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Sensitization to fungi: epidemiology, comparative skin tests, and IgE reactivity of fungal extracts

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Summary

Background Several fungal species are known to cause severe respiratory and cutaneous allergic diseases. Extracts from several allergenic fungi are used for *in vivo* and *in vitro* tests, as standard preparations are still not available.

Objective The aims are to define the pattern of *in vivo* and *in vitro* IgE reactivity to fungal species in an allergic population with respiratory symptoms; to determine the influence of different extract preparations on diagnostic results; and to evaluate whether there exists a relationship between the diagnostic pattern of reactivity and the pattern of specific IgE reactivity in immunoblots.

Methods Skin prick tests were applied to a cohort of 4962 respiratory subjects, aged 3–80 years. Fungal extracts from *Alternaria, Aspergillus, Candida, Cladosporium, Penicillium, Saccharomyces*, and *Trichophyton* were used, along with extracts from pollens, mites, and animal dander. Demographical and diagnostic data were recorded. IgE detection was carried out with the same allergenic extracts plus *Malassezia*. Comparative skin tests and IgE detection were carried out using extracts from three commercial suppliers. IgE immunoblots were carried out with the same panel of commercial fungal extracts and were compared with in-house extracts. Data analysis was carried out by grouping the population on the basis of their reactivity to a single, to two or to more than two, mould species.

Results Nineteen percent of the allergic population reacted to at least one fungal extract by means of the skin test. *Alternaria* and *Candida* accounted for the largest number of positive tests, and along with *Trichophyton* they were the main sensitizers in the subset of patients with an isolated sensitization. The prevalence of skin test reactivity increased for these three fungi in the subsets with two associated reactivities and, furthermore, in the subset showing reactivity to more than two mould species. In the latter group, a steady increase of the skin test reactivity was recorded for all the other fungal sources, suggesting a clustered reactivity. Comparative skin and IgE testing with different groups of subjects with a simple pattern of skin reactivity resulted in sensitivity differences between *in vivo* and *in vitro* tests, whereas discrepant results were recorded in the subsets of patients with multiple fungi sensitization. Although hampered by the limited reliability of fungal extracts, IgE immunoblots revealed differing patterns of reactivity when sera from the three subsets were used. This suggests a link between the diagnostic reactivity pattern and the IgE sensitization to extracts' components. Age and gender distribution differed among the *Alternaria*-, *Candida*-, and *Trichophyton*-sensitized subjects, but not in the subset with more than two fungi sensitizations.

Conclusions The preliminary assessment of a new classification of the mould-sensitized population has been reached. The limiting quality of fungal extracts requires future studies using an allergenic molecule-based approach. The diagnostic process and the definition of the reactivity pattern would thus be easy, and it could lead to a novel specific immunotherapy approach.

Keywords allergens, comparative study, epidemiology, fungal allergy diagnosis, fungal extract, IgE detection, immunoblotting, prevalence, skin tests.

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Introduction

Fungal allergy is a worldwide problem [1–6]. Mould spores can be found [2–6] in outdoor or indoor environments [2–8], but exposure can also occur by having contact with saprophytic species [9] or by ingestion of edible mushrooms [6, 10]. Exposure to allergenic moulds may lead to IgE-mediated rhinitis and asthma [11–13], atopic dermatitis [9, 12, 14], or, rather infrequently, to generalized reactions [10]. Inhalation of fungal spores carrying allergens has been claimed as a risk factor for severe asthma [15]. Differing from other allergenic species like pollens, mites, or animal dander, some of the allergenic fungi are pathogenic for

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humans, causing localized infections (e.g. *Candida, Malassezia, Trichophyton*) [16], more severe respiratory diseases (e.g. *Aspergillus*) [17], or systemic mycoses [18].

Several species and genera have been reported to cause fungal allergy. Epidemiological, environmental, and clinical research was focused on relevant species like *Alternaria* [19, 20], *Aspergillus* [21], *Cladosporium* [19, 22], and *Penicillium* [23]. Some studies reported the clinical relevance of *Candida* [3, 24], *Trichophyton* [25], and *Malassezia* [9] in either respiratory or skin allergic diseases. Allergy to spores of Basidiomycetes (e.g. Boletus, Coprinus, Pleorotus, Psilocybes) has been reported, and the relevance of their causative role in respiratory allergy has been documented [6].

During the last two decades, great efforts were made to optimize the allergenic extracts from several fungal sources [26, 27]. Nevertheless, problems still remain to obtain reliable preparations for diagnosis and therapy [28]. More recently, allergenic molecules have been cloned, identified, and characterized from *Alternaria* [19, 22], *Aspergillus* [21], *Candida* [29, 30], *Cladosporium* [19, 22], *Malassezia* [9], *Penicillium* [31, 32], *Saccharomyces* [33], and *Trichophyton* [34]. Allergens of Basidiomycetes have been identified, characterized, and cloned as well [6].

Several epidemiological and diagnostic studies reported variable prevalence of allergic reactivity to fungi by means of skin testing or IgE detection [3, 4, 28, 35]. Some of these studies reported both isolated reactivity to distinct species and co-reactivity to several species in the same patient [36, 37].

On the basis of the results of a previous study dealing with different skin test reactivity patterns to pollen extracts related to the sensitization to different allergenic molecules [38], we aimed to evaluate the prevalence of sensitization to seven major allergenic fungal sources by means of skin testing. Additionally, we wanted to define their possible association in terms of co-reactivity, to compare *in vivo* and *in vitro* IgE reactivity with extracts from different manufacturers, and to determine by means of immunoblotting whether the overall pattern of fungal sensitization corresponds to specific patterns of IgE reactivity to allergenic molecules.

