Analytical bias of cross-reactive polyclonal antibodies for environmental immunoassays of *Alternaria alternata*

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Background: *Alternaria alternata* is recognized as an important aeroallergen indoors and outdoors, and exposure to the fungus has been identified as a risk factor for asthma. Two recent publications concluded that 95% to 99% of American homes contained detectable amounts of *Alternaria* antigens when analyzed with a polyclonal antibody (pAb)–based ELISA.
Objectives: We investigated the cross-reactivity of the commercially available pAbs that were used in those studies.
Methods: Reactivity to 24 fungal species commonly found in indoor environments was analyzed by inhibition ELISA by using solid-phase *A alternata* antigen. The pAbs were also tested by immunoblotting and halogen immunoassay for a subgroup of fungi.
Results: Spores of 7 fungi including species of *Alternaria*, *Ulocladium*, *Stemphylium*, *Epicoccum*, *Drechslera*, and *Exserohilum* strongly inhibited the binding of the pAbs when tested by ELISA. Six other fungi reacted in the ELISA at a lower level, and 11 fungal species including several *Penicillium*, *Aspergillus*, *Fusarium*, and *Cladosporium* species failed to show inhibition. The immunoblots and the halogen immunoassay staining confirmed the cross-reactivity patterns of the ELISA.
Conclusion: The pAbs against *A alternata* were found to cross-react broadly with related and nonrelated fungi. The prevalence data previously reported for *A alternata* should be considered to be fungal-reactive rather than *A alternata*–specific.
(J Allergy Clin Immunol 2008;121:763-8.)

Key words: *Fungi, polyclonal antibodies,* Alternaria*, ELISA*

Abbreviations used
HIA: Halogen immunoassay
pAb: Polyclonal antibody
PBST: PBS with 0.05% Tween 20
PBSTM: PBS with 0.05% Tween 20 containing 1% nonfat dry milk powder
RT: Room temperature

Alternaria alternata is an important allergenic fungus worldwide.[1,2] Exposure to the fungus has been linked to the severity of asthma[3-5] and the pathophysiology of chronic rhinosinusitis.[6] *Alternaria* spores are commonly found outdoors especially during the summer and fall season.[7,8] Indoors, the fungus can be cultured from 50% to 75% of house dust samples.[9-14]

Traditional methods for the identification of fungi in the environment are based on the detection of viable and nonviable fungal components. Air or dust samples are either cultured or assessed by microscopy, and species are identified on the basis of morphological, biochemical, or cytological colony and/or spore characteristics. More recently, techniques based on PCR, and mAbs have also been developed.[15,16] However, a recent review by Trout et al[17] concluded that, to date, there is not enough scientific evidence for the routine application of immunoassays as a primary means of environmental sample analysis for fungi. Some of the challenges that need to be addressed before immunoassays can be successfully introduced for environmental exposure analysis include the development of representative sampling methods and the avoidance of ambiguities in assay interpretation because of antibody cross-reactivity.

Recently, a polyclonal antibody (pAb)–based inhibition ELISA was used to measure the prevalence of *A alternata* antigens in house dust samples from 831 housing units from 75 different locations throughout the United States.[18,19] This inhibition ELISA has also been used by other investigators to measure the amount of *A alternata* antigen in house dust samples.[20,21] However, the potential of the *A alternata*–specific pAbs to cross-react with other fungi has not been investigated. In this study, we investigated the reactivity of the pAb to 24 related and unrelated fungi commonly found in indoor environments by using a similar inhibition ELISA assay, immunoblotting, and the halogen immunoassay (HIA). Extensive cross-reactivity was observed for the *A alternata*–specific pAbs, demonstrating these assays measure fungal antigens rather than *Alternaria*-specific antigens.

METHODS

Cultivation of fungi

Twenty-four fungal isolates were purchased from certified culture collections (18), isolated from environmental samples (2), or provided by colleagues (4). The specific source for each fungal species is listed in Table I. Fungi were

From the Allergy and Clinical Immunology Branch, National Institute for Occupational Safety and Health.

