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Cork workers' occupational asthma: lack of association with allergic sensitisation to fungi of the work environment

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Abstract Objectives: To evaluate allergic sensitisation to *Chrysonilia sitophila*, *Penicillium glabrum*, and *Trichoderma longibrachiatum* in cork workers with asthma. **Methods:** Skin prick tests with a battery of common allergens and with the three fungi were performed on ten cork workers with asthma and eight non-exposed asthmatics. Based on serial peak expiratory flow measurements, five were classified as having occupational asthma (AO) and five as having non-occupational asthma (NOA). In exposed patients, specific antibodies for the three fungi were also studied by immunoblotting. **Results:** Two out of ten patients with occupational exposure and four out of eight of the control group showed positive results for skin prick tests for common allergens. Moreover, two out of five patients with OA and three out of eight controls exhibited sensitisation to storage mites. All exposed patients (with OA or NOA) had negative skin prick test results for the fungal extracts. In patients with asthma and occupational exposure, immunoblotting results confirmed the absence of specific IgE. However, specific IgG4 was present in some cases. **Conclusions:** Atopy does not seem to characterise occupational asthma in cork workers. Despite their long

exposure to moulds, we could not find evidence of IgE sensitisation to the three most prevalent cork fungi in patients with OA, which points to the search for other causative agents, such as cork chemical compounds or contaminants.

Keywords Occupational asthma · Immunoblotting · IgE · Fungi · Cork industry

Introduction

Cork workers are at risk of developing an occupational lung disease (suberosis) due to repeated exposure to mouldy cork dust (Pimentel and Ávila 1973). Usually, they present with an interstitial lung disorder (hypersensitivity pneumonitis) with a CD8+ lymphocytic alveolitis and IgG antibodies specific for *Penicillium glabrum* (formerly known as *Penicillium frequentans*), one of the fungi most frequently isolated from cork samples (Pimentel and Ávila 1973; Delgado et al. 1992).

In asthma sufferers in occupational environments traditionally associated with hypersensitivity pneumonitis, such as bird fanciers and farmers, the condition has been attributed to previously unrecognised antigens (Arlian et al. 1997; Colloff et al. 1997). However, in some cases, the same antigen may be implicated in both diseases (Hirakata et al. 2002).

We previously identified cases of occupational asthma in the cork industry, confirmed with serial peak expiratory flow recordings (Winck et al. 2001), but its aetiology is still unknown. Apart from *Penicillium glabrum*, a fungus that induces immunological sensitisation in cork workers (Pimentel and Ávila 1973), *Chrysonilia sitophila*, another prominent fungus that colonises cork (Danesh et al. 1997), has also been identified as a cause of occupational asthma in the wood industry (Cote et al. 1991; Tarlo et al. 1996).

Thus, the aim of our study was to search for IgE sensitisation to *Penicillium glabrum*, *Chrysonilia sitophila*

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and *Trichoderma longibrachiatum*, common fungi of the cork industry work environment, as a possible aetiology of cork workers' occupational asthma.

Patients and methods

During a 5-year period, from 96 cork workers referred to our outpatient clinic of occupational respiratory diseases, we selected ten patients (age 43.2 ± 9.8 years, five men and five women), with episodic wheezing or cough that they related to work (cork-stopper production). Eight consecutive patients referred to our allergy clinic, with the diagnosis of asthma, but with no exposure in the cork industry, were used as controls.

Lung function tests

All patients underwent spirometry (Erich Jaeger, Hoechberg, Germany), and reversible airflow limitation was considered when the improvement in the forced expiratory volume in 1 second (FEV₁), after a bronchodilator had been inhaled, was greater than 15% and exceeded 200 ml. We measured bronchial hyper-responsiveness by histamine challenge, using the protocol of Cockcroft et al. (1977).

Peak expiratory flow records

Patients with occupational exposure performed serial peak expiratory flow (PEF) measurements as previously described (Winck et al. 2001). They were asked to measure PEF every 2 h and to record the best of three attempts during at least 10 days at work and 10 days away from work, in a consecutive fashion. Mean, maximum, and minimum daily values were plotted on a graph, and the record was visually analysed and considered positive when two of the three physicians (without knowing the patient's identification) agreed that the PEF graph showed a work-related change.

Skin prick tests

All patients underwent skin prick testing with a panel of common allergens and storage mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Dactylis glomerata*, cat fur, *Acarus siro*, *Lepidoglyphus destructor*, *Euroglyphus maynei*, *Blomia tropicalis*, *Dermatophagoides microceras*, *Tyrophagus putrescentiae*). We also tested glycerol-saline extracts (2 mg/ml) obtained after reconstitution of freeze-dried samples of *Chrysonilia sitophila*, *Penicillium glabrum* and *Trichoderma longibrachiatum* isolated from the industrial environment (kindly provided by Dr. V. San Romão (Danesh et al. 1997) and prepared by CBF Leti, Madrid, Spain). A negative control solution and a positive control (0.1% histamine) were also used. Tests results were considered positive when the mean diameter (in millimetres) of the wheal and flare was $\geq$ positive control.

