exposure involve dispensing, sieving, weighing and mixing [55, 56, 60]. Exposures that exceed the maximum exposure limit for flour dust in the United Kingdom were identified in mixing, weighing [54], and dispensing operations [55]. The prevalence of sensitization to α-amylase is 9.9 times greater among workers in high-exposure categories compared to those workers in low-exposure categories [55]. Aerosolized particle size distribution analysis in baking environments demonstrated that workers are exposed to α-amylase particles within the inhalable size fraction [60]. OA is commonly identified in workers sensitized to α-amylase. After bronchial provocation with α-amylase, between 16 and 100% of sensitized workers were found to give a positive immediate response depending on the study [25, 35, 49]. Nasal provocation with α-amylase in skin prick test (SPT) positive workers also induced rhinitis [26]. Furthermore, positive associations between α-amylase SPT and work-related respiratory symptoms have been identified [23]. Interestingly, heating α-amylase has been shown to reduce enzymatic and allergenic activity of the enzyme [76]. Potential sensitization of bakers' family members due to α-amylase associated with clothes, shoes, and bakery textiles has also been reported by Vissers [77].

3.2. γ-Amylase. γ-amylase or glucoamylase is primarily obtained from *A. niger, A. awamori, and R. delemar.* Glucoamylase is used as a dough additive by bakers, often in association with α-amylase. The enzyme is also used in the production of high-glucose syrups [46]. Glucoamylase has a molecular weight of 68 kDa (Table 2) and can remain functionally active at elevated pH. Glucoamylase exposure has been primarily reported in baking occupations [10, 13, 18]; however, occupational exposure has also been reported in fruit and salad processing [52]. Sen et al. [52] demonstrated that three workers with shortness of breath, chest tightness, and wheeze had specific IgE to glucoamylase. Quirce et al.

[18] also demonstrated positive SPT to glucoamylase in all tested subjects ($n = 4$); however, only three of the four patients elicited an early asthmatic response following bronchial provocation. Airborne glucoamylase was shown in 9% of air samples from a bakery [59], and median levels were 10.3 ng m^{-3}. Moderate allergenic cross-reactivity has also been reported between glucoamylase and α-amylase [18].

3.3. Cellulase, Xylanase, and Hemicellulase. Cellulases are enzymes that hydrolize cellulose into glucose and are primarily used in the pharmaceutical, baking, detergent, and textile industries [6, 78]. Cellulase has been purified from several rhizosphere fungi including *A. niger* and *Trichoderma viride* [49], as well as *Humicola insolens* [41]. The molecular weight of cellulases ranges from 22 to 45 kDa (Table 2). Cellulases derived from these fungi are used in baking to break up roughage in dough and as a digestive aid in the food industry [13, 79, 80]. The first case of OA caused by cellulase was reported in 1981 in a plant pathologist [49, 81], and later these findings were confirmed in two pharmaceutical workers [80], four laboratory workers [14], and two bakers [49]. In each of these studies, the workers had specific IgE to the cellulase antigens. In 171 German bakers, the prevalence of sensitization to cellulase was 13% [13]. Airborne concentrations of cellulase have been quantified using a modified dot blot technique and were <180 ng m^{-3} in a flour mill, crisp bread factory, and a bakery [82]. OA has also been reported to cellulase in the baking industry [83] as well as from *H. insolens* used in the detergent industry [41].

Endo 1, 4-beta-D-xylanase and beta-xylosidase are major enzymes involved in xylan hydrolysis [13]. Collectively termed xylanases, these enzymes are a type of hemicellulase that breaks down hemicelluloses, a major component in plant cell walls [13]. Besides α-amylase, xylanases are the next most frequently used enzymes in the baking industry to remove pentosans from bread and increase bread volume [13, 49]. The prevalence of IgE sensitization to hemicellulase was reported to be 8% [19] and 11% for xylanase [13]. Sander and colleagues [13] found that 7 of 8 bakers had serum IgE to a 105 kDa protein in a xylanase ingredient. This protein was identified using mass spectrometry to be beta-xylosidase derived from *A. niger*. The allergen was designated Asp n 14 by the IUIS Allergen Nomenclature Subcommittee (Table 2). Airborne concentrations of xylanase have been reported to be <40 ng m^{-3} in a flourmill and crisp bread factory [82]. Concentrations as high as 200 ng m^{-3} were also reported in a bakery, but these values were associated with the natural xylanase activity of wheat [82]. Case reports have verified xylanase sensitization and the presence of an IgE mechanism in respiratory disease [15, 79]. OA has also been reported to xylanase in the baking industry [83], and in a case report, a baker had an immediate asthmatic response following inhalation challenge [15]. Cross-reactivity between cellulase and xylanase has been reported to be in the range of 80–90% but no cross-reactivity has been shown with α-amylase [13, 14]. Similarly, workers can also be monosensitized to cellulase and xylanase without concomitant sensitization to α-amylase [83].

3.4. Lactase. *A. oryzae* lactase is a high-molecular-weight protein that is involved in the hydrolysis of the disaccharide, lactose. Lactase is used in the pharmaceutical industry to develop dietary aids for patients intolerant to lactose. In a cross-sectional study of United States pharmaceutical workers, Bernstein and colleagues [38] identified 29% of lactase-exposed workers to have positive SPT response to lactase. Workers with a positive SPT were nine times more likely to have respiratory symptoms than workers with a negative SPT [38]. Interestingly, atopy was not associated with the development of respiratory symptoms. Occupational sensitization to lactase has been reported in workers formulating and packaging gastrointestinal consumer products [39]. In inhalational challenge studies conducted by Laukkanin and colleagues [40], lactase was identified to induce occupational IgE-mediated respiratory sensitization. Interestingly, lactase exposure has also been identified to cause contact skin reactions [40].

3.5. Lipase. Lipase is an essential catalyst that digests water-insoluble lipids. *A. oryzae* and *R. oryzae* lipase are used because of low extraction costs, thermal and pH stability, substrate specificity, and activity in organic solvents. Lipase is predominantly used in the manufacture of laundry detergents and in baking; however, other newer applications have been developed. For example, *Candida antarctica* lipase has recently been used as a biocatalyst for the biofuel industry [84]. The incidence of occupational sensitization to lipase, in industrial settings is understudied. In a preliminary analysis of detergent manufacturing workers, 3 workers were found to be sensitized to lipase and bronchial provocation tests provoked a reproducible asthmatic response [41]. A recent case study of a pharmaceutical manufacturing worker also demonstrated sensitization to fungal lipase derived from *R. oryzae* but not *A. oryzae* α-amylase [44].

3.6. Phytase. *A. niger* and *R. oligosporus* produce phosphatase that catalyzes the hydrolysis of phytate to lower-order phosphate esters [16]. Termed phytase, this enzyme enhances phosphate bioavailability in the digestive tract and has been utilized in the animal feed industry during the last two decades [17]. Phytase accounts for 0.1–1% of total extractable protein from *A. niger* [17]. 3-phytase B derived from *A. niger* is an 84 kDa protein that has been designated Asp n 25 by the IUIS Allergen Nomenclature Subcommittee (Table 2). Allergic sensitization to phytase has been reported in animal feed factory workers (7–90%), and sensitization is highest at sites where phytase is handled in powdered form [16, 17, 69, 85]. In a cross-sectional study of 53 technical center workers that produced *A. niger* phytase, 52% of workers in the high-exposure group and only 10% in the low-exposure group were sensitized to phytase [16]. Personal exposure to phytase has been shown to exacerbate OA, and inhalation challenge tests produced immediate asthmatic response [86]. It has been proposed that phytase is highly sensitizing and that direct contact should be avoided in this industry [16].

3.7. Enzymes Used in Health Care Settings: Biodiastase and Flaviastase. Fungal enzymes have a number of applications in the healthcare environment. Fungal enzymes derived from *A. niger* are used in powdered form with other enzyme extracts by pharmacists to prepare digestive powders. Biodiastase and Flaviastase are two examples of fungal enzymes that have been associated with sensitization in hospital workers and pharmaceutical workers handling these products [10, 12–21, 23–35, 38–44, 51–88]. To date, health effects in workers exposed to these enzymes remain poorly characterized.

3.8. EPg22 Protease: Aspartic Protease. The aspartic proteases produced by *Rhizomucor miehei* and *Cryphonectria parasitica* are utilized in almost half of the cheese production operations throughout the world [46]. The proteases assist in milk clotting and facilitate a change in cheese properties by hydrolyzing certain peptide bonds. Occupational exposure to these proteases has been associated with occupational sensitization in a rennet production plant [11]. Specifically 29% and 6% of workers had a positive skin prick test (SPT) to *R. miehei* and *C. parasitica* aspartic protease extracts, respectively [11]. Other novel enzymes with potential application in the food processing industry have been identified. Pg222 is a novel extracellular protease produced by *P. chrysogenum* (Pg222). The enzyme was isolated from dry-cured hams and was found to have a broad range of applications in industries that produce dry-cured meat products [89]. Although no occupational sensitization has been reported to this enzyme, it demonstrates that the introduction of any new enzyme could potentially represent an occupational hazard.

4. Emerging Occupational Fungal Enzyme Exposures

The utility of fungal enzymes to degrade xenobiotics and organic compounds in the industrial sector continues to be recognized [46]. Fungal enzymes are now being used for a variety of purposes across many different industries. Improved biochemical and molecular technologies have enabled the production of other potentially allergenic proteins [14]. According to Baur [10], more than 186 commercial enzymes were produced in the European Union in 2001, and many of these were produced by recombinant technology or had been genetically engineered. Table 1 provides a summary of the major fungal enzymes that are utilized in industrial settings. All of the aforementioned enzymes that are listed in Table 1 have been identified to be potent allergens in the workplace; however, the ability of the other listed enzymes to cause adverse health outcomes following occupational exposure remains unclear.

Several of the enzymes presented in Table 1, not identified as occupational allergens, have been identified as allergens associated with environmental bioaerosols. Catalase, a fungal enzyme utilized in hygiene products, pharmaceuticals, and textiles, has been identified as an allergen in the entomopathogenic fungus, *Metarhizium anisopliae* [90]. Pectinase is used in brewing and wine production, food processing, and paper industries and allergy to pectinase has been associated with occupational exposure [91]. Esterase has been identified as an allergen in *Hevea brasiliensis* (natural rubber latex) [92]. Beta-glucanase is used to improve the nutritional yield of animal feeds, and occupational exposure has been shown in a case study to significantly reduce forced vital capacity and forced expired volume in 1 second (FEV1) [86]. The worker in this case study was also SPT positive and had specific IgE to beta-glucanase [86]. In the biotechnology and pharmaceutical industries, glutathione-S-transferase (GST) has a number of applications. GST is an approximately 26 kDa protein that has been identified as a major *Alternaria alternata* allergen and is highly conserved across fungi [45, 93, 94]. The IUIS Allergen Nomenclature Subcommittee has designated this allergen Alt a 13 [93, 94]. Interestingly, alpha-galactosidase has been associated with delayed anaphylaxis, angioedema, or urticaria in sensitized patients following the ingestion of beef, pork, or lamb [95]. Although the role of alpha-galactosidase and these other enzymes following occupational exposure remains unclear, these studies provide preliminary insight into the possible potency of these allergens in industrial environments.

5. Immunodiagnostic Detection Methodologies

Occupational allergic sensitization to fungal enzymes is diagnosed clinically using available *in vivo* SPT reagents, or *in vitro* assays such as Phadia ImmunoCap [7]. However, SPT reagents for most of the fungal enzymes used in industrial settings are not commercially available and have to be either custom ordered or prepared individually by the investigator. Methods for SPT extract preparation that are used by investigators in the field have been previously described by Quirce et al. [49]. *In vitro* diagnostic tools that can quantify the amount of specific IgE to an occupational allergen are not readily available except in research laboratories where investigators prepare their own inhibition or radioallergosorbent enzyme-linked immunosorbent assay (ELISA) to quantify specific IgE [36, 49]. To date, α-amylase (k87) is the only fungal enzyme available on the Phadia ImmunoCap testing panel. To confirm OA caused by fungal enzymes, bronchial provocation tests can be undertaken to document immediate or late-phase responses to fungal enzymes [36, 49]. Positive immediate response criteria used in workers exposed to enzymes include a greater than 20% fall in FEV1, whereas a late-phase response has been considered positive when there is a 30% or greater fall in peak expiratory flow rate [49]. However, there are several limitations with bronchial provocation tests that should be considered; these are discussed in detail by Peden and Reed [7].

In order to better understand the relationships between occupational fungal enzyme exposure and clinical symptomology, accurate information on the distribution and quantity of the fungal enzyme in the occupational environment will be required. Immunodiagnostic methods that utilize antibodies could provide standardized methods for quantifying fungal enzyme biomarkers in a variety of

occupational environments. Following validation and interlaboratory comparison, the assays could be used for exposure assessment to determine the existence of exposure-response relationships [58, 96]. This information is critical for the development of future threshold limit values (TLVs) and other occupational standards.

Several antibodies and immunodiagnostic methods have been produced to detect industrial fungal enzymes, in particular α-amylase. These methods have been employed in field investigations and used to quantify the concentration of the enzyme from collected air samples. Bogdanovic et al. [97] used an enzyme immunoassay with a sensitivity of 25 pg/mL to quantify α-amylase in airborne and surface dust samples collected from five bakeries. In the same study, a lateral flow immunoassay for α-amylase was compared to the reference enzyme immunoassay. The sensitivity of the lateral flow assay was 1–10 ng/mL, and extracts with >5 ng/mL allergen were positive in the lateral flow assay [97]. In a study of 507 personal air samples, Houba and colleagues [60] used a rabbit IgG capture immunoassay to quantify α-amylase in specific baking job category. Concentrations of α-amylase up to 40 ng m^{-3} were quantified, and workers directly involved with dough preparation had the highest exposures [60]. Using the same rabbit IgG sandwich assay, Nieuwenhuijsen et al. [55] identified dispensing and mixing areas to have the highest α-amylase exposure in British bakeries and flour mills. Two monoclonal antibody- (mAb-) based ELISAs have been developed for the detection of α-amylase in the occupational environment. Assay sensitivities ranged from 0.2 ng/mL [98] to 0.6 ng/mL [99]. A quantitative mAb-mediated dot blot assay has also been previously described for cellulase and xylase; the detection limits reported were 20 ng m^{-3} and 2 ng m^{-3}, respectively [82]. mAbs to other fungal enzymes, such as xylanase have been produced and reported in the literature [100]. Similarly, the detergent industry has produced antibodies and immunoassays for several common fungal enzymes and these have been utilized in industrial hygiene safety programs to mitigate worker exposures [101–103]. Unfortunately, for many other fungal enzymes presented in Table 1, there are no commercially available antibodies to enable quantification in the occupational environment. The development of fungal enzyme-specific mAbs in combination with immunodiagnostic techniques will further our knowledge of the exposure-response relationships in occupational environments. Using these methods will also help enable the development of standards and focus on the prevention of sensitization in heavily contaminated work environments.

6. Allergen Avoidance and Directions for the Future

Exposure to fungal enzymes, in particular α-amylase, is a considerable health risk in a number of industries. Cross-sectional studies have shown that processing workers in high-exposure categories who handled fungal enzymes are up to ten times more likely to be sensitized to fungal enzymes than workers in the low-exposure category [55]. Highest concentrations of enzymes in the inhalable fraction were encountered among workers located in dispensing, mixing, weighing, and sieving occupations [54–56, 60]. Airborne concentrations as high as 40 ng m^{-3} and in some cases even higher (200 ng m^{-3}) have been reported for sensitized workers located in these handling areas [12, 55, 60]. Concentrations in the low ng m^{-3} range have been associated with an increased frequency of sensitization [58]. For other fungal enzymes, such as phytase, similar findings have been reported [16].

The continued utilization of other previously overlooked enzymes as well as new genetically engineered enzymes in various industries will continue to provide diagnostic challenges, even for the most seasoned occupational medicine professional. It is likely that new cases of occupational allergic disease will emerge following exposure to fungal industrial enzymes during the next decade. In response, identification of exposure-response relationships will be critical for the development of TLVs and occupational exposure levels. However, this will depend on the development of suitable diagnostic antibodies and immunoassays. Currently, subtilisin, a sereine endopeptidase derived from *Bacillus subtilis*, is the only enzyme for which the American Conference of Governmental Industrial Hygienists (ACGIH) has established a TLV value (60 ng m^{-3}). The European Union Directive also classifies the fungal enzymes cellulase and α-amylase with the risk phrase R42 (may cause sensitization by inhalation) [10]. There are currently no consensus standards for other industrially utilized fungal enzymes.

As a precautionary measure, it has been concluded that all enzymes should be regarded as an allergen that can exacerbate respiratory sensitization in susceptible populations [10, 59]. Baur [10] has further proposed that all enzymes should be classified as R42 according to the European Union Directive criteria. Although intervention in the bakery industry has had little to no effect [104], installation of engineering controls and implementation of personal protective equipment programs in animal feed workers exposed to phytase was shown to result in the immediate cessation of hypersensitivity symptoms [10]. Improvements in biotechnology have also included the encapsulation of some enzymes [105, 106] and proteins [107]. These engineering controls have been proposed to reduce occupational exposure to enzymes; however, encapsulation alone may not completely prevent enzyme-induced allergy and OA [108, 109]. To date, the detergent industry has implemented a derived minimal effect level (DMEL) of 60 ng m^{-3} for pure enzyme proteins [110]. Although this DMEL was provided as guidance by the ACGIH, other manufacturers have implemented their own occupational exposure guidelines (OEGs) for fungal enzymes such as α-amylase (5–15 ng m^{-3}), lipase (5–20 ng m^{-3}), and cellulase (8–20 ng m^{-3}) [110]. In addition, the detergent industry has developed a medical surveillance program to identify and correct elevated exposures before occupational illnesses occur [101–103, 111]. As a result, the incidence of occupational allergy has dropped substantially [101–103]. Implementation of DMELs and OEGs will further assist in the reduction of occupational exposure. Reducing worker exposure to fungal enzymes in industry by the implementation

of engineering controls and other allergen avoidance strategies will continue to mitigate personal exposure and further reduce the occupational health risk.

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Case Report

Vicia faba Hypersensitivity and ASA Intolerance in a Farmer: A Case Report

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The IgE-mediated allergic reactions to food are caused, generally, by ingestion. However, they can be rarely induced by exposure to airborne food particles through the handling or the cooking. *Vicia faba* is a vegetable which belongs to Legumes or *Fabaceae* family, *Fabales* order. Allergic reactions after ingestion of legumes and cases of asthma after exposure to the cooking vapors have been reported in the literature. A paper assessed the volatile substances (insect repellents) released by *V. faba*. The authors demonstrated that this plant produces several chemical substances, such as small quantities of methyl salicylate. We describe a case of occupational allergy, induced by handling during picking up of fresh broad beans, in a farmer with history of adverse reaction after eating the cooked and raw vegetable.

1. Introduction

The IgE-mediated allergic reactions to food are caused, generally, by ingestion. However, they can be rarely induced by exposure to airborne food particles through the handling or the cooking [1].

Vicia faba is a vegetable which belongs to Legumes or *Fabaceae* family, *Fabales* order. The name "legume or pod" derives from the fruit, which is composed from two symmetrical valves enclosing the seeds [2]. Several legumes were used as food, such as the kidney bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), broad bean (*Vicia faba*), lupin (*Lupinus albus*), chick pea (*Cicer arietinum*), peanut (*Arachis hypogaea*), soy bean (*Glycine max*), and lentil (*Lens culinaris*) [3]. Immunological cross-reactivity has been widely reported in this family but the clinical cross-reactivity is rare [4, 5].

The broad bean is cultivated in Italy, especially in the southern regions and the islands although it is originated in northern Africa and Asia. The plant grows erect and can reach a height of 1.8 m. The leaves are long with jagged edges, and the flowers are white with black stripes and a white and purplish halo with a black spot. The fruit is a green pod with a velvety consistency that gets darker as it matures. Each pod can contain 3–8 oval-shaped seeds that can be consumed both cooked and raw [6].

Allergic reactions, sometimes severe, after ingestion of legumes and cases of asthma after exposure to the cooking vapors have been reported in the literature [7–12].

The presence of 7S (from 10 to 70 kDa) and 11S globulin (from 20–25 to 35–50 kDa) has been demonstrated by Freitas et al. in a study on several legumes, including *V. faba* [13]. In addition, some authors extracted from *V. faba* a VfSBPL (*Vicia faba* sucrose binding-like protein) of 52 kDa that has a 58% homology with the 62 kDa protein contained in soy bean (*Glycine max*) [14].

A paper assessed the volatile substances (insect repellents) released by *V. faba.* The authors demonstrated that this plant produces several chemical substances, such as small quantities of methyl salicylate [15].

A IgE-mediated mechanism has been demonstrated in a series of six patients with referred adverse reactions after eating fresh raw and cooked broad beans and specific IgE to the proteins extracted from the vegetable in two of the six patients' serums. In the remaining four patients, the relationship between history of acetylsalicylic acid (ASA) intolerance and asthmogenic metabolites present in

the vegetable has been suggested as a cause of the *V. faba* reactions [16].

We describe a case of occupational allergy, induced by handling during picking up of fresh broad beans, in a farmer with history of adverse reaction after eating raw and cooked vegetable.

2. Materials and Methods

2.1. Patient. A 49-year-old woman, farmer, reported two episodes of adverse reactions induced by raw and boiled broad beans ingestion or handling of fresh vegetable.

The first episode presented as dyspnea, chest tightness, tachycardia, and malaise after the patient ate fresh raw and boiled broad beans; the second episode presented as a sensation similar to the previous associated with burning eyes and facial oedema while picking up fresh broad beans. On both occasions prompt treatment in an emergency unit relieved the symptoms after approximately two hours.

In addition, the patient had reported other episodes, related to the picking up of fresh broad beans pods, but in milder form.

The clinical history showed dyspnea and chest tightness after intake of ASA and nonsteroidal anti-inflammatory drugs (NSAIDs). These reactions were diagnosed as adverse drug reactions at another hospital and were prior to those caused by ingestion and handling of fresh broad beans.

The patient picked up the pods of fresh broad beans for 4-5 years, working many hours in large areas used for the cultivation of the vegetable; moreover, she began to show the symptoms during the harvest of the last two years, but after the reaction caused by raw and boiled broad beans ingestion. The respiratory symptoms reported in medical history were absent when she did not work in the picking up of fresh broad beans and when she was at home.

To explain the adverse reactions reported, the patient underwent *in vivo* and *in vitro* tests. In addition, the extracts from fresh cooked and raw broad beans diluted and undiluted were prepared to perform skin prick tests (SPTs) and immunoblotting (IB).

2.2. Protein Extraction from Boiled and Raw Fresh Broad Bean. To extract the proteins from the cooked legume, the seeds were boiled for approximately 30 min at 100°C. The extraction was done by homogenization, separately for the boiled and raw seeds, in PBS at 4°C for 24 h. Then the mixtures were centrifuged at 15000 rpm for 1 h at 4°C, and the supernatant was concentrated (Centrifugal Filter Devices, Millipore, Bedford, USA), performing a first centrifugation at 2500 g for 30 min at 4°C and an inverse centrifugation at 2000 g for a further 4 min, again at 4°C. The protein concentration was determined according to Bradford's method [17].

2.3. Gel Electrophoresis and Protein Spectrum. For electrophoresis, the extracts were set up in reducing conditions, according to the Laemmli method, adding β-mercaptoethanol and heating at 100°C for 5 min [18]. The proteins, contained in the extracts, were separated on a 4–12% polyacrylamide gel (Nupage Bis Tris, Invitrogen, Milan, Italy) for 1 h at 190 V and then stained with colloidal Coomassie blue [19].

2.4. Skin Prick Test. The patients underwent SPTs with common inhalant allergens, commercial food extracts including pea, peanut, kidney bean, soy bean (Stallergenes, Milan, Italy), chickpea, and lentil (Lofarma, Milan, Italy), and with extracts of fresh boiled and raw broad beans undiluted and diluted (1 : 1000, 1 : 100, 1 : 10 in saline solution and glycerol) [20]. Saline solution and histamine (10 mg/mL) were used as negative and positive control, respectively.

2.5. Immunoblotting. The proteins separated on polyacrylamide gel were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Biosciences, Germany) [21]. The membranes, after saturation, were incubated at 4°C overnight with the patient's serum. After several washes, the membranes were incubated with human anti-IgE conjugated with peroxidase for 2 h. Bound specific IgE was detected by chemiluminescence.

3. Results

3.1. Protein Extracts and Spectrum. The protein concentration from the raw and boiled seeds of *V. faba* extracts was 5.48 mg/mL and 4.43 mg/mL, respectively. Molecular analysis showed the presence of proteins weighing from 12, 16, 20, 32, 45 to 60–65 kDa (Quantity One Basic, BioRad, Milan, Italy).

3.2. Skin Prick Test. Prick tests with common inhalant allergens and commercial food extracts were negative. SPTs performed with extracts of fresh raw and boiled broad beans undiluted and diluted were stopped at raw broad beans extract diluted 1 : 1000, because the patient had positive wheal and symptoms, such as tachycardia, malaise, and hypotension. This reaction required prompt pharmacological therapy with remission of the symptoms.

SPTs performed with undiluted and diluted extracts of raw and boiled fresh broad beans in controls were negative.

3.3. Immunoblotting. The IB performed with the patient's serum showed specific IgE reactivity to the 60–65 kDa protein.

4. Discussion and Conclusion

Legumes are vegetables that are responsible for allergic reactions especially after ingestion, but they occasionally can induce occupational allergies, as in the case described by Bush and Cohen about asthma induced by exposure to soybean dust [22].

Our patient experienced dyspnea, chest tightness, tachycardia, and malaise both after ingestion and after handling of fresh broad beans while picking up. These clinical manifestations recurred during the performance of SPTs with extracts

of fresh raw broad beans diluted to a low concentration (1 : 1000) that required discontinuation testing and pharmacological therapy. Daroca et al. reported cases of respiratory symptoms appeared after handling of green beans or green beans and chards together in patients who ingested the same food without reactions. The IB performed with patient's serum showed several differences, although minimal, about the reactivity of IgE between IB with the raw extract and the blotting with boiled extract. This may be related to the lability of allergens of green beans to cooking, which would explain the tolerance to the ingestion of cooked foods [23]. Another case of asthma induced by handling and cooking vapors of green beans has been described by Igea et al. in a housewife who tolerates ingestion of vegetable. In this case, the presence of protein heat-labile in vegetable has been suggested [24]. In our case, the patient did not tolerate the ingestion of the native (raw) and boiled food and the proteins are well preserved also in the cooked extract.

The IB shows IgE reactive against the protein of molecular weight of 60–65 kDa, present in the fresh raw and boiled broad beans extract. This demonstrates that the reaction experienced by the patient can be IgE mediated.

The presence of salicylates in vegetable and the role of salicylic acid as defense response to pathogens are known in the literature [25–27]. In addition, after external stress stimuli, the *V. faba* produces small quantities of methyl salicylate and others substance that can be measured in the surrounding air [15]. A series of patients with ASA intolerance and reactions after ingestion of *V. faba* cooked and raw seeds had assumed to the authors a asthmogenic role of some metabolites of methyl salicylate present in the plant [16]. In several cases reported in the literature, the reactions caused by the inhalation of food allergens appear, generally, in domestic environment [7, 8, 23, 24] or in industrial environment [22].

Our patient reported history of adverse reactions after intake of NSAIDs and ASA, prior to those caused by ingestion and handling of fresh broad beans. Moreover, the reactions that the patient experienced during working, picking up the pods of *V. faba* (without the concomitant ingestion of seeds), were at first slight and after successive exposures had become more serious. The evolution of symptoms has suspected that the patient's ASA intolerance could have had a significant role. In fact, the methyl salicylate, released from the vegetable in the surrounding air and inhaled by the patient during the picking up of the pods, may have induced the respiratory symptoms due to previous sensitization to the ASA. After the investigations performed, by us the patient avoided her carrying out her work where *V. faba* was cultivated.

Furthermore, we have demonstrated an IgE-mediated mechanism of the reactions after ingestion of the seeds of broad beans, but we cannot exclude that the reactions during the picking up of broad beans could be caused by the inhalation of airborne allergens.

We concluded that the reactions after ingestion of fresh broad beans are IgE mediated, while the reactions that occurred during the harvest of vegetable can be explained by two diagnostic hypotheses:

(1) exposure to airborne allergens responsible for an immediate response,

(2) exposure to airborne salicylates responsible for a intolerance reaction.

Future studies could confirm these hypotheses and clarify this phenomenon.

We cannot exclude, however, that reports may increase over time, because the plants and the vegetables are increasingly exposed to aggressive agents and then tend to respond to these stimuli by increasing the synthesis of allergenic defensive proteins and volatile substances. This situation, therefore, could lead to an increase of cases of occupational allergies induced by volatile substances released from the vegetables in the surrounding air.

Conflict of Interests

The authors declare that they have no financial relationship with any biotechnology manufacturers that has an interest in the subject matter or materials discussed in the submitted paper.

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Case Report

Gum Arabic as a Cause of Occupational Allergy

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Background. Gum arabic is a potential sensitizer in food industry. *Methods*. We examined 11 candy factory workers referred to examinations due to respiratory and skin symptoms paying attention to exposure and sensitization to gum arabic. Skin tests, pulmonary function tests, and respiratory provocation tests were carried out as indicated by the symptoms and findings. *Results*. Occupational asthma, caused by gum arabic was diagnosed in 4/11 candy factory workers and two of them had also occupational contact urticaria and one had occupational rhinitis. One of them had oral symptoms associated with ingestion of products containing gum arabic. *Conclusions*. Airborne exposure to gum arabic may cause sensitization leading to allergic rhinitis, asthma, and urticaria.

1. Introduction

Gum arabic, or gum acacia is mainly derived from Acacia senegal tree. As a nontoxic material it is used as an emulsifier, a thickening agent, and as a stabilizer in foods, with E-code E414 [1]. It is useful in many kinds of foodstuffs because of its very low viscosity, complete solubility in water, and absence of any taste or odour. Due to technical properties gum arabic can be used in multiple applications like in pharmaceutical industry, lithography, and cosmetics.

Gum arabic is comprised of sugars and glucuronic acid residues in a long chain of galactosyl units with branched oligosaccharides attached to a polypeptide backbone. Protein content of gum arabic varies from 1 to 2%. IgE antibodies against polypeptide chains in gum arabic have been described to elicit asthma in occupational exposure [2]. Occasional cases of occupational asthma in printers [3], candy factory [4, 5], and pharmaceutical industry workers [2] have been described. Although gum arabic is extensively used in food industry ingestion of it is a rare cause of immediate allergy symptoms [1].

We describe several cases of occupational asthma caused by gum arabic among candy factory workers.

2. Methods

2.1. Patients. Eleven candy factory workers with respiratory and/or skin symptoms were referred to the Allergy Unit of Turku University Central Hospital (Table 1).

2.2. Workplace Description in the Candy Factory. This Finnish candy factory was a major producer of multiple goodies in Finland since 1910. Gum arabic was an important ingredient in many candies. In making soft pastilles, gum arabic was dissolved in water as kibbles without making dust in the air. Hard boiled candies, instead, were covered with spray dried gum arabic inside a rotating drum. Dry, powdered gum arabic, packed in 25 kg bags, was poured by workers into the drums. Cornstarch, used to cover liquorice, also made dust in the air.

Working clothes were a short-sleeve jacket and/or T-shirt and pants. The workers, who had to do cleaning used rubber gloves. Other protective equipment was not required but respiratory masks were available.

These patients were referred during the last operation year of the factory. In the preceding year the production of

Table 1: Patients of the candy factory examined for suspected occupational disease.

