

Original article

Marker antigens to assess exposure to molds and their allergens.

II. *Alternaria alternata*

The estimation of exposure to molds and their products in the indoor environment, which may lead to the occurrence of allergies or respiratory complaints, by means of enumeration of viable parts is inadequate. Therefore, other methods must be developed. When grown under various circumstances (22°C and 30°C, high and low water activity) under laboratory conditions, *Alternaria alternata* produces one antigen that can be found under all studied growth conditions in extracts of the water-soluble portion of the mycelium. This common antigen may serve as marker antigen for exposure to *A. alternata* and its allergens. In extracts of the culture filtrate, three antigens, designated index antigens, have been identified that together may have the function of marker for the exposure to allergens of *A. alternata*.

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Molds are ubiquitous organisms, some of which can cause various adverse effects in man and animals. These effects vary from relatively harmless cutaneous infections to often fatal systemic mycoses. Exposure to molds may also lead to the emergence of allergies. It is assumed that exposure of children to molds or their products at very young age has a stimulating effect on the development of allergies at a later date (1).

To date, the main method to determine exposure to molds is the detection of viable parts. However, this method is highly inadequate, as part of the exposure is to nonviable and thus nonculturable parts. Methods for the detection of constituents or excretion products of molds have been described (2–7). Such methods have their disadvantages: the detection of chitin is nonspecific because chitin is not solely produced by molds, and the determination of extracellular polysaccharides (EPS) is genus-specific, making a method to detect *Aspergillus* or *Penicillium* unable to detect *Alternaria*. Some of these methods have been tested as replacement for the culture test (7, 8).

Molds can grow under various conditions, the most important determinant for growth being moisture. Even in seemingly dry indoor conditions, moisture may be sufficiently available. Sharp drops in temperature and subsequent condensation of water may generate, even temporarily, enough moisture for germination and

growth (9). