

Original article

Antigen characterization of major cork moulds in Suberosis (cork worker's pneumonitis) by immunoblotting

Background: We characterized by immunoblotting the antigenicity of the most frequent fungi colonizing cork during its industrial processing, *Penicillium glabrum* and *Chrysonilia sitophila*. *Penicillium glabrum* is the main causative agent of Suberosis, a hypersensitivity pneumonitis of cork workers. *Chrysonilia sitophila* induces both IgE sensitization and occupational asthma in the wood processing industry.

Methods: Serum-specific IgG, IgG4 and IgE to *P. glabrum* and *C. sitophila* from nine cork workers with hypersensitivity pneumonitis (HP) and seven with asthma (four with occupational asthma) were analysed by immunoblotting.

Results: Both HP and asthmatic patients' sera showed immunoreactivity to several proteins resolved in the specific immunoblot strips. The frequency of specific IgG recognition to 12–13.5 and 33 kDa proteins of *P. glabrum* was significantly higher in HP patients. The sera of HP patients had significantly higher specific IgG recognition to 16 and 51–55 kDa proteins of *C. sitophila*. There was no specific IgE recognition in the sera of HP or asthmatic patients to both fungi.

Conclusions: Different patterns of antibody reactivity to *P. glabrum* and *C. sitophila* are seen in cork workers with hypersensitivity pneumonitis or asthma. The 12–13.5 and 33 kDa proteins of *P. glabrum* and the 16 and 51–55 kDa proteins of *C. sitophila* may be major antigens in Suberosis.

**J. C. Winck¹, L. Delgado², R. Murta³,
M. Lopez³, J. A. Marques¹**

¹Pneumology and ²Immunology Departments, Faculdade de Medicina, Universidade do Porto, Portugal; ³Diagnostics Products Corporation, Los Angeles, USA

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João Carlos Winck
Pneumology Department
Faculdade de Medicina
Universidade do Porto
Portugal

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Suberosis is a hypersensitivity pneumonitis (HP) resulting from repeated exposure to mouldy cork dust during its industrial processing (1). The main causative agent of Suberosis is *Penicillium glabrum* (formerly known as *Penicillium frequentans*), one of the most frequent fungi isolated in cork samples (2). Precipitating antibodies to *P. glabrum* as well as a high-intensity CD8+ lymphocytic alveolitis are characteristic of the cork workers' pneumonitis in Suberosis (3, 4).

Patients with HP (like farmer's lung and bird fanciers' disease) have specific antibodies for a range of antigens including proteins, glycoproteins and polysaccharides (5). However, their presence in asymptomatic exposed individuals makes their role in the pathogenesis unclear. While some authors claim that immuno-dominant antigens exist both in farmer's lung and bird fanciers' disease (5, 6), others state that these results are simply a reflection of the sensitivity of the immunochemical methods used. Qualitative differences in the antibodies produced by symptomatic and asymptomatic patients have also been found, suggesting that IgG subclass of specific antibodies should also be studied (7). In this context, specific IgG4 is

seen as a marker of chronic fungal exposure and, comparatively to other IgG subclasses, has a lower affinity for complement and Fc-receptors, with a consequent lower inflammatory potential (8).

Recently, we have also characterized cases of occupational asthma in the cork industry, confirmed with serial peak expiratory flow recordings, but could not find evidence for IgE sensitization to *P. glabrum* in other *in vitro* and *in vivo* tests (9). *Chrysonilia sitophila* has also been identified as a cause of IgE sensitization and occupational asthma in the wood processing industry (10, 11), and is also a common mould in cork industrial samples (12).

Immunoblotting has been extensively used in the investigation of major environmental antigens (6, 13) and of the fungal aetiology of new cases of HP (14). The aim of our study was to characterize the antigenic composition of *P. glabrum* and *C. sitophila* by immunoblotting and to identify the protein determinants to specific IgG, IgG4 and IgE antibodies in cork worker's respiratory disease, thus evaluating both patients with hypersensitivity pneumonitis and airways disease.