Patient sex/age years	Years in candy factory	Duration of symptoms	Symptoms	Diagnosis
F/41	8	6 months	Hives	Chronic nonoccupational urticaria
M/31	10	2.5 years	Hives	Work associated urticaria (carmine and house dust mites positive in skin prick tests)
F/32	8	2 months	Hives	Chronic non-occupational urticaria
F/50	16	9 months	Papular erythema of the hands	Occupational allergic eczema from rubber gloves
F/36	15	1.5 years	Dyspnoea, rhinitis, eye symptoms, hives	Occupational asthma, rhinitis and urticaria from gum arabic
F/54	10	6 years	Dyspnoea, rhinitis, eye symptoms, redness of the skin	Occupational asthma from gum arabic
F/43	13	3 years	Dyspnoea, rhinitis, eye symptoms, itching of the skin	Occupational asthma and urticaria from gum arabic
F/52	21	10 years	Dyspnoea, rhinitis, eye symptoms, hives	Occupational asthma from gum arabic
F/45	Unknown	2 months	Dyspnoea and cough	Laryngitis (non-occupational)
F/35	Unknown	4 months	Dyspnoea and cough	Allergic non-occupational asthma
F/38	10	3 years	Nasal congestion and secretion	Rhinitis (non-occupational)

the candy factory had been cut down stepwise before ending of the production. Hard boiled candies were the last products of the factory.

2.3. Lung Function Tests. Patients with lower respiratory tract symptoms underwent spirometries and bronchodilation tests with 0.4 mg inhaled salbutamol aerosol administered with a spacer. The dosimetric bronchial histamine challenge test using four stepwise increasing doses of histamine diphosphate solution (0.025, 0.1, 0.4 and 1.6 mg) was carried out using Spiro Electro 2 dosimeter (Spira Respiratory Care Center ltd, Hämeenlinna, Finland) as described by Sovijärvi et al. [6]. The patients were defined to have bronchial hyperresponsiveness if the provocative dose of histamine diphosphate causing a 15% fall in FEV1 (PD15) was 1.6 mg or less. Serial peak expiratory flow measurements (PEF) were carried out in every two hours during the awaketime for a minimum of two weeks period at work and home including at least two periods of free days [7]. PEF record was considered compatible for occupational asthma if there was at least 20% diurnal variation in two working days and less variation in free days and suggestive if there was not over 20% variation but the variation was clearly higher on working days. PEF record was not compatible with occupational asthma if no clear difference were found between working and free days. The fraction of nitric oxide in the exhaled air (eNO) was measured with Niox Mino portable device according to the instructions of the manufacturer.

2.4. Specific Bronchial Provocation Test. Specific bronchial provocation tests were performed in the Finnish Institute of Occupational Health in Helsinki with powdered gum arabic and by using lactose powder as a referent test. The provocation tests were done in a 8 m^3 challenge chamber according to international guidelines [8]. In both the active and the referent tests, the patient sat in the chamber for 30 minutes with the powder bowl in the front of her. The powder was dispersed in the air with compressed air once in every one to five minutes. PEF and FEV1 values were monitored for 24 hours after each challenge test with a pocketsize spirometer (One Flow, Sti Medical, Saint-Romans, France). A >20% fall in FEV1 or PEF values was considered as significant. The patient was also followed for clinical symptoms and lung auscultation.

2.5. Skin Prick Tests (SPT). SPTs were carried out with commercial common environmental allergens including birch, grass and mugwort pollens, cat, dog and horse epithelium, house dust mite, molds and latex (ALK-Abelló A/S, Hosholm, Denmark and concerning birch and timothy from February 2006 to October 2006 Allergopharma, Reinbek, Germany), and with gum arabic (Caesar & Loretz GmbH D-40721) 1 : 10 (w : v) in physiologic saline using a commercial one-peak lancet and prick-prick method. Depending on the exposure in the working place own powdered gum arabic and cornstarch and carmine red colour, all moisturized with saline, were also tested with SPT. Histamine dihydrochloride 10 mg/mL (ALK-Abello) was used as a positive control and the diluent (Soluprick, ALK-Abello) as a negative control. The largest diameter and the diameter opposite to it were measured at 15 min. A reaction was interpreted as positive

when the mean of the wheal diameters was at least 3 mm greater than that of the negative control.

2.6. Cutaneous Exposure Test. Open cutaneous application test was done on a skin area about 5 cm in diameter on the volar surface of the arm. Gum arabic powder (5 g) moisturized with saline was applied and gently removed at 15 min for the reading of the reaction. In addition to erythema an appearance of one or more wheals was interpreted as a positive reaction.

2.7. Patch Tests. Patch testing was carried out to one patient with eczematous skin disease according to standardized guidelines [9]. The allergens were derived from Chemotechnique (Vellinge, Sweden), and the application time was 48 hours. The final interpretation of the test reactions was done at 96 hours.

2.8. Serological Tests. Gum arabic specific IgE (Immuno CAP f297, Phadia) was measured in patients with suspected occupational asthma.

2.9. Definition of Occupational Asthma. The subject was defined to have occupational asthma due to gum arabic if the asthmatic symptoms worsened at the working place, there was positive skin prick test or specific IgE to gum arabic and a compatible PEF record with occupational asthma and/or positive challenge test. The aim was to confirm all cases by placebo-controlled bronchial challenge tests. One patient was not tested because her PEF recording was compatible with occupational asthma and she had strong oral symptoms of ingested gum arabic.

3. Results

3.1. Diseases of Candy Factory Workers. Six candy factory workers had occupational allergic disease (Table 1). Four patients had occupational asthma caused by gum arabic. Concomitant occupational contact urticaria was verified by the cutaneous exposure test in 2/4 of them and occupational rhinitis together with asthma in one of them in the specific bronchial provocation test. One other patient had occupational allergic contact dermatitis caused by thiuram chemicals. One patient had occupational urticaria caused by allergy to carmine red used in candies. There were no positive SPT reactions to cornstarch. No work-related allergies were found in five patients. Their symptoms were not clearly related to work and they were not sensitized to work-related allergens. One of them had atopic asthma, one had laryngitis probably caused by reflux disease and smoking and, one had rhinitis which was found not to be related to work. Two patients had chronic urticaria not related to work.

3.2. Occupational Asthma due to Gum Arabic in Four Candy Factory Workers. The workers with occupational asthma had been doing the same work for 10 to 21 years (mean 14.8 years) and experienced symptoms of the respiratory tract and skin for 1.5 to 10 years (mean 5.1 years). The characteristics of four candy factory workers with occupational asthma due to gum arabic are seen in Table 2. Figure 1 presents the change in PEF and Figure 2 the change in FEV1 in the challenge tests with lactose (negative control), gum arabic 10%, and gum arabic 100% in patients 1, 2, and 4.

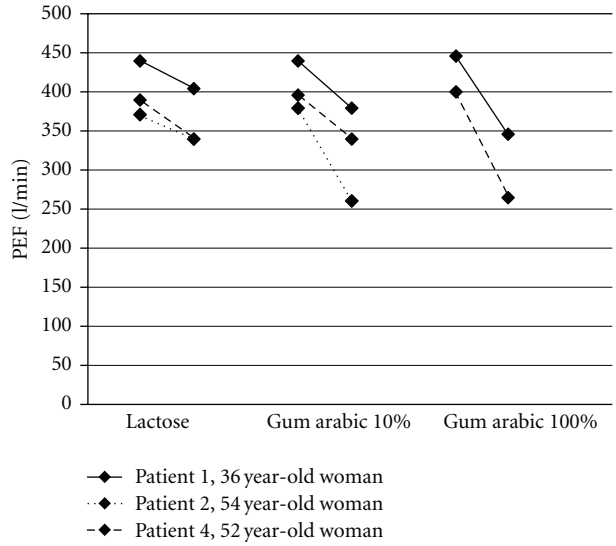

Figure 1: Change in PEF in the challenge tests with lactose (negative control), gum arabic 10%, and gum arabic 100%. All reactions to gum arabic were immediate reactions. The numbers of the patients refer to the numbers in Table 2.

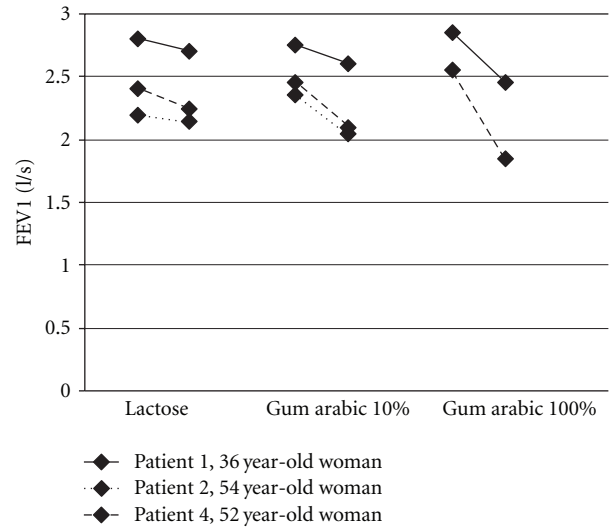

Figure 2: Change in FEV1 in the challenge tests with lactose (negative control), gum arabic 10%, and gum arabic 100%. All reactions to gum arabic were immediate reactions. The numbers of the patients refer to the numbers in Table 2.

3.3. Outcome of the Patients with Occupational Diseases. Patient 1 (in Table 2) with occupational asthma started re-education. The three other patients with occupational asthma continued to work in the factory until the production ended a few months later. They avoided exposure to gum

Table 2: The characteristics of four candy factory workers with occupational asthma due to gum arabic.

	Patient 1 36 year-old woman	Patient 2 54 year-old woman	Patient 3 43 year-old woman	Patient 4 52 year old woman
Duration of current work/duration of symptoms (years)	15/1.5	10/6	13/3	21/10
Sensitization to common allergens[1]	No	no	no	no
S-IgE (kU/l)[2]	78	39	104	338
SPT to gum arabic (mm)[3]	4	3	5	3
S-IgE to gum arabic (kU/l)	4.4	0.60	4.5	5.6
FEV1 liters (% of reference)	2.0 (64%)	2.5 (81%)	2.6 (86%)	2.3 (77%)
Compatibility of PEF records in working and free days to occupational asthma[4]	not compatible	suggestive	compatible	compatible
Exhaled NO (ppb)	43	21	20	19
Bronchial hyperresponsiveness in histamine challenge[5]	no	mild	mild	moderate
Bronchial challenge test with gum arabic	positive	positive	not done	positive

[1]Tree, grass and mugwort pollens, cat, dog and horse epithelium, house dust mite, molds, [2]Serum total immunoglobulin E level, [3]SPT = skin prick test, [4]PEF = peak expiratory flow, [5]strong hyperresponsiveness: histamine PD15 <0.1 mg, moderate hyperresponsiveness: histamine PD15 0.11–0.4 mg, mild hyperresponsiveness: histamine PD15 = 0.41–1.60 mg, no hyperresponsiveness: histamine PD15 >1.60 mg.

arabic. Six months after the work in the candy factory had ended patient 3 (in Table 2) was free of symptoms without asthma medication. She, however, experienced angioedema when ingesting gum arabic containing foods. In patients 1, 2, and 4 asthma was in control with medication, and they did not experience symptoms associated with gum arabic ingestion.

The patients with occupational skin diseases; caused by carmine red in one and by rubber chemicals in the other; were symptom-free when avoiding those allergens.

4. Discussion

Occupational asthma due to IgE-mediated allergy to gum arabic was diagnosed in four candy factory workers. Only one corresponding case has been reported in another Finnish candy factory [4], although the use of gum arabic in food industry is extensive [1]. Even though the occurrence of occupational allergy due to gum arabic is rare, it is possible that there is underreporting of the symptoms. In this study we did not survey the workers in the factory for sensitization to gum arabic and associated symptoms because of the approaching closure of the factory. We do not know whether more workers were sensitized to gum arabic and whether there were mildly symptomatic subjects who had not contacted a doctor.

Rhinitis is known to increase the risk of asthma by 3 to 5 times [10], and patients with occupational rhinitis have an increased risk of developing asthma [11]. Our patients had also rhinitis and skin symptoms. They had experienced symptoms for a variable, but rather long time before contacting the doctor. They probably did not contact the doctor until they had developed asthmatic symptoms.

All cases of occupational asthma in this factory were caused by gum arabic. In different candy factories carmine, pectin [12], milk, egg, nuts, seeds [13], spices, flavours, guar gum, and cornstarch may cause occupational allergy or nonspecific respiratory symptoms. In this study one worker, sensitized to carmine, was diagnosed to have work associated urticaria with minor respiratory symptoms. The workers who were diagnosed to have occupational asthma due to gum arabic had most symptoms at the working place when handling gum arabic powder.

SPTs with cornstarch were negative. Airborne cornstarch evidently caused mucosal irritation. We do not know whether exposure to cornstarch powder increased the symptoms due to gum arabic like airborne cornstarch seems to increase symptoms of latex allergy [14].

Latex must also be considered in candy factory workers as a cause of occupational urticaria and dermatitis, rhinitis, and asthma. In this study thiuram chemicals in rubber gloves had caused allergic contact dermatitis in one worker but all workers had a negative SPT to latex.

There is an exposure-response relationship between exposure to protein allergens such as wheat flour and alpha-amylase in bakeries and the development of occupational sensitization and symptoms [15, 16]. The production of this candy factory was concentrated on hard pastilles before closing the factory which increased the exposure to gum arabic. Increasing exposure to gum arabic probably caused the symptoms. The workers reported most symptoms in situations where exposure to gum arabic powder was the highest.

The sensitization route of these workers was the respiratory tract and/or the skin. Sensitization through the respiratory tract or skin to a food allergen may lead to the

subsequent development of symptoms during oral exposure as has been reported for sensitization to egg in bakery and confectionery workers [17], for lupine seeds in legume laboratory workers [18], for carmine in a worker engaged in dye manufacturing [19] and for fish [20]. Only one of these candy factory workers with occupational asthma reported oral symptoms associated with ingested gum arabic. Despite her asthma relieved after discontinuation of the exposure, the oral symptoms remained.

We have shown that gum arabic may cause occupational allergic rhinitis and asthma with urticaria symptoms. In this study the cases of occupational asthma in the candy factory appeared when there was an increase in the exposure. None of the patients had any previous atopic disease. Working methods which produce less powder and respiratory and skin protection are recommended. Early diagnosis of occupational allergy due to gum arabic is important in order to prevent the development of asthma.

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Review Article

Genetic Variability in Susceptibility to Occupational Respiratory Sensitization

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Respiratory sensitization can be caused by a variety of substances at workplaces, and the health and economic burden linked to allergic respiratory diseases continues to increase. Although the main factors that affect the onset of the symptoms are the types and intensity of allergen exposure, there is a wide range of interindividual variation in susceptibility to occupational/environmental sensitizers. A number of gene variants have been reported to be associated with various occupational allergic respiratory diseases. Examples of genes include, but are not limited to, genes involved in immune/inflammatory regulation, antioxidant defenses, and fibrotic processes. Most of these variants act in combination with other genes and environmental factors to modify disease progression, severity, or resolution after exposure to allergens. Therefore, understanding the role of genetic variability and the interaction between genetic and environmental/occupational factors provides new insights into disease etiology and may lead to the development of novel preventive and therapeutic strategies. This paper will focus on the current state of knowledge regarding genetic influences on allergic respiratory diseases, with specific emphasis on diisocyanate-induced asthma and chronic beryllium disease.

1. Introduction

Workplace allergens can be categorized as either high or low molecular weight allergens. Low molecular weight (LMW) allergens such as diisocyanates, acid anhydrides, and metallic salts are reactive chemicals with molecular weight less than 1000 kD. They act as haptens and can cause sensitization that may or may not be associated with specific immunoglobulin E (IgE). While some LMW agents such as acid anhydrides, platinum salts, and persulfates stimulate IgE antibodies, many others including isocyanates and glutaraldehyde rarely cause IgE-mediated sensitization [1]. On the other hand, high molecular weight (HMW) protein-derived agents (e.g., proteases, flour, and laboratory animal allergens) cause allergic sensitization through mechanisms mediated by IgE [2]. Early detection of sensitization is very important since sensitized individuals can have life-threatening reactions to future exposures even years after the cessation of exposure. Although the risk of sensitization for individuals with underlying atopy is higher for some exposures (particularly IgE-mediated responses), high prevalence (around 20%) of atopy in the general population indicates that atopy alone is not the determining factor [3]. Although the main factors that affect the onset of the symptoms are the types, duration, and intensity of allergen exposure, host genetic factors can modulate how individuals interact with these agents and induce a shift in the dose-response relationship [4]. Recent genetic epidemiology research focused on common gene variants and identified a number of genetic associations and gene-environment interactions for allergic respiratory diseases. Understanding gene-environment interactions is especially important to improve occupational and public health since environmental/occupational factors that influence genetic risk are modifiable. In this respect, the results of molecular epidemiology studies have the potential to be used in risk evaluation and to help determine more accurate safe occupational exposure levels, thereby contributing to improved protection of workers at high risk. This paper will

summarize the contribution of genetic variability to two important occupational respiratory diseases, diisocyanate-induced asthma (DA) and chronic beryllium disease (CBD).

2. Occupational Asthma Caused by Diisocyanates

Among LMW substances, the diisocyanates are the most frequently reported cause of respiratory sensitization in the workplace. These agents are widely used in polymerization reactions for manufacturing surface coatings, varnishes, paints, urethane foams, insulation, and adhesives. Workers in these industries and workers that use these end products may be influenced by potential adverse health effects of such chemicals. National Occupational Exposure Survey database, National Institute for Occupational Safety and Health (NIOSH), showed that at least 280,000 workers were potentially exposed to some form of isocyanates in the United States alone [5]. Isocyanates are the leading cause of occupational asthma (OA), estimated to cause asthma in 5–10% of chronically exposed workers [6–8]. Despite improved industrial hygiene efforts, new cases of OA continue to occur [9, 10]. The most common isomers used in industry are: the aliphatic agent 1,6-hexamethylene diisocyanate (HDI), used principally as a hardener in spray paints, 4,4-di-phenylmethane diisocyanate (MDI), and toluene diisocyanate (TDI). Early diagnosis of OA leads to favorable clinical outcomes (i.e., less risk of chronic and severe asthma) if affected workers are promptly recognized and removed from harmful exposure [9, 11]. In addition to early case detection, it is also important to more closely monitor the most susceptible workers at a preclinical stage.

Genetic epidemiologic studies have identified a number of susceptibility markers for a variety of asthma phenotypes including OA. Most of these genetic studies were hindered by difficulty in defining asthma, a complex phenotype representing allergic and nonallergic types. This has led to selection of intermediate or quantifiable phenotypes (e.g., airway hyperresponsiveness, lung function, and serum IgE levels) in some association studies. On the other hand, OA is a unique model in that the phenotype can be defined accurately by specific inhalation challenge (SIC) testing often considered the gold standard for diagnosing OA [12]. For this reason, OA is an excellent model for studying gene-environment interactions since the causal agent can be identified with SIC and the lag period between initial exposure and onset of sensitization and clinical symptoms can be followed [13].

Given its immune-inflammatory nature, OA phenotypes are likely associated with specific variants of immune-/inflammatory-related genes. Linkage studies have suggested a variety of candidate genes for asthma and related phenotypes in chromosomal regions 2q14-q32, 5q31-q33, 6p24-p21, 7p15-p14, 11q13-q21, 12q21-q24, 13q12-q14, and 20p13 [14–21]. In particular, variants of interleukin (IL)-4, IL-4RA, IL-13, β-adrenergic receptor (β-AR), tumor necrosis factor (TNF)-α, human leukocyte antigen (HLA)-DRB1, DQB1, the β-subunit of the high-affinity IgE receptor (FceRI), CD14, a disintegrin and a metalloproteinase 33 (ADAM33) genes have been consistently associated with asthma-related phenotypes in independent studies [22, 23]. As in other forms of asthma, inflammatory changes and allergen-specific T-lymphocytes are found in the airways of many patients with OA, along with eosinophils, cytokines, and serum IgE antibodies [24–26]. Thus, similar genetic associations as in immune-mediated asthma might be expected to occur in OA. Although a number of genetic association studies have been conducted on individuals with allergic asthma from environmental causes, there are only limited studies on OA.

The Human Leukocyte Antigen (HLA) class II molecules play a role in the presentation of intracellularly processed peptides to CD4+ T-helper cells. HLA class II molecules are highly polymorphic and the variations in their protein structure may determine the specific epitopes presented to T cells. Therefore, HLA class II molecules are also plausible candidates for controlling specific immunological responses to allergens. Genetic studies investigating the immunopathogenesis of OA have focused on HLA Class II alleles. Bignon et al. demonstrated that HLA DQB1*0503 and the allelic combination DQB1*0201/0301 were associated with susceptibility to DA [27] whereas the DQB1*0501 allele and the DQA1*0101-DQB1*0501-DR1 haplotype were considered protective. Subsequently, Mapp et al. confirmed the association with HLA-DQB1*0503 and reported that the DQA1*0104 allele was increased in DA compared with asymptomatic exposed workers. They also showed that "protective" alleles, HLA-DQB1*0501 and DQA1*0101, were increased in asymptomatic exposed workers versus those with DA [19]. In another study, a significantly higher proportion of subjects with DA were found to express the HLA-DQB1*0503-associated aspartic acid at residue 57 [28]. HLA associations with DA were also investigated in a population of Asian workers exposed to diisocyanates but the associations found in European workers were not entirely replicated. The HLA haplotypes DRB1*15-DPB1*05 and HLA DRB1*1501-DQB1*0602-DPB1*0501 were reported as a susceptibility marker for the development of TDI-induced asthma in Koreans [29, 30]. Bernstein et al., investigated association between known SNPs in immune response genes (IL-4Rα, IL-13, and CD14) and DA in a group of exposed workers undergoing SIC testing. The results demonstrated increased frequencies of IL-4RA I50V allele and combinatorial genotypes of IL4RA (I50V), IL-13 (R110Q), and CD14 (C159T) in HDI-exposed workers suggesting an exposure-specific interaction [31]. These finding supported the notion that immune mechanisms play an important role in the pathogenesis of DA.

Since isocyanates are known to cause oxidative injury to respiratory epithelial cells, variations within antioxidant defense genes have been examined in workers with DA [32]. Glutathione, a major antioxidant protein found in the bronchial lining fluid and in respiratory epithelial cells, is likely to serve a protective function by binding with free isocyanate molecules and, thereby, preventing damage to respiratory epithelial cells or intracellular binding to respiratory epithelial proteins or proteins in the bronchial lumen [33]. Piirilä et al. examined polymorphisms of the glutathione S-transferase (GST) genes (GSTM1, GSTM3, GSTP1, and GSTT1) in

workers with DA. GSTM1 null genotype was associated with a 1.89-fold risk of DA. Subjects with GSTM1 null and GSTM3 AA genotypes developed late reaction in the specific bronchial provocation test with diisocyanates, individually or in combination [34]. Later, Mapp et al. assessed the GSTP1 gene in TDI-exposed asymptomatic and asthmatic workers [18]. The frequency of the GSTP1 Ile105Val Val/Val genotype was lower in subjects with DA, and was significantly lower among subjects with airway hyperresponsiveness. In another study, the N-acetyltransferase (NAT1) slow acetylator genotype was associated with a 2.5-fold risk of OA among diisocyanate-exposed workers. Interestingly, a far greater 7.77-fold risk of OA was reported among workers exposed to TDI, suggesting an exposure-specific association. In addition, a gene-gene interactive effect was identified in diisocyanate-exposed workers with the combined NAT1 or NAT2 slow acetylator genotypes and GSTM1 null genotype [35]. Broberg et al. investigated the influence of variants in TDI-metabolizing genes on the associations between TDI in air (2,4-TDI and 2,6-TDI) and its metabolites toluene di-amine (2,4-TDA and 2,6-TDA) in plasma and urine. Their results showed that the GSTP1 Ile105Val variant modifies the association between 2,4-TDA in plasma and in urine, supporting the importance of GST system for the metabolism of TDI [36]. Based on the role of neurogenic inflammation in TDI-induced airway hyperresponsiveness, the association between neurokinin-2 receptor (NK2R) gene polymorphisms and TDI-induced asthma was investigated in a Korean population. An association was found between the NK2R 7853GG genotype and increased serum VEGF levels, suggesting that NK2R variants may modulate the airway inflammation conferred by VEGF [37]. Another Korean study investigated the possible role of β2-adrenergic receptor gene (ADRB2) polymorphisms in TDI-induced asthma. The Arg16Gly A>G, Leu134Leu G>A, and Arg175Arg C>A SNPs and haplotype [TTACGC] were found to be associated with specific IgE sensitization in TDI-exposed workers [38]. In another study, genome-wide association was performed to identify susceptibility alleles associated with asthma induced by TDI. The results showed significant association between genetic polymorphisms (rs10762058, rs7088181, rs4378283, and rs1786929) of catenin α3 (CTNNA3) and susceptibility to TDI-induced asthma [39]. The CTNNA3 variants have been suggested to influence TDI-induced asthma risk by increasing epithelial damage and airway inflammation.

3. Chronic Beryllium Disease

Chronic beryllium disease (CBD) is a serious granulomatous lung disease caused by beryllium (Be) exposure in the workplace. CBD continues to occur in industries where Be is manufactured and processed such as aerospace, nuclear, automotive, and electronics. NIOSH estimated that up to 134,000 workers in the United States were exposed to beryllium [40]. Be exposure leads to a cell-mediated hypersensitivity (delayed, type IV) reaction in which Be haptenates native proteins leading to the production of the specific allergen [41]. It is known that accumulation of Be-specific CD4(+) T cells and persistent lung inflammation play a key role in the immunopathogenesis of CBD. Prior to the development of CBD, many exposed workers become sensitized and many of those eventually develop CBD. Approximately 50% of sensitized individuals have CBD at initial clinical evaluation [42]. Be-specific T-cell proliferative responses are detected in the blood of exposed workers using the Be lymphocyte proliferation test (BeLPT) [43, 44]. The BeLPT has been shown to identify approximately 70 to 94% of cases of BeS and CBD and widely used in screening and surveillance of Be-exposed workers [45–47]. Epidemiological studies showed the prevalence rates of BeS and CBD to be between 5–21% and 3–21% among beryllium workers, respectively [48, 49]. The pathologic progression from BeS to CBD is not well understood warranting further research into the pathophysiological mechanisms and susceptibility markers of BeS and CBD. Such efforts will be important for early detection and disease prevention in Be-exposed workers.

A number of molecular epidemiology studies showed that the presence of glutamic acid in position 69 of the B1 chain of the HLA-DPB1 molecule confers an increased risk for both BeS and CBD [41, 50–54]. The HLA-DPB1Glu69 frequency was reported to be between 39–90% in sensitized workers and 53–97% in workers with CBD as compared to 19–48% in nonsensitized workers [41, 50–52, 55–61]. Importantly, studies have demonstrated a dose-dependent effect of HLA-DPB1Glu69 alleles suggesting that Glu69 is a potential marker of disease severity in addition to overall disease risk [41]. Although HLA-DPB1Glu69 is more frequent in individuals with BeS and CBD (73–95%), 30–40% of exposed workers carrying HLA-DPB1Glu69 do not develop CBD or BeS [41, 50, 62]. This suggests that other host and environmental factors likely play key roles in the pathogenesis of CBD. Studies investigating the interaction between the HLA-DPB1 Glu69 and Be exposure showed independent and additive effects of Glu69 carriage and Be exposure in the development of BeS and CBD [58, 63].

Rossman et al. reported that HLA-DQB1Gly86 and HLA-DRB1Ser11 alleles occurred more often in individuals with CBD [55]. Maier et al. found that HLA-DRB1*01 and DQB1*05 alleles were less frequent in workers with CBD. They also reported that DRB1*13 and DQB1*06 were associated with CBD in the absence of Glu69 [41]. A recent study showed that the DRβE71 allele is a risk factor for both CBD and BeS in the absence of Glu69 and highlighted the importance of interactions between peptides and T cells in the development of CBD [61]. Chemically specific Be-protein interactions were also investigated using a computational approach. Glu69 alleloforms with the greatest negative surface charge were found to confer the highest risk for CBD and irrespective of allele, equal risk for BeS [64]. Current HLA research includes investigating whether the risk is associated with any or only certain Glu69 alleles or allelic combinations.

Non-HLA genetic studies also identified some significant associations. Sato et al. investigated eight SNPs within CCR5 gene that is implicated in the chemotaxis and activation of leukocyte subsets. The results showed that CCR5-5663 and -3458 variants were associated with worsening pulmonary

Table 1: Examples of genetic associations for DA and CBD.

Disease	Gene	Variation	RR; P value; OR (95% CI)	Reference
DA				
	HLA-DQB1	*0503	RR = 9.8, $P < .04$	[27]
	HLA-DQB1	*0501	RR = 0.14, $P < .03$	[27]
	HLA-DQA1	*0104	$P = .008$	[19]
	HLA-DRB1-DPB1	*15*05	$P = .001$	[29]
	GSTM1	Null	1.89 (1.01–3.52)	[34]
	NAT1	Slow acetylator	7.77 (1.18–51.6)	[35]
	IL4RA, IL-13 R, CD14	I50V-R110Q-C159T	6.4 (1.57–26.12)	[31]
	CTNNA3	rs1786929	$P = .015$	[39]
CBD				
	HLA-DPB1	Glu69	9.4 (5.4–16.6)	[50]
	HLA-DQB1	G86	$P < .04$	[55]
	HLA-DRB1	S11	$P < .03$	[55]
	CCR5	-3458	$P < .0001$	[65]
	TGFβ1	-509	$P = .01$	[66]
	GCLC	TNR 7/7	0.28 (0.08–0.95)	[67]
	GCLM	-588 C/C	3.07 (1.00–9.37)	[67]
	IL-1A	-1142	3.02 (1.36–6.70)	[69]
		-3769	2.51 (1.21–5.19)	[69]
		-4697	2.56 (1.24–5.29)	[69]

RR: relative risk; OR: odds ratio.

function over time in CBD [65]. The -509C and codon 10T variants of the transforming growth factor-β1 (TGFβ) gene, a cytokine with a major role in the fibrotic/Th1 response, were found to be associated with more severe granulomatous disease in CBD [66]. Since glutathione has been reported to be increased in CBD, genetic variants of the glutamate cysteine ligase (GCL), a rate-limiting enzyme in GSH synthesis, were investigated. GCL consists of a catalytic subunit (GCLC) and modifier subunit (GCLM). GCLC trinucleotide repeat polymorphism (7/7 genotype) and the GCLM-588 SNP were found to be associated with altered susceptibility to CBD [67]. While Saltini et al. reported an association between the TNFα-308*02 variant and BeS and CBD, this result was not confirmed in a large population-based study [57, 68]. A recent study showed that IL-1α-1142, -3769, and -4697 variants were significantly associated with CBD compared to individuals with BeS or nonsensitized workers after adjusting for Glu69 status [69]. These results suggested that the formation of granulomas in CBD may require an independent inflammatory response controlled by genes unrelated to beryllium recognition. Table 1 lists some examples of associations found for both DA and CBD.

4. Conclusions

Genetic association studies can provide more accurate information on the interindividual variability, thereby contributing to establishment of more accurate exposure limits in the workplace. These efforts, in a larger perspective, provide opportunities to effectively target engineering controls, personal protective equipment, and intervention strategies to protect the health of high-risk workers. With the advances in high-throughput technologies and computational methodologies, this information could be used in designing better predictive models to incorporate genetic variability into risk evaluation and improving the regulation and redefinition of acceptable exposure levels in the workplace. Success of such approaches depends on how molecular epidemiology studies overcome some of the current challenges. Despite the rapid growth of published associations, some of the genetic associations lack consistency across different studies. The inconsistency in results might be explained by the differences in study populations, phenotype characterization, exposure assessment, characterization of other environmental exposure (e.g., air pollution, smoking), intermediate phenotypes (e.g., airway hyperresponsiveness), statistical inconsistencies and other potentially modifiable risk factors such as lifestyle. For example, allele/carrier frequencies of the HLA-DPB1Glu69 ranged between 0.21/0.38 to 0.33/0.59 across different ethnic populations [70]. This emphasizes the importance of replication studies in independent populations with a different genetic background. Although the genetics of allergic respiratory diseases including DA and CBD have yet to be fully characterized, summarized discoveries hold promise for the identification of susceptibility profiles and characterization of gene-environment interactions. It is to be hoped that future genetic association studies with large, well-characterized populations through national and international collaborations will increase the understanding of the pathogenesis of these diseases and help identify novel therapeutic targets and preventative/educational strategies for better identification and management of occupational diseases.