In vitro tests (immunoblotting)

In eight cork workers with asthma and work-related symptoms [three with non-occupational asthma (NOA) and five with occupational asthma (OA)], we searched for antibodies (IgE, IgG4) specific for the three moulds (using the same antigenic material source as the "in vivo" tests) by immunoblotting (AlaSTAT-Ala-BLOT System, DPC, Los Angeles, Calif., USA). *Chrysonilia sitophila*, *Penicillium glabrum* and *Trichoderma longibrachiatum* allergen extracts were separated by polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to nitrocellulose

membranes, which were cut into the strips provided in the Ala-BLOT kits. A serum pool of four atopic patients with a high score (> 17.5 IU/ml, class 4 microplate AlaSTAT) for IgE specific for several mould allergens was used as a positive control serum in the production of AlaBLOT strips.

AlaBLOT strips specific for each antigen were used to identify specific IgE or IgG4 antibodies in patients' sera. Briefly, antigen strips were incubated with 500 μ l of diluted patient serum (1:10) for 2 h at room temperature, and, after being washed, the strips were then probed with 0.5 ml of enzyme-labelled anti-IgE Ab (or anti-IgG4) for 30 min. After the strips had been washed again the recognised antigens were then visualised by addition of 0.5 ml substrate solution for 15 min, and colour development was stopped by distilled water washing. The developed strips were scanned (HP Scanjet 7400 C) and analysed with the help of Quantiscan software (Biosoft, Cambridge, UK), which provided intensity and molecular weight for each resolved band in a range from 6 to 250 kDa.

Results

The group of workers we studied had a long history of exposure in different cork factories (18.8 ± 8.5 years) and, in all, symptoms began after a long latency period (5.3 ± 4.5 years). All patients worked in cork-stopper production, most of them in punching and/or sorting of bottle corks. Apart from episodic wheezing/cough after exposure, all had reversible airflow obstruction (four had moderate—FEV₁ 60 to 80%—and one had severe—FEV₁ $< 60\%$ —airflow obstruction) and/or significant bronchial hyper-responsiveness (PC20 histamine < 16 mg/ml in five out of six subjects tested) (Table 1).

On the basis of the visual assessment of the mean, maximum, and minimum daily PEF, five patients had patterns indicative of OA and five had negative studies (NOA).

Positive results for skin prick tests for common allergens were shown by two out of ten patients with occupational exposure and four out of eight in the control group (Table 2). Moreover, two out of five patients with OA and three out of eight controls exhibited sensitisation to storage mites. While one polysensitised (house dust and storage mites) asthmatic patient from the control group had a positive response to skin prick tests for fungi (*Penicillium glabrum* and *Chrysonilia sitophila*), all patients with OA and NOA had negative results for skin tests for the three fungi.

Table 1 Demographic characteristics, lung function and bronchial hyper-responsiveness in cork workers with asthma (F female, M male, RV residual volume, BHR bronchial hyper-responsiveness)

Characteristic	Occupational asthma (n = 5)	Non-occupational asthma (n = 5)
Age (years)	40.9 $\pm$ 8.6	44.9 $\pm$ 8.9
Gender	3F/2M	2F/3M
Symptom duration (years)	6.8 $\pm$ 6.0	3.8 $\pm$ 2.2
Exposure (years)	15.8 $\pm$ 6.5	21.8 $\pm$ 10.0
FEV ₁ (%)	89.4 $\pm$ 22.7	74.0 $\pm$ 19.1
RV (%)	123.4 $\pm$ 20.4	166.8 $\pm$ 48.4
BHR	3/3	2/3

Table 2 Positive skin prick test results in cork workers' asthma and non-exposed asthmatic controls. The number of positive tests (mean diameter of the wheal $\geq$ positive control) is shown. None of the exposed patients showed skin reactivity to *Dactylis glomerata* and cat fur