Materials and methods

Patients

We studied 16 cork workers, with a long history of exposure in the cork stoppers industry (18 ± 10 years), referred to our department due to respiratory symptoms that related to work (punching and/or sorting of bottle corks).

Nine patients had a typical hypersensitivity pneumonitis (age: 34.4 ± 11.1 years, seven males and two females; exposure: 19.4 ± 11.7 years) with work-related symptoms of cough, exertional dyspnoea and weight loss. All had a high-intensity CD8+ lymphocytic alveolitis (bronchoalveolar lavage fluid total cells $9.1 \pm 6.0 \times 10^5 \text{ ml}^{-1}$, $78.4 \pm 9.1\%$ lymphocytes, CD8+ $55.5 \pm 11.6\%$), a restrictive pattern or lung diffusion impairment (5/9, 56%) and/or 'ground-glass' attenuation (7/7, 100%) in high-resolution computed tomography scans.

As controls, we included patients with the same exposure characteristics, in which we could clearly exclude an interstitial lung involvement. These seven cork workers had asthma (age: 45.4 ± 10.6 years, five males and two females; exposure: 17.1 ± 8.4 years), and complained of wheezing and dyspnoea worsening with occupational exposure. Two of them had reversible airways obstruction and five a significant bronchial hyper-responsiveness (PC20 histamine $< 16 \text{ mg/ml}$). Patients performed serial Peak Expiratory Flow Records (PEFR), as previously described (9): patients 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 three physicians (without knowing the patient identification) agreed that the PEFR showed a work-related change. Accordingly, four were classified as having occupational asthma (positive PEFR) and three as nonoccupational asthma.

Immunoblotting

Specific antibodies (IgG, IgG4 and IgE) to *P. glabrum* and *C. sitophila* were searched by immunoblotting method (AlaSTAT-AlaBlot System, DPC, Los Angeles, CA, USA) in aliquoted serum samples stored frozen at -20°C . Extracts of lyophilized mould isolates, obtained from different stages of cork processing, identified and kindly provided by Dr V San Romão (12), were separated by polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes, which were cut into the strips provided in the AlaBLOT kits. A serum pool of four atopic patients with high specific IgE values ($> 17.5 \text{ IU/ml}$, Class 4 Microplate AlaSTAT) to several mould allergens was used as a positive control serum in the production of AlaBLOT strips. For the AlaBLOT *P. glabrum* specific strips, a bronchoalveolar lavage pool with positive IgG in fluoro-enzymo-immuno assay was also used as a positive control. In each run, one antigen strip was reserved for carbohydrate (DIG Glycan Detection Kit, Roche Molecular Biochemicals, Indianapolis, IN, USA) and another for protein staining (Colloidal Gold, Biorad, Hercules, CA, USA).

Immunoblot strips specific for each antigen were used to identify specific IgG, IgG4 or IgE antibodies in the sera of patients. Briefly, antigen strips were incubated with 500 μl of diluted patient serum (1 : 10) for 2 h at room temperature and, after washing, the strips were then probed with 0.5 ml of enzyme-labelled anti-IgG, IgG4 or IgE Ab for 30 min. After washing, the recognized antigens were then visualized by the addition of 0.5 ml substrate solution for 15 min, and colour development stopped by washing with distilled

water. The developed strips were scanned (HP Scanjet 7400 C) and analysed with the help of QUANTISCAN software (Biosoft, Cambridge, UK), which provided the intensity and the molecular weight for each resolved band in a range from 6 to 200 kDa.

Statistics

All data were analysed with the statistical package spss® for Windows® (SPSS Inc., Chicago, IL, USA). Descriptive analysis was used and, unless otherwise stated, data are reported as mean $\pm$ SD and frequencies as n (%). Differences between band intensities of the two patient groups (hypersensitivity pneumonitis and asthma) were analysed using Mann-Whitney's U-test, while Fisher's exact test was used for comparison between band frequencies. Significance was accepted at 5%.