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Review Article

Haptenation: Chemical Reactivity and Protein Binding

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Low molecular weight chemical (LMW) allergens are commonly referred to as haptens. Haptens must complex with proteins to be recognized by the immune system. The majority of occupationally related haptens are reactive, electrophilic chemicals, or are metabolized to reactive metabolites that form covalent bonds with nucleophilic centers on proteins. Nonelectrophilic protein binding may occur through disulfide exchange, coordinate covalent binding onto metal ions on metalloproteins or of metal allergens, themselves, to the major histocompatibility complex. Recent chemical reactivity kinetic studies suggest that the rate of protein binding is a major determinant of allergenic potency; however, electrophilic strength does not seem to predict the ability of a hapten to skew the response between Th1 and Th2. Modern proteomic mass spectrometry methods that allow detailed delineation of potential differences in protein binding sites may be valuable in predicting if a chemical will stimulate an immediate or delayed hypersensitivity. Chemical aspects related to both reactivity and protein-specific binding are discussed.

1. Introduction

The term, "hapten," was coined by Landsteiner and Jacobs [1] and is derived from the Greek "hapten", meaning "to fasten." Haptens are low molecular weight (LMW; <1000 daltons) chemicals that must bind to a carrier molecule to be antigenic. The carrier is usually an endogenous or exogenous protein to which the LMW chemical is covalently bound. The hapten hypothesis was originally proposed to explain both humoral and cellular immune responses to LMW chemicals observed by Landsteiner and Jacobs [1] in their research. The absolute requirement for covalent binding of a hapten to a protein for immune recognition in the development of all drug/LMW chemical allergies has been challenged in recent years [2], but substantial evidence exists for this to be a prominent mechanism through which chemicals and drugs or their metabolites become antigenic.

The role of chemical reactivity has been proposed to be one of the major determinants in allergic contact dermatitis (ACD). Over the years, extensive databases containing representative chemicals that are skin sensitizers have been published [3–5]. In the context of occupational health, predictive toxicology, and ensuring overall safety of manufactured products, it is important that skin sensitization potential of new and existing chemicals be assessed. The use of guinea pigs has been the experimental model of choice in evaluating the skin sensitization potential of chemicals [6, 7] until about a decade ago when the local lymph node assay (LLNA) was adopted after extensive interlaboratory validation [8].

Although ACD is a Type IV hypersensitivity response, the ability of a chemical to bind to macromolecules is also thought to be important for immediate (Type I) hypersensitivity sensitization and reactions in both the skin and lung. Presently, why a specific LMW chemical predominately skews the immune system toward Type I versus Type IV hypersensitivity is not known. Electrophilic reactivity alone does not seem to distinguish respiratory and dermal sensitizers such as toluene diisocyanate and dichlorobenzene, respectively. Selective protein targets or sites on a protein may be important and recent advances in protein mass spectrometric analysis now provide the capability to better explore how and where such chemicals bind. The present paper discusses these physical chemical aspects related to formation of the hapten-carrier protein complex.

2. Electrophiles and Reactivity

The hapten hypothesis was developed from the interaction of nucleophilic moieties on proteins with chemicals that are electrophilic. Adduct formation has been demonstrated to be more feasible with electrophilic chemicals [9]. In the following description, haptenation within the skin is discussed, as most of the research knowledge gained has been through examining the relationship between chemical reactivity and allergenicity in this organ system. An analysis of two published databases [4, 5] containing more than 300 chemicals demonstrated to be allergens by the LLNA reveals that approximately 40% of the skin sensitizers have at least an electrophilic center that is amenable to nucleophilic attack. From an organic chemistry perspective, formation of such adducts is via covalent bonds and to a certain extent coordination bonds. This is chiefly because covalent and coordination bonds have bond energies ranging from 200 to 420 kJ/mol compared to hydrophobic, dipolar, and ionic interactions with bond energies <50 kJ/mol. The high bond energies enable covalent adducts to survive the intracellular antigen processing of the haptenated protein into short peptides for cell surface expression by MHC complexes.

Both guinea pig ACD models and LLNA data have been used to develop a number of structural-activity-relationship models relating chemical reactivity and hydrophobicity to skin sensitization potency [10]. Hapten reactivity data generated from our laboratory [11] supports a central role for chemical reactivity in allergic sensitization. Reactivity rate constants (k) were obtained for twenty five electrophilic haptens using a thiol-based probe, 4-nitrobenzenethiol (NBT). This k is a measure of the speed at which an electrophilic hapten will bind to a nucleophilic center. A very high correlation was obtained between allergic potency (EC3) of electrophilic haptens as determined by the LLNA and the reactivity rate constants ($r^2 = 0.74$, independent of reactivity domain). More recently, we have developed a similar assay using an amine-based probe, and preliminary results suggest that a similar relationship between EC3 and reactivity with a nucleophilic amine exists [12].

Employing chemical reactivity as an endpoint to probe target toxicity is neither restricted to skin sensitization nor is it a new concept. For example, the ability of a chemical to react with biomolecules has been used as a predictor for aquatic toxicity [13, 14] and carcinogenesis [15]. Landsteiner and Jacobs [1] noted correlations between a chemical's ability to cause skin sensitization in guinea pigs with its reactivity to aniline [1]. Interestingly, Landsteiner and Jacobs optimized their chemical reactions by altering pH or elevating the reaction temperature to observe reaction to nucleophiles like butylamine and aniline. It has to be noted that many covalent adducts that would otherwise form under harsh conditions are not formed as reaction conditions (i.e., temperature, pH) and medium are adjusted to physiological-like conditions. More recently, reactivity assays utilizing cysteine, lysine, glutathione, and several model peptides have been developed and efforts are underway to validate them as alternative *in chemico* methods for screening skin sensitizers [16–19]. The underlying concept for all these assays is electrophile-nucleophile interaction.

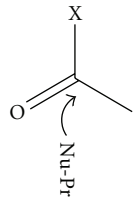
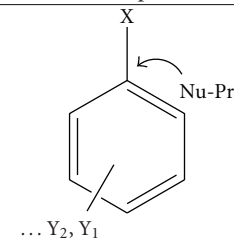
An understanding of the chemistry of electrophiles is required to produce qualitative and quantitative data and also for determination of an appropriate solvent system for reactivity assessment. For example, cinnamic aldehyde (EC3 = 3; [20]) and bromohexadecane (EC3 = 1.75; [21]) which are both moderate sensitizers in the LLNA require different solvent systems (*in vivo, in vitro, and in chemico)* for assessment of allergenicity. While all chemicals with reactive electrophilic centers will form covalent adducts with proteins, mechanistic pathways are different for different chemicals and may determine the type and strength of adduct formed. Table 1 illustrates the five common mechanistic domains and the electrophilic centers amenable to nucleophilic attack. An extensive analysis of mechanistic domains, their subcategories, and "special cases (domain not clearly defined or >1 domain for a single chemical)" has been discussed in recent reviews [9, 22, 23]. Mechanistic domains for protein interaction should not be confused with chemical classes, which are structural-based classifications. Mechanistic domains are functional reaction groups that are targeted as alerting pointers to a chemical's possible reaction with a protein and thus classify chemicals based on their reaction chemistry. For example, benzoquinone and 2,3-butanedione are both ketones but they belong to the Michael acceptor (MA) and Schiff base former (SBF) mechanistic domains, respectively.

Use of chemical classes instead of mechanistic domains has been noted to have a number of limitations. The relative alkylation index (RAI) of Roberts and Williams was developed using chemical classes and could only predict reactions of a select group of chemical classes [24]. When the RAI was modified to predict protein haptenation based on mechanistic domains, it attained applicability to a wider range of chemicals as discussed by Patlewicz et al. [10]. Our initial experience with 19 chemicals spanning three mechanistic domains (MA, S_N1/S_N2, AA) suggests that restricting correlations between k and LLNA data to mechanistic domains was not necessary to provide a good prediction of an electrophilic hapten's allergenic potency [11]. Despite a good prediction of allergenic potency that was obtained across all binding mechanisms, separation of allergens by mechanistic domain improved the correlations from linear regression analysis. A further comparison of MA and S_N1/S_N2 to EC3 by regression analysis reveals similar slopes, but different Y-intercepts ($P = .006$), confirming that the mechanism of electrophilic binding does influence allergenic potency.

3. Hapten Bioavailability and Reactivity Methods Development

Another major physical chemical consideration involves the bioavailability of the hapten. For skin, this is penetration of the chemical across the stratum corneum and is thought to be mainly a function of the chemical's solubility (octanol/water partitioning; $\log P$). Models for prediction of skin sensitization take this into consideration

Table 1: Common mechanistic domains.

Mechanistic domain				
Michael acceptors	Acylating agents	Schiff base formers	S_NAr electrophiles	S_N1/S_N2 electrophile
				
X = electron withdrawing	X = electron withdrawing and a good leaving group.	Attacking nucleophile is an amine (NH_2-)	X = usually a halogen or pseudohalogen. Y_1,Y_2 = electron withdrawing	X = usually electron withdrawing and a good leaving group.

and usually include both reactivity and hydrophobicity parameters. While the inclusion of penetration rate may be ideal for accurate prediction of skin sensitization potential, in practice, it is not a significant parameter [11, 25] as hapten reactivity rates alone highly correlate with LLNA potency. Most electrophilic haptens tested in our model fall into the desired range of log P values (-1.4 to 4) for skin absorption and lipophilicity. It is possible that bioavailability may exert a greater influence on allergenic potency for extremely hydrophilic and lipophilic haptens. Roberts and Natsch [26] included both reactivity and hydrophobicity parameters in their modeling of allergenic potency and noted that the influence of reactivity was greater than that of solubility for predicting allergenic potency.

Methods for the assessment of electrophilic chemical allergen binding to (protein) nucleophiles developed in recent years have been primarily non-kinetic-based assays that measure the loss of the unconjugated nucleophile. Development of these methods is based on the assumption that hapten bioavailability for chemicals with log P values between -1.4 and 4 is approximately the same. The nucleophilic probes reported include glutathione [16, 18, 27] or model peptides with a free cysteine thiol or lysine amine [17, 28]. These assays report percent depletion as the reactivity index for a given chemical. The rationale for design of a particular synthetic peptide, including the choice of neighboring amino acids in the hepta-peptides and why only seven amino-acid peptides are used, has not been delineated for most of the peptide probes proposed. The exception is the peptide, Ac-NKKCDLF (Cor1-C420) [29], derived from AA417-423 of the human Coronin 1 protein, where the Cys420 is thought to be highly reactive to electrophiles [30]. The original HPLC-based peptide reactivity assay has since had numerous modifications and improvements including the inclusion of LC-MS to characterize the adducts [29, 31] and configuring it to high throughput kinetic profiling for more accurate determination of rate constants [26]. The modifications seek to interrogate the chemistry behind the peptide depletion by the electrophilic skin sensitizers and also begin to move towards high throughput assay development. The generation of reaction kinetics data was another important aspect that had been lacking in the original peptide reactivity assay. Data on peptide depletion based on varying initial electrophile concentrations [26] results in more accurate determination of reactivity constants as opposed to derivation of the RC50 (2 h assay) [18, 27] and peptide depletion (24 h assay) [32] as reactivity indices. These endpoint determinations do not adequately capture the nature of the chemical kinetics involved in these electrophile-nucleophile interactions. The fact that values are measured at fixed time points under pseudo-first-order conditions (electrophile $\gg$ peptide) is a limitation of these assays. Fixed time points of several hours do not take into consideration the initial reaction and chemical kinetics involved which have a bearing on whether the reaction with the peptide is going to be linear or not throughout its duration.

The high throughput kinetic profiling (HTKP) method [26] was able to address some of the shortcomings of earlier reactivity assays with respect to reaction times and the chemistry of electrophiles that do not adhere to pseudo-first order kinetics. Measurements of peptide depletion/reactivity were done at several time points for varying initial concentrations of the sensitizers, and compensations were made for the "drowning out effects" [26] and loss of test chemicals due to evaporation [26]. While this presented a breakthrough in terms of determining more accurate rate constants that could be tied to LLNA potency of the electrophiles, determining rates of rapidly reacting sensitizers such as benzoquinone and nitrobenzyl bromide still presented challenges and the rate constants for these sensitizers had to be estimated rather than measured. The application of stopped flow techniques [11] to measure the rate constants of these rapid electrophile-nucleophile interactions introduced a novel chemoassay that was superior with respect to the detectable range of electrophilic reactivity. Other confounders such as potential loss of nucleophile due to evaporation and even oxidation were eliminated ensuring the measurement of reaction rates from solution kinetics. Using this technique, the modeling and correlations of reactivity constants to LLNA data were able to utilize measured rate constants instead of estimates.

Our current method utilizes the depletion of NBT with the assumption that the electrophile-nucleophile reactions are characterized by adduct formation. Peptide binding

studies [31], which have included characterization of the chemical products formed, have noted oxidation of the peptide thiol producing an apparent loss of parent peptide whenever there was absence of adduct formation. The buffered organic media precluded oxidation of nucleophile to species other than the disulfides of which the rate would have been slower than the electrophile-nucleophile reaction. We are currently evaluating an amine-based probe, pyridoxylamine (PDA) that will better assess amine-selective electrophiles. This assay, which captures adduct formation without interference from side reactions like oxidations, might be of greater utility than the previously reported assays where complications arose from side reactions. Several advantages in screening for hapten potency by binding to NBT and PDA include the ability to quickly obtain both initial and overall binding rates for both extremely fast or slow reactions, increased accuracy of rate constants, analysis which can be conducted on relatively inexpensive stopped-flow spectrophotometers, and the ability to model potential reaction mechanisms, competing mechanisms, and intermediate products.

4. Nonelectrophilic Haptens

The allergenicity of nonelectrophilic compounds and metals, which have not been shown to be metabolically bioactivated cannot be explained by direct electrophile-nucleophile interaction chemistry. When the potential for chemicals to induce mutations in *Salmonella* was used as a surrogate for electrophilicity [33], correlation of electrophilicity with occurrence of ACD in humans from 355 randomly chosen chemical allergens demonstrated that only 30%–40% of the contact allergens were electrophilic. Nonelectrophilic compounds have been studied including thiols such as the rubber accelerator allergens, mercaptobenzothiazole (MBT), mercaptobenzothiazole disulfide (MBTS), zinc diethyldithiocarbamate (ZDEC), and tetraethylthiuram disulfide (TETD). Metabolic activation to electrophilic metabolites may account for the potency of a portion of these allergens; however, haptenation mechanisms other than electrophile-nucleophile interactions have been proposed. Guinea pig studies with MBT and several structural analogues demonstrated that sensitization was most likely a result of protein haptenation via disulfide formation [34]. ACD cases which have also been reported for chemicals like diallyl disulfide [35] and lipoic acid [36] which are thiols further indicate that disulfide formation may be a common mechanism for chemical thiol haptenation. The conclusion that MBT/MBTS haptenates proteins via disulfide formation was supported by enzyme inhibition and protein-binding studies where binding of MBTS to enzymes (reductases) and other protein cysteine residues was through disulfide formation [37]. Bioactivation of MBT, which could potentially lead to an electrophilic hapten was not observed [37], suggesting that MBT is not a metabolically activated prohapten. To date, there are no reports of MBT bioactivation by cutaneous cytochrome P450s (CYPs) enzymes.

TETD has also been shown to haptenate proteins through the formation of disulfide linkages [38]. As a strong ligand, TETD binds coordinatively to metalloproteins, the mechanism by which it inactivates aldehyde dehydrogenase [39] and anhydrases [40]. Whether the same properties (binding and inactivating dehydrogenase) that make TETD a suitable alcohol abuse deterrent apply to skin sensitization biology is yet to be determined. If accessible, chemicals like TETD and ZDEC (through transmetallation) will chelate the metal ions in a porphyrin center. Absorbance measurements, dialysis experiments, and mass spectrometry after haptenation of zinc/copper-superoxide dismutase (SOD) with ZDEC indicated that the DEC from ZDEC were strongly chelated to the copper ion on SOD [38, 41, 42]. The lack of binding between ZDEC and the apoenzyme was confirmatory of the chelation chemistry being the probable mechanism of haptenation [38]. It has to be noted though, that ZDEC and TETD, unlike MBT, can potentially be metabolized to electrophilic species, through sulfoxidation [43], which would then haptenate proteins through electrophile-nucleophile interactions. Human cytochrome P450 enzyme that can metabolize the thiocarbamates has been identified [43]. Contact allergens may also undergo nonenzyme catalyzed, air oxidation to electrophilic intermediates. Lepoittevin [44] suggested the separate classification term, prehapten, to refer to chemicals that undergo nonenzymatic transformation to the active form.

5. Metal Allergens

The formation of coordinate bonds has been touted as the mechanism behind metal ion-induced allergies. Commonly encountered metal allergens are transition and trace metals which include nickel, cobalt, chromium, beryllium, platinum, and gold [45]. Binding of metals to proteins stems from the polarized nature of the metal atoms which allows them to accept electrons from electron rich ligands. Metals are capable of forming geometrically, highly defined coordination complexes with four or six electron donors. The electron donors are mainly nitrogen or oxygen in amino acid side chains of appropriate proteins or peptides [46]. The binding of nickel to albumins was shown to have the capacity to stimulate Ni-reactive T cells in the presence of appropriate antigen presenting cells (APC) [47–49]. The comparable response of T cells to determinants formed by hapten peptides in a major histocompatibility complex- (MHC-) binding groove versus the Ni–MHC–peptide complexes strongly suggested that the coordinate binding is a feasible mechanism for metal-induced allergies [50]. Thus metals represent nonclassical haptens in the sense that coordinate bonds which form metal-protein complexes are not sufficiently strong to survive antigen processing that classical haptens undergo. The binding of metals like Ni to cell surface proteins like MHC would indicate a more plausible mechanism as it bypasses intracellular antigen processing steps. This type of protein binding would suggest that sensitization to metals is protein independent as long as the cell surface protein is able to chelate the metal and present it to T cells. This protein/peptide independence can also be attributed to the observed cross-reactivity between different metal ions [51, 52] and in the case of Ni it was proposed that

Ni may link T cell receptors (TCR) and MHC in a peptide-independent manner [51]. Another mechanism that has been postulated for metals, Ni in particular, is binding to specific carrier proteins that ensure survival of the critical Ni-peptide complex throughout the transport and processing through the epidermis and dermis, and then transfer of the metal to short-lived, high-affinity coordination sites created within certain TCR–MHC contact zones [49].

6. Pharmacological Interaction Mechanism

Additional allergenic compounds that are otherwise chemically inert (unable to directly haptenate proteins) have been shown to cause lymphocyte proliferation [53] suggesting a different mechanism by which they are able to stimulate TCR. The chemicals, which are usually drugs associated with adverse hypersensitivity reactions, have been shown to bind to the MHC on the basis of their conformation rather than reactivity. This kind of binding, referred to as pharmacological interaction (p-i), is labile and more effective when it is on the MHC and within proximity of the TCR [53]. Experiments that involved washing steps after binding of the MHC proteins and before stimulation of T cells resulted in lack of stimulation indicating that the weak protein binding is reversible. Washing was ineffective with chemicals that were covalently bound to MHC peptides. Other evidence supporting the model for direct interaction of the sensitizer with both TCR and the MHC includes the kinetics of T-cell activation which happens much faster than would be feasible if antigen processing was occurring. The p-i concept has been used to explain hypersensitivity of drugs such as lidocaine, sulfamethoxazole, mepivacaine, celecoxib, carbamazepin, lamotrigine, and ciprofloxacin [54–58] which are not haptens/prohaptens but still elicit an immune response because their conformation allows them to fit into the MHC-TCR sandwich. The chemical p-phenylenediamine (PPD) has been shown to stimulate TCR via this model in addition to its ability to haptenate proteins [59]. The large number of TCR available ($>10^{12}$) [53] makes it plausible that some chemicals will have conformations that allow them to associate with the TCRs.

7. Prohaptens

Prohaptens are chemicals that are not protein reactive unless they are metabolically activated to electrophilic species. It has also been proposed that prohapten chemicals that undergo air oxidation to reactive species be classified separately as prehaptens, but the criteria on when a chemical is a pro- or prehapten is confusing [44]. Many chemicals such as PPD, isoeugenol, and limonene are assigned to the pro- or prehapten category on evidence indicating that they are either metabolized or undergo air oxidation. With an estimated one third of known skin sensitizers needing metabolic or abiotic activation to react with skin proteins [60], it is important that a clear distinction be made between haptens and prohaptens. The recent emphasis on alternative screening methods to avoid animal use for screening of both contact and respiratory allergens depends on identification of prohapten mechanisms to minimize false negative classification of sensitizing chemicals. While guinea pig tests and the LLNA are able to identify prohaptens, nonanimal assays have to include a metabolizing system to bioactivate these otherwise nonsensitizing chemicals. With animal-based assays, the lack of false negatives with prohaptens attests to the fact that the skin is an important site of metabolizing enzymes even though its metabolic capability has not been fully characterized [61]. Bioactivation of prohaptens commonly involves oxidative processes, with the cytochrome P450 system (CYPs) playing a major role in the biotransformation of the majority of prohaptens to sensitizers. CYP enzymes that have been detected at the mRNA level in the skin include CYP1A1, 1B1, 2B6, 2E1, and 3A5 [62]. While the proteins were not shown, this mRNA expression was consistent with expression levels that were found for normal human epidermal keratinocytes (NHEKs) and dermal fibroblasts [63–65]. Other metabolic enzymes identified in the skin include monooxygenases, dehydrogenases, esterases, amidases, and Phase II enzymes which are mainly transferases [66, 67]. Keratinocytes have also been shown to possess prohapten metabolizing capacity [63, 68]. To date, there have been few studies on the characterization of prohapten bioactivation by CYP enzymes. Prohaptens that have been studied include the polyaromatic hydrocarbons (PAHs), cinnamic alcohol, carvone oxime, isoeugenol, and diphenylthiourea [62, 69–72]. Once bioactivated to an electrophilic species, haptenation of proteins proceeds via one of the previously discussed mechanisms (Table 1).

Metabolic activation has so far proven to be the Achilles' heel for many *in vitro* and *in chemico* assays. The need to exhaustively interrogate the use of metabolic systems where prohaptens are concerned has been discussed in detail [73]. Recent *in vitro* studies [62, 74, 75] have included metabolic systems to detect prohaptens with marked success, but more work needs to be done to identify metabolic systems that are more representative of skin metabolism.

8. Protein-Selective Haptenation Targets: The Diisocyanate-Albumin Example

Exposure to diisocyanates in the workplace is one of the leading causes of occupational asthma. It is hypothesized that isocyanate acts as a hapten by reacting with protein carriers via nucleophilic attack; however, the ultimate form of these protein-isocyanate conjugates that functions as allergens *in vivo* is, as yet, unknown. The diverse functional groups present in proteins (amines, amides, thiols, alcohols, carboxylic acids) present a large number of potential reaction sites for the diisocyanate (dNCO). However, previous studies have suggested that under physiological conditions, these are limited to N-terminal α-amines, the sulfhydryl group of cysteine, the hydroxyl groups of serine and tyrosine, the ε-amine of lysine, and the secondary amine of the imidazole ring of histidine [76]. Understanding the products formed by reaction of allergenic dNCOs such as methylene diphenyldiisocyanate (MDI) and toluene diisocyanate (TDI)

with biological molecules is critical to understanding the mechanisms by which these chemicals affect living systems. Tandem mass spectrometry performed on a quadrupole time-of-flight (qTOF) mass spectrometer [77] is particularly well suited for the characterization of chemically modified proteins. Proteins of interest may be digested with a proteolytic enzyme (such as trypsin) and the resultant peptides analyzed with high sensitivity and mass accuracy. Because covalent modification of an amino acid residue results in a change in that residue's mass, accurate mass determination of the fragment ions produced by collision-induced dissociation [78] allows unambiguous assignment of the site of modification. Such experiments have become routine for the analysis of posttranslational modifications such as acetylation, glycosylation, and phosphorylation, among many others [79].

Recent efforts in our laboratory [81, 90] and others [80] have begun to focus on harnessing the power of tandem mass spectrometry to determine how and where dNCOs modify model peptides and proteins. Hydrolysis of the isocyanate functional group to a primary amine is a competing reaction under aqueous conditions. These hydrolyzed isocyanate amines may then undergo nucleophilic addition to another dNCO molecule. Therefore, conjugation products observed upon reaction of dNCOs with model peptides and proteins *in vitro* results in a complex variety of different reaction products, including intra- and intermolecular crosslinking, dNCO self-polymerization, and dNCO hydrolysis. Our initial study focused on determining the site of dNCO modification on model bioactive peptides conjugated under aqueous conditions [81]. Analysis of these conjugates by tandem mass spectrometry revealed that the dNCO was bound preferentially to the N-terminal amine of each of the peptides examined. Furthermore, when a peptide with an N-terminal residue containing a side chain amine (lysine, arginine) was reacted with dNCO, intramolecular crosslinking with the side chain amine becomes competitive with hydrolysis, however, the reactivity decreases as the residue is displaced further from the N-terminus. The results of this peptide study suggested agreement with a long-held hypothesis that the N-terminal amine of protein chains is a likely target for isocyanate conjugation [76, 82]. Studies of the kinetics of isocyanate binding with protein functional groups [83] determined that at pH 7, reaction with an N-terminal amine should proceed approximately 100 times faster than the ε-amine of the lysine side chain. The difference is due to the relative pK_a of the two functional groups (α-NH_3^+ $pK_a \sim 9$ versus Lys ε-NH_3^+ $pK_a \sim 10.5$), as dNCO conjugation proceeds through the neutral $-NH_2$ rather than the charged $-NH_3^+$ species.

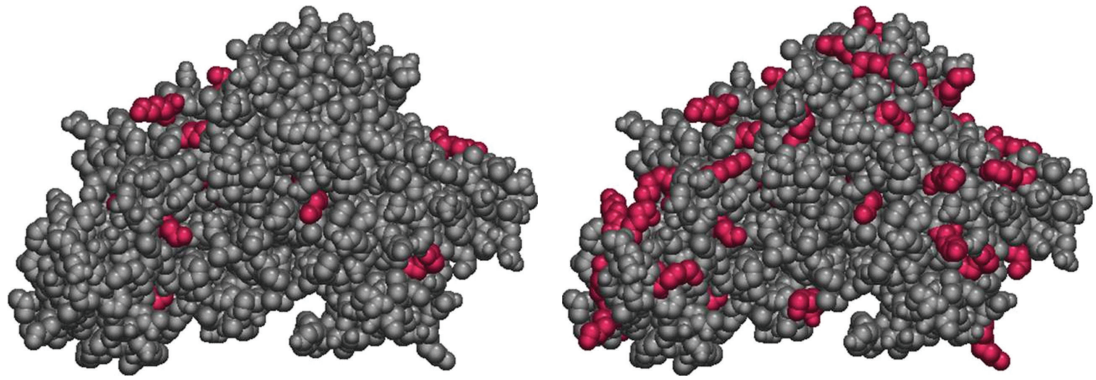
Table 2

Residue	MDI	TDI
Asp1*	X	X
Lys4*	X	X
Lys12	X	X
Lys73	X	X
Gln104		X
Lys106		X
Lys136	X	X
Lys137*	X	X
Lys159		X
Lys190	X	X
Gln196		X
Lys199*	X	X
Lys205		X
Lys212		X
Lys262*		X
Lys274		X
Lys276		X
Lys281		X
Lys351*	X	X
Lys378		X
Lys402		X
Lys413*	X	X
Lys414*	X	X
Lys432*	X	X
Lys436*	X	X
Lys439*	X	X
Lys444*	X	X
Lys524	X	X
Lys525*	X	X
Lys534		X
Lys536	X	X
Lys541*	X	X
Lys545		X
Lys557		X
Lys560		X
Lys573		X
Lys574		X

* MDI binding sites indentified in [80].

9. Conclusion

Although tandem MS studies of dNCO-conjugated peptides are useful for determining the diverse chemical species produced when dNCO binds amino acids, they do not produce the unique chemical microenvironments presented by the complex three-dimensional structure of proteins. In order to understand how dNCOs react in these complex environments, studies on model proteins are essential. Serum albumin is an appropriate model protein, as it is monomeric and its sequence and three dimensional structure have been well defined. It is naturally abundant (35–50 mg/mL in serum), found in most tissues, and has been identified as a target of dNCO binding *in vivo* [84–89]. Recently, our laboratory completed an extensive analysis of the binding sites of TDI on human serum albumin [90]. At high (40 : 1 dNCO : protein) ratios, near-stoichiometric binding was observed; TDI binds at thirty-seven sites on the protein, including the N-terminal amine on aspartic acid at position one and the side chain of thirty-four lysine residues. At lower conjugation ratios (1 : 2 dNCO : protein), a small subset of



(a) (b)

Figure 1: (a) Lysine residues observed bound to MDI and TDI on human serum albumin. (b) All lysine residues on human serum albumin.

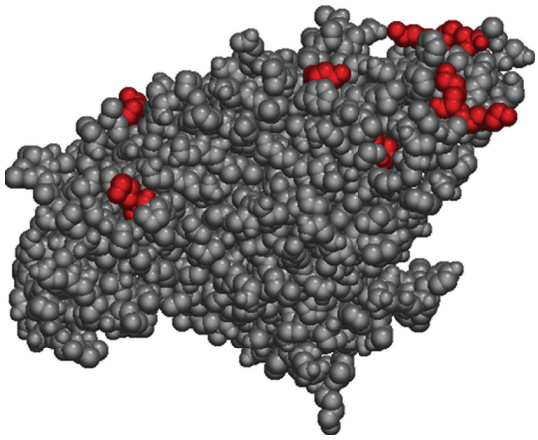


Figure 2: Lysine residues reactive to TDI but not MDI.

these thirty-seven sites is conserved, with binding observed at the N-terminus and four lysine residues, suggesting these sites are preferred binding sites. Kristiansson and coworkers [91] determined that at a tenfold molar excess, HHPA bound to thirty-seven sites on human serum albumin, including the N-terminal aspartic acid and thirty-six lysine residues.

Interestingly, all 59 lysine residues of human serum albumin have been determined to be solvent accessible, but only 37 are reactive toward TDI, while 19 are reactive toward MDI (Hettick and Siegel, unpublished data). Human serum albumin is a highly charged molecule, in part accounting for its high solubility. For example, Figure 1 provides two views of human serum albumin based on its crystal structure [92]. In Figure 1(a), the lysine residues that bind MDI and TDI are highlighted, whereas in Figure 1(b), all 59 lysine residues are highlighted. Steric effects are insufficient to explain the difference between accessibility and observed binding. It is therefore likely that the microenvironment of the binding site(s) determines whether or not a certain lysine residue is reactive toward isocyanate. Lysine 199, which is known to bind hydrophobic anions such as aspirin and benzyl penicillin, was also determined to be a predominant binding site for both TDI and MDI. Gerig and Reinheimer [93] determined that the pK_a of the aspirin binding site (later determined to be Lys199) of albumin was 7.9. These authors hypothesized based on the reactivity of human serum albumin with dinitrofluorobenzene that there exist two lysines on HSA that have a pK_a as low as 7.9. In addition, Lys199 has been shown by molecular dynamics calculations to be predominantly uncharged, undergoing proton transfer with the nearby Lys195 [94]. This study elegantly suggests the reason we observed dNCO bound to Lys199, and not the nearby the Lys195. Other sites noted to be abundant binders of dNCO, such as Lys439 and Lys525, have been observed to undergo nonenzymatic glycosylation *in vivo*. Glycosylation is generally observed to occur at lysine residues located near another amino group, presumed to be charged [95]. Lys439 is located in a region with two other nearby lysine residues (Lys432 and Lys436) and Lys525 is part of a dilysine motif.