Supported in part by an Interagency Agreement from the National Institute of Environmental Health Sciences (Y1-ES-0001)-Immunotoxicity of Workplace Xenobiotics and with internal National Institute for Occupational Safety and Health funding.
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.
Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.
Received for publication August 24, 2007; revised September 21, 2007; accepted for publication September 24, 2007.
Available online November 26, 2007.

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0091-6749
doi:10.1016/j.jaci.2007.09.046

TABLE I. The number of spores that result in 50% inhibition in the ELISA

Fungal species	Origin of isolate	Range of spores/mL tested	50% inhibition: spores/mL
S botryosum	ATCC 26881	500-256,000	7,332
E nigrum	ATCC 34929	1000-512,000	9,605
Drechslera spp	Gift	2000-1,024,000	12,456
A brassicae	ATCC 58169	250-128,000	14,185
E rostratum	ATCC 26856	500-256,000	15,522
U botrytis	UAMH 7841	2000-1,024,000	28,566
A alternata	ATCC 11612	1000-512,000	32,860
U chartarum	UAMH 5703	2000-1,024,000	61,084
A brassicicola	ATCC 96836	1000-512,000	92,041
B spicifera	ATCC 46101	2000-1,024,000	198,789
C lunata	ATCC 34477	1000-512,000	508,896
M olivacea	UAMH 6968	4000-2,048,000	545,795
M verrucaria	NRRL 2003	2000-1,024,000	1,498,537
A strictum	ATCC 46646	4000-2,048,000	BD
A versicolor	ATCC 44408	2000-1,024,000	BD
A fumigatus	House dust	2000-1,024,000	BD
C cladosporioides	House dust	2000-1,024,000	BD
C herbarum	ATCC 6506	2000-1,024,000	BD
Doratomyces microsporus	Gift	4000-2,048,000	BD
F moniliforme	Gift	2000-1,024,000	BD
M echinata	NRRL 2373	2000-1,024,000	BD
P variotii	ATCC 66705	4000-2,048,000	BD
P chrysogenum	NRRL 1951	4000-2,048,000	BD
Z masonii	Gift	4000-2,048,000	BD

BD, Below detection limit of the inhibition ELISA.

grown for 2 to 3 weeks at room temperature (RT) in standard unsealed Petri plates containing 5 mL malt extract agar (2% dextrose, 0.1% peptone, 2% malt extract, 2% agar; DIFCO; Becton Dickinson, Sparks, Md).

Spore preparation

Spores were harvested from sporulating Petri plate cultures by adding 15 mL 0.01 mol/L PBS (pH 7.4) supplemented with 0.05% Tween 20 (PBST; Sigma, St Louis, Mo) to each plate and suspending the spores by repeated pipetting. The suspension was centrifuged at 4100*g* for 5 minutes at 4°C, and the spore pellet was resuspended in 0.2 mol/L borate buffer, pH 8.2. Uniform suspensions for ELISA analysis and spore counting by hemocytometer (Hausser Scientific, Horsham, Pa) were obtained by sonication for 15 seconds at amplitude 6 using a Sonicator 3000 (Misonix, Inc, Farmingdale, NY).

Alternaria reagents

The *A alternata* extract and the anti–*A alternata* pAbs were purchased from Greer Laboratories (Lenoir, NC). The commercial extract (lot #XPM1-D22-140) was prepared by extracting *A alternata* (ATCC 11680) cellular antigens in 0.01 mol/L ammonium bicarbonate buffer under current good manufacturing practices. The rabbit pAb (lot #ZA4-4L) was produced by using an extract of *A alternata* (ATCC 11680) as the antigen, and the antibodies were partially purified by ammonium sulfate precipitation. To rule out whether the *A alternata* extract may be contaminated with other fungi, 7 mAbs that do not react with *A alternata* were tested by indirect ELISA. Four of these mAbs were produced against *Aspergillus versicolor*, and 3 mAbs were produced against *Penicillium chrysogenum*. Although these mAbs show cross-reactivity with many *Aspergillus* and *Penicillium* species, none reacted with the commercial *A alternata* extract (data not shown).