Results

Specific antibodies to *Penicillium glabrum*

All tested sera from cork-exposed patients showed IgG reactivity to several components of *P. glabrum*, with reactivity clearly more intense in the group with HP (Fig. 1). A total of 11 components of *P. glabrum* ranging in molecular weights from 12 to $\geq 100 \text{ kDa}$ bound to IgG antibodies. We observed that there was a significant difference in the frequency of IgG binding to 12–13.5 and 33 kDa proteins of *P. glabrum* antigens between HP and asthmatic patients; only two of the seven asthmatics reacted to 12–13.5 and 33 kDa proteins, and all HP patients reacted to the above protein bands ($P = 0.007$ and 0.035, respectively, Fisher's exact test). Moreover, the 33 kDa component was also reactive in the immunoblot of a control BALF pool (data not shown). Some of the higher molecular weight components reacted with all tested sera from both groups, such as the 48–52 kDa and the 88–92 kDa antigens. The 37 and $\geq 100 \text{ kDa}$ components reacted with all HP sera and also with the majority of asthmatic patients (86%).

The QUANTISCAN software also allowed comparison of the relative band intensities of the IgG-binding *P. glabrum* antigens recognized by both patient groups. The intensity of IgG binding was significantly higher in the HP group for eight of the 11 antigens.

All tested sera of HP patients also showed IgG4-specific binding to the different molecular weight components between 14 and 92 kDa, with frequencies of immunoreactivity ranging from 33 to 78% of the cases. The frequency of specific IgG4 recognition in HP patients to 14–17, 24, 37 and 57–62 kDa proteins was significantly higher than in asthmatics ($P < 0.05$, Fisher's exact test). The asthmatic group showed immunoblot reactivity to only four of the eight components, with lower frequency and binding intensity, except for the 33 kDa antigen that was positive in 71% of cases (vs 33% in HP patients).

There was no observed specific IgE recognition in our occupationally exposed patients (data not shown).