Materials and methods

Allergenic extracts

Commercial fungal extracts (*Alternaria alternata, Aspergillus fumigatus, Candida albicans, Cladosporium herbarum, Penicillium notatum, Trichophyton mentagrophytes*, Allergopharma, Reinbeck, Germany; *Saccharomyces cerevisiae*, Stallergenes, DHS, Antony, France) were used to select sensitized patients by means of skin prick tests (SPT). As non-overlapping results have been reported by comparing commercial extracts from different manufacturers [28], the chosen preparations were those currently used in routine diagnostic procedures [38]. A complete panel of allergenic extracts from pollen and non-pollen sources, already used in a previous study [38], was skin tested as well. The same panel of fungal extracts plus the *Malassezia furfur* extract were used for the detection of specific IgE antibodies (Pharmacia, Uppsala, Sweden). Commercial *Alternaria alternata, Aspergillus fumigatus, Candida albicans, Cladosporium herbarum, Penicillium notatum, Saccharomyces cerevisiae*, and *Trichophyton mentagrophytes* extracts (Stallergenes) were used for comparative purposes in the SPT. The same panel of mould extracts, supplied by Allergopharma and Stallergenes, together with *Alternaria alternata* and *Cladosporium herbarum* extracts from in-house preparations [39], were used for comparative IgE immunoblots. The batches of the extracts available from the commercial suppliers were different from those used in the screening and in the comparative SPT.

The panel of fungal extracts used in the present study was defined on the basis of data reported in the literature, as mentioned in the Introduction section. Only the genera names are used in the paper to refer to the fungal extracts mentioned above (e.g. *Alternaria* for *Alternaria alternata*).

Patients, skin testing, grouping criteria, and sera

Unselected consecutive subjects ($N = 4962$) presenting respiratory symptoms (rhinitis and asthma) that suggested an allergic disease, and referred to the Allergy Unit, were enrolled. Demographical (age and gender) and clinical data (atopic dermatitis, urticaria, conjunctivitis, rhinitis, and asthma) were recorded for each patient. Patients underwent SPT, with a standardized procedure [38] for the fungal and non-fungal extracts. A result was considered positive when a weal of at least 3 mm greater than the negative control was recorded 15 min after the application of the tested substance. None of the patients received a specific immunotherapy course for any of the mould species considered in the study.

Patients were divided into three subsets on the basis of their SPT reactivity to a single (ONE-FS), two (TWO-FS), or more than two fungal sources. The criteria to include subjects in the third group (more than two positive SPT to fungal extracts) were defined on the basis of preliminary studies showing the SPT reactivity spreading to most of the mould extracts when more than two clear-cut reactivities were recorded (multiple fungi sensitization, MFS).

Following the SPT, sera were obtained from 431 patients who consented to blood sampling for an *in vitro* diagnostic procedure. The subgroup of patients who underwent blood drawing matched the original fungi-sensitized cohort in terms of age and gender. Sera were stored at $-20\,^{\circ}C$ until required.

Informed oral consent for skin testing and blood sampling was obtained from patients or caregivers during the allergy consultation.

IgE detection and immunoblotting

Allergen-specific IgE were detected by the ImmunoCAP system following the instructions of the manufacturer (Pharmacia). Values $\geqslant 0.4\,kU_A/L$ were considered positive in order to obtain clear positive or negative results. ImmunoCAP IgE detection was carried out on all the available sera.

IgE immunoblots were performed as previously reported [19, 39]. IgE detection by immunoblotting was carried out on selected sera. Sera used in the Fig. 2 experiments were those showing the highest IgE values. In the case of Fig. 3, the highest number of unselected sera available was used for the immunoblots.

Statistics

Data were stored by means of customized databases and retrieved by means of study-tailored query programs. Statistical analyses were carried out by means of the Prism package (Graphpad Software, version 3.0, San Diego, CA, USA). The Fisher's exact test and the Mann–Whitney test were applied where appropriate. Statistical significance was accepted at $P<0.05$.

Results

Prevalence data

Six hundred and twenty-one subjects (19.1% of the whole allergic population) were SPT-positive to at least one fungal source. Ages ranged from 3 to 80 years, with a male/female ratio of 1.2. Patients sensitized to at least one pollen were 2395 (73.7% of the allergics), 1775 either to mites or to cockroaches (54.6%), and 775 to at least one of the mammal epithelia (23.9%). Ninety-four of the fungi-sensitized subjects (15.1%) had no other associated sensitization, whereas an associated sensitization to at least one pollen species was recorded in 461 patients (74%), to mites or cockroaches in 355 patients (57.2%), and to at least one of the mammal epithelia in 16 patients (2.6%).

Table 1 reports the prevalence of different patterns of fungal sensitization. The group of ONE-FS accounted for about 77% of the subjects showing fungal sensitization. *Alternaria*, *Candida*, and *Trichophyton* were positive in 98% of the subjects, whereas the isolated sensitivity to the other moulds tested was rather seldom. A further increase in the prevalence of the three main mono-sensitizers was recorded in the second subset of patients (TWO-FS), where two associated sensitizations were considered (10% of the subjects showing fungal sensitization). A slight increase of the prevalence of the other mould sources was recorded, but only *Cladosporium* reached the *Trichophyton* value (19%). The association of *Alternaria*/*Candida* or *Alternaria*/*Trichophyton* was most commonly recorded. In the MFS subgroup (12.4% of the subjects showing fungal sensitization), the prevalence of almost all the fungal sources increased above 50%. *Alternaria* prevalence was 92.2%, *Aspergillus, Candida*, and *Cladosporium* were above 80%, but *Trichophyton* reached only 37.7%.

As no reference standards exist for fungal extracts, comparative tests in the ONE-FS and TWO-FS subsets were evaluated arbitrarily assuming that the Allergopharma extracts correspond to 100%. Table 2a reports the comparative reactivity to fungal extracts (*Alternaria, Candida, Trichophyton*) from the three manufacturers by both *in vivo* and *in vitro* methods. In the case of *Alternaria* extract, all the patients showing a positive SPT reactivity to the Allergopharma extract also reacted with the Stallergenes extract. Moreover, 94% of the *Candida*-sensitized patients were positive for the *Candida* extract of Stallergenes and 85% reacted positively with the *Trichophyton* extract of Stallergenes. The sensitivity of the IgE assay was lower for the three fungal species (*Alternaria* 89%, *Candida* 75%, and *Trichophyton* 61%). Table 2b reports the reactivity to the fungal extracts in 48 consecutive subjects belonging to the MFS subset. The MFS selection criteria considered the pattern of SPT reactivity of the patients rather than the specific reactivity to pre-defined allergenic species. Comparative results varied, depending on either the mould species or the extracts' manufacturer. An increase of the SPT reactivity to the Stallergenes extracts was recorded for *Alternaria* and *Trichophyton* (Table 2b). Further increase of the prevalence was recorded by means of IgE detection in the *in vitro* test. More than 70% of the subjects in the MFS subset reacted with the tested fungi (Table 2b). Moreover, testing for IgE to the *Malassezia* extract in the same subset led to 70% positive results, whereas negative results were obtained in a random sample of control sera from the ONE-FS and TWO-FS

Table 1. Prevalence of fungal allergy based on skin prick testing (SPT) of the whole population with suspected respiratory allergy

	Allergics*	Fungi*	ONE-FS*	TWO-FS*	MFS*
Total	3248 (65.5)	621 (19.1)	482 (77.6)	62 (10.0)	77 (12.4)
Alternaria	410 (12.6)	410 (66.1)	288 (59.8)	51 (82.6)	71 (92.2)
Aspergillus	78 (2.4)	78 (12.6)	3 (0.6)	11 (17.7)	64 (83.1)
Candida	275 (8.5)	275 (44.3)	162 (33.6)	44 (71.0)	69 (89.6)
Cladosporium	81 (2.5)	81 (13.1)	4 (0.8)	12 (19.3)	65 (84.4)
Penicillium	50 (1.5)	50 (8.1)	1 (0.2)	10 (16.1)	39 (50.6)
Saccharomyces	46 (1.4)	46 (7.4)	2 (0.4)	3 (4.8)	41 (53.2)
Trichophyton	63 (1.9)	63 (10.2)	22 (4.6)	12 (19.3)	29 (37.7)

Studied population = 4962 subjects.
All data are expressed as number of subjects and percentage (in brackets).
Percentages have been calculated following the subset divisions for the first row, and within the subsets for each column.
*Allergics: SPT positive to at least one allergenic source; fungi: positive to at least one fungal source; ONE-FS: one positive SPT to a single fungal species; TWO-FS: two associated positive SPT to fungi; MFS: more than two positive SPT to fungi (multiple fungi sensitization).

Table 2. Comparative reactivity to fungal extracts

	SPT Allergopharma	SPT Stallergenes	IgE* Pharmacia
(*a*) *Comparative SPT and IgE reactivity to* Alternaria, Candida, Trichophyton *extracts using commercial extracts in subjects with isolated or two associated sensitization*			
Alternaria	100%	100% (72)	88.8% (160) 12.7 kU_A/L 0.4/68.4
Candida	100%	93.7% (60)	75.0% (80) 4.0 kU_A/L 0.4/88.0
Trichophyton	100%	84.6% (13)	60.9% (10) 2.8 kU_A/L 0.4/18.9
(*b*) *Comparative SPT and IgE reactivity to all the fungi sources using commercial extracts in 48 subjects with multiple fungi sensitization (MFS)*			
Alternaria	65.4%	84.6%	96.2% 26.8 kU_A/L 0.4/96.8
Aspergillus	65.4%	57.7%	91.8% 5.1 kU_A/L 0.4/36.7
Candida	84.0%	68.0%	88.7% 4.9 kU_A/L 0.4/20.5
Cladosporium	69.2%	61.5%	76.0% 4.3 kU_A/L 0.4/20.9
Malassezia	NT	NT	69.6% 2.4 kU_A/L 0.4/26.2
Penicillium	46.1%	26.9%	80.0% 3.2 kU_A/L 0.4/10.8
Saccharomyces	NT	56.2%	76.7% 3.9 kU_A/L 0.4/9.4
Trichophyton	32.0%	36.0%	73.9% 3.1 kU_A/L 0.4/14.2

Values are expressed as percentage of positive SPT or IgE tests. SPT: skin prick test.
*Median and range IgE values are also reported. NT: not tested.
Section (a): the numbers in brackets refer to the number of subjects who were tested with the fungal extracts of Stallergenes and Pharmacia vs. Allergopharma.
Section (b): MFS subjects were positive to more than two SPT to fungal extracts.

subsets. Only one patient within the MFS subset having IgE to the *Malassezia* extract was affected by the typical head and neck atopic dermatitis [9].

Demographical data

Figure 1a reports the age distribution of the subjects in the different subsets. A statistically significant difference was recorded for the group sensitized to fungi vs. the whole allergic cohort, the former being younger than the latter. This was mainly due to the large number of *Alternaria* sensitization among younger patients leading to a mean age of 16.5 years. Conversely, *Candida*- (29.6 years) and *Trichophyton*- (28.5 years) sensitized patients were older than the mean of the fungi cohort (22.8 years). Statistically highly significant differences were recorded when both the allergenic fungi were compared with the entire 'Fungi' group and the '*Alternaria*' subset (Fig. 1a). Such differences were much more pronounced when the subset of ONE-FS was considered (Fig. 1b). The mean ages of *Alternaria*-, *Candida*-, and *Trichophyton*-sensitized subjects were 15.7, 36.4, and 33.9, respectively. Statistically significant differences were retained only by the *Trichophyton*-sensitized subset in the TWO-FS group (Fig. 1c), and were completely absent in the MFS group (Fig. 1d)

Differences in gender distribution were found by the differential analysis of the data. Although the entire cohort of subjects had more female than male subjects (M/F ratio 0.83), the sensitized subjects were equally subdivided (ratio = 1). Male subjects were slightly but significantly more present in the whole fungi-sensitized cohort (ratio = 1.19), whereas no other statistically significant differences were recorded for the other subsets. In the ONE-FS subset, the *Alternaria* M/F ratio was 1.23 and did not differ from that of the ONE-FS subset. The higher prevalence of female patients gave a *Candida* M/F ratio of 0.8, differing significantly from *Alternaria* and *Trichophyton* gender distribution, and being very close to significance vs. the ONE-FS subset ($P<0.07$). The opposite was recorded for *Trichophyton*, where the M/F ratio of 3.4 was significantly different from the ONE-FS, *Alternaria*, and *Candida* subsets. Only *Trichophyton* gender distribution differences were retained in the TWO-FS group, whereas no statistically significant values were obtained by the analysis of the M/F ratio in the MFS subset, although a higher number of male subjects was recorded in this subset.

IgE immunochemical data

Differences of IgE reactivity among the subsets tested were explored by means of comparative IgE immunoblots using different fungal extracts. Sera from *Alternaria-, Candida-*, and *Trichophyton*-sensitized subjects from the ONE-FS and TWO-FS groups were tested on *Alternaria, Candida*, and *Trichophyton* extracts. None of the sera recognized any proteins on heterologous extracts. Only serum 9 from a *Candida* mono-sensitized patient and serum 4 from an *Alternaria* mono-sensitized patient had weak reactivity to a 64 kDa band in the *Alternaria* extract and to a 42 kDa band in the *Candida* extract, respectively (data not shown).

The in-house *Alternaria* extract showed the highest diversity of allergenic protein contents, whereas the Allergopharma extract predominantly contains a 14 kDa component (identified as Alt a 1 by means of IgE inhibition experiments using sera pre-incubation with rAlt a 1; data not shown) and a second one at about 30 kDa (either another allergen or an Alt a 1 dimer) (Fig. 2a). The Stallergenes extract seems to lack the 14 kDa component, but contains an IgE-binding protein with a slightly higher molecular weight. Reactivity patterns of the in-house and the Allergopharma extracts correlated better than the in-house and the Stallergenes extract. The sera from *Alternaria*-sensitized subjects selected within the TWO-FS subsets had a more complex IgE reactivity pattern, differing in quality and quantity when compared with the *Alternaria* mono-sensitized subjects. Differences were also observed

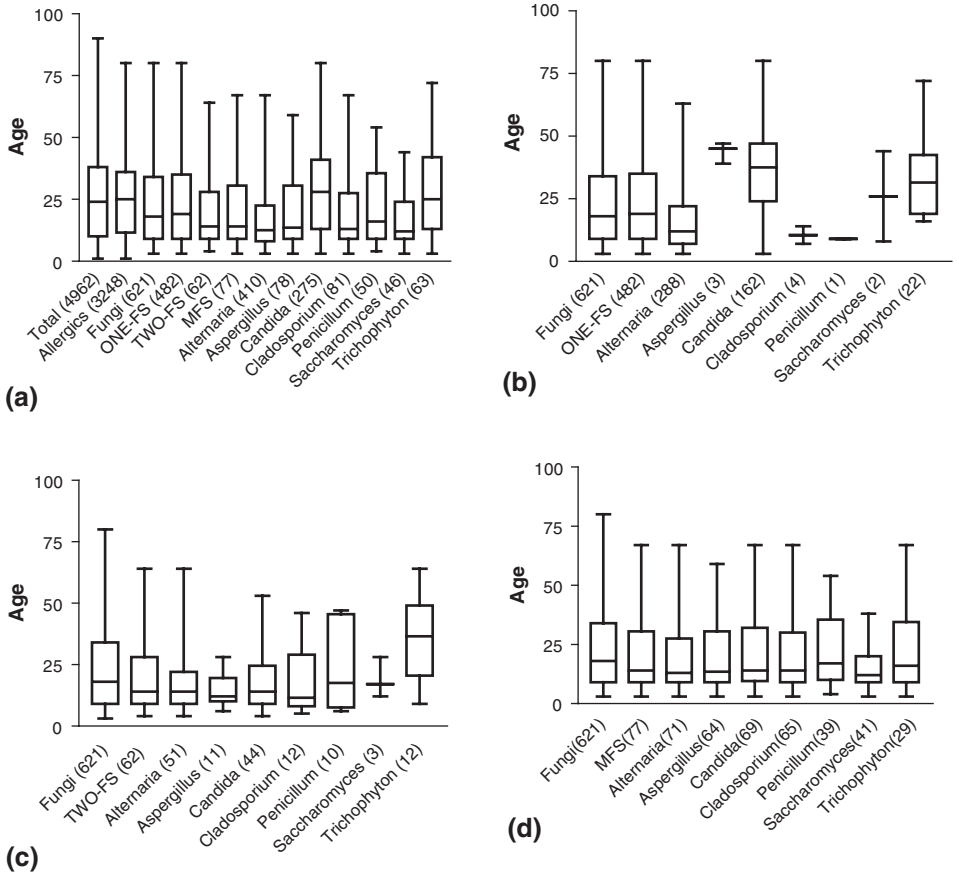

Fig. 1. Age distribution of subjects sensitized to fungi. Statistics: Mann–Whitney test. (a) Total subjects: fungi vs. allergics $P<0.0001$; *Alternaria* vs. fungi $P<0.0001$; *Candida* vs. fungi $P<0.0001$; *Trichophyton* vs. fungi $P<0.007$; *Alternaria* vs. *Candida* $P<0.0001$; *Alternaria* vs. *Trichophyton* $P<0.0001$. (b) Subjects sensitized to a single fungal species (ONE-FS): *Alternaria* vs. one fungi $P<0.0001$; *Candida* vs. one fungi $P<0.0001$; *Trichophyton* vs. ONE-FS < 0.002; *Alternaria* vs. *Candida* $P<0.0001$; *Alternaria* vs. *Trichophyton* $P<0.0001$. (c) Subjects sensitized to two fungal species (TWO-FS): *Trichophyton* vs. TWO-FS $P<0.005$; *Alternaria* vs. *Trichophyton* $P<0.001$; *Candida* vs. *Trichophyton* $P<0.003$. (d) Subjects with multiple fungi sensitization (MFS). No statistical differences.

between extracts from in-house and commercial preparations (Fig. 2a).