Allergen	Occupational asthma (n = 5)	Non-occupational asthma (n = 5)	Controls (n = 8)
House dust mites			
<i>Dermatophagoides farinae</i>	1/5	0/5	3/8
<i>Dermatophagoides pteronyssinus</i>	1/5	0/5	4/8
Total	2/5	0/5	4/8
Storage mites			
<i>Acarus siro</i>	2/5	0/5	1/8
<i>Lepidoglyphus destructor</i>	1/5	0/5	3/8
<i>Euroglyphus maynei</i>	1/5	0/5	3/8
<i>Blomia tropicalis</i>	1/5	0/5	1/8
<i>Dermatophagoides microceras</i>	1/5	0/5	3/8
<i>Tyrophagus putrescentiae</i>	2/5	0/5	2/8
Total	2/5	0/5	3/8
Fungi			
<i>Penicillium glabrum</i>	0/5	0/5	1/8
<i>Chrysionilia sitophila</i>	0/5	0/5	1/8
<i>Trichoderma longibrachiatum</i>	0/5	0/5	0/8
Total	0/5	0/5	1/8

Immunoblotting tests on the sera of three NOA cases and five OA cases confirmed the absence of IgE antibodies to *Penicillium glabrum* (Fig. 1, left), *Chrysionilia*

sitophila and also *Trichoderma longibrachiatum* (data not shown). However, we could demonstrate IgG4 antibodies to *Penicillium glabrum* (Fig. 1, right: lanes 5, 6, 8 and 12), and also to *Chrysionilia sitophila* (data not shown) but without a specific pattern of reactivity for occupational asthma.

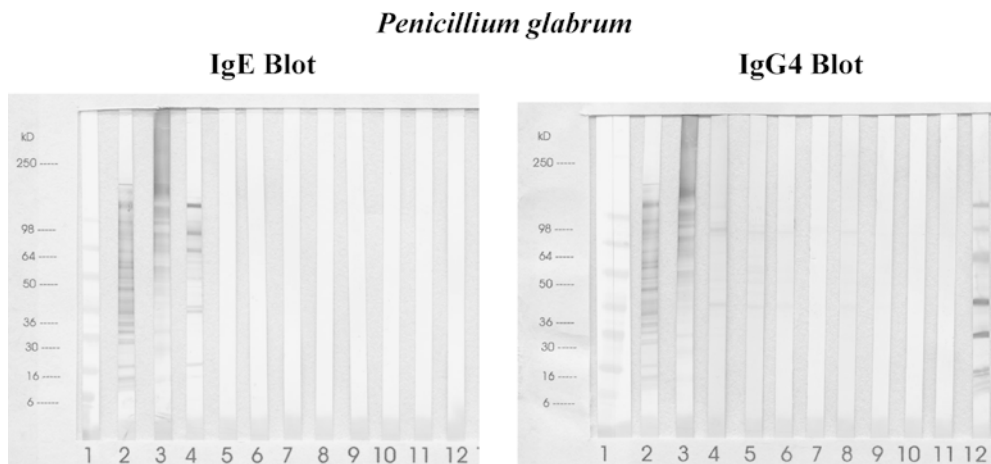
Discussion

Workers in the cork industry are exposed to dusts and moulds that can result in development of an occupational respiratory disorder—suberosis (Pimentel and Ávila 1973; Delgado et al. 1992). Traditionally, as in other occupational settings such as those of bird fanciers and farmers, this exposure leads to the development of hypersensitivity pneumonitis. However, description of cases of asthma in these settings is now more common, and its aetiology needs some clarification (Arlian et al. 1997; Colloff et al. 1997; Winck et al. 2001; Hirakata et al. 2002). In the absence of an identifiable aetiological agent, the assessment of peak expiratory flow rates, at and away from work, is a valuable diagnostic method to ascertain the occupational nature of a worker's asthma (Winck et al. 2001). Accordingly, we have shown that the visual analysis of the mean, maximum, and minimum daily PEF values may be the procedure of choice to support the occupational nature of asthma in the cork industry.

As in bakers' asthma, fungal and mite allergens can be possible causes (Heederik and Newman Taylor 1999). Previous studies with bronchoalveolar lavage have shown that cork workers' asthma is associated with eosinophilic bronchoalveolar inflammation, a finding similar to that for occupational asthma caused by low molecular weight agents, and for atopic asthma (Winck et al. 2002).

Fungal spores are known to be related to the incidence of asthma and to produce IgE-mediated hypersensitivity (Licorish et al. 1985; Lehrer et al. 1994). Moreover, fungal allergens have also been implicated as causative agents of occupational asthma (Cote et al.

Fig. 1 IgE (left) and IgG4 (right) immunoblots of *Penicillium glabrum* from individual sera of subjects with non-occupational asthma (lanes 5–7) and occupational asthma (lanes 8–12). The molecular weight standard (lane 1), protein stain (lane 2) and carbohydrate stain (lane 3) profiles are also shown. On lane 4 there is a positive serum pool. The IgG4 immunoblotting profile showed several protein bands from 13 to 136 kDa that were reactive to IgG4 from cork workers with both types of asthma. More intense binding was seen between 45–53 kDa and 86–92 kDa (easily seen in lanes 5, 6, 8 and 12)



1991; Tarlo et al. 1996). Apart from *Penicillium glabrum*, a fungus implicated in the early descriptions of suberosis, recently, *Chrysonilia sitophila* has been also identified throughout the manufacturing process of cork (Danesh et al. 1997) and, as in the logging industry, may play a role in cork workers' occupational asthma (Tarlo et al. 1996).