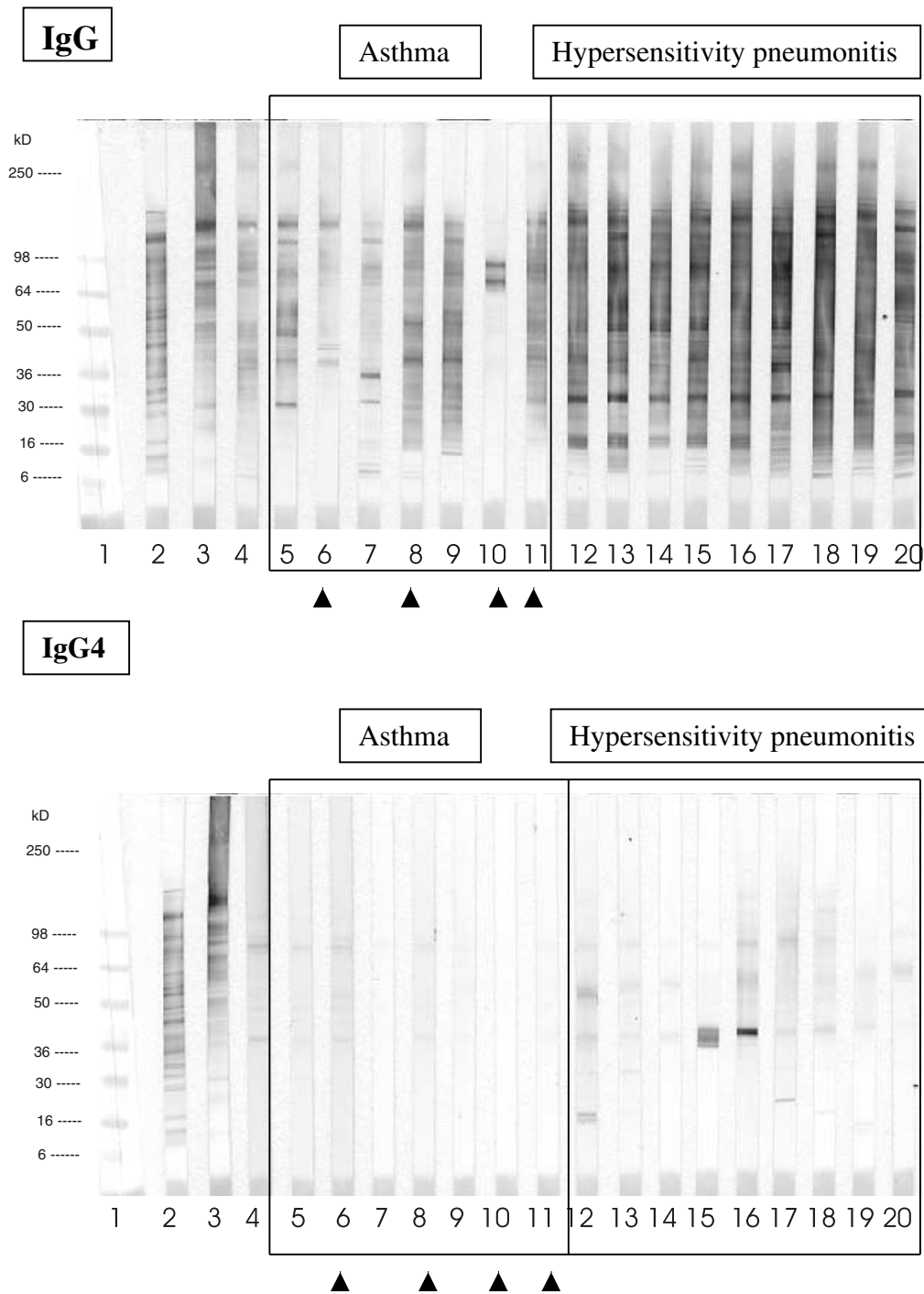


Figure 1. IgG (top) and IgG4 (bottom) immunoblots of *Penicillium glabrum* with individual sera of asthma cases (lanes 5–11) and hypersensitivity pneumonitis (lanes 12–20). The molecular weight standard (lane 1), protein stain (lane 2) and carbohydrate stain (lane 3) profiles are also shown. On lane 4, there is a positive serum pool of mould-allergic patients. Patients with occupational asthma are indicated by black triangles.

Specific antibodies to *Chrysosilia sitophila*

All tested sera from cork-exposed patients showed IgG reactivity to several components of *C. sitophila* (Fig. 2, top). A total of 13 components of *C. sitophila* ranging

in molecular weights from 12 to ≥ 100 kDa bound to IgG antibodies and, to most of them (11/13), there was a high frequency of positivity in both HP (from 67 to 100%) and asthma cases (from 57 to 100%). The 16 kDa component was only reactive with HP