Performing IgE immunoblots with the *Candida* extract, we showed that the two commercial extracts had a comparable reactivity, although the Stallergenes extract had a slightly stronger IgE reactivity on some bands (29 kDa) and a more complex pattern with some sera (serum 6). The sera from the TWO-FS subset showed reactivity to fewer bands (predominantly to a 47 kDa protein) than the sera from the ONE-FS subset. Sera from *Candida*-sensitized subjects within the ONE-FS subset had a more complex IgE reactivity pattern if compared with the *Alternaria* patterns.

A single serum of a *Trichophyton* mono-sensitized patient was tested on *Trichophyton* extracts from Allergopharma and Stallergens showing a different non-overlapping pattern of reactivity. Only a 43 kDa band was detected in both extracts (Fig. 2a).

Sera from the MFS group were tested on available extracts from *Alternaria*, *Aspergillus, Candida, Penicillium, Saccharomyces*, and *Trichophyton*. All the sera tested displayed IgE reactivity to several protein bands of the *Alternaria* extracts, although the number of components recognized and the intensity of the immunostaining varied within the three extracts (Fig. 2b). A variable number of components was identified on the *Aspergillus, Candida*, and *Cladosporium* extracts (Fig. 2b), whereas either weak or no reactivity at all was recorded for *Penicillium, Saccharomyces*, and *Trichophyton* extracts (data not shown).

To further verify the differences of IgE reactivity to fungal proteins by the sera from the ONE-FS or TWO-FS subsets and those from subjects with MFS, a larger panel of sera was tested with in-house *Alternaria* and *Cladosporium* extracts (Figs 3a and b). The pattern of reactivity to the *Alternaria* extract by the *Alternaria* mono-sensitized sera differed from those belonging to the MFS subset. In fact, the former mainly showed an IgE reactivity to a single band (Alt a 1), whereas the latter displayed a multiple component reactivity (Fig. 3a). Results on *Cladosporium* extract differed, as fewer bands were recognized by MFS sera as compared with the *Alternaria* extract (Fig. 3b). Such sera most frequently recognized components at 30 and 46 kDa.

Discussion

In the present study, an epidemiological survey of an allergic population with skin and respiratory symptoms has been carried out. A 19.1% prevalence of SPT reactivity to at least

one of the seven selected fungal species was found. Grouping the mould-sensitized patients according to the pattern of SPT reactivity showed that the largest subgroup of mould-sensitized patients was reactive to only one mould species, in most cases to either *Alternaria*, *Candida*, or *Trichophyton*. *Alternaria* prevalence was similar to those already reported in other national population studies [35], whereas no comparative data on large populations are available for *Candida* and *Trichophyton*. When this subset of fungal-sensitized subjects was comparatively tested using three commercial extract preparations, minor discrepancies were recorded between extracts used in the SPT, whereas a lower sensitivity was recorded for the IgE assay. *Alternaria* IgE immunoblots showed reactivity to either a single (Alt a 1) or a few proteins in the extracts. Comparing the IgE reactivities between commercial and in-house *Alternaria* extracts led to discrepant results. Furthermore, a lower reactivity was recorded with one of the commercial extracts, which instead matched the positive results in the comparative SPT. These data would suggest that batch-to-batch variations could be present, as the SPT and immunoblots were performed with different preparations [28]. However, the trend to an isolated reactivity toward a single component of the *Alternaria* extract was confirmed by testing a larger number of mono-sensitized sera.