More recently, we have begun an investigation to compare the binding sites of TDI and MDI on serum albumin under identical conditions (40 : 1 dNCO : HSA; 50 mM NH_4HCO_3, pH 7.9). Under these conditions, MDI is observed to bind to a subset of 19 of the 37 sites observed for TDI (see Table 2). Although it would be tempting to attribute differences in binding between TDI and MDI to steric effects, as seen in Figure 2, many of the lysine residues that are reactive toward TDI but not MDI are open and highly accessible. We therefore hypothesize that the difference in observed binding between TDI and MDI is attributable to a combination of steric effects and the increased reactivity of TDI. The electron withdrawing character of the second N=C=O group on the aromatic ring of TDI significantly increases the reactivity of the first isocyanate. In contrast, the reactivity of the isocyanate functional group(s) on MDI is lower because the p-ethyl phenylisocyanate substituent is less electron-withdrawing. Wisnewski and coworkers also examined the reaction products between MDI and human serum albumin by HPLC-MS/MS. Their data indicated 14 binding sites on albumin, including 12 lysine and 2 asparagine residues, in relatively good agreement with the results presented in Table 2. In addition, these authors suggested that the four "dilysine" (KK) motifs in human serum albumin are important binding sites, and that MDI shows reactive specificity for the second lysine. As discussed previously, the ability of a lysine residue to transfer its proton

to a nearby lysine or histidine residue may, in fact, lead to increased reactivity toward dNCOs. However, as dilysine motifs account for 8 of 36 TDI binding sites and 6 of 19 MDI binding sites, it is clear that two lysine residues in a "KK" arrangement are not essential to binding.

It has been recognized for over 80 years that the ability of a hapten to react to a protein was central to its ability to produce allergic sensitization. Haptenation of a protein can occur by multiple mechanisms (primarily electrophilic attack) and is dependent on many factors such as chemical properties, bioavailability, and site of exposure. Recent studies have greatly expanded the knowledge in this area by demonstrating that it is the rate at which an electrophilic hapten reacts with a nucleophile center that is a central determinant in its dermal sensitization potency. In addition, the chemical mechanism of binding (i.e., mechanistic domain) also influences allergenic potency. These physical chemical factors, however, have not been shown to be related to skewing of the immunological response toward Th1 versus Th2. Studies are currently utilizing modern proteomic mass spectrometry to identify hapten binding sites on proteins and to identify specific hapten target proteins. It is possible that the protein-specific factors may play a role in the ultimate nature of the immune response.

Abbreviations

AA:	Acylating agents
ACD:	Allergic contact dermatitis
CYPs:	Cytochrome P450s
dNCO:	Diisocyanate
EC3:	Concentration of allergen producing a 3-fold lymph node cell proliferation = LLNA threshold
HPLC-MS/MS:	High performance liquid chromatography-tandem mass spectrometry
HSA:	Human serum albumin
LLNA:	Murine local lymph node assay
LMW:	Low molecular weight
log P:	Octanol/water partitioning
MA:	Michael acceptor
MHC:	Major histocompatibility complex
MBT:	Mercaptobenzothiazole
MBTS:	Mercaptobenzothiazole disulfide
NBT:	4-nitrobenzenethiol
MDI:	Methylene diphenyldiisocyanate
PPD:	p-phenylenediamine
qTOF:	Quadrupole time-of-flight
RAI:	Relative alkylation index
SBF:	Schiff base former
S_N1/S_N2:	Nucleophilic substitution unimolecular or bimolecular
SOD:	Superoxide dismutase
TCR:	T-cell receptors
TETD:	Tetraethylthiuram disulfide
TDI:	Toluene diisocyanate
ZDEC:	Zinc diethyldithiocarbamate.

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Review Article

The LLNA: A Brief Review of Recent Advances and Limitations

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Allergic contact dermatitis is the second most commonly reported occupational illness, accounting for 10% to 15% of all occupational diseases. This highlights the importance of developing rapid and sensitive methods for hazard identification of chemical sensitizers. The murine local lymph node assay (LLNA) was developed and validated for the identification of low molecular weight sensitizing chemicals. It provides several benefits over other tests for sensitization because it provides a quantitative endpoint, dose-responsive data, and allows for prediction of potency. However, there are also several concerns with this assay including: levels of false positive responses, variability due to vehicle, and predictivity. This report serves as a concise review which briefly summarizes the progress, advances and limitations of the assay over the last decade.

1. The Murine Local Lymph Node Assay

Allergic disease continues to be an important environmental and occupational health concern. Allergic contact dermatitis (ACD) is the second most commonly reported occupational illness, accounting for 10% to 15% of all occupational diseases. This poses a significant public health burden with combined annual costs of up to $1 billion for medical costs, workers compensation, and lost time from work. This highlights the importance of developing rapid and sensitive methods for hazard identification of chemical sensitizers. Historically, guinea pig tests (GPT; i.e., the guinea pig maximization (GPMT) and the Buehler assay (BA)) were used for this purpose. The human repeated insult patch test (HIRPT) is still used in many countries as a confirmatory test for skin allergens; however, ethical concerns and the existence of reliable alternative testing procedures have largely eliminated the justification for the HIRPT [1]. The murine local lymph node assay (LLNA) was developed in 1989 [2] and continues to undergo refinement as an alternative for the evaluation of sensitizing potential of low molecular weight (LMW) chemicals. The LLNA has been evaluated extensively in the context of both national and international interlaboratory trials. This data has been reviewed and validated in the USA by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [3, 4] and in Europe by the European Center for the Validation of Alternative Methods (ECVAM) [5] resulting in the LLNA becoming the preferred method for assessing skin sensitization potential by various regulatory authorities [6, 7]. In 2002, the LLNA was adopted by the Organization for Economic Co-operation and Development (OECD) as a standalone method (OECD 429). Recently the LLNA has been designated as the initial requirement for sensitization testing with the new registration, evaluation, authorization and restriction of chemical substances (REACH) regulation in the European Union.

The basic principle underlying the traditional LLNA is that chemical sensitizers induce a primary proliferation (induction phase) of lymphocytes in the lymph nodes draining the site of chemical application which can be quantified using measurement of radiolabeled thymidine incorporation into the lymph node cellular DNA. Low molecular weight (LMW) chemical sensitizers, referred to as haptens (or prohaptens), are themselves too small to be allergenic and must bind to a protein to be allergenic. Three major cells types, keratinocytes, Langerhans cells, and T-lymphocytes, have been identified as central in the induction phase of ACD. The role of keratinocytes in both the induction and elicitation

phases has been recently reviewed [8]. Haptens can directly stimulate keratinocytes present in the epidermis of the skin to release inflammatory mediators such as interleukins 1, 6 and 18, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-α. The chemokine CCL2, which can recruit dendritic cells into the site of inflammation, is also upregulated in keratinocytes following hapten exposure. Langerhans cells (LCs), immature dendritic cells (DCs) present within the epidermis, take up and process haptenated protein within the major histocompatibility complex (MHC II). In the presence of the proper cytokine-signaling milieu, LCs migrate from the skin through the afferent lymphatics to lymph nodes draining the site of contact and become mature DCs during that process. DCs then present the haptenated peptide to responsive T-lymphocytes [9, 10]. Activated T-lymphocytes divide and differentiate into both T-effector and T-memory cells which starts the central phase of sensitization [11], and it is this allergen-driven proliferation response that is quantified in the LLNA.

In the performance of the LLNA (based on the original guidelines), mice (female CBA/Ca or CBA/J preferred strain; minimum of four per group) are topically exposed to accepted vehicle, increasing concentrations (minimum of three) of LMW chemical, or accepted positive control on the dorsal surface of each ear once a day for three consecutive days. On day 6, mice are injected, intravenously, via the lateral tail vein with ^{3}H-thymidine (^{3}H-T). Five hours later, following sacrifice of the left and right draining auricular lymph nodes located at the bifurcation of the jugular vein are excised and pooled for each animal. Lymphocyte proliferation, determined by quantification of radioactive (^{3}H-T) incorporation in the draining lymph nodes, is evaluated using a liquid scintillation analyzer. A chemical is classified as a sensitizer if at one or more test concentration it induces a three-fold or greater increase in draining lymph node cell proliferation compared with concurrent vehicle-treated control mice and data follows dose-response kinetics. The data generated from the LLNA has been demonstrated to provide a simple means of obtaining an objective, quantitative evaluation of sensitization. From the analysis generated during the review process, the LLNA was determined to be 86% accurate (N = 97), 82% specific (N = 33), 87% sensitive (N = 93) with positive predictivity of 93% (N = 87) when compared to GPT [4]. More detailed descriptions of the LLNA are reported elsewhere [4, 12].

2. Benefits over Previously Used Assays for Skin Sensitization

There are many advantages to the LLNA in comparison to GPT (OECD test guideline 406, [13]. The LLNA provides a quantitative endpoint, dose-responsive data, allows for prediction of potency (EC3; effective concentration for a SI of 3 in proliferation of lymph node cells) and does not require the use of an adjuvant. GPT, which evaluate the elicitation phase of skin sensitization provide a qualitative endpoint which tends to be highly variable in part due to its subjective nature, and these tests are not typically used for estimations of potency. Evaluation of the sensitization phase as an endpoint results in a reduced time for animals to be on study and eliminates the discomfort associated with the development of inflammation in the elicitation phase of the response. Compared to GPT, the LLNA reduces animal numbers needed, improves animal welfare, and decreases experimental time and costs. This report is not intended as an exhaustive update on the progress of the LLNA, but rather a concise review which will briefly summarize the progress, advances, and limitations of the assay over the last decade.

3. The Potential Use of the LLNA in Risk Assessment

Although the LLNA was originally validated for the purpose of hazard identification, much attention and debate have been recently focused on the potential use of the LLNA in human quantitative risk assessment [14]. Risk assessment is generally viewed as a four-step process: hazard identification, dose-response assessment, exposure assessment, and risk characterization. The LLNA provides information for use in the first two steps. Lymphocyte proliferation has been proven to be related both causally and quantitatively to chemical sensitization. Potency estimation can therefore be made by comparing the concentration of chemicals necessary for the acquisition of sensitization. The EC3, effective chemical concentration required for a SI = 3, can be mathematically derived by linear interpolation of dose-response data [15]. The EC3 value has been shown to be highly reproducible, consistent among laboratories and stable over time [16–18]. While the EC3 value, is not a measure of absolute po-tency which can be directly extrapolated to humans, it is an objective measure of relative allergenic potency of one potential sensitizer with that of another [19]. In 2003, after extensive laboratory investigations, four categorizes of chemical sensitization potency, with 10-fold difference in EC3 value (extreme (<0.1%), strong (≥0.1–<1.0%), moderate (≥1.0–<10%), and weak (≥10%)), were determined by the ECETOC (European Center for Ecotoxicology and Toxicology of Chemicals) [20]. Recently, these classification schemes for regulatory purposes have been reevaluated by ICCVAM and the European Chemical Bureau, and their findings will be released later this year [14].

The results from numerous studies support the use of the LLNA in quantitative risk assessment by demonstrating an overall association between EC3 and relative potency of chemical allergens in humans [21–25]. EC3 values of 26 chemicals were found to have a linear relationship with the threshold for the induction of sensitization derived from human repeated insult patch test [21]. From the analysis generated during its review process, the accuracy of the LLNA versus human tests (human maximization test and human patch test allergen) was 72% (N = 74) while the positive predictivity was 96% (N = 51) [4].

There are concerns about extrapolating data generated from the LLNA for potential use in risk assessment. The LLNA is based on the induction phase of the hypersensitivity response following acute (3 consecutive days) exposure.

The complexity of the induction-elicitation response and the degree to which skin sensitization influences the dose of chemical necessary to elicit a reaction are important factors to consider. Theoretically, elicitation thresholds are lower than those required for induction. Furthermore, the dose required for induction may be dependent on duration frequency and site of exposure. Human chemical exposure may be the result of an incidental single contact, repeated exposure, or continual exposure. These types of scenarios can present difficulties when trying to classify weak (high EC3) versus strong (low EC3) sensitizers. In general, chemicals with high EC3 are considered to be of low risk to human while chemicals with a low EC3 value present a much higher risk. While this scenario usually holds true, there are other factors that need to be considered such as: is there a greater risk for allergy when there is frequent exposure to a weak sensitizer versus infrequent exposure to a strong or extreme sensitizer? For example, studies have found that although methyl methacrylate is a weak sensitizer (EC3 value of 60–90%), numerous cases of skin sensitization have been reported in individuals exposed to plastic materials [26]. This finding could be related to factors such as duration of exposure, exposure concentration, and route of exposure. In occupational settings, workers have a greater potential for exposure to pure, undiluted chemicals than the general public which would most likely be exposed to a diluted version in a consumer product. While attempts are being made to use data generated from LLNA studies toward utilization in risk assessment, all of these factors need to be carefully considered.

4. Limitations of the LLNA

From the analysis generated during its review process, the accuracy of the LLNA versus GPMT/BA was 89% ($N = 97$), LLNA versus all GPT was 86% ($N = 126$), the LLNA versus human data was 72% ($N = 74$), GPMT/BA versus human was 72% ($N = 57$), and all guinea pig tests (GPT) versus human's was 73% ($N = 62$) [4]. In terms of accuracy, sensitivity, specificity, and positive and negative predictivity, the performance of the LLNA was found to be similar to that of the GPMT/BA. Equally important, the performance of the LLNA and the GPMT/BA was similar when each assay was compared to human data. No predictive toxicology tests will ever be 100% accurate, and because of this, it is important to understand the limitations of each assay [27]. Inconsistencies between LLNA and human patch test data have been documented [28]. While the mouse has been identified as the optimal experimental model for the LLNA, rodents have been shown to have increased skin penetration of chemicals compared to humans [29, 30]. These types of interspecies differences may contribute to some of the inconsistencies between animal and human skin sensitization tests, therefore, confounding interpretation of the results especially with respect to potency determination. This section will briefly describe some of the limitations that have been identified for the LLNA.

4.1. Level of False Positives. Irritants and sensitizers can both induce lymphocyte proliferation. While sensitizers generate antigen-specific lymphocyte proliferation, this response is nonspecific for irritants. The use of ^{3}H-T incorporation for measurements of lymphocyte proliferation in the LLNA does not allow for differentiation of the two. For this reason, it has long been debated that the LLNA may give an unacceptable number of false positives when nonsensitizing irritating chemicals are tested [17, 31]. The determination of an SI value of 3 as indicative of skin sensitization potential was made after extensive evaluations of chemical datasets. It is a threshold set as a precautionary measure to try and account for background fluctuations in lymphocyte proliferation [15]. For example, topical application of the well-studied surfactant sodium lauryl sulfate (SLS) has been shown to test positive in the LLNA with SI values above the threshold limit (3-fold increase) [9, 17, 31–34]. In contrast to the scenario presented for SLS, when numerous nonsensitizing skin irritants were evaluated using the LLNA, the majority tested negative [35]. However, positive responses, occasionally conflicting with data generated from other studies, to other nonsensitizing irritants have been reported and include: chloroform/methanol, Triton X-100, oxalic acid, methyl salicylate, and nonanoic acid [34, 36]. Similar to SLS, the SI values obtained for these compounds in the LLNA were most often low and close to the threshold level. More recently, 7/9 nonsensitizing irritating compounds (oleic acid, linoleic acid, linolenic acid, undecylenic acid, maleic acid, squalene, and octinol) tested positive in the LLNA with the highest SI value of each substance between the range of 4.4–16.1 [37]. It is important to point out that these types of limitations are not unique to the LLNA and have also been associated with GPT for skin sensitization [38] as well as with human patch test studies [39].

Numerous methods have been developed based on the mechanisms underlying the induction of sensitization to try and distinguish between sensitizing and irritating compounds. These include but are not limited to: measurements of antigen expression on Langerhans cells [40], cytokine production [41–43], DC activation [44], and lymph node cell phenotyping [45–48]. At this time, these modifications of the standard LLNA are intended for use as research tools and are not validated for the purpose of hazard identification.

4.2. Variability due to Vehicle. Lymphocyte proliferation has also been shown to be influenced by several factors including vehicle selection [49]. OECD recommended vehicles include: acetone/olive oil (AOO: 4 : 1 v/v), dimethylformamide, methyl ethyl ketone, propylene glycol, dimethyl sulphoxide, and dimethyl sulfoxide (DMSO). Several of these vehicles including AOO, DMSO, and propylene glycol have been shown to augment the LLNA response of certain chemicals. For example, AOO has been shown to give highly variable results when used as a vehicle in the LLNA [50, 51]. In addition, research suggests that olive oil itself may cause contact allergy [52, 53]. DMSO is a polar solvent that is known as a penetration enhancer and may augment bioavailability of the allergen across the stratum cornea. Another commonly used LLNA vehicle propylene glycol has been shown to suppress the proliferative effects of certain chemicals such as 2,4-dinitrochlorobenzene (DNCB) [54]. Select

vehicles with the ability to enhance or suppress proliferative responses may be an important consideration for weak sensitizers with high EC3 values. Jowsey et al. [55] reported that solvent selection was very important when conducting the LLNA. They tested 15 different solvents with multiple allergens and found that when propylene glycol was used as the vehicle, the EC3 obtained for the chemicals varied by >10 fold compared to the other vehicles used. This is consistent with our study of bromoalkanes [56]. An approximate 3-fold difference in lymph node cell stimulation for C18 and C19 bromoalkanes was observed when dissolved in AOO versus butanol/tetrahydrofuran (1/1). In addition, the importance of other physical/chemical considerations for solvent selection and the potential influences on test results were noted. While allergens may be soluble in AOO, the acetone quickly volatilizes away during application of allergen to the skin. Test compounds such as bromohexane may be lost due to volatility, while the longer chain bromoalkanes result in large particulate-olive oil slurries that are poorly retained on the skin with application in AOO. However, while these are important factors to considerer, the degree of augmentation due to vehicle selection has not typically been shown to affect the category of sensitization because the SI value is based on increase in lymphocyte proliferation over vehicle control.

4.3. Inability to Distinguish Specific Type of Hypersensitivity Response. There are two types of chemical allergy which are of greatest relevance for occupational and consumer exposures: skin sensitization causing allergic contact dermatitis (Th1-type immune response) and sensitization of the respiratory tract associated with allergic rhinitis and asthma (Th2-immune response). In addition to identifying contact sensitizers, it is generally accepted that LMW respiratory sensitizers also test positive in the LLNA because the initial sensitization or induction phase of allergy is similar for both types of allergic responses [57]. Based on this concept, a LMW chemical testing negative in the LLNA can be classified as nonsensitizing for urticarial, contact, and respiratory allergies. However, there are currently no validated methods to distinguish between these two types. Modifications of the LLNA have been developed to try and classify the type of chemical sensitizer. These include but are not limited to methods that evaluate serum IgE levels (representative of a Th2-type immune response) [57], cytokine fingerprinting (analysis of Th1 versus Th2 cytokines) [58–60], the mouse ear swelling test (MEST) [61], and immune cell phenotyping [48, 62].

Although there is mounting evidence that lymphocyte proliferation can be used to identify contact and respiratory allergens, dermal application is the only route of exposure validated for the LLNA. While there is significant evidence suggesting that dermal exposure to sensitizers such as the isocyanates and acid anhydrides can induce respiratory tract sensitization [63–65], there are currently no validated test methods to identify compounds, including high molecular weight protein allergens that cannot pass through the skin. Attempts are currently being made to address these issues and will be discussed in greater detail later in this paper.

5. What Is in Store for the Future?

5.1. Updated OECD Guidelines. The OECD Guidelines for the testing of chemicals are periodically reviewed as a result of nominations of new methods highlighting scientific progress, changing regulatory needs, and animal welfare considerations. Many modifications to the OECD guidelines have been recently published [66] and will be briefly described in this section.

5.2. Nonradioactive Alternatives. Two modifications of the LLNA to utilize nonradioactive endpoints have been developed. Advantages of these assays include the elimination of occupational exposure to radioactivity and issues related to radioactive waste. The LLNA: DA and LLNA: BrdU have recently been reviewed, validated, and recommended by an international peer review panel as useful for identifying skin sensitizing and non-sensitizing substances, with certain limitations [66]. These methods are considered to be of equal merit to the standard LLNA and may be employed as an alternative to GPT or the standard LLNA. Positive or negative results no longer require additional confirmation. As with all the validated tests discussed, both positive and negative (solvent) controls must be run in parallel to the test substance. The concept for the LLNA: BrdU method (OECD 429B) is similar to that of the standard LLNA and is based on the incorporation and quantification of BrdU into proliferating cells in the auricular lymph nodes following topical chemical exposure [67]. BrdU incorporation is measured by peroxidase-labeled BrdU-specific antibody. Following addition of a substrate, reaction with the peroxidase produces a colored product that is quantified at a specific absorbance using a microtiter plate reader. A chemical is considered a sensitizer if an SI value ≥ 1.6 is obtained.

The second approved nonradioactive method is the LLNA: DA (OECD 442A). The LLNA: DA (developed by Daicel Chemical Industries, Ltd.) uses quantification of adenosine triphosphate (ATP) content (known to correlate with living cell number) measured using bioluminescence as an indicator of increased lymphocyte proliferation [68]. The method utilizes the luciferase enzyme to catalyze the formation of light from ATP and luciferin, which is measured using a luminometer and linearly related to the ATP concentration [66]. A chemical is considered a sensitizer if an SI value ≥ 1.8 is obtained. Although both of these assays provide quantitative data suitable for dose-response assessment, the results may not be directly compared to the EC3 values obtained for the LLNA. The thresholds to determine sensitization (SI) for these assays are lower than that established for the standard LLNA, and SI values for equivalent doses of allergen also tend to be lower. It has not been established, to our knowledge, if this shift in basal and allergen-induced SIs will provide comparable EC3 determinations to the traditional LLNA. As with any assay, there are limitations to these modifications. Certain chemicals, such as ones that affect ATP levels, have been determined to be inappropriate for use with these types of assay. In addition, ATP is very labile and the assay, as presently validated, requires immediate analysis after sample recovery.

5.3. Reduced LLNA. The reduced LLNA (rLLNA) is a validated and accepted modification to the standard LLNA which was purposed in an effort to reduce experimental animal use for the assessment of skin sensitization potential of chemicals as well as to address the demand for increases in sensitization testing of chemicals required by REACH [66]. This alteration of the original protocol requires only a single, high-concentration test group (the highest dose that does not produce significant irritation) and a positive control group [69, 70]. When used to test a substance for the potential to cause allergic contact dermatitis, the rLLNA uses fewer animals than the LLNA to provide a "yes-no" result. ICCVAM has recommended that the rLLNA be used routinely to determine the allergic contact dermatitis hazard potential of chemicals and products before conducting the multidose LLNA in cases that do not require dose-response information. Since the rLLNA uses only a negative control group and a high-dose group, use of the rLLNA can reduce the number of animals needed for each test by 40% compared to the multidose LLNA. Clear justification and scientific rationale must be provided before utilization of this method because it cannot generate dose-response or potency data that could be used for risk assessment.

5.4. Testing of Formulations. The standard LLNA was not originally evaluated for the testing of formulations. However, ICCVAM recently recommended, due to a nomination by the US Consumer Product Safety Commission, to reevaluate the LLNA applicability domain. This would allow the LLNA to be used to test any chemical or product, including pesticide formulations, metals, substances in aqueous solutions, and other products such as natural complex substances and dyes unless the chemical or product to be tested has properties that may interfere with the ability of the LLNA to detect skin-sensitizing substances [71]. This conclusion was based on the compilation of data from previously described research as well as newly generated data obtained for the purpose of this evaluation. For immunotoxicological evaluation of investigational new drugs, the FDA requires that "when a murine LLNA is conducted to support the safety of clinical trials, the sensitizing potential of the drug substance, clinical excipient, and clinical formulation should be evaluated" [72]. This modification expanded the use and application of the LLNA to test formulations found in occupational settings and consumer products as well as individual chemicals.

5.5. Nonanimal Alternatives to the Standard LLNA. Much focus has been placed on the use of *in vitro, in chemico*, and *in silico* alternatives due to the increasing public and political concerns regarding the use of animals in research. The successful development, evaluation, and validation of these nonanimal alternatives for evaluation of skin sensitization will depend heavily on the precision and accuracy with which they can predict the *in vivo* classification of sensitizing chemical. They must be able to predict the complex interaction of the chemical with all aspects of the immune response. Numerous methods are being developed for this purpose and are based on specific mechanistic steps that occur during skin sensitization including but not limited to: protein/peptide binding and haptenization, activation of keratinocytes and DC, and T-cell proliferation [73].

A number of *in silico* methods currently exist and aim to predict a novel chemical reactivity based on the known *in vivo* reactivity of existing structurally similar chemicals. This kind of theoretical computer modeling is referred to as structural activity relationship (SAR) or quantitative structural activity relationship (QSAR). Derek for Windows and TOPKAT are examples that have been used for several years. This type of predictive tool allows the user to input a chemical structure and obtain a readout of the chemical constructs that could potentially lead to sensitization [74]. Challenges with this type of modeling include the analysis of chemicals requiring metabolic activation. Although these models may be helpful as an initial screen, inconsistent results have been observed when compared to animal models of sensitization [75, 76].

Computer and skin models are also being developed to evaluate chemical epidermal bioavailability based on physical/chemical data [77]. A chemical must react with host proteins to produce an altered or haptenated selfprotein before sensitization can occur [78–80]. Additionally, some chemicals termed prohaptens require metabolic or chemical conversion before they can react with a protein. Efforts have also focused on investigating whether the intrinsic sensitizing potential of a chemical can be predicted from its electrophilic reactivity [81]. The direct peptide reactivity assay (DPRA) investigates peptide reactivity kinetics to evaluate sensitization and is currently undergoing validation by ECVAM. It aims to model protein haptenation *in chemico*, by measuring the depletion of two synthetic peptides which are typical reaction targets [82].

There are numerous *in vitro* tools being developed for prediction of skin sensitizers. Several are based on critical steps in chemical sensitization including keratinocyte and DC activation [73, 77]. These pivotal points of sensitization result in numerous cellular and molecular processes related to antigen processing and presentation which can be evaluated and measured. These include upregulation of costimulatory molecules (CD83, CD86, CD40, and CD80) and various cytokines (IL-1β, IL-8, TNF-α, and IL-10) which can be measured by methods such as flow cytometry and quantitative real-time PT-PCR. Models are being developed using LC-like dendritic cells derived from human bone marrow, cord blood, or peripheral blood precursors [83, 84] as well as DC-like cell lines including THP1 [84, 85], U937 [86], KG-1 [87, 88] and MUTZ-3 [89]. Limitations with these types of protocols include: donor-to-donor variability (blood-derived DC-), complexity, expense, and lab-to-lab variability.

Two *in vitro* test methods, myeloid U973 skin sensitization test (MUSST) and human cell line activation test (h-CLAT) have been evaluated and accepted for prevalidation as alternatives for evaluating skin sensitization [77] by ECVAM. Limited success has also been obtained when antigen-proliferative responses by naïve T-lymphocytes have been evaluated *in vitro* following coincubation with chemical sensitizer-treated DCs or LCs [90–92]. These types of

assays have been able to identify strong but not weak sensitizers.

It is anticipated that in the next several years a multiparameter system will be available as a nonanimal alternative for the prediction of skin sensitization. Increased confidence for a method would be based on inclusion of analysis of multiple phases of chemical sensitization.

5.6. Modifications of the LLNA to Identify Respiratory Allergens. Given that positive results can be obtained in the LLNA for contact and respiratory allergens [57], numerous *in vitro* and animal models have been investigated to differentiate these responses. However, none are widely applied or fully accepted, most probably due to the complexity of the system and lack of validation efforts [93, 94]. Respiratory allergens are defined by their ability to provoke a Th2-type immune response. While a negative result in the LLNA typically excludes a LMW chemical as a respiratory sensitizer, there is currently no validated screening for the identification of chemicals or proteins that result in allergic sensitization of the respiratory tract. Numerous methods and endpoints have been purposed for the identification of respiratory sensitizers and will briefly be described here.

It has long been debated which is the best route of exposure when investigating respiratory sensitizers. Although topical application of select strong respiratory sensitizers has been shown to result in sensitization of the respiratory tract, [63–65] this does not hold true for all LMW chemical sensitizers or high molecular weight protein allergens that cannot pass through the skin. Alternative routes of sensitization including intranasal, intratracheal, oropharyngeal, and intradermal have been examined to try and address this issue [95–97]. However, there are several disadvantages with these routes of exposure including chemical solubility (most are not water-soluble), vehicle selection, species variability, requirements for sophisticated equipment and expense have made this a difficult task [57]. These complexities have prevented a general consensus on the best exposure route for evaluation of respiratory sensitizers.

Several endpoints have been examined to try and distinguish respiratory sensitizers from contact sensitizers and are typically based on differences between Th1-(allergic alveolitis/hypersensitivity pneumonitis) and Th2-immune response (allergic asthma and/or rhinitis) [57]. These include the analysis of total serum IgE levels and cytokine finger printing [98–100]. While IgE levels tend to be a hallmark of respiratory sensitization and allergic asthma, they do not always correlate to clinical manifestation of asthma [101–103]. This may be due to antigen specificity or failure of the detection of antigen-specific IgE. Identification of respiratory allergen has also been attempted through cytokine profiling. Respiratory allergens generally induce increases in the Th2 cytokines, IL-4, IL-5, IL-10, and IL-13, while contact allergens have been associated with increases in the Th1 cytokines, INF-γ and TNF-α [58, 104]. This differentiation is not absolute as some respiratory sensitizers have also been shown to increase INF-γ [105]. Other asthma models examine lung function along with histopathology, following dermal sensitization and respiratory challenge, for the evaluation of respiratory sensitization [106, 107]. The above-mentioned factors along with method variability have complicated the development of a standardized assay for the identification of respiratory sensitizers. Similar to the LLNA, limitations with these types of models have also been identified and include false positives associated with exposure to respiratory irritants and difficulties with potency measurements [57].

6. Conclusions

In conclusion, it should be restated that no toxicologypredictive test is perfect, and each will always require a balance between sensitivity and specificity. The LLNA has been recognized as a gold standard for hazard identification of LMW sensitizer for the last decade, and most of the identified limitations are not unique to the LLNA itself but rather to the use of an animal model. Many modifications to the original LLNA OECD guidelines have been published, and others are currently being developed. Among the biggest challenges ahead are maintaining predictive value while moving from whole animal to *in vitro* systems.

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Research Article

Inhalation of *Ortho*-Phthalaldehyde Vapor Causes Respiratory Sensitization in Mice

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Ortho-Phthalaldehyde (OPA) has been approved for high-level sterilization of heat-sensitive medical instruments and is increasingly being used as a replacement in the healthcare industry for glutaraldehyde, a known sensitizer. Numerous case reports have been published indicating workers and patients experiencing respiratory problems, anaphylaxis, skin reactivity, and systemic antibody production. Our laboratory previously demonstrated that OPA is a dermal sensitizer in mice. The goal of the present study was to determine if OPA is a respiratory sensitizer following inhalation exposure. Mice were exposed to OPA vapor and airway and lymph nodes were examined for cytokine gene expression and alterations in lymphocyte populations. Inhalation of OPA for 3 days resulted in a concentration-dependent increase in lymphocyte proliferation, mainly B lymphocytes, in the draining lymph nodes. A secondary challenge of mice with OPA resulted in a dramatic increase in the population of B lymphocytes expressing IgE. Expression of Th2 (IL-4, IL-5, and IL-13) and anti/proinflammatory (IL-10, TNFα, and IL-1β) cytokine genes was upregulated in the lymph nodes and the nasal mucosa. Mice exposed to the higher concentrations of OPA-produced OPA-specific IgG_1 antibodies indicating systemic sensitization. These findings provide evidence that OPA has the potential to cause respiratory sensitization in mice.

1. Introduction

Ortho-Phthalaldehyde (OPA) is an aromatic dialdehyde, used as a high-level antimicrobial disinfectant for medical equipment which is sensitive to normal heat or steam sterilization processes, including endoscope, cystoscopes, and certain dental instruments. For 40 years, glutaraldehyde, another dialdehyde, has been the primary choice for disinfecting heat-sensitive medical devices; however, it has been reported to be a chemical sensitizer. Glutaraldehyde is known to have high affinity for biological amines, and its use as a tissue fixative capitalizes on this property. As such, glutaraldehyde and dialdehydes as a chemical class can bind to native proteins, thus, altering their presentation to the immune system. Haptenization of native proteins can lead to an aberrant immune response and the development of allergy. Several human studies have demonstrated the presence of IgE antibodies specific for glutaraldehyde adducts in the serum of exposed workers with respiratory disease [1, 2]. Importantly, workplace exposure to glutaraldehyde is known to induce occupational asthma [2–4] and allergic contact dermatitis [5] suggesting the need for safer alternatives. OPA has shown superior antimycobactericidal activity as compared to glutaraldehyde [6], allowing for its use at lower concentrations. In addition, low volatility and no need for activation have increased the use of OPA as a more practical alternative to glutaraldehyde.