Inhibition ELISA

Maxisorp 96-well ELISA plates (Nalge-Nunc International, Rochester, NY) were coated with 100 μL 1 μg/mL *A alternata* extract (Greer Laboratories) in carbonate buffer (60 mmol/L sodium carbonate, 140 mmol/L sodium bicarbonate, pH 9.6) by overnight incubation at RT. Excess antigen was removed by 3 consecutive 5-minute washing steps with PBST, and then the plates were blocked for 1 hour at RT with 200 μL PBST containing 1% nonfat dry milk powder (PBSTM). The anti–*A alternata* pAb (Greer Laboratories) was used at a 1:100,000 dilution, and inhibition curves for each fungus were established by testing 2-fold serial dilutions of fungal spores in borate buffer. A 2-fold dilution series of 1 μg/mL *A alternata* extract (Greer Laboratories) was also included on each ELISA plate as a standard curve. pAb diluted in borate buffer without any inhibitor was also included on each ELISA plate to establish baseline antibody reactivity for the calculation of the degree of fungal inhibition. Negative assay controls on each ELISA plate included wells that were incubated with all reagents except the pAbs and the inhibitor.

Eppendorf tubes containing the antibody/antigen mixtures were incubated on a Labquake shaker (Barnstead/Thermolyne, Dubuque, Iowa) for 1 hour at RT to allow immune complex formation before centrifugation at 10,000*g* for 5 minutes at 4°C. Aliquots 100 μL of the resulting supernatants were transferred to blocked ELISA plates and incubated for 1 hour at RT on a Titer Plate Shaker (Lab-Line Instruments, Inc, Melrose Park, Ill) at speed setting 5. The plates were washed and incubated with 100 μL/well of a 1:5000 dilution of biotin-SP–conjugated AffiPure goat antirabbit IgG secondary pAbs (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa) in PBSTM for 1 hour at 37°C. Bound secondary antibodies were detected by incubation with 100 μL of a 1:5000 dilution of alkaline phosphatase–conjugated streptavidin (Jackson ImmunoResearch Laboratories) in PBSTM at 37°C for 1 hour. Reactivity was detected by incubation at RT with 100 μL/well of *p*-nitrophenyl phosphate substrate (0.5 mg/mL) in development buffer (1 mol/L diethanolamine, 0.5 mmol/L $MgCl_2$, pH 9.8). ODs were determined spectrophotometrically at 405 nm after 30 minutes by using a microplate reader (ELx800; BIO-TEK Instruments, Inc, Winooski, Vt). All ELISA results represent the average of 4 replicates, and the 50% inhibition levels for all 24 species tested were calculated by logarithmic regression analysis.

Mycelial extracts and immunoblot analysis

Mycelia were extracted in borate buffer, and the protein concentration was determined according to the bicinchoninic acid method (Pierce, Rockford, Ill). Extracts (5 μg/lane) were separated on a 10% TRIS-HCl gel (Bio-Rad, Hercules, Calif) under denaturing conditions and transferred to a nitrocellulose membrane (0.2 μm; Bio-Rad). Membranes were blocked with 3% BSA in PBS for 1 hour and incubated for 1.5 hours with a 1:20,000 dilution of pAb. After washing, the blot was reacted for 1 hour with a 1:2000 dilution of alkaline phosphatase–conjugated goat antirabbit IgG (Promega, Madison, Wis). Immuno reactive proteins were visualized by using Nitroblue tetrazolium and Bromo-chloro-indolyl phosphate (Promega). Prestained broad range markers (Bio-Rad) were used for molecular mass determinations.