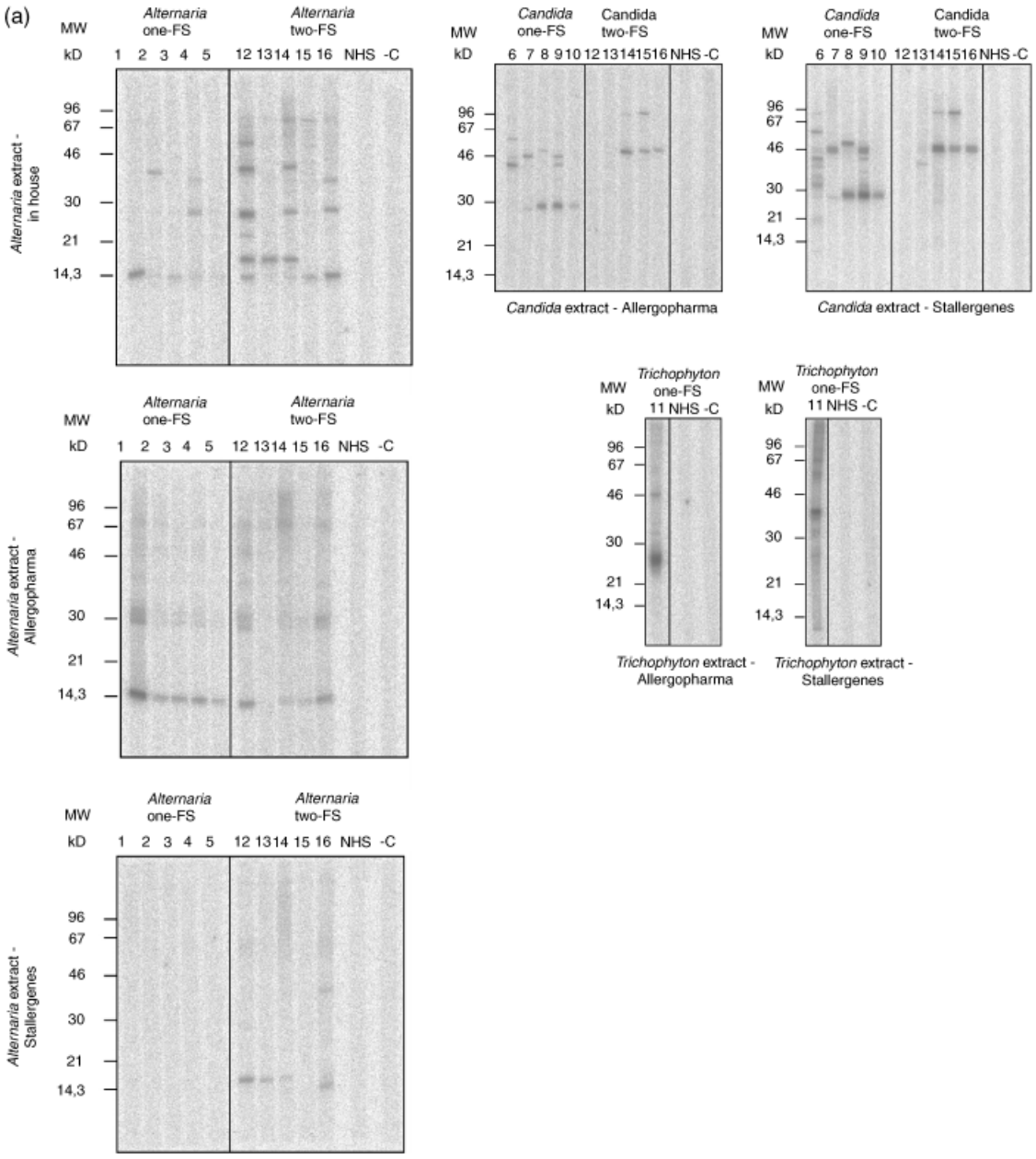

Fig. 2. (a) IgE immunoblotting with representative sera from the ONE-FS and TWO-FS subsets showing SPT and IgE reactivity to *Alternaria, Candida*, and *Trichophyton* extracts. Commercial and in-house extracts were used. Numbers (1–16) correspond to the patients tested. Lanes NHS and -C represent controls. In case of NHS (normal human serum) the membrane strip was incubated with a serum of a non-allergic subject, whereas in lane -C the membrane strip was decorated solely with the second rabbit-anti-human IgE-antibody. (b) IgE immunoblotting with representative sera from the multiple fungi-sensitized group showing SPT and IgE reactivity to more than two different fungi extracts. Commercial and in-house extracts were used.