It is estimated that 3253 workers were potentially exposed to OPA compared to 376,330 for glutaraldehyde from 1981–1983 [7]. If OPA was fully adopted as an alternative for glutaraldehyde, it is a reasonable assumption that more than 300,000 US workers could now be exposed. The estimated use of OPA in 2002 was between 10,000 and 500,000 pounds [8]. OPA is commonly considered as a "safe" alternative to

glutaraldehyde despite a paucity of information regarding the toxicity of this chemical and the potential health effects associated with exposure. Very few toxicology studies are available in the published literature to establish the safety of OPA. The few toxicity studies that have been performed suggest that OPA may be a chemical irritant and sensitizer and may act as an adjuvant for other allergens [9–12]. Currently there are no regulations regarding proper use and safe exposure levels of OPA in spite of the potential of exposure for a large number of healthcare workers and their patients. Concentrations of OPA ranging from 1.0 to 13.5 ppb have been detected in air samples collected from an endoscope cleaning unit of a hospital that used OPA as its primary disinfectant [13–15].

Several case reports have been presented in the literature questioning the safe substitution of OPA as a high-level sterilant in the healthcare industry. Fujita et al. [15] investigated a case involving a female nurse who exhibited slight dyspnea and dry cough that began a few months after switching to OPA for high-level sterilization in the endoscopy unit. The patient was subsequently diagnosed with bronchial asthma and experienced episodic attacks when working in the endoscopy unit. Another report identified four patients who experienced nine episodes of anaphylaxis with associated respiratory symptoms after a urology practice switched from using glutaraldehyde to OPA for cystoscope disinfection [16]. In a separate report, anaphylactic reactions with respiratory involvement occurred in two bladder cancer patients following repeated cystoscopic examination of their tumors [17] and a woman receiving repeated checkups by laryngoscopy [18, 19]. Two potential cases of occupational asthma in healthcare workers disinfecting endoscopes and similar devices with OPA have also been reported [20]. These case reports demonstrate that occupational and medical exposure to OPA can induce systemic anaphylaxis as well as pose a risk of respiratory sensitization.

Toxicity data derived from animal studies will be important for regulating and setting occupational exposure limits for OPA. Our laboratory recently demonstrated that mice dermally exposed to OPA tested positive in the local lymph node assay (LLNA) with associated increases in total and OPA-specific IgE levels, suggesting an IgE-mediated allergic mechanism [9]. The EC3 value for OPA was 0.051%, ten-fold lower than the working concentration for disinfection, establishing this chemical as a strong dermal sensitizer. The goal of the present studies was to determine the respiratory sensitization potential of inhalation exposure to OPA vapor.

2. Methods

2.1. OPA Exposure System. In order to study the potential for the inhalation of OPA to cause respiratory sensitization, a nose-only exposure system was developed to minimize skin contact. The OPA exposure system consists of two major components, the vapor generator and the nose-only inhalation tower. Briefly, OPA (0–1000 ppb) was dissolved in ddH_2O as a vehicle for delivery to the generator. The OPA generator consisted of a stainless steel septum T that was heated with a variable resistance heat rope to an internal air temperature of 105°C, a temperature that would ensure complete vaporization of the water vehicle. Air flow through the generator and exposure system was set at 10 LPM, and temperature of the system was controlled at 74 ± 2°C. This flow rate equated to 1 LPM/active exposure port on the nose-only tower and is sufficient to ensure adequate removal of exhalation gases to prevent dilution of the OPA atmosphere and rebreathing of respiratory gases, namely, CO_2 [21]. A KDS100 syringe pump (KD Scientific Inc., Holliston, MA) was used to inject the OPA/vehicle (water) into the generator at a flow rate of 117 μL/min conditioning the system air to 50 ± 3% relative humidity. Temperature and humidity were monitored using an HMP243 humidity/temperature transmitter (Vaisala Inc., Woburn, MA) placed in a nose-only restrainer to position the sensor in the breathing zone. The concentration of the OPA injection solution was adjusted to provide the desired OPA vapor concentration given the fixed injection rate of 117 μL/min. The OPA/air mixture then entered a mixing chamber prior to the nose-only inhalation tower. The exposure atmosphere was pumped into the directed-flow nose-only exposure tower (InTox Products, Moriarty, MN). A vacuum was applied to the exhaust plenum to maintain a constant negative pressure of −0.1 inches water across the exposure tower controlled by a real-time electronic pressure controller (Alicat Scientific, Tucson, AZ) that monitored and dynamically adjusted the pressure within the exhaust plenum. This minimized leaks to atmosphere as well as ensured sufficient removal of respiratory gases from the breathing zone. Mice were exposed to OPA using nose-only restrainers. The calculated OPA vapor concentration was confirmed empirically using a laboratory assay based upon the fluorescence of OPA-protein conjugates. Air samples from the inhalation tower were sampled from the breathing zone of a free exposure port using a midget impinger. The sample was used to detect a fixed concentration of L-alanine as the amine acceptor, and the OPA concentration was determined relative to an OPA standard curve monitored at 442 nm.

2.2. Experimental Animals. Female specific-pathogen-free inbred C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 6 to 7 weeks of age. Upon arrival, the mice were quarantined for 2 weeks and acclimated to a 12-hour light/dark cycle. Animals were housed in ventilated microisolator cages in environmentally controlled conditions at NIOSH animal facilities in compliance with AAALAC-approved guidelines and an approved IACUC protocol. The animal rooms were monitored for specific pathogens through disease surveillance and a sentinel animal program. Food and water were provided *ad libitum*. Mice were randomized across two exposure paradigms (Figure 1) each with a control (filtered and conditioned air) and four concentrations of OPA (125–1000 ppb). The sensitization exposure paradigm involved 4-hour inhalation exposures (4 hours/exposure) on day 1–3 followed by euthanasia 48 hours after final exposure. The sensitization/challenge exposure paradigm consisted of inhalation exposure on days 1–3 and again on days 16–18 followed by sacrifice 48 hours later.

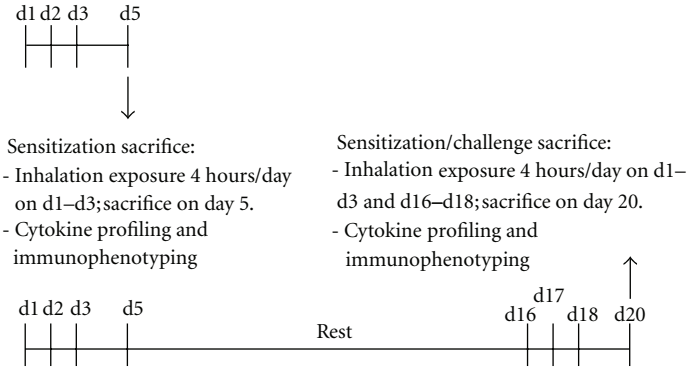

Figure 1: OPA inhalation and sacrifice schedule.

The exposure environment was maintained at 74 ± 2°C and 50 ± 3% relative humidity.

2.3. Tissue Collection. Mice were sacrificed via pentobarbital overdose (200 mg/kg, i.p.) 48 hours following the final exposure. Blood was collected from the abdominal aorta and serum was isolated and frozen at −80°C until assessment of antibody production. For gene expression analysis, the head was removed and the nasal cavity was flushed with RNAlater (Qiagen, Valencia, CA), the skin and fur, top of the skull, brain and lower jaw were removed and the remaining tissue was stored in 10 volumes of RNAlater at 4°C until dissection and tissue removal (3 days). The left mandibular lymph nodes and the lungs were removed, lungs were inflated with RNAlater, and then the tissues were stored in 10 volumes of RNAlater at −20°C until RNA extraction for PCR analysis. The nasal cavity was opened by removing the nasal bones and flattening the skull along the anterior-posterior axis. Using blunt dissection in RNAlater, the nasal mucosa lining the maxilloturbinates and lateral wall were removed as a single sample and stored at −20°C in RNAlater until processed for microarray and PCR gene expression analysis. The right mandibular lymph nodes were removed and placed in 3 mL PBS at 4°C and quickly processed for flow cytometric phenotyping as described below.

2.4. OPA-Specific Antibody Detection. OPA-specific immunoglobulin G_1 (IgG_1) and IgE serum antibodies were detected using an ELISA procedure as previously described [9]. Briefly, Immulon-4 microtiter plates (Nunc, Thermo Scientific) were coated overnight at 4°C with mouse serum albumin (MSA; 10 μg/mL in carbonate buffer, pH 9.5). Plates were washed 3 times with 0.05 M borate buffer followed by the addition of 0.5% OPA in distilled deionized water for 1 hour at room temperature. Plates were washed 3 times with PBS/0.05% Tween-20 wash buffer, and nonspecific binding sites were blocked with 1% BSA in PBS/0.05% Tween-20 for 30 minutes. A two-fold dilution series (1/10 to 1/5120) of serum was added to wells coated with MSA-only and wells with OPA-conjugated MSA and incubated for 2 hours at 4°C. Plates were washed 3 times with PBS/0.05% Tween-20, and then biotin-conjugated antibodies specific for mouse IgG_1 or IgE (BD Biosciences, San Jose, CA) were added for 1 hour at 4°C. Finally, plates were washed 4 times, and avidin-HRP was added for 30 minutes at room temperature followed by 4 washes. TMB-Turbo substrate (Thermo Fisher Scientific, Pierce, Rockford, IL) was added for 30 minutes followed by the addition of 2 M H_2SO_4 stop solution. Absorbance was read on a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA) at 650 nm during color development and at 450 nm following addition of stop solution.

2.5. Assessment of Nasal Mucosa Gene Expression. Total cellular RNA was extracted from the nasal mucosa using the Qiagen RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions following homogenization in RLT buffer using a TissueLyser II (Qiagen, Valencia, CA) bead mill system. One μg of RNA was reverse-transcribed using random hexamers and 60 U of Superscript II (Life Technologies, Grand Island, NY). Real-time PCR primer/probe sets for murine IL-1β, IL-4, IL-5, IL-13, IFNγ, TNFα, and 18s were purchased from Applied Biosystems (Foster City, CA). Real-time PCR was performed using Taqman Universal Master mix with Amperase in an Applied Biosystems 7900HT (Applied Biosystems, Foster City, CA) for 1 cycle at 50°C for 2 minutes, and 95°C for 10 minutes, followed by 60 cycles at 95°C for 15 seconds and 60°C for 1 minute. Relative differences in mRNA expression between control and treatment groups were determined by the relative quantification method developed by Pfaffl [22]. This method uses gene specific PCR efficiencies to more accurately generate relative changes based on threshold cycle. Target gene expression was normalized to the housekeeping gene 18s/rRNA.

2.6. Flow Cytometric Phenotyping of Draining Lymph Nodes. All antibodies and isotype controls for phenotyping T and B lymphocytes were purchased from BD Pharmingen (San Jose, CA). The right mandibular lymph nodes that drain the nasal mucosa were collected in 3 mL PBS and dissociated using the frosted ends of two microscope slides. Cell counts were performed using a Coulter Counter (Z2 model, Beckman Coulter, Brea, CA), and 1×10^6 cells per sample were added to the wells of a 96-well plate. Cells were washed using staining buffer (0.2% bovine serum albumin/0.1% sodium azide in DPBS; BD Pharmingen, San Jose, CA) and then incubated for 10 minutes with Fc

block (clone 2.4G2). The cells were then incubated with anti-CD3 (APC, clone 145-2C11)/anti-CD4 (FITC, clone RM4-5)/anti-CD8 (PE, clone 53-6.7) or anti-CD45RA/B220 (PE, clone RA3-6B2)/anti-IgE antibodies (FITC, clone R-35-72) or the appropriate isotype controls diluted in staining buffer for 30 minutes. Cells were washed, incubated with propidium iodide (PI) for 5 minutes to stain dead cells. After a final wash, cells were resuspended in staining buffer and analyzed with a FACSCaliber flow cytometer (Becton Dickinson, San Jose, CA) using a PI viability gate. Data for a total of 10,000 cells were collected based on the forward-side scatter lymphocyte gate.

2.7. Statistical Analysis. The OPA inhalation exposure system is capable of exposing mice to a single concentration with an equivalent inhalation tower used for the concurrent control group. Therefore, all concentrations for each exposure paradigm (Figure 1) required its own concurrent control, and all data analysis was performed relative to the concurrent control. Treatment effects were determined using a Student's *t*-test comparing a single OPA concentration to the concurrent control. Differences were considered significant at $P < .05$. For clarity of presentation, data have been normalized to the concurrent control group and shown as fold change, unless otherwise stated.

3. Results

3.1. OPA Inhalation Did Not Induce Overt Signs of Systemic Toxicity and Respiratory Distress. Inhalation exposure to OPA did not cause observable clinical signs of systemic toxicity or respiratory distress throughout the exposure dose response. All mice actively groomed following each exposure period and were observed to eat and drink shortly after exposure. However, mice treated with 1000 ppb OPA for 3 days followed by sacrifice on the 5th day (sensitization exposure; Figure 1) had lost approximately 10% of their initial body weight versus 1.9% in the controls. Over the rest period, mice regained weight at a similar rate as the control group. Subsequent inhalation challenge with the same OPA concentration (sensitization/challenge exposure; Figure 1) resulted in a smaller body weight loss (5% loss from initial body weight). Similar body weight loss was observed in mice exposed to 500 ppb OPA but concentrations below 500 ppb showed similar body weight changes as observed in the control mice (data not shown). No mice died from OPA inhalation prior to the scheduled study termination.

3.2. Inhalation Exposure to OPA Vapor Induced the Production of OPA-Specific Antibodies. Serum was collected and evaluated to determine if mice exposed to OPA developed systemic sensitization resulting in the production of antibodies specific for OPA-conjugated protein. No OPA-specific antibodies were observed in any of the control mice or mice exposed to OPA using the sensitization exposure. In contrast, inhalation challenge of mice to ≥500 ppb OPA vapor resulted in OPA-specific IgG_1 production. The OD_{450} values were 1.76 ± 0.02 and 1.35 ± 0.35 for a 1/20 dilution of serum from mice treated with 500 or 1000 ppb OPA, respectively, versus a background OD_{450} in the control sera of 0.086 ± 0.02. Serum from mice treated with 500 and 1000 ppb OPA also showed mild antibody specificity for native MSA as observed in our previous studies following dermal exposure to OPA [9]. OPA-specific IgE was not detected in the serum of any mice (data not shown).

3.3. OPA Inhalation Induces Cytokine Gene Expression in the Airways and Draining Lymph Nodes. Gene expression analysis of key cytokine genes can provide insight into the immunotoxicity of inhaled chemicals especially when examined in the target mucosal tissue lining the airways and the associated draining lymph nodes. Pro/anti-inflammatory cytokines (IL-10, TNFα, IL-1β) are important in the initial stages of an immune response to a chemical sensitizer and can shape the developing immune response towards Th1 immunity (IFNγ), Th2 immunity (IL-4, IIL-5, IL-13), or a combination. Sensitization and allergy are supported mainly by Th2 cytokine responses although Th1 cytokines have been shown to be important for chemical sensitizers [23]. Lymph nodes in the cervical region drain the skin of the head and the nasal mucosa. Since OPA is a highly reactive chemical, the mandibular lymph nodes draining the nasal mucosa, a target site, were examined for gene expression changes. The inhalation of OPA during sensitization resulted in concentration-dependent upregulation of IL-4 and IL-5 expression (significant increases ≥500 ppb OPA; Table S1) in the mandibular lymph nodes (Figure 2). The expression of TNFα, IL-1β, and IL-10 were also increased in the lymph nodes of mice treated with 1000 ppb OPA. Subsequent OPA challenge for an additional 3 days resulted in increased IL-4 gene expression throughout the concentration range tested (Figure 2; Table S1). The expressions of IL-10 and IL-1β were also increased in the lymph nodes following challenge with OPA although to a lesser extent than following sensitization alone (Figure 2; Table S1).

The inhalation of OPA also increased cytokine gene expression in the mucosal tissue lining the upper airways (Figure 3; Table S1). Increased IL-4 expression in mice exposed to 1000 ppb OPA and a concentration-dependent stimulation of IL-1β in the nasal mucosa occurred in mice exposed to OPA during sensitization only suggesting acute inflammation. Challenging mice after an 11-day rest period to the same concentration of OPA vapor for an additional 3 days caused marked concentration-dependent increases in all of the cytokine genes interrogated. IL-4, IL-5, and IL-1β were upregulated at all concentrations of OPA while IL-13, IFNγ, TNFα, and IL-10 were increased at ≥500 ppb. Mice that inhaled the higher concentrations of OPA also showed increased expression of IL-4, IL-5, IL-13, and IL-1β in the lung tissues although not statistically significant (Figure 4; Table S1).

3.4. Respiratory Exposure to OPA Induces Lymphocyte Proliferation and an Allergic Phenotype in the Mandibular Lymph Nodes. A hallmark characteristic of respiratory allergy is proliferation of lymphocytes, namely, B lymphocytes in the lymph nodes draining the target tissue. OPA inhalation

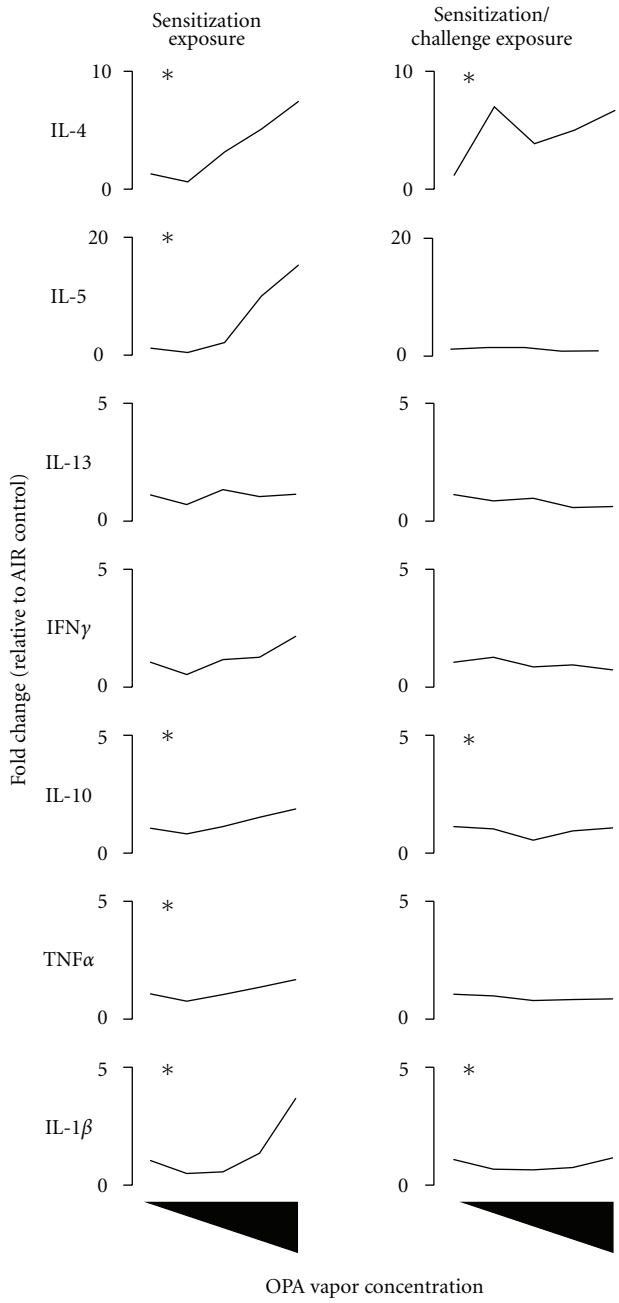

Figure 2: *Inhalation of OPA vapor induces the expression of Th2 and pro/anti-inflammatory cytokines in the draining lymph nodes of mice.* Mice were exposed to OPA (125, 250, 500, 1000 ppb) or filtered air according to the schedule shown in Figure 1. Two days following the final exposure, the mandibular lymph nodes from the left side of the neck were removed and processed for gene expression analysis. Data are presented as mean ($n = 5$) and represent fold change relative to the concurrent control group. *Indicates that the cytokine was significantly increased at one or more of the OPA concentrations. Refer to Table S1 of the supplementary material available online at doi: 10.1155/2011/751052 for the empirical gene expression data.

resulted in a concentration-dependent increase in total lymphocytes in the mandibular lymph nodes with a maximum increase of approximately 4 fold over the control mice (Figure 5). Both T lymphocytes and B lymphocytes were increased in the lymph nodes of OPA-exposed mice, however, the increase in B lymphocytes was much greater. The ratio of T : B lymphocytes was reduced in a concentration-dependent manor in mice treated with OPA reflecting the greater proliferation of B lymphocytes relative to T lymphocytes (Table 1). The ratio of CD4:CD8 T lymphocytes in the draining lymph nodes did not change in any of the treatment groups relative to the controls (data not shown).

3.5. OPA Inhalation Induces Isotype Switching to IgE$^+$ B Lymphocytes in the Draining Lymph Nodes. An important characteristic of an allergic response is the isotype switch to

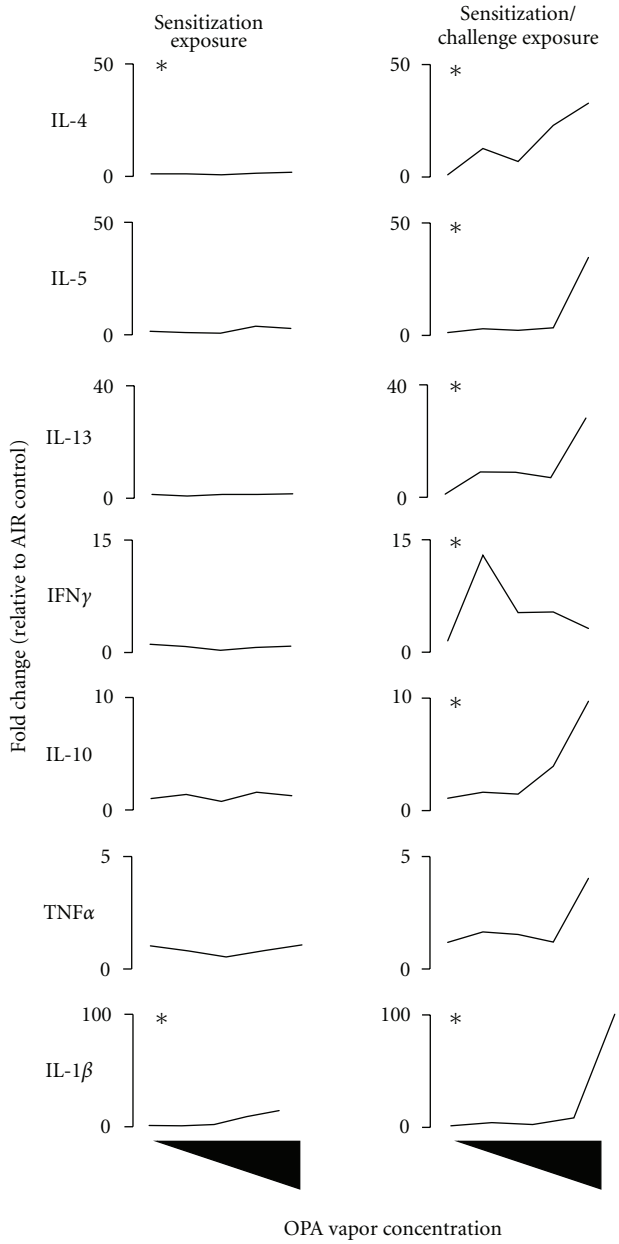

Figure 3: *Inhalation of OPA vapor induces the expression of Th2, Th1, and pro/anti-inflammatory cytokines in the nasal mucosa of mice.* Mice were exposed to OPA (125, 250, 500, 1000 ppb) or filtered air according to the schedule shown in Figure 1. Two days following the final exposure, the mucosal tissue lining the maxilloturbinates and lateral wall of the nasal cavity were removed and processed for gene expression analysis. Data are presented as mean ($n = 5$) and represent fold change relative to the concurrent control group. *Indicates that the cytokine was significantly increased at one or more of the OPA concentrations. Refer to Table S1 for the empirical gene expression data.

IgE producing B lymphocytes. We utilized multicolor flow cytometry to identify the B lymphocyte population in the draining lymph nodes and enumerated the IgE^+ and IgE^- populations using an anti-IgE antibody. Figure 6 shows that inhalation exposure to OPA during sensitization only did not induce isotype switching to IgE^+ B lymphocytes at any concentration tested. In contrast, inhalation challenge 11 days later to the same OPA concentration resulted in a marked increase in the number of B lymphocytes expressing IgE indicating isotype switching to IgE (Figure 6). The percentage of total B-lymphocytes producing IgE in the mandibular lymph nodes increased from $7.32 \pm 1.08\%$ in the control group to 11.38 ± 0.99, 34.01 ± 5.06, 41.62 ± 8.72, and $22.67 \pm 2.09\%$ in mice exposed to 125, 250, 500, and 1000 ppb OPA, respectively. These effects were significant at all concentrations of OPA tested.

4. Discussion

Ortho-Phthalaldehyde has been approved by the FDA for the high-level disinfection of reusable heat sensitive medical/dental instruments [24] and is increasingly being substituted

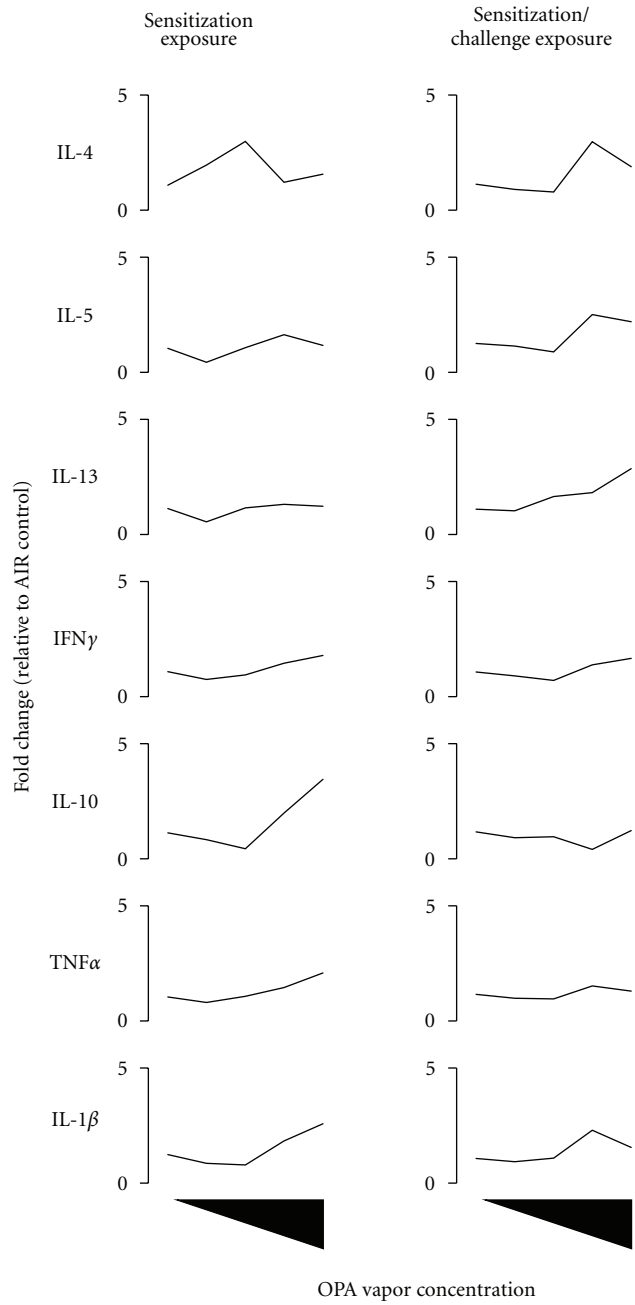

Figure 4: *Effect of respiratory exposure to OPA vapor on the expression of Th2, Th1, and pro/anti-inflammatory cytokines in the lungs of mice.* Mice were exposed to OPA (125, 250, 500, 1000 ppb) or filtered air according to the schedule shown in Figure 1. Two days following the final exposure, the lungs were removed, inflated with RNALater, and processed for gene expression analysis. Data are presented as mean ($n = 5$) and represent fold change relative to the concurrent control group. *Indicates that the cytokine was significantly increased at one or more of the OPA concentrations. Refer to Table S1 for the empirical gene expression data.

for glutaraldehyde in the healthcare industry. Approval was rendered despite a lack of toxicity data supporting OPA as a safe alternative. There have been numerous case reports on skin and respiratory complications in healthcare workers and patients exposed to OPA suggesting similar health risks to glutaraldehyde. The goal of the present studies was to determine if OPA has the potential to act as a respiratory sensitizer following inhalation exposure. Our previous studies demonstrated that OPA was a potent dermal sensitizer following skin exposure [9]. In order to study the potential for the inhalation of OPA to cause respiratory sensitization, a nose-only exposure system was developed to minimize skin contact. Cytokine gene expression and lymphocyte phenotyping in the respiratory mucosa and draining lymph nodes provide evidence that OPA is a respiratory sensitizer in mice.

OPA is a highly reactive chemical with an affinity for biological amines, a property exploited in its use as a biocidal

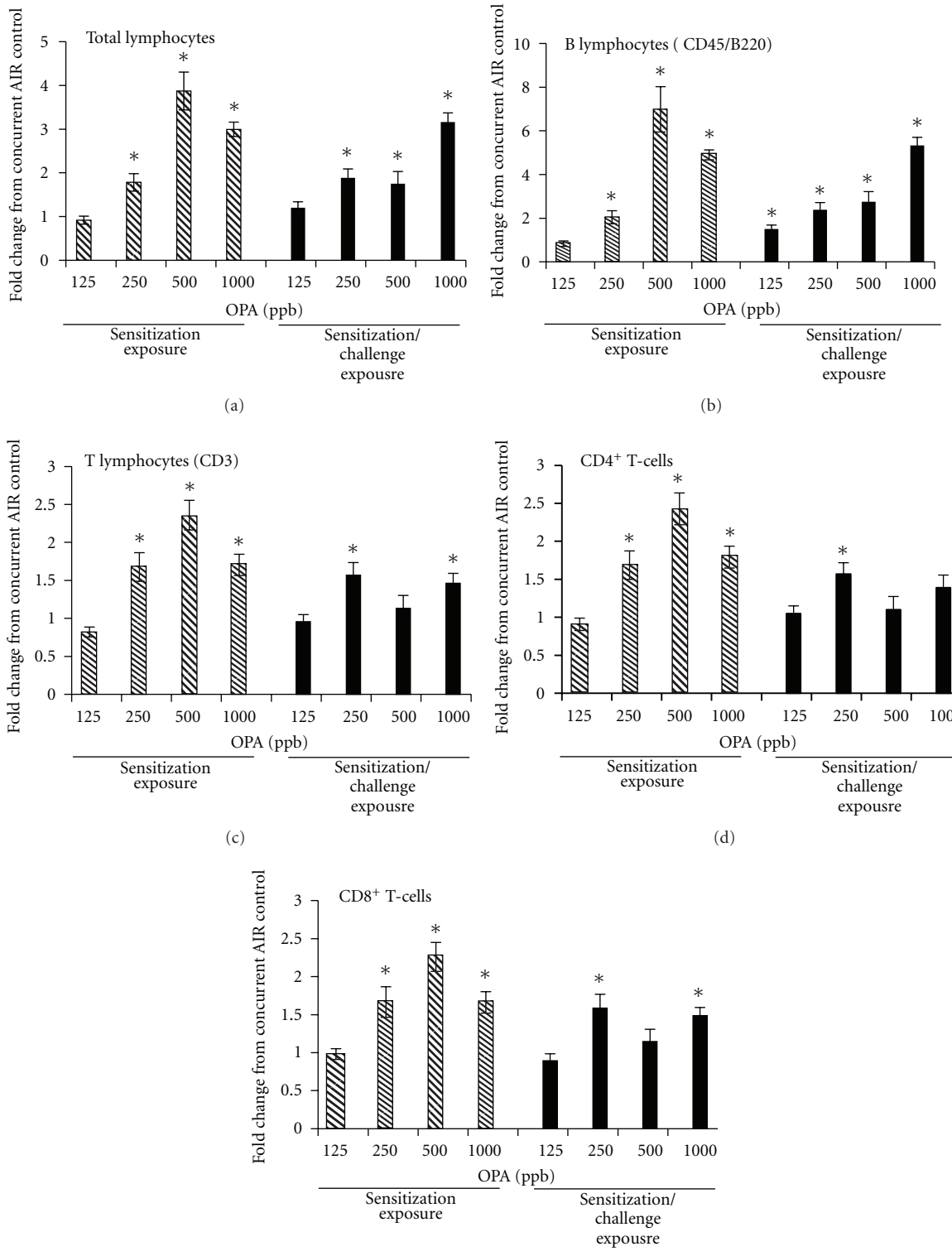

Figure 5: *OPA inhalation stimulates B- and T-lymphocyte proliferation in the draining lymph nodes of mice.* Mice were exposed to OPA or filtered air according to the schedule shown in Figure 1. Two days following the final exposure, the mandibular lymph nodes from the right side of the neck were removed and immediately processed into single-cell suspensions. Scatter properties were used to identify lymphocytes followed by fluorescent antibodies labeling to identify B lymphocytes, T lymphocytes (total, $CD4^+$ and $CD8^+$) using flow cytometry. Data are presented as fold change relative to the concurrent control. Statistical analysis was performed on the absolute cell counts for each lymphocyte population. Hatched bars represent mice that were exposed for 3 days followed by sacrifice on day 5 (sensitization exposure, Figure 1). Solid bars represent mice that were exposed on d1–d3, d16–d18 and sacrificed on d20 (sensitization/challenge exposure, Figure 1). Mean ± SEM ($n = 5$). *Significantly different from concurrent control group at $P < .05$. Absolute cell counts are presented in Table S2.