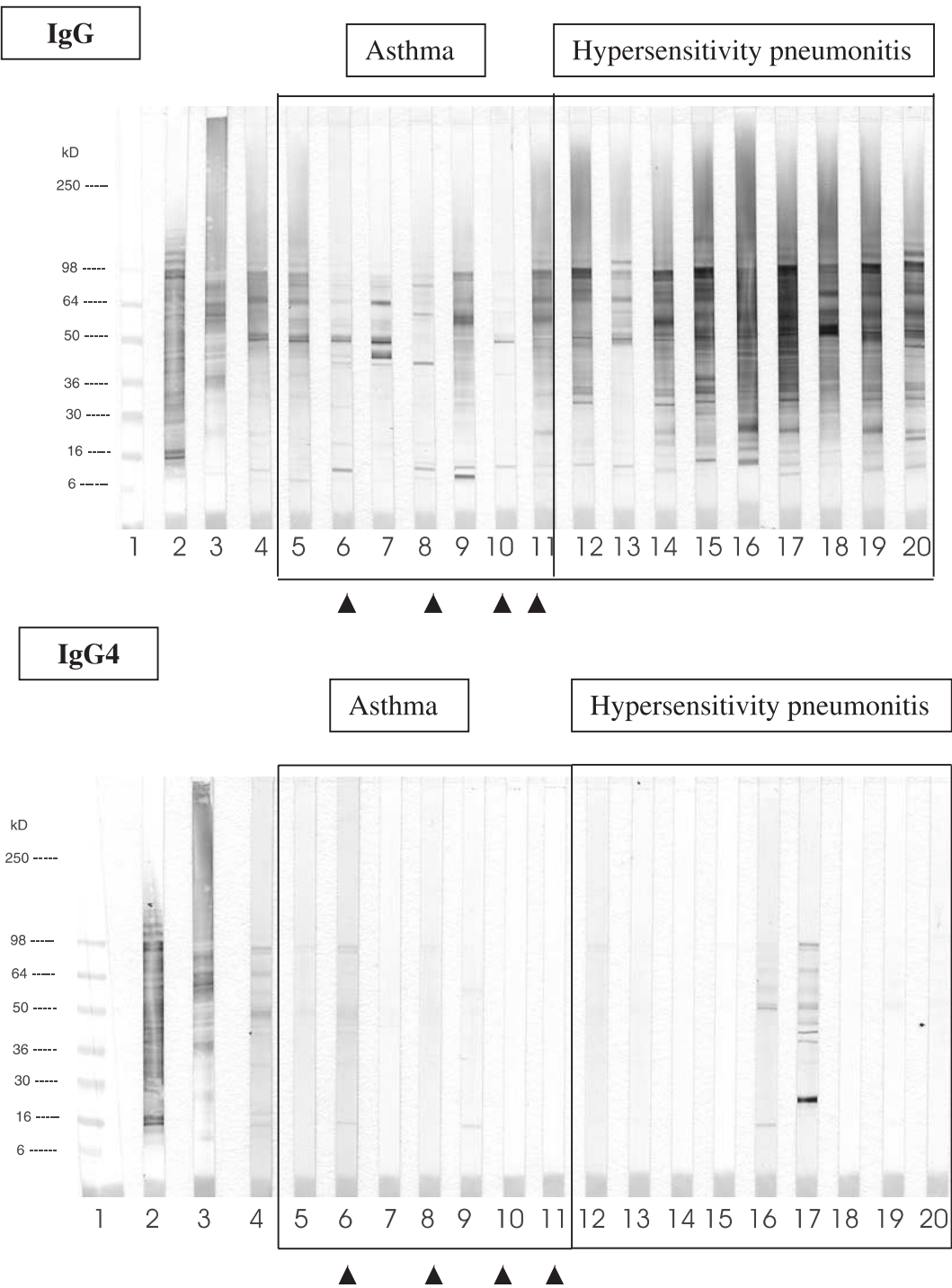


Figure 2. IgG (top) and IgG4 (bottom) immunoblots of *Chrysonilia sitophila* with individual sera of asthma cases (lanes 5–11) and hypersensitivity pneumonitis (lanes 12–20). The molecular weight standard (lane 1), protein stain (lane 2) and carbohydrate stain (lane 3) profiles are also shown. On lane 4, there is a positive serum pool of mould-allergic patients. Patients with occupational asthma are indicated by black triangles.

sera, and the 51–55 kDa antigen was recognized by most (89%) of HP sera, but only by one of the asthmatic patients ($P = 0.001$ and $P = 0.035$, respectively, Fisher's exact test), and band intensities were

significantly higher in HP for nine of the 13 components (Table 1).