HIA

Fungal particles were aerosolized by directing a jet of air across 2-week-old sporulating fungal cultures, and airborne particles were collected by suction onto mixed cellulose ester protein-binding membranes (0.45 μm pore size; Millipore Corp, Bridgewater, Mass). Impacted fungal particles were processed as described previously.[22] In brief, clear adhesive/glass cover slip laminated samples were incubated in 0.2 mol/L borate buffer, pH 8.2, at RT for 3 hours, blocked in 5% BSA in PBS for 1.5 hours, and then incubated overnight at 4°C with the anti–*A alternata* pAb (Greer Laboratories) diluted 1:1000 in PBST containing 5% BSA. Negative control treatments were processed in parallel by substituting the anti–*A alternata* pAb with an anti–*Aspergillus fumigatus* pAb (lot #ZE-AF06-1-1; Greer Laboratories). The membranes were rinsed 3 times in PBST and incubated for 1.5 hours with Alexa Fluor 488-conjugated goat antirabbit IgG (green fluorescence; Molecular Probes, Inc, Eugene, Ore) diluted 1:500 in PBST containing 5% BSA. The membranes were rinsed 3 times in distilled H_2O and mounted on a microscope slide in ProLong Gold (Molecular Probes, Inc) antifade reagent. Confocal laser scanning images were captured by using a Zeiss LSM 510 laser scanning confocal

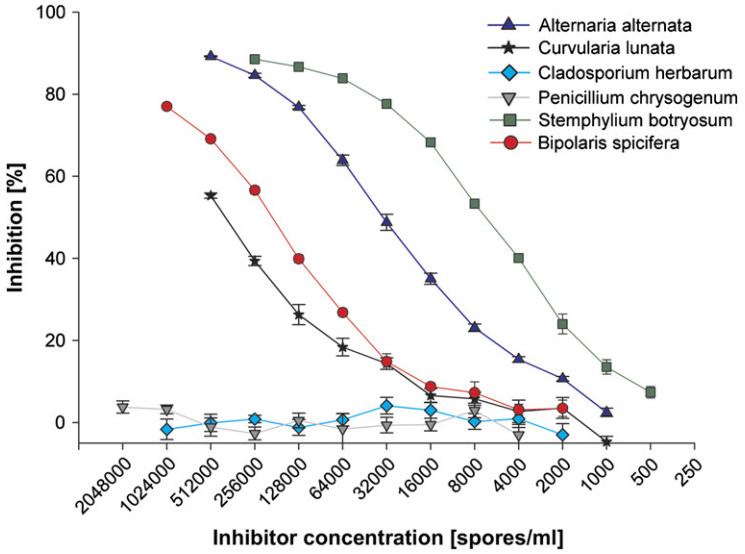

FIG 1. Representative inhibition curves of pAb binding to solid-phase *A alternata* extract by spores commonly found in indoor environments. The results represent the average of 4 replicates, and the *error bars* indicate the SEMs.

system (Carl Zeiss Inc, Thornwood, NY). The images were captured by using 488-nm excitation and a narrow emission filter bandwidth (505-550 nm). Fluorescent and differential interference contrast images were captured by using Zeiss software version 3.2 (Carl Zeiss Inc). All settings on the confocal laser microscope remained constant throughout the analysis.