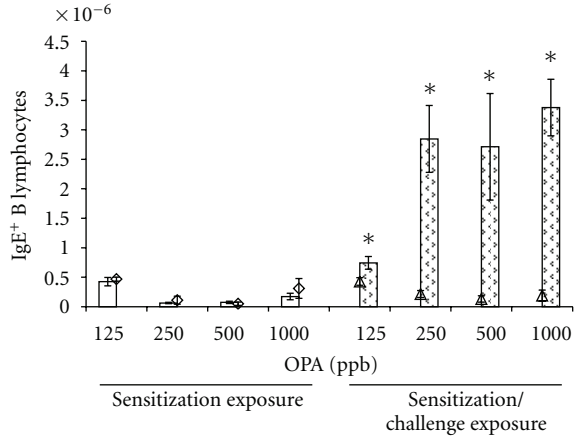

Figure 6: *OPA inhalation induces isotype switching in B lymphocytes to IgE.* Mice were exposed to OPA or filtered air according to the schedule shown in Figure 1. Two days following the final exposure, the mandibular lymph nodes from the right side of the neck were removed and immediately processed into single-cell suspensions. Scatter properties were used to identify lymphocytes followed by fluorescent antibodies labeling to identify B lymphocytes using flow cytometry. Fluorescent anti-IgE antibodies were used to identify B-lymphocyte expressing IgE on their membrane. Open bars and open diamonds represent mice that were exposed OPA or filtered air, respectively, for 3 days followed by sacrifice on day 5 (sensitization exposure, Figure 1). Stippled bars and open triangles represent mice that were exposed OPA or filtered air, respectively, on d1–d3, d16–d18 and sacrificed on d20 (sensitization/challenge exposure, Figure 1). Mean ± SEM ($n = 5$). *Significantly different from concurrent control group at $P < .05$.

Table 1: Inhalation of OPA skews the lymphocyte population heavily towards B lymphocytes in the mandibular lymph nodes.

OPA (ppb)	T-lymphocyte/B-lymphocyte Ratio (Fold change from concurrent control)
Sensitization Exposure	
125	1.19 ± 0.09[a]
250	−1.41 ± 0.19
500	−3.22 ± 0.24*
1000	−3.20 ± 0.29*
Sensitization/Challenge Exposure	
125	−1.58 ± 0.08*
250	−1.56 ± 0.11*
500	−2.39 ± 0.29*
1000	−4.13 ± 0.20*

* Significantly different from concurrent control group at $P < .05$.
[a]Mean ± SEM ($n = 5$).

agent. This property is also exploited for the detection of biomolecules since upon interaction with amines, OPA becomes highly fluorescent [25]. By virtue of its ability to react with proteins, OPA may also act as a hapten in biological systems and could possibly lead to aberrant immune and allergic reactions. These hapten reactions form the basis for sensitization resulting from exposure to reactive low molecular weight chemicals in the workplace. The present work shows that the inhalation of OPA results in the systemic production of IgG_1 antibodies that are specific for OPA-MSA, supportive evidence that OPA-protein conjugates formed *in vivo* are immunogenic. The production of IgG_1 in by B lymphocytes requires support from $CD4^+$ T lymphocytes producing IL-4 and IL-5 which facilitate isotype switching and maturation, respectively [26]. Passive transfer of chemical-specific IgG antibody to naïve mice has been shown to induce respiratory symptoms following exposure to toluene diisocyanate [27] and trimellitic anhydride [28] indicating a potential role for IgG in the pathogenesis of chemical-induced airway allergy. It is also possible that OPA-specific IgG_1 may represent a marker of exposure as has been suggested for diisocyanates [29, 30].

OPA-specific IgE was not detected following inhalation exposure; however, aggressive dermal exposure of mice to OPA resulted in the production of OPA-specific IgG_1, IgG_{2a}, and IgE isotypes supporting an allergic immune response to this chemical [9]. Similar chemical-hapten-specific antibody responses have been observed in rodent models for other low molecular weight chemicals including toluene diisocyanate [23, 31] and trimelitic anhydride [32]. OPA-specific antibodies have been detected in the serum of healthcare workers experiencing respiratory and dermal symptoms resulting from workplace exposure to OPA [18]. The presence of OPA-specific antibodies in the serum of sensitized workers may result from dermal and/or airway exposure. Importantly, basophils from healthcare workers were shown to have bound OPA-specific IgE as *in vitro* culture with 0.55% OPA solution resulted in histamine release that was similar to treating the cells with anti-IgE antibody [18]. This demonstrates a functional importance of OPA-specific antibodies.

A hallmark feature of chemical sensitizers is a positive reaction in the local lymph nodes assay (LLNA). Our laboratory showed that OPA tested positive in the LLNA with an EC3 of 0.051% [9]. This suggests that OPA is a powerful dermal sensitizer and may cause health risks when in contact with the skin at concentrations as much as 10 fold below the effective working concentration (0.55%) for disinfecting medical devices. Recently, a similar approach to the dermal LLNA has been presented for the identification of respiratory sensitizers. The respiratory LLNA utilizes inhalation as the route of exposure and examines the lymph nodes that drain the nasal mucosa for lymphocyte proliferation [33] and cytokine production [34]. Although the traditional LLNA endpoint of H^3 incorporation as a metric of lymphocyte proliferation was not used in the present study, mice that were exposed to OPA by inhalation showed a concentration-dependent increase in the absolute and relative lymphocyte counts in the mandibular lymph nodes. This response was significant at concentrations ≥250 ppb OPA following the 3 days of inhalation as well as in mice that were reexposed for an additional 3 days. B lymphocytes accounted for the majority of the proliferation suggesting a shift towards a humoral immune response. Although T-lymphocyte numbers also increased in the mandibular lymph nodes, the ratio of CD4 : CD8 T cells remained unchanged (data not shown).

Isotype switching to IgE is a cardinal feature of a type I hypersensitivity response. The serum of mice exposed to OPA via inhalation was negative for IgE antibodies specific for OPA-MSA although our previous work showed that dermal exposure to OPA did induce OPA-specific IgE [9]. The lack of systemic IgE may be due to the sensitivity of the ELISA assay or may result from a less aggressive sensitization response following inhalation exposure relative to dermal. Phenotyping the B-lymphocyte population in the draining lymph nodes can provide clues to the isotype specificity of the proliferating population. It has been shown that B lymphocytes from the lymph nodes of mice exposed to Th2 respiratory sensitizers are positive for IgE on their plasma membrane [35–38]. The inhalation of OPA vapor during sensitization did not increase the population of IgE^+ B lymphocytes at any concentration tested despite the dramatic proliferation observed for the B-lymphocyte population. In contrast, there was a significant increase in the number of IgE^+ B-lymphocytes in the mandibular lymph nodes at all concentrations of OPA following OPA challenge. Nearly 50% of the B lymphocytes from mice exposed to 500 ppb OPA were positive for IgE indicating isotype switching to an allergic phenotype, evidence that OPA may cause type I hypersensitivity.

Isotype switching to IgE is regulated by Th2 cytokines, namely, IL-4 [39] which is produced primarily by $CD4^+$ T lymphocytes [40]. There was increased proliferation of T lymphocytes following the inhalation of OPA, and these cells likely play an important role in shaping the humoral immune response to this chemical. The inhalation of OPA resulted in a concentration-dependent increase in IL-4 and IL-5 gene expression in the mandibular lymph nodes even following sensitization exposure regimen. Mice that were challenged with OPA showed significant upregulation of IL-4 gene expression at all concentrations of OPA supporting class switching to IgE in the B-lymphocyte population. In contrast, Th2 cytokine gene expression was unaltered in the nasal mucosa and lung following sensitization exposure. Inhalation challenge with OPA caused a marked concentration-dependent increase in the expression of the Th2 cytokine IL-4, IL-5, and IL-13 and to a lesser degree, the Th1 cytokine IFNγ. This strong shift towards Th2 cytokine production in the target mucosal tissue further supports an allergic response to OPA inhalation. In addition to these cytokines, the proinflammatory cytokine IL-1β was upregulated and is known to play an important role in priming antigen presenting cells. Th2 cytokine gene expression in the lungs was also upregulated in mice that received the challenge exposure to OPA although not statistically significant. The ability of OPA to stimulate Th2 and proinflammatory cytokines may be one mechanism responsible for its adjuvant-like properties observed in a murine OVA allergy model [11].

Overall, the findings of this study demonstrate that OPA has the potential to induce respiratory sensitization following inhalation exposure. Characteristics of the immune response to OPA suggest that exposure to this chemical may cause a type I hypersensitivity response as there was a large increase in the IgE^+ B-lymphocyte population. The immune response to OPA is similar to other low and high molecular weight chemicals that are known to cause occupational rhinitis and asthma, indicating the need for the regulation of OPA in the workplace. The concentration range tested in the present study is at least 10 fold higher than air concentrations that can be found in the workplace [14]. Therefore, further studies with lower concentrations and longer exposure periods are important to better characterize the immunotoxicity of OPA and to facilitate risk assessment. Additionally, the understanding the interaction between skin and respiratory exposure will be important for risk assessment for OPA. Previous work with other low molecular weight sensitizers have shown that skin exposure can prime the immune system to respond to future airway exposures [41–44]. There is a high potential for skin exposure during manual reprocessing of medical devices with OPA. The potent sensitizing response to dermal exposure [9] may significantly lower the threshold air concentration of OPA required to elicit an immune response in the airways. Our laboratory is currently examining the interaction between skin and airways in the development of OPA-induced allergic respiratory disease using the mouse model.

Disclosure

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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Research Article

Allergic Potential and Immunotoxicity Induced by Topical Application of 1-Chloro-4-(Trifluoromethyl)Benzene (PCBTF) in a Murine Model

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The purpose of the studies in this paper was to evaluate the allergic potential, immunotoxicity, and irritancy of the occupationally relevant chemical, 1-chloro-4-(trifluoromethyl)benzene, also known as parachlorobenzotrifluoride (PCBTF), following dermal exposure in a murine model. Evaluation of the sensitization potential, conducted using the local lymph node assay (LLNA) at concentrations ranging from 50% to 100%, identified a dose-dependent increase in lymphocyte proliferation with a calculated EC3 value of 53.1%. While no elevations in total or specific IgE were observed after exposure to any concentration of the chemical, significant increases in IFN-γ protein production by stimulated draining lymphoid cells were observed, indicating a T-cell-mediated response. Dermal exposure to PCBTF was not found to alter the immune response to a T-cell-dependant antigen. These results demonstrate that PCBTF has the potential to induce allergic sensitization following dermal exposure and based on LLNA results would be classified as a weak sensitizer.

1. Introduction

1-Chloro-4-(trifluoromethyl)benzene, a fluorinated toluene, also known as parachlorobenzotrifluoride (PCBTF), is a chemical used as an intermediate in a wide range of organic reactions for the synthesis of dyes, pharmaceuticals, pesticides, insecticides and herbicides [1]. It is primarily used as a solvent in commercial surface finishes, such as vapor degreasing, precision wipe cleaning, cold cleaning and electronics cleaning, and is manufactured in both pure and blended formulations based upon specific cleaning requirements [2, 3]. It is also used as an ink solvent in the printing industry and is a component (5–12%) of low volatile organic compound (VOC) compliant polyurethane finishes. PCBTF is not considered to be an air toxin or ozone depleter. It has therefore recently been considered exempt from VOC regulations [4], which has led to an increase in its use as a replacement for other solvents previously used in the manufacture of a variety of commercially-available paints, inks, and other products and finishes (Oxsol 100, Occidental Chemical Co.) [1].

PCBTF was recently nominated by the National Toxicology Program (NTP) for toxicological characterization due to its unknown chronic toxicity profile and changes in its industrial and consumer use [1]. In addition, its improper use or disposal may lead to an increase in public exposure outside of the occupational context. There are currently no Occupational Safety & Health Administration (OSHA), National Institute for Occupational Safety and Health (NIOSH), or American Conference of Governmental Industrial Hygienists (ACGIH) limits regulating PCBTF exposure [1].

Although the health effects of PCBTF have not been thoroughly tested, epidemiological studies in workers have reported increases in respiratory and stomach cancers [5]. Animal studies investigating the health effects of PCBTF exposure are limited. In a 13-week inhalational study in rats, no changes were identified in any measured clinical chemistry parameter, at doses up to 252 ppm, and no adverse

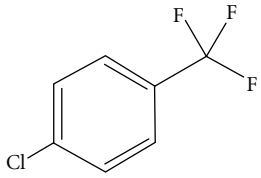

Figure 1: Chemical structure of PCBTF.

observations were recorded during exposures or during detailed weekly clinical evaluations [6]. Subchronic studies were negative for precancerous hematological changes and other histopathological indicators of carcinogenicity [7]. However, exposure to PCBTF did increase relative liver weights between dose groups. Subchronic inhalational and oral exposure to higher concentrations of PCBTF in rats produced clinical signs of toxicity that included salivation, tremors, altered hematological, and hepatocellular profiles [7]. These signs, however, were only noted at 1000 mg/kg/day, which is greatly outside of the range expected in a potential spill of PCBTF-containing paint products. PCBTF has low subchronic oral toxicity as well, and neither pathological nor adverse biochemical effects were found at doses up to 10 mg/kg/day, which have been described as the no-observable-effect level of PCBTF [8].

Although it is a primary route of occupational exposure, very few dermal exposure studies have been conducted on this chemical [2]. In addition, immunotoxicological studies are lacking. The recent increase in occupational use, along with the potential for dermal exposure warrants the evaluation of the immunotoxicity of PCBTF following dermal exposure.

2. Material and Methods

2.1. Test Articles and Chemicals. 1-Chloro-4-(trifluoromethyl)benzene (98%) (PCBTF; Figure 1) [CAS no. 98-56-6], alpha-hexylcinnamaldehyde (HCA) [CAS no. 101-86-0], 2,4-dinitrofluorobenzene (DNFB) [CAS no. 70-34-8], toluene 2,4-diisocyanate (TDI, CAS 584-84-9) and cyclophosphamide [CAS no. 50-18-0] were all purchased from Aldrich Chemical Company, Inc. (Milwaukee, Wis).

2.2. Species Selection. Female BALB/c and B6C3F1 mice were used in these studies. BALB/c mice have a Th2 bias and are commonly used to evaluate potential IgE-mediated sensitization. They were therefore used in the hypersensitivity studies [9, 10]. B6C3F1 mice are the strain of choice for immunotoxicity studies and were used to evaluate the IgM response to SRBC [11]. The mice were purchased from Taconic (Germantown, NY) at 6–8 weeks of age. Upon arrival, the animals were allowed to acclimate for a minimum of 5 days. Each shipment of animals was randomly assigned to a treatment group, weighed, and individually identified via tail marking using a permanent marker. A preliminary analysis of variance on body weights was performed to ensure a homogeneous distribution of animals across treatment groups. The animals were housed at a maximum of 5 per cage in ventilated plastic shoebox cages with hardwood chip bedding. NIH-31 modified 6% irradiated rodent diet (Harlan Teklad), and tap water was provided from water bottles, *ad libitum*. The temperature in the animal facility was maintained between 68 and 72°F and the relative humidity between 36 and 57%. The light/dark cycle was maintained on 12-hour intervals. All animal experiments were performed in the AAALAC accredited NIOSH animal facility in accordance with an animal protocol approved by the Institutional Animal Care and Use Committee.

2.3. Concentration Range Finding Studies. Concentration range finding studies were performed to select the concentrations of PCBTF to be used for dermal exposures. BALB/c mice were exposed topically to acetone vehicle or increasing concentrations of PCBTF up to 100% in acetone on the dorsal surface of each ear (25 μL per ear) for three consecutive days. Animals were allowed to rest for 2 days following the last exposure and then weighed and examined for signs of toxicity, such as loss of body weight, fatigue/lack of activity, and ungroomed fur. The maximum concentration selected for the subsequent studies was based on limits of toxicity.

2.4. Combined Local Lymph Node and Irritancy Assay. To determine the irritancy and sensitization potential of PCBTF, a combined local lymph node assay (LLNA) was conducted. PCBTF dosing concentrations (50–100%) and vehicle (acetone) were selected based on solubility and preliminary concentration range finding studies. The LLNA was performed according to the method described in the ICCVAM Peer Review Panel report (1999) with minor modifications [12]. Briefly, mice (5 per group) were topically treated with acetone vehicle, increasing concentrations of PCBTF, or positive control (30% alpha-hexylcinnamaldehyde; HCA) on the dorsal surface of each ear (25 μL per ear) once a day for three consecutive days. 2,4-dinitrofluorobenzene (DNFB) was used as a positive control for irritancy. Irritancy measurements were performed as previously described [13]. The thickness of the right and left ear pinnae of each mouse was measured using a modified engineer's micrometer (Mitutoyo Co.) before the first chemical administration and 24 hours following the final exposure. The mean percentage of ear swelling was calculated based on the following equation: [(mean postchallenge ear thickness − mean prechallenge ear thickness)/mean prechallenge thickness] × 100. Animals were allowed to rest for 2 days following the last exposure. On day 6 mice were injected intravenously, via the lateral tail vein, with 20 μCi ^{3}H-thymidine (Dupont NEN; specific activity 2 Ci/mmol). Five hours after ^{3}H-thymidine injection, animals were euthanized via CO_2 inhalation, and the left and right superficial parotid draining lymph nodes (DLNs), located at the bifurcation of the jugular vein, were excised and pooled for each animal. Single cell suspensions were made and incubated overnight in 5% trichloroacetic acid (TCA), and samples were counted using a Packard Tri-Carb 2500TR liquid scintillation analyzer (Perkin Elmer). Stimulation indices (SI) were calculated by dividing the mean disintegrations per minute (DPM) per test group by

the mean DPM for the vehicle control group. EC3 values (concentration of chemical required to induce a 3-fold increase over the vehicle control) were calculated based on the equations from Basketter et al. [14].

2.5. Phenotypic Analysis of Lymphocytes. Lymphocyte phenotypes were analyzed using flow cytometry as described by Manetz and Meade [15]. For the phenotypic analysis, mice were topically exposed to acetone or increasing concentrations of PCBTF (up to 100%) on the dorsal surface of each ear (25 μL per ear) once a day for four consecutive days. Animals were allowed to rest for 6 days after the final treatment and then euthanized on day 10 by CO_2 inhalation. Animals were weighed and examined for gross pathology at the end of the experiment. The following organs were removed, cleaned of connective tissue and weighed: liver, spleen, kidneys, and thymus. DLNs (two nodes/animal/tube) and spleens were also collected separately in 3 mL PBS and were dissociated using the frosted ends of two microscope slides. Cell counts were performed using a Coulter Counter (Z2 model, Beckman Coulter), and 1×10^6 cells per sample were added to the wells of a 96-well plate. Cells were washed using staining buffer (1% bovine serum albumin/0.1% sodium azide in PBS) and then incubated with Fc block (clone 2.4G2). For IgE+/B220+ analysis, the cells were incubated with anti-CD45RA/B220 (PE, clone RA3-6B2) and anti-IgE antibodies (FITC, clone R-35-72) or appropriate isotype control diluted in staining buffer. For analysis of T-cell subsets, cells were incubated with anti-mouse CD3e antibody (APC, clone 145-2C11), anti-mouse CD4 antibody (FITC, clone RM4-5), and anti-mouse CD8a antibody (PE, clone 53-6.7) or the appropriate isotype controls diluted 1 : 100 in staining buffer. All antibodies and isotype controls were purchased from BD Pharmingen. Cells were then washed and incubated with propidium iodide (PI). After a final wash, cells were resuspended in staining buffer and analyzed with a Becton Dickinson FACSCalibur flow cytometer using a PI viability gate.

2.6. Total Serum IgE. For analysis of total IgE, PCBTF was tested at concentrations up to 100%. Mice were treated with acetone, increasing concentrations of PCBTF, or, as a positive control, 1.5% TDI on the dorsal surface of each ear (25 μL per ear) once a day for four consecutive days. Animals were allowed to rest for 6 days after the final treatment and were euthanized on day 10 by CO_2 inhalation. Following euthanasia, blood samples were collected via cardiac puncture. Sera were separated by centrifugation (10 min at 10,000 $\times$ g) and frozen at -20°C for next day analysis of IgE by ELISA. A standard colorimetric sandwich ELISA was performed as previously described [16]. All antibodies and isotype controls were purchased from BD Pharmingen. In brief, 96-well flat bottom plates (Dynatech Immulon-2) were coated with purified monoclonal rat anti-mouse IgE antibody (clone R35-72; 2 μg/mL, diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6), sealed with plate sealers, and incubated overnight at 4°C. The plates were washed 3 times with PBS/Tween 20 and then blocked for 1 hour with diluent (2% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, Utah) and 0.05% sodium azide) at room temperature. Serum samples were diluted 1 : 10 in diluent and IgE control standards (mouse IgE anti-TNP, clone C38-2) were prepared (highest concentration: 500 ng/mL). The diluted serum samples and IgE control standards were then serially diluted (1 : 2) through 8 wells, added to the coated plates in a 100 μL volume and incubated at room temperature for 1 hour. The plates were washed 3 times with PBS/0.05% Tween 20. Biotin-conjugated rat anti-mouse IgE (clone R35-92; 2 μg/mL) was added in a 100 μL volume and plates were incubated at room temperature for 1 hour. The plates were then washed 3 times with PBS/0.05% Tween 20. Streptavidin-alkaline phosphatase was added (100 μL of a 1 : 400 in diluent) and plates were incubated for 1 hour at room temperature. P-Nitrophenyl phosphate (Sigma) was used as the alkaline phosphatase substrate and added to the plates in a 100 μL volume. The plates were allowed to develop for up to 30 minutes at room temperature or until the optical density (OD) reading of the highest standard reached 3.0. Absorbance was determined using a Spectramax Vmax plate reader (Molecular Devices) at 405–605 nm. Data analysis was performed using the IBM Softmax Pro 3.1 (Molecular Devices), and the IgE concentrations for each sample were interpolated from a standard curve using multipoint analysis.

2.7. Analysis of Cytokine Production by Draining Lymph Node Cells. To determine cytokine protein production by lymphocytes, DLNs of mice used for the analysis of total IgE were collected (two nodes/animal/tube) in 2 mL PBS and dissociated using the frosted ends of two microscope slides. Cell counts were performed using a Coulter Counter (Z1 model, Beckman Coulter), and cells were adjusted to 1×10^6 cells/mL using sterile RPMI media containing 10% FBS. Cells were added to a 48-well plate in a 500 μL volume, stimulated with α-CD3 and α-CD28 (2 μg/mL of each; BD Pharmingen) and incubated for 24 hours at 37°C and 5% CO_2. Supernatants were analyzed for IL-4 and IFN-γ production using an OptEIA ELISA kit purchased from BD Biosciences according to the manufacturer's instructions. Supernatants collected from each culture (2 stimulated and 2 unstimulated for each mouse) were added to the plates in triplicate along with serial dilutions of the standards. Plates were read at 450 nm [OD values for standards ranging from 0.77–1.93] using a SpectraMax M2 spectrophotometer (Molecular Devices). Cytokine concentration was extrapolated from the standard curve. The final data are expressed as the mean value generated when the concentration identified for the unstimulated cultures was subtracted from the value generated from the stimulated cultures for each mouse.

2.8. In Vivo IgM Response to the T-Cell-Dependent Antigen, SRBC. The primary IgM response to sheep red blood cells (SRBC) was enumerated using a modified hemolytic plaque assay of Jerne and Nordin [17]. B6C3F1 mice were dermally exposed to PCBTF (6–100%) for 14 days (25 μL/ear). Four days prior to euthanasia (day 11), the mice were immunized

with 7.5×10^7 SRBC by intravenous injection in a 200 μL volume. All SRBC for these studies were drawn from a single donor animal (Lampire Laboratories, Pipersville, Penn). On the day of sacrifice, mice were euthanized by CO_2 asphyxiation, body and organ weights were recorded and spleens were collected in 3 mL of Hanks Balanced Salt Solution (HBSS). Single cell suspensions of the spleens from individual animals were prepared in HBSS by disrupting the spleen between the frosted ends of microscopic slides. To identify the total number of spleen cells, 20 μL of cells were added to 10 mL of isoton buffer (1 : 500) and two drops of Zap-o-globin were added to lyse red blood cells. Cells were then counted using a Coulter counter. 1 : 30 and 1 : 120 dilutions of spleen cells were made. One hundred μL of the dilutions were added to a test tube containing a 0.5 mL warm agar/dextran mixture (0.5% Bacto-Agar, DIFCO; and 0.05% DEAE dextran, Sigma), 25 μL of 1 : 1 ratio of SRBC suspension, and 25 μL of 1 : 4 dilution (1 mL lyophilized) guinea pig complement (Cedarlane Labs). Each sample was vortexed, poured into a petri dish, covered with a microscope coverslip, and incubated for 3 hours at 37°C. The plaques (representing antibody forming B-lymphocytes) were viewed and quantified after this incubation. Results were expressed as specific activity (IgM PFC per 10^6 spleen cells) and total activity (IgM PFC per spleen).

2.9. Statistical Analysis. For analysis of the data generated from the described animal studies, the data were first tested for homogeneity using the Bartlett's Chi Square test. If homogeneous, a one-way analysis of variance (ANOVA) was conducted. If the ANOVA showed significance at $P < .05$ or less, the Dunnett's Multiple Range t test was used to compare treatment groups with the control group. Linear trend analysis was performed to determine if PCBTF had exposure concentration-related effects for the specified endpoints. Statistical analysis was performed using Graph Pad Prism version 5.0 (San Diego, CA). Statistical significance is designated by $*(P \leq .05)$ and $**(P \leq .01)$.

3. Results

3.1. In Vivo Studies Identified PCBTF to Be an Allergic Sensitizer. Dermal exposure to PCBTF was not found to be toxic at any concentration tested (data not shown). For this reason, concentrations of PCBTF up to 100% were tested in the subsequent studies. No ear swelling was observed in mice after dermal exposure to PCBTF (Figure 2), suggesting that PCBTF is nonirritating. DNFB (0.03%) was used as a positive control for irritancy studies and resulted in an average significant increase of 84% in ear swelling after application (data not shown). In the LLNA, dose-dependent (Linear Trend test; $P < .01$) increases in DLN proliferation were observed after treatment with PCBTF, with counts from the 75% and 100% PCBTF exposed animals being significantly elevated over the vehicle control animals (Figure 3). SI values were 2.6, 5.3, and 5.3 for the 50%, 75%, and 100% exposure groups, respectively. An EC3 value of 53.1% (Figure 3) was calculated. HCA (30%) was used as a positive control for

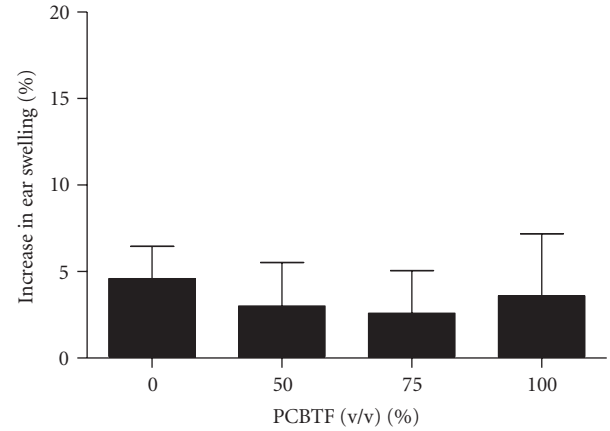

Figure 2: Ear swelling as a result of topical application of PCBTF. Analysis of irritation after topical application of PCBTF. Bars represent mean ± SE of 5 mice (10 ears) per group.

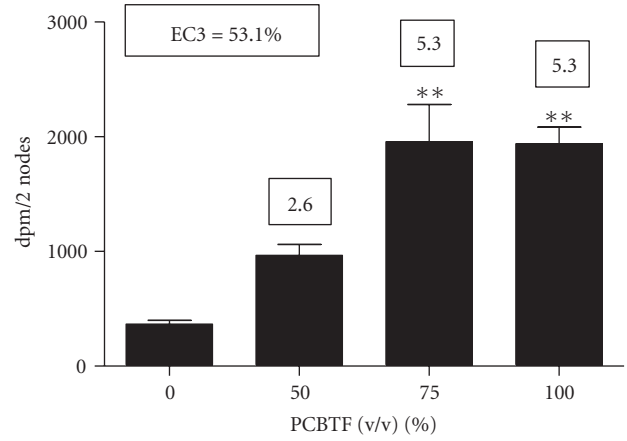

Figure 3: Allergic sensitization potential after dermal exposure to PCBTF. Analysis of the allergic sensitization potential of PCBTF using the LLNA. ^{3}H-thymidine incorporation into draining lymph node cells of BALB/c mice following exposure to vehicle or concentration of PCBTF. Numbers in boxes appearing above the bars represent the stimulation indices for each concentration tested. Bars represent mean ± SE of 5 mice per group. Levels of statistical significance are denoted $**(P < .01)$ as compared to acetone vehicle.

these experiments and resulted in an average SI value of 24.5 (data not shown). Consistent with the LLNA results, PCBTF exposure also significantly elevated the cellularity of the DLN (Figure 4) following exposure to all concentrations.