The frequencies of IgG4 binding to *C. sitophila* were less than 15% for all components except for the 12–14 kDa

Table 1. Specific IgG to *Cryphonilia sitophila*: frequency of IgG binding (%) to different molecular weight (kDa) components and respective band intensity (mean of band area and height as determined by the QuantiScan software). Note that the 16 kDa component was only present in hypersensitivity pneumonitis cases and the 51–55 kDa antigen was recognized by most hypersensitivity pneumonitis cases but in only one case with asthma (the differences being significant: $P = 0.001$ and $P = 0.035$, respectively, Fisher's exact test)

MW (kDa)	Hypersensitivity pneumonitis		Asthma	
	Frequency (%)	Area (height)	Frequency (%)	Area (height)
12–14	100	178 (36)	86	167 (56)
16	89	111 (25)	0	0 (0)
18–24	100	653* (100)**	86	87* (18)**
25–30	100	1089* (202)**	86	163* (28)**
33	89	504* (91)**	71	169* (27)**
38–43	100	546 (91)	71	247 (59)
45–48	100	904* (165)**	100	422* (86)**
51–55	89	785 (155)	14	255 (56)
59–62	100	1081* (112)**	100	934* (55)**
70–77	100	1002* (149)**	86	313* (47)**
81–90	67	487 (103)**	71	277 (47)**
91–94	78	824 (124)**	57	363 (58)**
≥100	100	2579* (265)**	57	551* (57)**

* and ** Significantly different (Mann–Whitney U -test).

(29% of asthmatics) and 38–43 kDa (33% of HP) proteins. The IgG4 reactivity showed low intensity in all samples except for some of the HP cases (Fig. 2, bottom).

No IgE-binding to *C. sitophila* was found in any of the cork-exposed patients we studied.

Discussion

Suberosis is an immunological lung disease caused by repeated inhalation of airborne cork particles and spores of different moulds colonizing cork slabs during its industrial processing (2). The major mould species isolated from the *Quercus suber* bark after harvest (12) and from air samples in cork factories (2) include *P. glabrum* and *C. sitophila*.

The pathogenesis of this HP is possibly related to an initial immune complex-mediated lung injury followed by cell-mediated hypersensitivity interstitial damage (15). In fact, high titres of specific IgG to *P. glabrum* in bronchoalveolar lavage as well as diffuse mononuclear cell infiltration with granuloma formation in histopathological samples are prominent features of Suberosis (1). More recently, we could demonstrate, by bronchoalveolar lavage studies, that the alveolar inflammation mainly involves T-cells and an increase of CD8+ cytotoxic lymphocytes (4).

The presence of high titres of antibodies to mould antigens is a useful evidence of exposure in the context of characteristic clinical, radiological and functional findings. However, as in other HP forms, the diagnostic usefulness of antibody detection decreases due to its

presence in many exposed workers with no apparent lung disease. In the case of *P. glabrum*, the seropositivity ranges from 7% of unaffected cork workers to 81% in HP forms and 27% in asthmatics (1, 2).

The finding of 'disease-specific' antigens only detected in sera of symptomatic subjects by precipitin reactions or immunoenzymatic assays is controversial (5, 6) and may be linked to the poor sensitivity of the immunochemical tests used (16). Immunoblotting has been used to identify major antigenic components in several forms of HP, such as the glycoproteins from *Saccharopolyspora rectivirgula* (5, 17) and *Aspergillus umbrosus* (18) in farmer's lung, mannoproteins of *Trichosporon asahii* (19) in summer type hypersensitivity pneumonitis (SHP) and a 21 kDa protein from pigeon dropping extracts (20). More recently, in pigeon breeders' disease and SHP, the immunoblotting technique allowed the identification of antigen components that could induce the specific proliferation of peripheral blood mononuclear cells only in affected patients (20, 21).