RESULTS

Inhibition ELISA

To characterize the cross-reactivity profile of the pAbs, multiple dilutions of spores of 24 different fungal species were tested for their potential to inhibit the binding of the anti–*A alternata* pAbs to a commercial *A alternata* extract by inhibition ELISA. Individual ELISA plates for all fungi also included inhibitions with the *A alternata* extract itself as positive assay controls. The average for all *A alternata* controls resulted in a linear range of inhibition for the ELISA assay between 15.6 ng/mL (21 ± 6.3% inhibition) and 250 ng/mL (74 ± 4.9% inhibition). Table I identifies the range of spore concentration tested and the number of spores that resulted in 50% inhibition of the pAb binding. The inhibition values for *Stemphylium botryosum*, *Epicoccum nigrum*, *Drechslera* spp, *Alternaria brassicae, Exserohilum rostratum,* and *Ulocladium botrytis* were found to be below the value of *A alternata* (32,860 spores/mL). Spores of *Acremonium strictum*, *Doratomyces microsporus*, *Fusarium moniliforme*, *Memnoniella echinata*, *Zygosporium masonii*, *Penicillium chrysogenum*, and several *Aspergillus* and *Cladosporium* species were not found to inhibit the binding of the pAbs. Spores of *Ulocladium chartarum*, *Alternaria brassicicola*, *Bipolaris spicifera*, *Curvularia lunata*, *Microsphaeropsis olivacea*, and *Myrothecium verrucaria* were also found to inhibit the binding of the pAbs, but to a lesser extent than *A alternata*. The strongest 50% inhibition values were observed for *S botryosum* (7332 spores/mL) and *E nigrum* (9605 spores/mL). Fig 1 shows representative inhibition curves of 6 selected fungi tested at multiple spore concentrations. Equal numbers of spores were tested when possible; however, the range of spore numbers tested varied for different fungi because they did not produce enough spores or they produced spores too large to be tested at higher numbers. Equal numbers of spores of different fungi were found to inhibit the binding of the pAbs to different degrees. For example, 16,000 spores/mL of *A alternata*, *S botryosum*, and *Bipolaris spicifera* resulted in about 40%, 70%, and 10% inhibition, respectively.

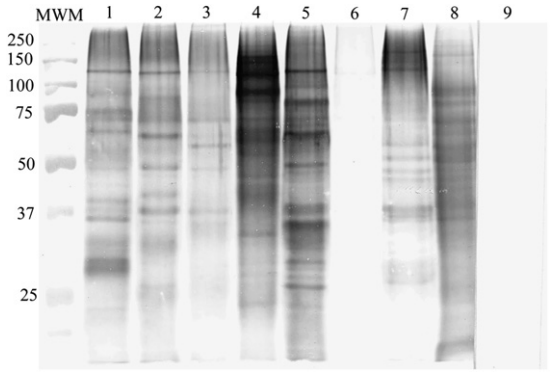

FIG 2. Immunoblot analysis of pAb reactivity to mycelial extracts of selected fungi. *MWM*, Molecular weight markers; 1, *S botryosum*; 2, *U chartarum*; 3, *A alternata*; 4, commercial *A alternata* extract, 5, *A brassicicola*; 6, *Cladosporium cladosporioides*; 7, *C lunata*; 8, *P variotii*; 9, *P variotii* with no pAb (negative control).

Immunoblot analysis

The cross-reactivity of the pAb was also analyzed by immunoblotting using the commercial *A alternata* extract and mycelial extracts of 7 other selected fungi. As shown in Fig 2, the pAb reacted to multiple bands in the commercial *A alternata* extract (lane 4) and also to multiple bands in 6 other fungal extracts including *S botryosum* (lane 1), *U chartarum* (lane 2), and *A brassicicola* (lane 5). Interestingly, several species such as *C lunata* (lane 7) and *Paecilomyces variotii* (lane 8) with spores that were weakly or nonreactive in the ELISA were cross-reactive in