3.2. Exposure to PCBTF Did Not Induce an Increase in Local or Systemic IgE Levels. No changes in body or organ weights were observed after exposure to PCBTF for these studies (data not shown). The mechanisms of PCBTF sensitization were further investigated using phenotypic analysis of B220+ and IgE+B220+ expressing cells in the DLNs and spleen. No increases were observed in the B220+ cell or IgE+B220+ cell populations (Table 1) in the DLN or spleen. No changes in the percentage of CD4+ or CD8+ T cells in the DLN or

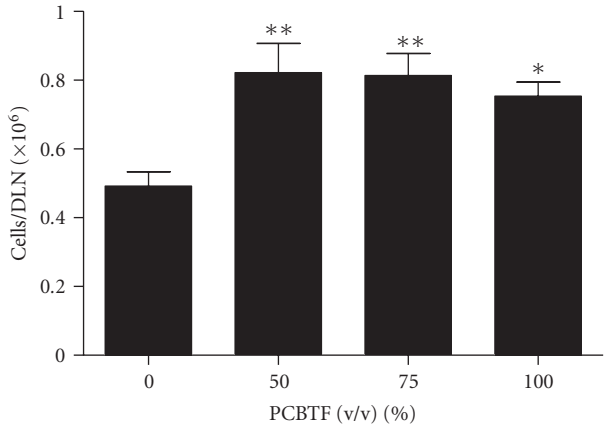

Figure 4: Increased cellularity of DLN after PCBTF exposure. Analysis of lymphocyte cellularity (cells/DLN) following exposure to PCBTF. Bars represent means ± SE of 5 mice per group. Levels of statistical significance are denoted as $^{*}(P \leq .05)$ and $^{**}(P \leq .01)$ as compared to acetone vehicle.

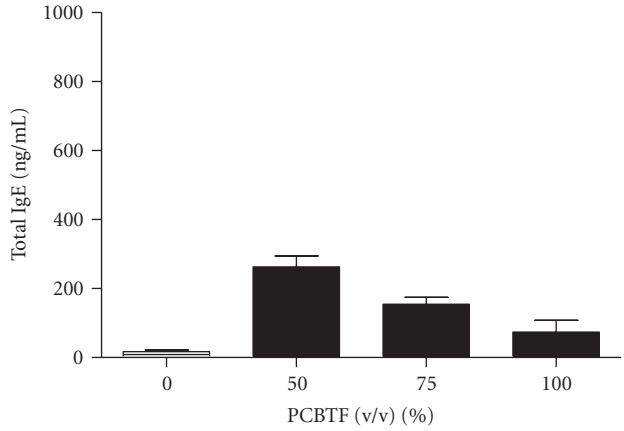

Figure 5: Lack of increase in serum IgE following PCBTF exposure. Analysis of total serum IgE after exposure to PCBTF. Bars represent means ± SE of 5 mice per group.

spleen were observed after exposure to any concentration of PCBTF (data not shown). Consistent with the IgE+B220+ results, exposure to PCBTF did not elevate total serum IgE levels after exposure to any treatment groups (Figure 5). Dermal exposure to the respiratory sensitizer TDI (1.5%) significantly elevated the B220+ (19.8 ± 2.3%) cell population, IgE+B220+ (13.3 ± 2.8%) cell populations, and total IgE (1587 ± 109 ng/mL) levels (data not shown).

3.3. Exposure to PCBTF Increased Production of IFN-γ, but Not IL-4 by Stimulated DLN Cells. Levels of IL-4 and IFN-γ cytokine production by stimulated draining lymphoid cells were analyzed to evaluate the effect of PCBTF exposure on Th1/Th2 balance. A dose-responsive (Linear Trend test; $P < .01$) increase in IFN-γ protein production by the DLN was observed after dermal exposure to PCBTF. Significant elevations in cytokine production were observed at PCBTF concentrations 50% and greater (Figure 6(a)). The maximum increase in INF-γ protein expression was calculated to be 1,174 ± 169 pg/mL. No significant alterations in IL-4 production were detected at any dose concentration (Figure 6(b)). To confirm the lack of Th2 cytokine induction following PCBTF exposure, cytokine mRNA levels were also analyzed in the DLNs of dermally exposed animals. Consistent with the protein data, no increase in IL-4 or IL-13 expression was observed (data not shown). Dermal treatment with 1.5% TDI elevated IL-4 and IL-13 mRNA expression in the DLN and significantly enhanced IL-4 protein production by stimulated draining lymphoid cells (496 ± 20 pg/mL) (data not shown). The T-cell-mediated sensitizer HCA (30%) significantly elevated IFN-γ expression (2,122 ± 67 pg/mL) (data not shown).

3.4. Dermal Exposure to PCBTF Did Not Alter the IgM Response to SRBC. To evaluate immunosuppressive potential, the murine splenic IgM response to SRBC was examined following a 14-day exposure to PCBTF. No changes in total (PFC/spleen) or specific (PFC/10^6 cells) IgM antibody activity to SRBC were observed after exposure to any concentration of PCBTF (Figure 7). Animals exposed to the positive control, cyclophosphamide, had a significantly reduced specific spleen IgM response (67%) and total IgM response (54%) compared to the acetone control animals. No changes in body or organ weights were observed for these animals (data not shown).

4. Discussion

More than 13 million workers in the United States are potentially exposed to chemicals that can be absorbed through the skin. Dermal exposure to chemicals in the workplace has a great potential to affect immune function. Contact dermatitis is the second most commonly reported occupational illness responsible for up to 30% of all cases of occupational disease in industrialized nations [18]. This may result in considerable social and economic implications, including time off from work, loss of workplace productivity, reduced quality of life, and medical and worker's compensation costs, accounting for the loss of billions of dollars [19].

Results from these studies suggest that PCBTF is a weak T-cell-mediated sensitizer. This was evidenced by an increase in lymphocyte proliferation and IFN-γ protein production by stimulated draining lymphoid cells, in the absence of elevations in markers of IgE-mediated sensitization, such as total IgE and IL-4 (mRNA and protein) production, following dermal exposure in mice. The EC3 value for PCBTF, 53.1%, falls within the working concentration for this chemical, as it is used at concentrations up to 100% as a solvent and as a chemical intermediate in the production of other chemicals. This suggests a greater predicted risk for worker sensitization. Immune suppression and other immunotoxic effects were not observed after exposure to this chemical.

Although exposure can occur through inhalation and dermal contact during its production and use [2], there

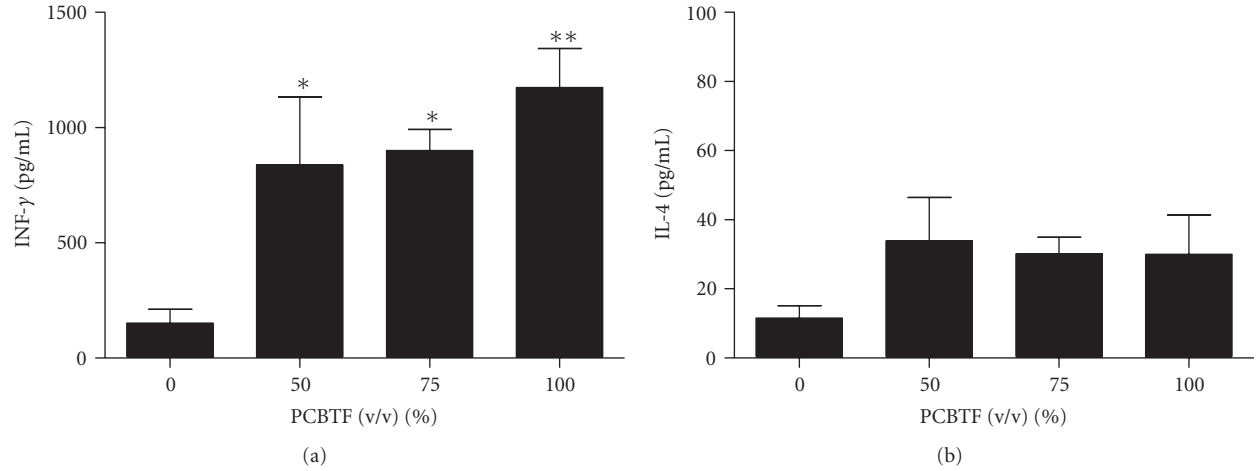

Figure 6: Increased IFN-γ protein production by DLN. Analysis of cytokine protein expression (a) IL-4 and (b) IFN-γ generated by stimulated DLN after dermal exposed to PCBTF. Bars represent mean fold change ± SE of 5 mice per group. Levels of statistical significance are denoted as $^{*}(P < .05)$ and $^{**}(P < .01)$ as compared to acetone vehicle.

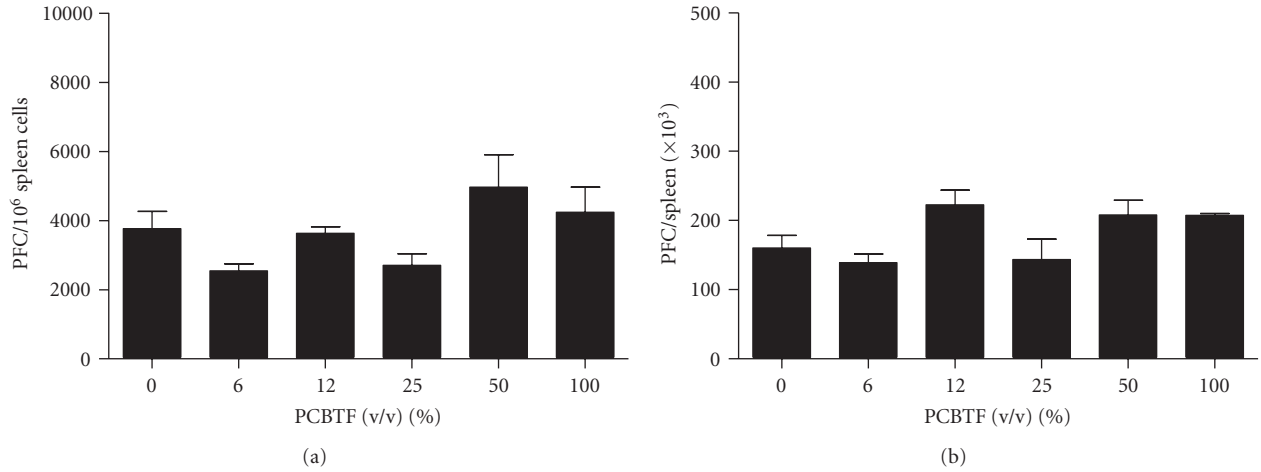

Figure 7: PCBTF exposure does not alter the IgM response to SRBC. Analysis of antibody producing spleen cells after a 14-day dermal exposure to PCBTF did not produce alternations in the total (a) and specific activity (b) IgM response to SRBC. Bars represent mean fold change ± SE of 5 mice per group.

Table 1: Phenotypic analysis after *in vivo* PCBTF treatment.

Dose group	IgE+B220+ (% lymphocyte population)		B220+ (% lymphocyte population)	
	%	Cells × 10^6	%	Cells × 10^6
DLN				
Acetone	1.1 ± 0.3	0.09 ± 0.02	11.5 ± 1.9	1.1 ± 0.16
PCBTF				
50%	4.5 ± 2.1	0.13 ± 0.02	14.0 ± 3.1	2.0 ± 0.53
75%	3.5 ± 0.4	0.12 ± 0.02	12.6 ± 0.9	1.8 ± 0.24
100%	2.4 ± 0.6	0.14 ± 0.01	12.0 ± 1.4	1.8 ± 0.23
Spleen				
Acetone	4.2 ± 2.4	2.1 ± 1.01	43.4 ± 4.7	2.3 ± 1.06
PCBTF				
50%	4.7 ± 2.5	2.2 ± 1.33	38.9 ± 1.8	1.7 ± 1.12
75%	1.4 ± 0.1	1.7 ± 1.58	42.6 ± 1.7	1.9 ± 0.96
100%	2.2 ± 0.9	2.1 ± 0.98	40.1 ± 1.4	2.1 ± 1.21

are no standards in place to limit occupational exposure to PCBTF [1]. For occupational exposures, an 8-hour time-weighted average (TWA) permissible exposure level of 20 ppm has been suggested by the Kowa American Corporation, the United States chemical importation company that has renominated PCBTF for toxicological evaluation [1, 6]. In additional to occupational exposure, there is also the potential for exposure of the general public through PCBTF-containing products and PCBTF in and around ground water [20]. Detectable levels of PCBTF have been identified in workers (<1 ppm) as well as wildlife (0.17–2.0 ppm) [21, 22].

PCBTF is a halogenated solvent. These types of solvents are usually considered more toxic to humans and usually capable of causing greater environmental damage. Examples of other halogenated solvents include trichloroethylene, tetrachloroethylene, 1,1,1-trichloroethane, carbon tetrachloride, and dichloromethane. The majority of these solvents are classified as carcinogens and central nervous system depressants and can enter the body through respiratory or dermal exposure [23]. In addition to the effects to the central nervous system, workplace exposure to many of these solvents have been associated with toxic effects in the liver and kidney [24], as well as immune cell activation [25–31]. The effects of PCBTF exposure on immune parameters are consistent with those for other halogenated solvents, such as trichloroethylene, which has been shown to enhance activation markers on and IFN-γ secretion by splenic CD4+ T-cells [25–27]. Enhanced T-cell activation due to trichloroethylene exposure has been demonstrated to enhance autoimmune-related responses in both humans and mouse models [28, 29]. Due to the wide range of health effects associated with halogenated solvent exposure, occupational exposure limits have been set for these compounds, which have resulted in more stringent ventilation controls and personal protective equipment use by workers.

5. Conclusion

These are the first studies to indentify the sensitization potential of PCBTF following dermal exposure. Results from these studies indicate the importance of avoiding dermal contact with PCBT, due to its potential to function as a T-cell-mediated sensitizer and encourages the need to determine appropriate PPE to prevent worker exposure.

Disclosure

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

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Review Article

Occupational Asthma in Antibiotic Manufacturing Workers: Case Reports and Systematic Review

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Background. The risks of occupational asthma (OA) from antibiotics are uncertain. We report 4 new cases and a systematic review of the literature. *Methods*. Cases were identified through a specialist clinic, each underwent specific provocation testing (SPT). We subsequently reviewed the published literature. *Results*. The patients were employed in the manufacture of antibiotics; penicillins were implicated in three cases, in the fourth erythromycin, not previously reported to cause OA. In two, there was evidence of specific IgE sensitisation. At SPT each developed a late asthmatic reaction and increased bronchial hyperresponsiveness. 36 case reports have been previously published, 26 (citing penicillins or cephalosporins). Seven cross-sectional workplace-based surveys found prevalences of 5–8%. *Conclusions*. OA in antibiotic manufacturers may be more common than is generally recognised. Its pathogenesis remains unclear; immunological tests are of uncertain value and potential cases require confirmation with SPT. Further study of its frequency, mechanisms, and diagnosis is required.

1. Introduction

Among the several hundred workplace agents implicated as causes of occupational asthma (OA), several are encountered in the pharmaceutical manufacturing industry [1]. These include antibiotics which, through inhalation, may induce asthma in exposed employees. The role of dermal exposure and the detailed pathogenesis of the condition—as with many other low molecular mass causes of OA—remain unclear. Immunological tests are of uncertain value and most diagnoses require confirmation with specific provocation testing.

The first case of occupational antibiotic allergy was described in 1953, but a relatively small number of individual case reports have been published subsequently. Very large quantities of antibiotics are produced in almost every country but the frequency of OA in those who manufacture them remains unclear. While prevalence estimates of around 10% have been reported [2–4], clinical experience suggests a far less common problem. Here we report three new cases of occupationally-induced penicillin allergy and, for the first time, a case of asthma induced by work in erythromycin manufacture. The reports are supplemented by a systematic literature review of antibiotic occupational asthma.

2. Methods

2.1. Case Reports. New cases were seen and diagnosed at Royal Brompton Hospital in London, UK between 1995 and 2009. Specific IgE measurements, where attempted, were carried out by either the radioallergosorbent test (RAST) method or the commercial ImmunoCAP assay. Each patient underwent controlled, single-blind, specific provocation testing using a dust-tipping method, with small quantities of the relevant antibiotic powder mixed with a larger amount of dried lactose; active and control exposures were carried out on sequential days with the patients having stopped any asthma medications prior to the tests. Responses were assessed using serial FEV1 measurements following challenge and by changes in bronchial responsiveness to inhaled histamine using the Yan technique [5].

2.2. Systematic Review of Literature. We were not able to identify any previous systematic review of this subject. We searched the published literature using the Medline database between 1953 and February 2010. Both key word- and text word-based searches were performed with combinations of the terms "occupational asthma", "asthma", "respiratory sensitisation", and "antibiotics". In addition, we examined the reference lists of relevant articles. Both case reports (n = 21 papers) and workforce studies (n = 7) were retrieved (one case report in Russian was not included.) and their information extracted on to a standard form independently by two reviewers.

We used a quantitative structure-activity relationship (qSAR) model to examine the potential for each published antibiotic to act as a respiratory sensitising agent. "Hazard indices" for each were calculated, where possible, using the Chemical Asthma Hazard Assessment Program [6] which generates a risk prediction for organic compounds with a molecular mass of less than 1 kDa.

3. Findings

3.1. Case Reports (Table 1, Figure 1). Each of the four cases was involved in the primary manufacture or formulation of antibiotics in the United Kingdom and had presented with new onset, work-related asthmatic symptoms. Three worked with penicillins and their derivatives, the fourth with a variety of medicines including erythromycin. This last, a woman of 52, developed asthma 22 years previously, two years after starting work on the packaging lines. She reported that her symptoms worsened with exposure to granulated erythromycin and on two occasions in the year prior to referral had required treatment with oral corticosteroids. At referral, she had normal spirometry, was demonstrated to be atopic on skin prick testing, and completed a series of peak flow measurements which showed significant variability on both work and rest days, with no clear work-related pattern.

All four patients underwent single-blind, controlled specific provocation testing using a dust-tipping method. Each of the four developed a (predominantly) late asthmatic reaction (Figure 1) accompanied by increased bronchial hyperresponsiveness as demonstrated by a fall in histamine PC_{20} concentration following challenge; these findings were repeatable on further testing but not seen after identical exposure to lactose powder alone. Following diagnosis, each patient avoided further exposure at work to the causative antibiotic with improvement or resolution of their symptoms. They were advised to avoid therapeutic use of the relevant antibiotic.

3.2. Literature Review (Table 2). Previous published case reports, including a total of 37 patients, are summarised in Table 2. All but one of the cases was employed in the manufacture of antibiotics. In 25 individuals, the diagnosis was supported by specific inhalation testing which prompted early, late, and dual asthmatic responses in 14 (56%), six (24%), and three (12%) patients, respectively, with the results of the remaining two positive tests being unspecified. A hazard index was calculated for all bar two of the implicated antibiotics (the exceptions being vancomycin and colomycin, both of which have a molecular mass greater than 1 kDa) and was in all cases between 0.99 and 1.00.

Three reports describe patients sensitised to penicillins. In the first, published in 1953, Eaton Roberts described two employees in a US factory with clinical evidence of OA ascribed to penicillin; apart from skin prick testing with procaine penicillin (negative), he did not perform any objective investigations [7]. Similarly, Tara in 1957 [8] and Gaultier et al. in 1960 [9] reported a total of six French workers with clinical evidence of new-onset asthma after exposure to penicillin dust.

Synthetic penicillins have also been reported as a cause of work-related asthma. Davies et al. [10] used specific provocation testing to confirm OA from inhaled ampicillin in three UK employees; in each case skin prick tests with ampicillin and a variety of penicillin antigens were negative. Oral challenge with ampicillin induced asthmatic and other allergic symptoms in two of the three cases. Losada et al. described two cases of dyspnoea after inhalation of semisynthetic penicillins in workers in the manufacture of antibiotics [11]; in neither case was there objective evidence of immune sensitisation to these antibiotics. In Germany, Wuthrich and Hartmann [12] described a single case of OA from ampicillin; specific IgE antibodies to benzylpenicilloyl were detected and cumulative inhalation testing with lactose, tetracycline, ampicillin, and chloramphenicol caused a delayed asthmatic reaction. A factory worker in Belgium developed OA from amoxicillin [13] and subsequently, while employed as a nurse, to latex. Piperacillin, a semisynthetic penicillin, has also been reported to cause OA, the diagnosis supported by both skin prick testing and an immediate asthmatic response to specific inhalational testing [26].

There are several reports of cephalosporins and associated precursors and derivatives as causative agents for OA; 11 cases are summarised in Table 2 with supporting evidence from specific provocation testing in ten, albeit most frequently reported as immediate asthmatic responses only. Serum and skin prick tests of immunological sensitisation were only occasionally positive [15, 19, 20], and oral challenges, both positive, were reported in just two patients [11, 18].

The remaining eight reported cases developed OA during the manufacture of antibiotics other than penicillins or cephalosporins. Most have been in workers exposed to the macrolide spiramycin, an antibiotic widely used in the livestock industry and particularly with poultry so that traces may be found in some chicken eggs. Davies and Pepys [23] described the case of a pharmaceutical worker in whom the diagnosis of OA from spiramycin was confirmed by specific inhalation challenge; he reported symptoms on eating eggs but had negative skin prick tests to egg extracts. Six other cases, in Canadian and Italian manufacturers [4, 25], have been reported, in each case following specific inhalation testing. In one case [25], an immediate asthmatic response to provocation testing was also elicited by adipic acid, an additive used to bind spiramycin. In the only case of reported antibiotic OA outside the manufacturing sector,

Table 1: Cases of occupational asthma from antibiotics identified at Royal Brompton Hospital in the period 1995–2009.

case	Year of diagnosis	Workplace exposure	Allergic symptoms	Latency	Specific IgE	Bronchial provocation test		
						Agent	FEV1 response	Increase in histamine reactivity
a	1995	penicillin	wheeze	19 years	not done	penicillin	late	Yes
b	1996	amoxicillin	wheeze, cough	27 years	penicilloyl G (+) penicilloyl V (+)	amoxicillin	late	yes
c	2000	amoxicillin	wheeze, cough	27 years	amoxicilloyl (+)	amoxicillin	late	yes
d	2009	erythromycin	wheeze, rhinitis	2 years	erythromycin ethylsuccinate (−)	erythromycin ethylsuccinate	late	Yes

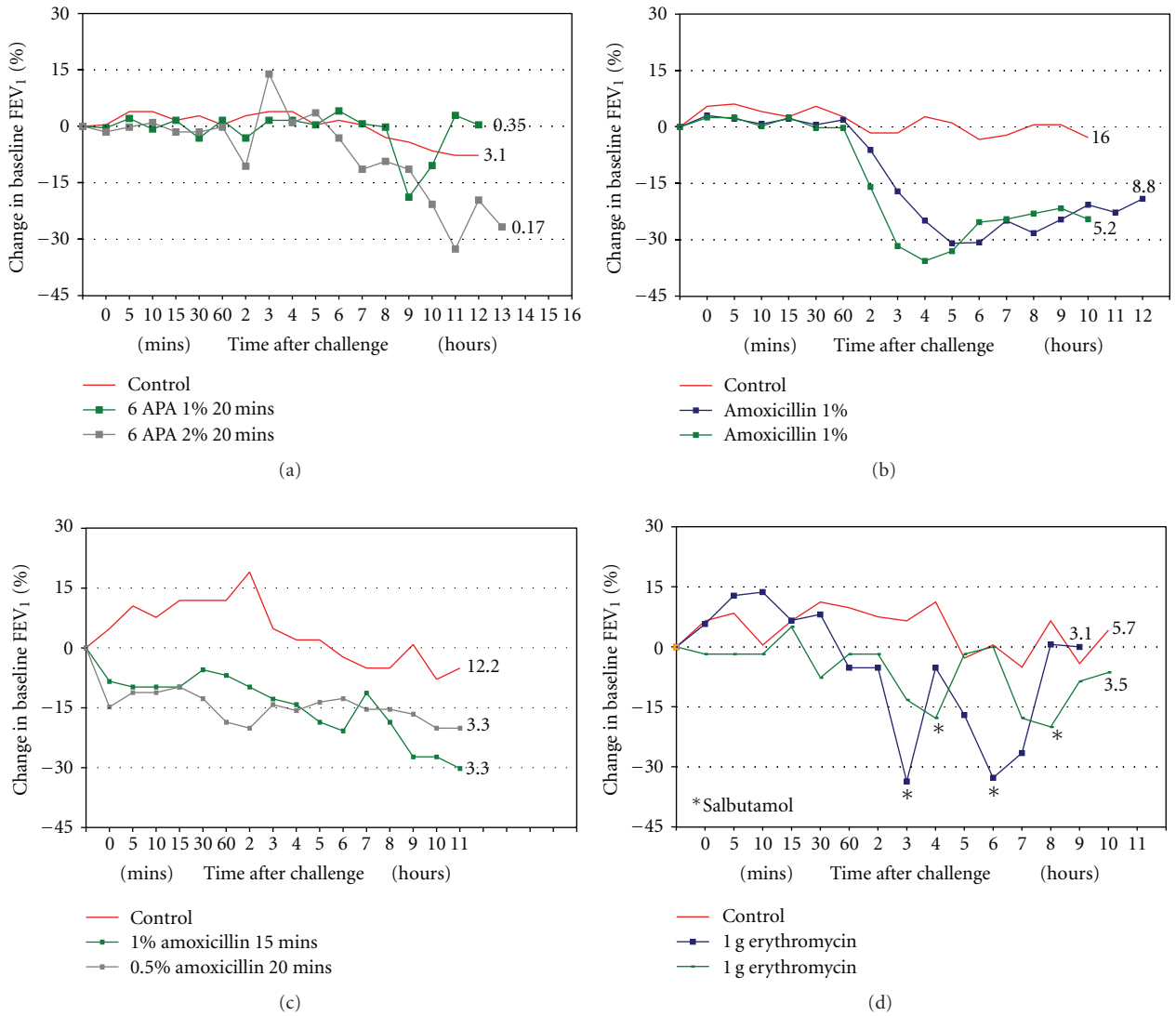

Figure 1: Changes in FEV_1 and histamine reactivity following bronchial provocation testing in four antibiotic manufacturing workers. Serial FEV_1 measurements (y-axis) are plotted against time after provocation (x-axis); a fall of greater than 15% from baseline indicated a significant response. A late reaction (greater than two hours after challenge) is seen in all cases; an early reaction was also seen in case c. The numerical value associated with each challenge plot is the postchallenge histamine PC_{20} result (mg/ml histamine): the lower the PC_{20} the greater the degree of bronchial hyperresponsiveness.

Table 2: Published cases of occupational allergy to antibiotics 1953–2009.

Penicillins										
Reference	Year	Country	No.	Exposure	Latency	Respiratory symptoms	Skin test	Specific IgE	Bronchial provocation test	Oral challenge
[7]	1953	USA	2	penicillin	#1: "weeks" #2: 1 year	#1: cough, rhinitis #2: cough, rhinitis, wheeze	Procaine penicillin (+)	not reported	not reported	not reported
[8]	1957	France	4	penicillin	#1: "years" #2: 3 months #3: 1 year #4: 1 year	#1: cough, rhinitis, dyspnoea #2: dyspnoea, eczema #3: dyspnoea #4: asthma, dyspnoea, eczema	not reported	not reported	not reported	not reported
[9]	1960	France	2	penicillin	#1: 1 year #2: 7 years	#1: asthma, urticaria #2: asthma	#1: PMP* (+) #2: penicillin (+)	not reported	not reported	not reported
[10]	1974	UK	3	ampicillin BP* 6 APA*	#1: 2 years #2: 2 years #3: 2 years	asthma ($n = 3$) rhinitis ($n = 2$) eczema ($n = 2$) conjunctivitis ($n = 1$)	ampicillin (−) ampicillin polymer (−) BPP* (−) MDM* (−)	not reported	#1: ampicillin (+LR*)6APA* (+/−) #2: ampicillin (+LR*) BP*(+LR*) commercial 6APA* (+LR*) purified 6APA* (−) BPP* (−) #3: ampicillin (+LR*) BP* (+LR*) 6APA* (−)	#1: ampicillin (−) #2: ampicillin (+LR*) and intestinal symptoms #3: BP* (+ER*) and urticaria
[11]	1980	Spain	2	amoxicillin ampicillin	#1: 1 year #2: 1 year	#1: rhinitis, dyspnoea, wheeze #2: cough, wheeze	not reported	negative	not reported	not reported
[12]	1982	Germany	1	ampicillin	NS*	cough, rhinitis, dyspnoea, fever	ampicillin (−)	BPP* (+)	antibiotic mix (+LR*)	not reported
[13] ne	1997	Belgium	1	amoxicillin	6 months	cough, wheeze, rhinitis	not reported	not reported	amoxicillin (+ER* LR*)	not reported

Table 2: Continued.

[14]	1998	Spain	1	amoxicillin	27 years	cough, rhinitis, wheeze, dyspnoea	amoxicillin (−) ampicillin (−) BP* (−) BPP* (−) MDM* (−)	amoxicillin (+) ampicillin (−) (penicillin V (−)	amoxicillin: (+ER*) penicillin V (−)	amoxicillin (+LR*) penicillin V (−)
Cephalosporins										
Reference	Year	Country	No.	Exposure	Latency	Allergic symptoms	Skin test	Specific IgE	Bronchial provocation test	Oral challenge
[11]	1980	Spain	1	cephalexin,	3 months	cough, wheeze	PP* (−) penicillin G (−)	negative	not reported	cephalosporin (NS) (+) and rhinitis, urticaria
[15]	1981	UK	2	7ACA* 7CTD*	#1: NS* #2: 10 years	#1: cough, rhinitis, chest tightness #2: chest tightness, dyspnoea	#1: 7ACA (+) 7CTD (+) cefalexin (−) #2: cephalexin (+)	not reported	#1: 7ACA (+ER*) 7CTD (+ER*) cephalexin (−) #2: cephalexin (+ER*)	not reported
[16]	1995	UK	1	ceftazidime	1 year	rhinitis, dyspnoea	not reported	not reported	ceftazidime (+ER* LR*)	not reported
[17]	1996	Italy	1	cefmetazole 7-ACA*	1 year	cough, rhinitis, bronchospasm	cefmetazole (−) 7-ACA* (−)	penicillin G (−) penicillin V (−)	cefmetazole (+NS*) 7-ACA (+NS*)	not reported
[18]	1999	Spain	1	cefadroxil	9 months	cough, rhinitis, dyspnoea, chest tightness	PP* (−) MDM* (−) BP* (−) amoxicillin (−) cefadroxil (−)	penicillin G (−) penicillin V (−) amoxicillin (−) ampicillin (−) cefaclor (−)	cefadroxil (+ER*)	amoxicillin (−) cephalexin (+ER*)
[19]	2003	Korea	2	cefteram	NS*	NS*	#1: cefteram (+) #2: cefteram (+)	#1: cefteram-HSA* (+) #2: cefteram-HSA* (+)	#1: cefteram (+ER*) #2: cefteram (+ER*)	not reported
[20]	2004	Korea	2	7-ACA* ceftriaxone	2 years	#1: rhinitis, respiratory symptoms #2: rhinitis, respiratory symptoms	#1: 7-ACA* (+) ceftriaxone (+) #2: 7-ACA* (−) ceftriaxone (−)	#1: 7-ACA-HSA* (+) #2: 7-ACA-HAS* (−)	#1: 7-ACA* (+ER*) ceftriaxone (−) #2: 7-ACA* (+NS*) ceftriaxone (−)	not reported
[21]	2009	Italy	1	7-TACA* Cephalosporins	8 months	cough, rhinitis, dyspnoea	not reported	not reported	7-TACA*: (+ER*)	not reported

Table 2: Continued.