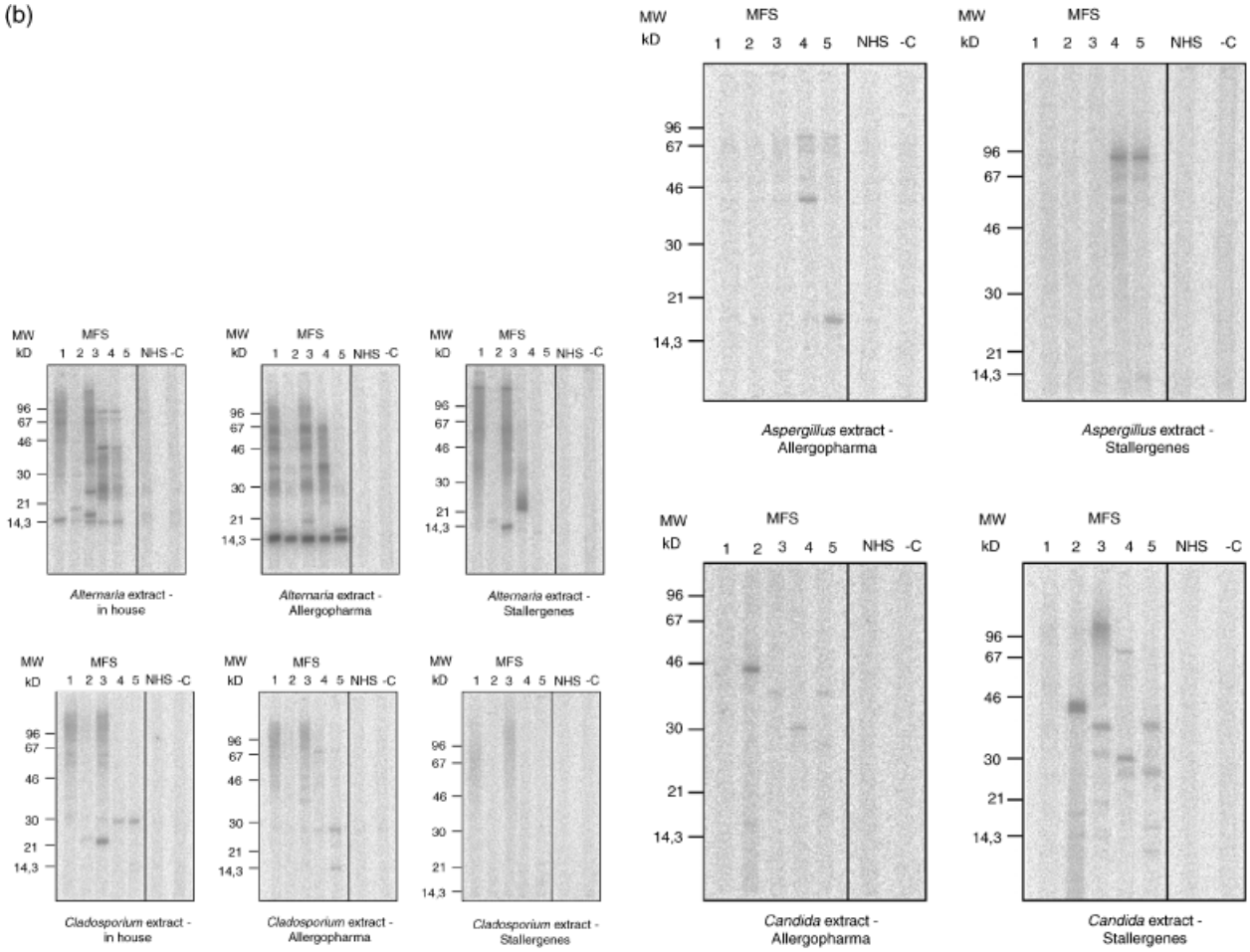

Fig. 2. (cont'd.)

Because of the fact that *Alternaria* mono-sensitized patients predominantly react with Alt a 1, the major *Alternaria* allergen, and a few additional allergens, makes the use of native or recombinant allergens for the immunotherapy of this large subset of patients reasonable. The pattern of IgE reactivity to *Candida* extracts by *Candida* mono-sensitized patients was much more complex and heterogeneous than that of *Alternaria* mono-sensitized patients. Similar results have been reported by other authors testing serum IgE from atopic dermatitis patients with *Candida* sensitization [14]. A good agreement was obtained in the comparative immunoblot between available extracts. The single serum of a patient mono-sensitized to *Trichophyton* and tested on two different preparations gave two different patterns of reactivity, presumably because of fungal strain differences.

TWO-FS sera, associating *Alternaria* and *Candida* sensitizations, tested on *Alternaria* and *Candida* extracts gave two different trends of reactivity. *Alternaria* TWO-FS sera showed a more complex pattern of IgE reactivity than the *Alternaria* sera in the ONE-FS subset. Several bands other than Alt a 1 were recognized and the pattern of reactivity differed among the sera, with a good agreement in the comparative evaluation of the extracts. *Candida* TWO-FS sera showed a reduced number of recognized bands when compared with the ONE-FS subset. Both results could be in agreement with differences in the demographical features of the TWO-FS subjects, where the prevalence for older female subjects was not recorded within the *Candida*-sensitized patients.

The prevalence of the three main sensitizers further increased when more than two mould extracts were positive in the SPT. A higher prevalence of SPT reactivity to all the remaining fungal extracts was recorded in the MFS subset. Such a clustering of fungal reactivity has been previously reported by other authors [36, 37] and was ascribed to the co-reactivity to distinct species rather than to the cross-reactivity of the different fungal species. On comparing the MFS subset with the ONE-FS and TWO-FS subsets in the comparative tests, discrepancies were recorded. Furthermore, the highest prevalence values in the MFS subset were obtained by means of the IgE assay. The IgE detection further documented the clustering of the fungal reactivity in the MFS subset, supported by the specific reactivity to the *Malassezia* extract. Similar results were obtained by Scalabrin et al. [12] for severely asthmatic patients. To evaluate the *in vitro* results correctly, IgE reactivity to cross-reactive carbohydrate components among fungal extracts should be determined [14, 40]. The comparative evaluation of the immunoblot results showed divergent patterns of reactivity between the sera and extracts tested. The testing of MFS sera with