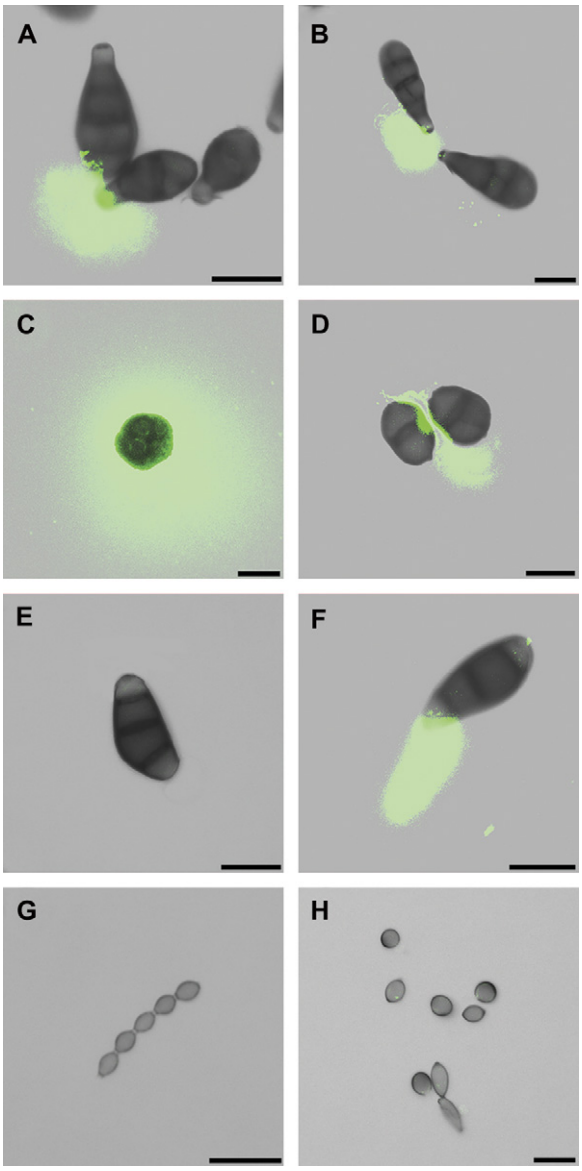

FIG 3. High-resolution images of halogen immunostaining (green fluorescence) of spores of **(A and B)** *A alternata*, **(C)** *E nigrum*, **(D)** *U chartarum*, **(E)** *Curvularia lunata*, **(F)** germinated *C lunata*, **(G)** *P variotii*, and **(H)** *Cladosporium herbarum* using an anti–*A alternata* pAb. Scale bar, 10 μm.

the immunoblot. The immunoblot uses significantly more antigen than the ELISA, and this could represent reactivity to minor or underrepresented antigens. Alternatively, it suggests that these species express additional mycelial antigens not necessarily present on spores, which are also cross-reactive.

HIA

To confirm further the cross-reactivity of the pAbs, HIAs with fungal spores of 6 selected species were performed. Fig 3 demonstrates that spores of *A alternata*, *E nigrum*, and *U chartarum* showed pronounced halos of staining. Spores of *E nigrum* produced intense staining all around the entire spore (Fig 3, *C*), whereas *A alternata* staining was mostly restricted to the beak portion of the spores (Fig 3, *A and B*). Spores of *Curvularia lunata* were found to stain only after overnight germination (Fig 3, *F*), and the staining was restricted to the germ tube (Fig 3, *F*). No staining was observed for spores of *P variotii* and *Cladosporium herbarum*.

DISCUSSION

In this study, we have examined the potential for cross reactivity of a polyclonal anti–*A alternata* antibody that was previously used for environmental monitoring of *Alternaria* in US homes.[18-21] Using several different immunoassays, our results clearly demonstrate that the commercially available polyclonal anti–*A alternata* antibodies extensively cross-react with multiple fungi commonly found in indoor environments.

Using an inhibition ELISA assay, we observed that spores of multiple species inhibited the antibody recognition of solid phase *A alternata* cellular antigens. For some fungi, the extent of the reactivity on a per spore basis was much higher than the reactivity with *A alternata* itself. This may be a result of a larger spore size or higher relative densities of epitopes expressed by spores of those fungi. This differential reactivity of normalized numbers of spores from different fungi is in agreement with our previous work on the detection of spores of *A versicolor* and *Stachybotrys chartarum* with mAbs.[16,23]

In immunoblots we observed multiple antigens, some of which appeared to be common, but we did not specifically identify the cross-reactive molecules. Recent comparative genomics studies of fungal allergens[24] have suggested that allergens may be widely shared between fungi. A recent study by Hong et al[25] amplified Alt a 1 allergen homologs from 52 species within the Pleosporaceae family, and another study by Sáenz-de-Santamaria et al[26] showed that several of those species produce Alt a 1 at the protein level. However, we did not test the pAbs for their reactivity with recombinant Alt a 1. The immunoblot data also indicate that there are cross-reactive antigens present in mycelia that may not be expressed in spores, as, for example *P variotii* showed multiple protein bands in the immunoblot but failed to inhibit the binding of the pAbs by inhibition ELISA.