Miscellaneous										
Reference	Year	Country	No.	Exposure	Latency	Allergic symptoms	Skin test	Specific IgE	Bronchial provocation test	Oral challenge
[22]	1977	India	1	tetracycline	1 year	cough, wheeze, dyspnoea	not reported	not reported	tetracycline (+ER*)	tetracycline (+ER*) and urticaria
[23]	1975	UK	1	spiramycin	1 year	cough, rhinitis, dyspnoea, dermatitis	spiramycin (+)	not reported	spiramycin (+LR*)	not reported
[24]	1979	Italy	1	spiramycin	1 year	cough, asthma, dermatitis	spiramycin (+)	not reported	chick feed with spiramycin (+LR*)	not reported
[25]	1984	Italy	2	spiramycin adipid acid	#1:14 years #2:7 months	#1: dyspnoea #2: cough, dyspnoea	not reported	not reported	#1: spiramycin adipate (+ER* LR*) #2: spiramycin adipate (+ER*) spiramycin base (+ER* LR*)	not reported
[26]	1995	Italy	1	piperacillin	22 months	rhinitis, dyspnoea, wheeze, rash	piperacillin (+)	not reported	piperacillin (+ER*)	not reported
[27]	2006	Korea	2	thiamphenicol	NS*	#1: rhinitis, asthma #2: rhinitis, asthma	#1: thiamphenicol (+) #2: thiamphenicol (+)	#1: thiamphenicol (+) #2 thiamphenicol (+)	#1: thiamphenicol (+ER*) #2: thiamphenicol (+ER*)	not reported
[28]	2009	Korea	1	vancomycin	5 months	rhinitis, chest tightness	vancomycin (−)	vancomycin-HSA* (−)	not reported	not reported
[29]	2010	Spain	1	colomycin	3 months	rhinitis, cough, wheeze, dyspnoea	not reported	negative	colomycin (+ER*)	not reported

*NS: not specified, *ER: early (asthmatic) response, *LR: late (asthmatic) response.
*HSA: human serum albumin, *MDM: minor determinant (penicillin) mix, *BP: benzylpenicillin.
*(B)PP: (benzyl)penicilloyl polylysine, *PMP: phenoxymethyl penicillin, *6APA: 6 amino penicillanic acid, *7-ACA: 7aminocephalosporanic acid, *7CTD: tosylate dihydrate derivative of 7ACA.
*7-TACA: 7-amino-3thiometihyl-3-cephalosporanic acid.

Paggiaro et al. [24] described dermatitis and asthma due to spiramycin in a chick breeder; a skin prick test to spiramycin was positive as was specific inhalation testing with chick feed containing the antibiotic.

Menon and Das [22] documented in an Indian worker immediate asthmatic reactions to intradermal, inhalation and oral testing with tetracycline but no response to inhalation testing with two antibiotics (nystatin and chloromycetin) to which he was not exposed at work. Three cases of OA from thiamphenicol—a methyl-sulfonyl analogue of chloramphenicol—were reported by Ye et al. [27]. Vancomycin has been described as a cause of OA in a single patient [28], the diagnosis confirmed by serial peak flow measurements. The most recently reported case was of OA due to the polymyxin antibiotic colomycin in an antibiotic transport and storage worker, who demonstrated an early asthmatic reaction following specific inhalation challenge [29]; no evidence of specific IgE was found despite extensive in vitro immunological testing.

3.3. Workforce Studies. We identified seven reports of epidemiological studies carried out in antibiotic manufacturing sites; all but one was of cross-sectional design. In several cases, the absence of detailed information on the size of the exposed populations precludes any estimate of disease prevalence.

Briatico-Vangosta et al. [2] surveyed 91 Italian workers exposed to cephalosporins with a combination of a symptoms questionnaire, skin prick and intradermal testing, and "on-off" testing of asthma symptoms and pulmonary function. On this basis, OA was reported for seven (8%) employees. Skin testing produced immediate-type responses in five workers with OA (in three cases with prick testing) suggesting to the authors that the asthma had, in some cases at least, arisen through a specific IgE-related mechanism.

Chida and Uehata [30] surveyed by interview 24 employees of a pharmaceutical factory which produced two antibiotics (ampicillin and cephalexin) as well as an antispasmodic and three anti-inflammatory drugs. Those with respiratory symptoms ($n = 18$) underwent skin prick, serum, and pulmonary function testing. Probable antibiotic-related asthma was claimed for four employees, two of whom had immediate responses to skin testing with ampicillin. In the absence of any clear information on the population at risk, a prevalence estimate is not available.

Phenylglycine acid chloride (PG-AC) is a highly reactive compound used chiefly in the manufacture of ampicillin and other antibiotic side chains. A survey of 24 workers involved in the production of PG-AC [3] included a symptoms questionnaire, examination of occupational health records, skin testing, and spirometry. Seven workers were felt, on the basis of their history alone, to have a respiratory allergy to PG-AC; all had positive responses to prick and/or intradermal skin testing with the conjugated compound. Two were admitted to hospital for specific inhalation testing with positive findings. A further seven employees were deemed to have "irritant" respiratory responses to PG-AC (all skin tests negative) and in nine the history was considered "equivocal"; two of this last group had positive skin test results. The authors commented that the amino groups in PG-AC predispose to the formation of hapten-protein conjugates and hence its allergenicity. Again, in the absence of any information on the size of the exposed workforce, an estimate of prevalence in this setting is unavailable.

Carnevale et al. [31] studied 67 workers employed in manufacturing and encapsulating antibiotics in Italy. Following questionnaire, clinical and laboratory investigations, two (3%) cases of OA due to ampicillin were recorded. In both cases skin tests were negative. Post- and preshift urine testing confirmed the systemic absorption of ampicillin by these workers. A similar survey was reported by Carlesi et al. [32]. Among 26 employees in an antibiotic manufacturing plant, eight (31%) claimed respiratory symptoms which in two cases (8%) were suggestive of asthma. A further three employees had positive skin prick tests to penicillin G and/or amoxicillin.

Two surveys of workers involved in spiramycin manufacture have been reported. Malo and Cartier [4] investigated all 51 employees at a processing plant in Canada. Twelve, on the basis of a compatible history or evidence of (work-related) bronchial hyperreactivity, underwent specific inhalation testing with positive findings in four cases. The measured prevalence of 8% was considered by the authors, to be a minimum estimate. In Italy, a 12-year prospective study of 305 workers in a spiramycin-manufacturing plant [33] suggested work-related asthma in 15 (5%), all of whom had positive epicutaneous or intradermal skin tests to spiramycin. Four had additional symptoms of rhinitis and one of urticaria. In the full study population, 41 employees (13%) had positive skin tests, 37 (90%) of them with allergic symptoms of some kind.

4. Discussion

Our cases add three to the previously reported eight cases of OA attributed to inhalation of synthetic penicillins during their manufacture, and the first case of disease arising from workplace exposure to erythromycin. An additional seven cases of penicillin OA were reported to a UK national surveillance scheme between 1989 and 2009 (THOR personal communication).

An examination of published findings from surveillance schemes in other parts of the world found specific reference to antibiotics in none although the Propulse scheme in Quebec collated seven cases of OA (2.4% of the total) attributed to "medical drugs" [34]. Our systematic review of the remaining literature revealed a total of 37 cases published over a period of almost 50 years, although others were identified during the course of seven epidemiological studies, the most recent of which was published over 20 years ago. It was often difficult, in reviewing the workplace surveys, to obtain meaningful estimates of prevalence but what information was available suggests that about 10% of those surveyed had disease suggestive of OA. Thus since the first report in 1953 there appears to have been only sporadic attention paid to what may be a significant occupational risk.

The clinical features of the published cases are similar to those found in other examples of allergic OA and are broadly

indicative of an immediate-type respiratory hypersensitivity. A latent period of asymptomatic exposure was reported for all, usually of fewer than 24 months although occasionally far longer, and rhinitis was a frequent accompaniment to asthma symptoms. In those cases where specific inhalation testing was used in diagnosis and the findings reported in full, a late or dual asthmatic response was reported in 43%. In most cases of isolated early asthmatic responses to specific provocation, there was evidence of immunological sensitisation on skin or serum testing. Each of the implicated antibiotics had a very high hazard index derived from a quantitative structure-activity relationship analysis [35]; in the clinical context of a patient with characteristic symptoms, index values such as these have a high positive predictive value for OA [36]. Finally, Roberts [7] reported the apparently successful, subcutaneous desensitisation of two patients with OA from penicillin.

Nonetheless, as with many other low-molecular-weight causes of the disease, the immunological details of antibiotic-related OA remain unclear. While an IgE-associated mechanism is likely in many cases, the possibility of sensitisation arising from an alternative mechanism, perhaps through cross-linking of cell surface receptors, cannot be ruled out. Even when, as is not always the case, their techniques are described, the variety of skin test methods used in the published reports makes their interpretation and diagnostic significance uncertain. In any case, the results were often negative and where they were not there is no systematic information on the findings among exposed but non-asthmatic employees. Similar comments apply to the use of tests for serum-specific IgE antibodies which were less often performed and more often negative although some success has been recorded for in-house assays using cephalosporin conjugates [20, 21] or thiamphenicol [27]. Thus, and probably in contrast to their use in oral antibiotic allergy [37], the value of available tests for evidence of immunological sensitisation in this context remains unclear.

Penicillins and cephalosporins are common causes of oral drug allergy which in most cases is attributed to an IgE-associated immune response to one or more hapten-protein conjugates. Penicillins are composed of betalactam and thiazolidine rings with one or more differentiating side chains; the instability of the betalactam causes its carbonyl group to form amide-bonds with the amino groups of lysine residues from nearby proteins. "Minor" antigenic determinants, some formed from side chain-protein conjugates, may also induce IgE immune responses. 7 aminocephalosporanic acid, the active nucleus of cephalosporins, is structurally similar to the active nucleus of the penicillins, consisting of betalactam and 6 dyhydrothiazolidine rings. Hapten-protein conjugates of the cephalosporin betalactam are relatively unstable. IgE antibodies that react to cephalosporins detect a large number of specific antigens derived from protein conjugates formed from the side chain(s), the side chain plus a portion of the betalactam ring, or the complete cephalosporin. On the basis of ELISA inhibition assays in two patients with OA from cefteram, Suh et al. [19] suggested that specific IgE responses to the cephalosporin may involve haptens, new antigenic determinants, or both and are likely to vary between patients.

Seven of the identified case reports describe oral challenges to the causative antibiotic (in six cases a penicillin or cephalosporin) in patients with OA. All but one produced an allergic response. The three cases reported by Davies et al. [10] had subtly different responses to inhaled and oral (negative in one patient) provocation tests suggesting the possibility that individually they were responsive to different hapten-protein antigens. The patient described by Jimenez et al. [14] had allergic responses to both inhaled and oral amoxicillin, but not to oral penicillin V suggesting to the authors that the responsible antigenic determinant lay in the aminohydroxyphenyl side-chain of amoxicillin. In contrast, Sastre et al. [18] reported the case of a patient with OA attributed to cefadroxil, a cephalosporin that shares a side chain with amoxicillin. Oral challenge with cefadroxil, but not with amoxicillin, produced an asthmatic response suggesting that her sensitisation was to the dihydrothiazine cephalosporin ring rather than to its side chain. Losada et al. [11] reported a further patient with asthmatic responses to both inhaled and oral cephalosporin but not to penicillin G. Oral allergy may also develop in patients with OA attributed to other antibiotics [22]. While the evidence base is weak, and we cannot exclude the possibility of bias in those selected for oral challenge or in publication, we suggest that there is sufficient experience to advise that patients with OA from an inhaled antibiotic encountered at work should avoid taking the same—or closely related—drug by mouth unless its safety has been established by a carefully supervised provocation test.

We have been unable to find valid information on the numbers of exposed employees in any parts of the world but we expect these to number many thousands. Much manufacture is carried out in conditions where exposures to employees are very low but this is not always the case, and we note the general movement of pharmaceutical manufacturing away from its traditional base in western Europe and North America. We note also that most published evidence relates to older types of antibiotic. This is likely to reflect the number and exposures of those involved in their manufacture rather than any intrinsic hazard; the hazard indices for newer antibiotics such as flucloxacillin (0.909), clarithromycin (0.997), tobramycin (1.0), and azithromycin (1.0) are no lower. While cases of OA arising from antibiotic manufacture are rarely published, the available epidemiological evidence, admittedly scanty, sometimes deficient and all of it dated, suggests that the risks may be higher than many appreciate. We suggest that further workplace-based surveys, of careful design and supported by improvements in immunological diagnosis, are required.

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Clinical Study

Exercise-Induced Bronchoconstriction and Exercise-Induced Respiratory Symptoms in Nurses

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In order to assess prevalence and characteristics of exercise-induced respiratory symptoms (EIRSs) and exercise-induced bronchoconstriction (EIB) in health care workers, we performed a cross-sectional study including 48 female nurses from primary care settings and an equal number of female office workers studied as a control group. The evaluation of examined groups included completion of a questionnaire, skin prick tests to common inhalant allergens, spirometry, and exercise and histamine challenge. We found a similar prevalence of EIRSs and EIB in both groups. EIB was closely related to asthma, atopy, family history of asthma, and positive histamine challenge in either group, while the association between EIB and daily smoking in nurses was of borderline statistical significance. Bronchial reaction to exercise was significantly higher in nurses than in controls with EIB. With the exception of exercise induced wheezing, EIRSs were weakly associated with EIB in both groups with a large proportion of false positive results.

1. Introduction

Exercise-induced bronchoconstriction (EIB), also referred to as exercise-induced asthma (EIA), is a manifestation of bronchial hyperresponsiveness (BHR) that occurs in the majority of patients with current symptomatic asthma, especially in the patients with moderately to severely increased responsiveness [1–3]. The current thinking about mechanisms by which EIB develops emphasizes the loss of heat and/or water from the airways during exercise that leads to release of proinflammatory mediators [4]. Airborne particles and pollutants, as well as airborne allergens, are considered as stimulants that contribute to EIB [5]. A fish oil supplementation may have a protective effect on EIB, which is probably attributed to its anti-inflammatory properties [6].

Results from several studies indicated that BHR prevalence is higher in females than in males [7, 8]. The mechanisms responsible for a higher susceptibility of the airways in females to nonspecific stimuli include lower airway calibre, lower body weight, greater cholinergic irritability, and hormonal factors [9].

On the other side, data from the studies carried out in the last two decades suggest an increased risk for asthma among health care workers, yet only a few specific determinants have been elucidated [10–13]. As Delclos et al. [14] have suggested, the contribution of occupational exposures to respiratory impairment and asthma in health care professionals is not trivial, meriting both implementation of appropriate controls and further studies.

To our knowledge, so far, there is no study assessing exercise-induced respiratory symptoms (EIRSs) and EIB in health care professionals. In the present study, we assessed effects of occupational exposure on EIRSs and EIB among health care workers by comparison of their prevalence and characteristics between females working as nurses in primary care settings and female office workers.

2. Materials and Methods

2.1. Study Design and Setting. A cross-sectional survey was carried out in a university research laboratory, that is,

Department of Cardiorespiratory Functional Diagnostics at the Institute for Occupational Health of R. Macedonia, Skopje—WHO Collaborating Center for Occupational Health and GA^{2}LEN Collaborating Center.

2.2. Subjects. We examined 48 females aged 24 to 51 years (mean age 37.8 ± 7.4) working as nurses in primary care settings with duration of employment 5 to 25 years (mean duration 14.7 ± 5.7).

The work shifts of the nurses lasted 8 hours per day, and their working tasks included completion of the medical documentation, assistance in medical interventions, administration of parenteral and aerosolized medications, and medical instruments cleaning. The workplace exposure included several types of cleaning products, disinfectants, adhesives, solvents, latex, and medications, some of which are in spray form. According to the classification of occupational muscular work, their work was classified as a light muscular work [15]. During the work shift, they use protective clothing, masks, and powdered latex gloves.

In addition, an equal number of female office workers matched to nurses as a group by age and smoking status were studied as a control group. According to the classification of occupational muscular work, their work was classified as a sedentary work.

In either group, there were no subjects in whom exercise challenge or histamine challenge were contraindicated [16, 17], nor there were subjects with the upper respiratory viral infection within three weeks before the challenge test was performed. None of the subjects took asthma medications or antihistamines at least one month before the challenge tests and skin-prick tests. Daily smokers were asked to restrain from smoking at least 3 hours before testing.

2.3. Questionnaire. The questionnaire was designed using the proposed model of the National Jewish Medical and Research Center, Denver, USA [18].

Subjects were considered having exercise-induced respiratory symptoms (EIRSs) if one or more symptoms were reported: coughing during or after exercise, wheezing during or after exercise, inability to get deep breath after exercise, noisy breathing after exercise, and chest tightness after exercise.

Detailed smoking history, asthma diagnosed by physician, family history of asthma and allergic diseases (taking into account the first-degree relatives), accompanying disease, and medication use were also evaluated.

Classification of smoking status was done according to the World Health Organization (WHO) guidelines on definitions of smoking status [19].

Daily smoker was defined as a subject who smoked at the time of the survey at least once a day, except on days of religious fasting. In daily smokers, lifetime cigarette smoking and daily mean of cigarettes smoked were evaluated. Pack years smoked (one pack year denotes one year of smoking 20 cigarettes per day) were calculated according to the actual recommendations [20].

Ex-smoker was defined as a formerly daily smoker who no longer smokes.

Passive smoking or exposure to environmental tobacco smoke (ETS) was defined as the exposure of a person to tobacco-combustion products from smoking by others [21].

2.4. Skin-Prick Tests. Skin-prick tests (SPTs) to common inhalant allergens were performed in all subjects on the volar part of the forearm using commercial allergen extracts (Torlak, Serbia, and Montenegro) of birch (5000 PNU), grass mixed (5000 PNU), plantain (5000 PNU), fungi mixed (4000 PNU), *Dermatophagoides pteronyssinus* (3000 PNU), dog hair (4000 PNU), cat fur (4000 PNU), and feathers mixed (4000 PNU). All tests included positive (1 mg/mL histamine) and negative (0.9% saline) controls. Prick tests were considered positive if the mean wheal diameter 20 min after allergen application was at least 3 mm larger than the size of the negative control [22]. Atopy was defined as the presence of at least one positive SPT [23].

2.5. Spirometry. Spirometry, including measures of forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), FEV_1 / FVC ratio, and maximal expiratory flow at 50%, 25%, and 25–75% of FVC (MEF_{50}, MEF_{25}, and $MEF_{25–75}$, resp.), was performed in all subjects using spirometer Ganshorn SanoScope LF8 (Ganshorn Medizin Electronic GmbH, Germany) with recording the best result from three measurements the values of FEV_1 of which were within 5% of each other. The results of spirometry were expressed as percentages of the predicted values according to the European Community for Coal and Steel (ECCS) norms [24].

2.6. Histamine Challenge. The histamine challenge test was performed according to the actual European Respiratory Society (ERS)/American Thoracic Society (ATS) recommendations [16, 17]. Concentrations of 0.5, 1, 2, 4, and 8 mg/mL histamine (Torlak, Beograd) were prepared by dilution with buffered saline. The doses of aerosol generated by Pari LC nebulizer with output rate 0.17 mL/min were inhaled by mouthpiece. Subjects inhaled increasing concentrations of histamine using a tidal breathing method until FEV_1 fell by more than 20% of its base value (provocative concentration 20—PC20) or the highest concentration was reached.

According to the ATS recommendations, bronchial hyperresponsiveness (BHR) was categorized as moderate to severe BHR (PC20 < 1.0 mg/mL), mild BHR (PC20 = 1.0–4.0 mg/mL), and borderline BHR (PC20 > 4.0 mg/mL) [17]. The test was considered positive if PC20 was equal or less than 4 mg/mL [16, 17].

2.7. Exercise Challenge Tests. The constant submaximal exercise challenge test (ECT) was performed in all subjects using cycle ergometer Hellige-dynavit Meditronic 40 (Hellige GmbH, Germany). ECT was conducted in an air-conditioned room with ambient temperature of 20–25°C and relative air humidity of 50% or less. According to the actual recommendations, subjects exercised

Table 1: Demographics of the study subjects.

Variable	Nurses ($n = 48$)	Office workers ($n = 48$)
Age (years)	37.8 ± 7.4	39.1 ± 9.2
BMI (kg/m^2)	25.4 ± 3.9	26.7 ± 4.3
Duration of employment (years)	14.7 ± 5.7	15.4 ± 7.8
Asthma diagnosed by physician	3 (6.3%)	2 (4.2%)
Family history of asthma	4 (8.3%)	4 (8.3%)
Family history of allergies	6 (12.5%)	8 (16.6%)
Daily smokers	14 (29.2%)	15 (31.2%)
Smoking experience (years)	17.7 ± 5.8	19.4 ± 7.9
Cigarettes per day	14.4 ± 6.9	16.8 ± 8.3
Pack years smoked	12.5 ± 3.1	13.4 ± 3.8
Daily smokers with less than 12 pack years smoked	6 (12.5%)	7 (14.6%)
Ex-smokers	5 (10.4%)	4 (8.3%)
Passive smokers	8 (16.6%)	10 (20.8%)

Numerical data are expressed as mean value with standard deviation and frequencies as number and percentage of study subjects with certain variable.
BMI: body mass index; kg: kilogram; m: meter.

8–10 min achieving 90% of predicted maximal heart rate (HR_{max} =220 − age) in the last 4 min of exercise [16, 17]. Heart rate was monitored continuously throughout the exercise and for 5 minutes after its completion from a three-lead electrocardiographic configuration. The measurements of FEV_1 were performed before and 1, 3, 5, 7, 10, and 15 min after the exercise with inhaled bronchodilator (200 mcg salbutamol) application upon completion of the protocol.

The response to exercise was expressed as fall index FEV_1 (100 × [pre-exercise FEV_1 − lowest postexercise FEV_1]/pre-exercise FEV_1). EIB was defined as fall index $FEV_1 \geq 10\%$ [16].

2.8. Statistical Analysis. Continuous variables were expressed as mean values with standard deviation (SD) whereas the nominal variables as numbers and percentages. Analyses of the data involved testing the differences in prevalence, comparison of the means, and testing the association between EIRSs and EIB and studied variables. Chi-square test was used for testing difference in the prevalence. Comparison of spirometric measurements and fall index FEV_1 values was performed by independent samples t-test. Chi-square test (or Fisher's exact test where appropriate) was used for testing association between EIRSs and EIB and studied variables. A P value less than .05 was considered as statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 11.0 for Windows.

3. Results

Demographic characteristics of the study subjects were similar in both examined groups (Table 1).

Table 2: Prevalence of EIRSs in nurses and controls.

EIRSs	Nurses ($n = 48$)	Office workers ($n = 48$)	P value*
Any exercise-induced respiratory symptom	19 (39.6%)	17 (35.4%)	.673
Cough	11 (22.9%)	13 (27.1%)	.637
Inability to get deep breath	14 (29.1%)	11 (22.9%)	.585
Wheezing	5 (10.4%)	4 (8.3%)	1.000
Chest tightness	9 (18.8%)	8 (16.7%)	.726
Noisy breathing	4 (8.3%)	6 (12.5%)	.740

Data are expressed as number and percentage of study subjects with certain variable.
EIRSs: exercise-induced respiratory symptoms.
*Tested by Chi-square test (or Fisher's exact test where appropriate).

Table 3: Spirometric parameters in the study subjects.

Spirometric parameter	Nurses ($n = 48$)	Office workers ($n = 48$)	P value*
FVC (% pred)	88.9 ± 9.8	91.6 ± 10.4	.102
FEV_1 (% pred)	84.3 ± 7.9	86.2 ± 9.6	.180
FEV_1/FVC%	76.1 ± 4.9	78.4 ± 5.8	.126
MEF_{50} (% pred)	64.8 ± 12.7	68.1 ± 9.8	.083
MEF_{25} (% pred)	60.8 ± 10.1	64.9 ± 8.9	.069
$MEF_{25–75}$ (% pred)	72.8 ± 14.7	75.1 ± 10.9	.094

Data are expressed as mean value with standard deviation.
FVC: forced vital capacity; FEV_1: forced expiratory volume in one second; MEF_{50}, MEF_{25}, and $MEF_{25–75}$: maximal expiratory flow at 50%, 25%, and 25–75% of FVC, respectively; % pred: % of predicted value.
*Compared by independent samples t test.

Prevalence of EIRSs, total and individual, was similar in both examined groups. Inability to get deep breath after exercise and cough during or after exercise was the most frequent EIRSs in either group (Table 2).

EIRSs were nonsignificantly associated with age and smoking in both examined groups. The association between EIRSs in nurses and duration of employment was also nonsignificant.

Prevalence of subjects with positive SPT to common inhalant allergens was similar in both nurses and controls (33.3% versus 37.5%, $P = .670$; Chi-square test). Mite sensitization was the most important individual common allergen with no statistical difference between sensitized subjects in both groups (22.9% versus 25.0%, $P = .811$; Chi-square test).

Spirometric parameters were lower in nurses, but statistical significance was not found for any parameter (Table 3). Spirometric parameters were nonsignificantly lower in the subjects with asthma diagnosed by physician as compared to nonasthmatics in both nurses and controls.

Prevalence of overall subjects with BHR was nonsignificantly higher in nurses (12.5% versus 8.3%; $P = .740$),

Table 4: Characteristics of the ECT in nurses and controls.

BHR	Nurses ($n = 48$)	Office workers ($n = 48$)	P value*
Moderate to severe BHR	2 (4.2%)	1 (2.1%)	1.000
Mild BHR	1 (2.1%)	1 (2.1%)	1.000
Borderline BHR	3 (6.3%)	2 (4.2%)	1.000

Data are expressed as mean value with standard deviation.
BHR: bronchial hyperresponsiveness.
*Compared by Fisher's exact test.

whereas the prevalence of subjects with moderate to severe and mild BHR, that is, prevalence of subjects with positive histamine challenge, was similar in both examined groups (Table 4).

We found similar prevalence of EIB in both nurses and controls (8.3% versus 6.3%, $P = 1.000$; Fisher's exact test). The EIB severity, expressed as fall index FEV_1, was significantly higher in nurses (28.1% versus 22.7%, $P = .033$; independent-samples t-test). Characteristics of the ECT performed in study subjects are shown Table 5.

EIB in both examined groups was significantly related to asthma diagnosed by physician, positive family history for asthma and allergies, and positive histamine challenge, whereas association with other variables was nonsignificant. Association between EIB and daily smoking in nurses was of borderline statistical significance ($P = .062$; Fisher's exact test), while association between EIB and pack years smoked (less or more than 12) was nonsignificant ($P = .097$; Fisher's exact test). These associations in controls were statistically nonsignificant.

Association between EIB and exercise-induced respiratory symptoms, with exception of exercise-induced wheezing in both nurses ($P = .037$; Fisher's exact test) and controls ($P = .034$; Fisher's exact test), was statistically nonsignificant. The frequency of false positive results was high in both nurses (84.3%) and controls (88.2%).

4. Discussion

According to the recent data, occupational exposures in health care professionals increase the risk of work-related asthma. Medical instruments cleaning, general cleaning, use of solvents/adhesives in patient care, use of powdered latex gloves, and aerosolized medication administration were identified as occupational risk factors associated with the development of asthma in nurses [10, 14, 25].

On the other hand, EIB is a common condition close related to asthma that is often unrecognized and uncontrolled leading affected subjects to avoid general and occupational physical activities and sports. We performed the present study on EIB among nurses in primary care settings as a continuum of our investigations on the effects of specific occupational exposures on the EIB occurrence and characteristics [26–28]. According to the results of several studies [11–13], the lowest risk of respiratory impairment and asthma was found in administrative workers, so this "unexposed" occupation was used as a control group.

Table 5: Characteristics of the ECT in nurses and controls.

Variable	Nurses ($n = 48$)	Controls ($n = 48$)
Exercise load (Watt)	102.1 ± 20.3	106.3 ± 16.1
Positive ECT	4 (8.3%)	3 (6.3%)
ΔFEV_1 in the subjects with EIB (%)	28.1 ± 3.4	22.7 ± 1.5
Time of EIB occurrence (minutes after exercise)	6.1 ± 2.1	6.9 ± 2.7

Numerical data are expressed as mean value with standard deviation and frequencies as number and percentage of study subjects with certain variable.
ECT: exercise challenge test; ΔFEV_1: a fall in FEV_1 of pre-exercise value; FEV_1: forced expiratory volume in 1 second; EIB: exercise-induced bronchoconstriction.

In the present study, both examined groups included subjects with similar demographic characteristics. In either group, there was a large proportion of daily and passive smokers similar to its prevalence among females in R. Macedonia documented in our previous studies [29, 30]. The prevalence of ex-smokers in both groups was low, suggesting insufficient smoking cessation activities. The situation in the developed countries seems to be somewhat different. In the study conducted in 12 European countries as well as Australia and the USA, Janson et al. [31] reported that both active and passive smoking rates have declined since the early 1990s but indicated lower quitting rates and higher risk of passive smoking among people with fewer qualifications and less skilled occupation groups.

We found high prevalence of EIRSs in both examined groups that is similar to the findings of several studies which investigated EIB in different subpopulations of both sexes [32, 33] as well as to the findings of our studies among workers with different occupational exposures [26–28]. The prevalence of atopy and the pattern of allergic sensitization to common aeroallergens in both examined groups was comparable to that we had previously observed among adults in R. Macedonia [34, 35]. All spirometric parameters were lower in nurses, but statistical significance was not achieved for any of them. The prevalence of BHR was nonsignificantly higher in nurses than in office workers that is similar to the findings obtained in our previous studies on BHR prevalence among workers with specific occupational exposures (herbal tea processors, cooks, and cleaners) and office workers as a control group [36, 37].

Several studies indicated that the occurrence of EIB depends on degree of bronchial hyperresponsiveness (alias underlying chronic inflammation), exercise intensity, and ambient conditions [38, 39]. There are many studies about EIB occurrence in selected groups of general population (children, school children, adolescents, and recruits) as well as in recreative and elite athletes. On the contrary, there is a limited number of studies on EIB associated with specific workplace exposures. The EIB prevalence in elite athletes varies from 12% of basketball players to 55% of cross-country skiers [40, 41]. In the present study, we found similar EIB prevalence in both nurses and controls (8.3% and 6.3%, resp.). According to the results of our previous studies, the

EIB prevalence among workers with specific occupational exposures ranged from 6.4% in herbal tea processors, 6.9% in bakers, 7.1% in agricultural workers, 8.9% in textile workers, to 9.3% in agricultural workers. Bronchial reaction to exercise in the subjects with EIB was significantly higher in nurses than in controls. Significantly higher bronchial reaction to exercise in comparison to office workers we also found in ECT-positive female cleaners, whereas the difference in mean fall index FEV_1 did not differ significantly between workers exposed to organic dusts and office workers with EIB [26–28]. This difference may be due to the presence of the study subjects of both sexes among workers exposed to organic dusts as well as to the different occupational exposures (i.e., dominant exposure to chemical compounds in cleaners and nurses).

We found significant association between EIB and asthma, family history of asthma, and atopy in both examined groups. Contribution of genetic factor in the EIB development is confirmed in a number of studies [39, 42, 43]. We also found a significant association between positive ECT and positive histamine challenge in both examined groups. Data from the studies which compared results of two bronchial challenge types are somewhat inconsistent. Some authors reported significant association between the results of exercise and histamine challenge [26–28, 44]. On the contrary, other authors reported a weak association, which was explained by different pathomechanisms of BHR to histamine and EIB [45]. Correlation between EIB and daily smoking was nonsignificant in controls, whereas in nurses, it was of borderline statistical significance. A similar finding, suggesting possible interaction of tobacco smoke and occupational exposure in EIB development, was obtained in our previous study on EIB in female cleaners [28].

In the present study, there was no positive association in both examined groups between EIB and overall and individual EIRSs with exception of exercise-induced wheezing. This finding, confirmed in a number of studies, was not unexpected, as the EIRSs may be triggered by many conditions and diseases other than BHR (e.g., physical unfitness, medical side effect of angiotensin-converting enzyme inhibitors and beta-blockers, anxiety, vocal cord dysfunction, arterial hypertension, gastroesophageal reflux, etc.) [26–28, 46–48].

The present study has some limitations. First, relatively smaller number of the subjects in the study groups could have certain implications on the data obtained and its interpretation. Second, we did not perform SPT to workplace allergens (e.g., latex), so we could not document relationship between sensitization to workplace allergens and EIB. Third, environmental measurements were not performed, so we could not document the effect of the type and the level of exposure on EIB. The strength of the study is the extensive examination of lung function in the study subjects with the possibility for comparison of the results of different tests.

5. Conclusions

In conclusion, in a cross-sectional study including nurses and office workers, we found a similar prevalence of EIRSs and EIB in both examined groups. EIB was strongly related to asthma in both nurses and controls. In addition, EIB was closely related to atopy, family history of asthma, and positive histamine challenge in either group as well as to daily smoking in nurses. Bronchial reaction to exercise in ECT-positive nurses was significantly higher than in ECT-positive controls. EIRSs were weakly associated with EIB in both examined groups, with a large proportion of false positive results. Our study confirms the need of regular medical examinations in order to identify affected workers and to implement adequate preventive measures.

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