In our study, we investigated the immunochemical characteristics and antigenicity of *P. glabrum* and *C. sitophila* by immunoblotting technique, studying serum samples of 17 patients with respiratory symptoms associated with occupational exposure in the cork industry. In addition, a specific software (QuantiScan, Biosoft, UK) allowed the determination of each band height and area, which identified low-intensity reactivity to particular components, avoiding the subjectivity of immunoblotting interpretation (22) and quantitatively comparing different antibody responses to discrete antigens.

Penicillium spp. are universal saprophytes that produce abundant spores and can induce both allergic asthma (23) and occupational lung diseases (2, 24). High numbers of airborne spores of *P. glabrum* are detectable in air samples, mainly in cork storage and cutting sections (2). In our study, using a sensitive method such as immunoblotting, we could detect IgG reactivity to different components of *P. glabrum* in all cases of both HP and asthma. Four of the 11 antigenic components reacted to more than 85% of the tested sera in both groups. As expected, the intensity of IgG binding was significantly higher in HP. In addition, the 12–13.5 and 33 kDa components were significantly associated with HP and may represent major antigens for these patients. Moreover, the 33 kDa component was also recognized by our BALF pool control, a feature that usually characterizes major HP antigens (20, 21).

IgG4 reactivity to *P. glabrum* was more intense in HP, suggesting that, like in *Aspergillus*-induced lung disorders, chronic antigen exposure in the cork industry may favour IgG4 production (8, 25). Surprisingly, in the asthmatic group, although IgG4 reactivity was weaker (Fig. 1, bottom), the frequency of reactivity to the 33 kDa component was higher (71 vs 33% in HP). Although we could not find a corresponding IgE response to this antigen, studies of different *Penicillium* spp. allergic

patients found IgE binding to components of 33 kDa (26, 27). The reason why our asthmatic patients, chronically exposed to high numbers of *Penicillium* spores, produce specific IgG4 but not IgE is difficult to explain, as IgE and IgG4 are both part of a Th2 response, primarily regulated by IL-4 (28). This may be another example of what has been proposed as a 'modified Th2 response' in the context of high antigen exposure. In fact, a similar response has also been described in animal handlers who, over the years, develop specific IgG antibodies to urinary allergens without producing IgE (29). On the other hand, asthma complaints may be related to the exposure to different volatile organic compounds of microbiologically attacked cork (30) that can potentially trigger or induce asthma.

Chrysonilia sitophila, another highly prevalent fungus in the cork industry (2, 12), has been linked to occupational asthma in the logging industry (10). It has an enzymatic activity involved in the biodegradation of cellulose and lignin, main constituents of wood matrix (31, 32), which may have an important role in the maturing process of cork.

IgG binding to several antigens of *C. sitophila* was present both in HP and asthmatic patients, but two components (16 and 51–55 kDa) were particularly

associated with the former. This may suggest that different microbial antigens may be involved in the hypersensitivity pneumonitis of cork workers. Similarly, in Farmer's Lung, it has been shown that apart from *Sacharopolyspora rectivirgula* (the classic antigen of the disease), other moulds (like *Absidia corymbifera*) may also be implicated (33). Alternatively, IgG binding of *C. sitophila* may represent cross-reactivity with *P. glabrum*, as has been suggested in the earlier studies of Suberosis; cross-inhibition of the immunoblotting reactivity with these two moulds may clarify this hypothesis.

In conclusion, immunoblotting with detection of IgG, IgG4 and IgE reactivity was a suitable tool in the evaluation of the antigenicity of major cork moulds in Suberosis. HP shows intense IgG and IgG4 responses to various *P. glabrum* and *C. sitophila* components, while asthmatics produce weaker IgG and IgG4 levels and no specific IgE to both moulds. Some of the antigens that proved to be more characteristic of hypersensitivity pneumonitis may deserve further purification and studies of mononuclear cell proliferation, which may shed more light on the immunopathogenesis of Suberosis and help in the diagnosis of the disease.

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