The HIA results confirmed the cross-reactivity and show that spore germination is also an important consideration for the interpretation of immunoassays. Ungerminated spores of *C lunata* failed to be stained in the HIA, but after germination, intensely stained halos were observed. Germination has been shown to increase the release of fungal allergens.[27,28] However, germination varies with incubation time, buffer, and the presence of competitive fungi. Thus, for environmental monitoring, ELISA incubation times should be kept short (<1 hour) to avoid spore germination–associated analytical variability. Longer incubation times would require the use of metabolic inhibitors to prevent germination.

Most of the fungi that were recognized by the pAbs taxonomically belonged to the Pleosporaceae family or other closely related dematiaceous fungal genera such as *Epicoccum*. The Pleosporaceae family is made up of 17 genera with 111 species[29] including *Alternaria*, *Ulocladium*, and *Stemphylium* spp. Several of these species are known to be prevalent in indoor environments. For example, Verhoeff et al[30] detected *A alternata*, *U chartarum*, and other *Alternaria* spp in approximately 35% of house dust samples. Hicks et al[10] found *Alternaria* spp, *Ulocladium* spp, and *Epicoccum* spp in 64% to 72% of low traffic dust samples in normal residences. Using PCR, Vesper et al[15] identified *A alternata* and *E nigrum* in 52% and 51% of dust samples from 52 homes, and Horner et al[31] reported *A alternata* and *E nigrum* as

the 15th and 8th most abundant fungi in dust samples from 50 homes without mold problems.

Antigenic and allergenic cross-reactivity of *A alternata* with other fungi has been extensively documented in the past by using serum IgE in RAST and immunoblot inhibition assays.[32-35] Studies with mAbs that were produced against *A brassicae*[36] and pAbs that were produced against *A alternata*,[37] *E nigrum*,[38] or *Fusarium solani*[39] also demonstrated extensive cross-reactivity with *A alternata* and related fungi. Also, recent work from our laboratory with mAbs produced against *A versicolor*[23,40] and *S chartarum*[16] clearly demonstrate that antigenic cross-reactivity among fungi is very common and needs to be considered in the development of environmental and clinical detection assays.

We agree that *A alternata* is one of the major allergenic fungi. However, our data question the conclusion of environmental monitoring studies that *A alternata* is present in 95% to 99% of US homes.[18,19] The reported prevalence should be considered to represent fungal antigens rather than *Alternaria* antigens. Our findings clearly demonstrate that equal concentrations of spores of various fungi reacted very differently with the pAbs. The results of any environmental analysis, therefore, may be a result of combinations of weakly and/or highly cross-reactive fungi rather than exclusive contamination with *A alternata*. The extent of the cross-reactivity suggests that monitoring results obtained with these pAbs should be critically re-evaluated and that these pAbs should not be used for the exclusive detection of *A alternata* as previously suggested.[20] The widespread sharing of antigens and allergens among fungi necessitates that information on antibody specificity is reported adequately to avoid analytical bias and ambiguities in result interpretation.

The development of standardized species-specific antibody or PCR-based assays for sample analysis will be critical before these techniques can provide successful and accurate alternatives to current techniques of sample analysis in fungal exposure assessments and ultimately improve patient management and care in clinical practice.

We thank Dr Elliott Horner from Air Quality Sciences, Marietta, Ga, for providing several fungal isolates.

Clinical implications: The specificity of any antibody to be used in clinical and environmental exposure assessment needs to be determined before its application. This will avoid analytical bias and ensure the accuracy and reproducibility of the monitoring results.

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