In our study, using skin testing and immunoblotting for the detection of specific IgE antibodies to *Penicillium glabrum*, *Chrysonilia sitophila* and *Trichoderma longibrachiatum*, the most prevalent moulds in the manufacturing process of stoppers (Danesh et al. 1997), we did not find evidence of allergic sensitisation in patients with asthma in the cork industry, although an antibody response (IgG4) was evident in some cases (Fig. 1). In fact, specific IgG4 has been previously associated with non-IgE mediated cases of occupational asthma to both organic (Devey and Panzani 1975) and inorganic agents (Park and Hong 1991; Yokota et al. 1998), but its biological significance is still unknown (Cullinan 1998). In our series, the finding of IgG4 binding bands in both occupational and non-occupational asthma cases (Fig. 1) supports the notion that antibody production is related to long-term exposure to fungal antigens. As IgE and IgG4 are both primarily regulated by IL-4 (Zhang et al. 1994), this pattern of specific antibody production may be another "modified Th2 response" to a high antigen exposure (Platts-Mills et al. 2001), as seen in animal handlers who develop IgG specific for urinary proteins, with no IgE production (Platts-Mills et al. 1987).

The patient with more intense IgG4 binding bands (Fig. 1, lane 12) combined clinical features of both occupational asthma and hypersensitivity pneumonitis. This patient, with asthma symptoms, an obstructive defect with normal diffusing capacity and a positive serial PEF, had an uncommon bronchoalveolar lavage pattern, evocative of hypersensitivity pneumonitis (44.1% lymphocytes, with a CD4/CD8 ratio of 0.86). In fact, the combination of occupational asthma associated with bronchoalveolar lavage findings and specific antibodies suggestive of hypersensitivity pneumonitis has also been described in rare cases with exposure to decaying wood (Halpin et al. 1994; Hirakata et al. 2002), and this association has been found in epidemiological studies of farmers (Karjalainen et al. 2002).

Although we have studied a small sample of asthmatic patients with proven work-related changes in PEF, atopy and fungal allergy do not seem to be aetiological factors in these patients' asthma. We cannot exclude in our study that the absence of IgE antibodies to moulds in these long-term exposed cork workers could be a "healthy workers effect". The extending of this study to a larger population of cork workers, irrespective of the diagnosis, could add to the knowledge on the general prevalence of mould sensitisation in this industry. In a previous study using a fluorometric immunoassay, we also did not find IgE specific for *Penicillium glabrum* in another sample of asthmatic cork

workers (Delgado et al. 1995). In the present study we included other common cork moulds and used a more sensitive technique to search for significant IgE.

The lack of IgE sensitisation to fungi known to colonise cork during its industrial processing points to the search for other causative agents, such as cork contaminants or volatile organic compounds of microbiologically attacked cork (Rocha et al. 1996). Some contaminants, such as isocyanates, are known to be used in some areas of the cork industry (Alegre et al. 1990), e.g. cork floor-tile production. However, that was not the case in the population included in our study, who were all involved in the punching and finishing of cork stoppers.

Two occupational asthma cases had positive results in skin tests for storage mites (*Acarus siro* and *Tyrophagus putrescentiae*) suggesting that, as in other forms of occupational asthma, they might be implicated in some cases of cork workers' asthma (Warren et al. 1983; Blainey et al. 1989; Alvarez et al. 1999). Humid conditions, such as those seen in cork warehouses, may favour the growth of storage mites and facilitate sensitisation to these mites in cork workers (Blainey et al. 1989). These patients were also sensitised to common house dust mites (one to *Dermatophagoides pteronyssinus* and another to *Dermatophagoides farinae*), and at least some response to storage mites may result from allergic cross-reactivity (Luczynska et al. 1990). The findings of a similar prevalence of sensitisation to mites in our controls suggest that storage mites are widespread in the environment and not an occupational allergen specific to cork workers.

In conclusion, although long-standing exposure to high levels of mould spores occurs, and in spite of evidence of immunological sensitisation with specific IgG4 production in some patients, in patients with occupational asthma we could not find IgE sensitisation to the three most prevalent fungi in the cork industry. Our results may indicate that other mechanisms are responsible for inducing this disease. In fact, cork workers are chronically exposed to a wide diversity of chemicals and particles, potential airway irritants, which can have a role in the aetiology of cork workers' asthma. New studies exploring this hypothesis are warranted.

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