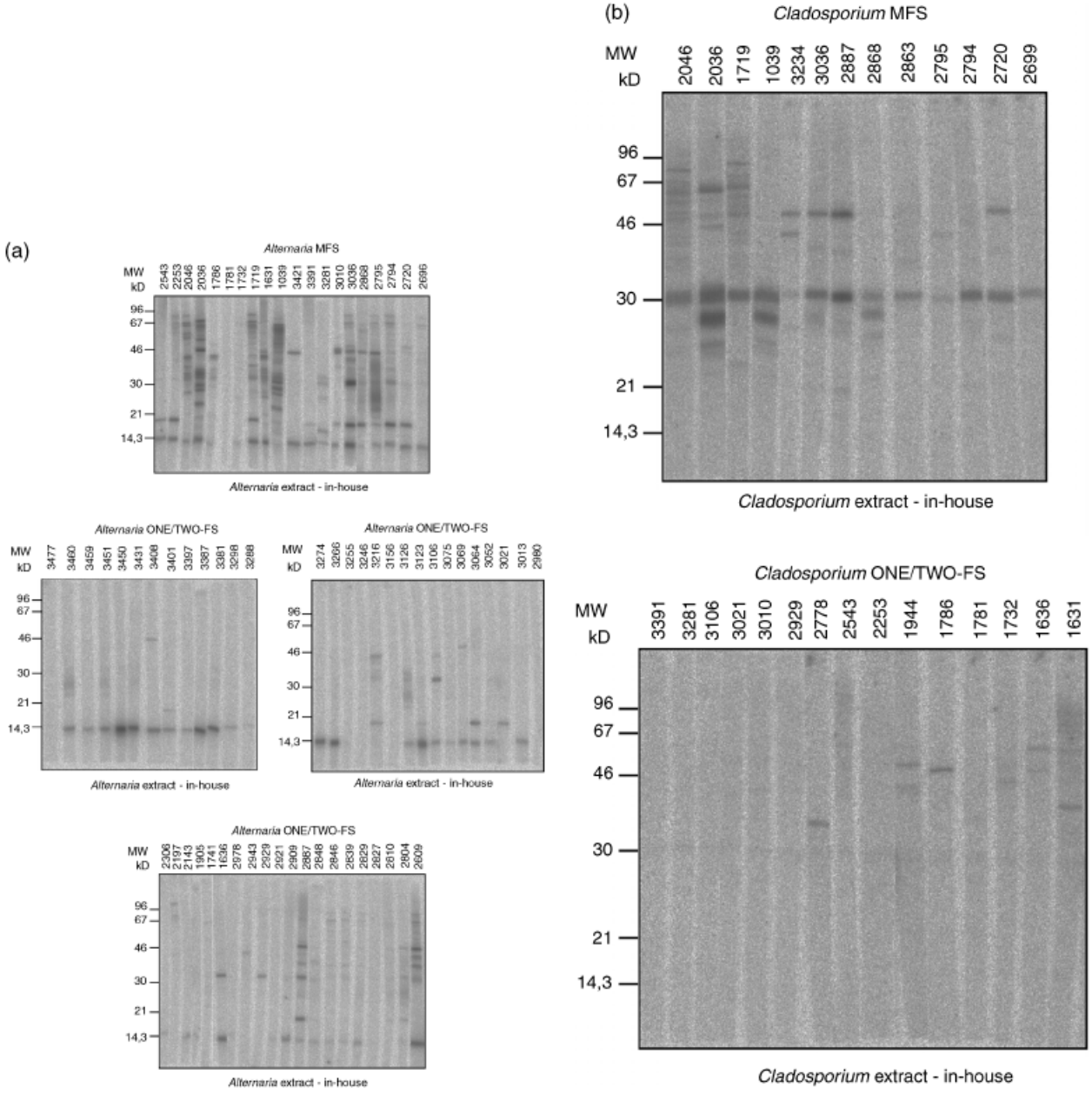

Fig. 3. (a) IgE immunoblots of *Alternaria* extract tested with a panel of sera from the ONE-FS or TWO-FS subsets, or to multiple fungal sensitization (MFS). An *Alternaria* in-house extract was used. (b) IgE immunoblots of *Cladosporium* extract tested with a panel of sera from the ONE-FS or TWO-FS subsets, or to MFS. A *Cladosporium* in-house extract was used. Numbers on the top of the lanes represent different patients.

additional fungal extracts clearly demonstrated a specific reactivity to some proteins of *Aspergillus* and *Cladosporium*, which in part have already been described [19, 21]. Data obtained using *Penicillium, Saccharomyces*, and *Trichophyton* extracts were limited by the low amount of allergenic components present in almost all the preparations and the weak reactivity of the selected sera. The IgE immunoblot reactivity pattern on the *Alternaria* extract was relatively more complex in the MFS population than in the ONE-FS and TWO-FS groups, both in the selected sera assay and in the larger IgE immunoblot survey of the mould-sensitized population. Such a trend of reacting to several components in patients with multiple sensitizations has already been reported for pollen allergy [38, 41]. Moreover, when similar grouping criteria are applied to a pollen-allergic population, the IgE reactivity to highly homologous molecules, like profilins or calcium-binding proteins (panallergens), can be recorded only in the multiple pollen-sensitized subset [38, 41]. Such findings suggest that if a subject develops IgE reactivity to a highly conserved allergenic molecule, the patient's serum could also react with the cross-reactive component of a divergent mould species. The hypothesis that even in the fungal sensitization shared allergenic components could play the role of a panallergen must therefore be considered. The immunoblot showed IgE reactivity to components (about 46 kDa) that are compatible with some already characterized

molecules (e.g. enolases). Enolases have been described in almost all the allergenic sources used in the present study [39, 42, 43] and their cross-reactivity has been documented [33, 39]. Other fungal panallergen-like molecules could be involved in the SPT reactivity of the MFS subset. Either the 30 kDa component in the *Cladosporium* extract described in the present study [19] or the mannans [44] could be good candidates. An IgE reactivity to components already detected by sera of the ONE-FS group was found even in the sera of the MFS subset (non-panallergen or family-restricted molecules). As for the study on pollen-reactive patients [38, 41], a complete panel of allergenic molecules will be necessary to define the MFS reactivity completely [19, 21, 22, 34].

Subjects sensitized to *Candida* and *Trichophyton*, but belonging to the MFS subset, missed defined epidemiological features, like those evidenced for ONE-FS and TWO-FS subsets. Such differences along with the different pattern in the IgE immunoblots further suggest that different components acting as allergens could lead to different sensitization phenotypes. The clinical picture of the sensitization to either indoor or outdoor mould species and the following possible exposure to the same allergen released by skin or mucosal pathogens deserve further studies.

Age and gender distribution of the *Alternaria-, Candida-*, and *Trichophyton*-sensitized patients allowed us to identify risk factors for developing distinct sensitization. Younger patients seem to be more at risk for *Alternaria* sensitization caused by IgE reactivity to Alt a 1. Such an observation further confirms Alt a 1 as a risk factor for developing childhood asthma [12]. Adult women seem to be more prone to *Candida* sensitization. *Candida* sensitization in respiratory patients could coincide with the increased risk of local infections [45]. These data should lead to further investigations on the relationship between vaginal exposure to *Candida* and the development of a respiratory allergic disease [46]. Adult men seem to have a greater risk of developing a sensitization to *Trichophyton* allergens [25], which could lead them to subtle respiratory allergy symptoms [11]. The eradication or reduction of the pathogens as infectious agents by means of pharmacological treatment could lead to a resolution of the respiratory disease [47]. Overlooking either *Candida* or *Trichophyton* sensitizations could lead to the diagnosis of a non-IgE-mediated allergy-like respiratory disease.

In conclusion, to circumvent the still limited reliability of fungal extracts in terms of constant content of allergenic components, a novel molecule-based strategy should be chosen. In a clinical study dealing with *Alternaria* sensitization, it has been shown that recombinant allergens are superior to fungal extracts with respect to positive and negative predictability of *Alternaria* sensitization [48]. The most complete molecule-based diagnostic approach to mould-sensitized patients could ease the diagnostic process, the definition of the reactivity pattern, and lead to a novel specific immunotherapy approach, where, for instance, young asthmatic patients could be treated with a single or a few allergenic molecules.

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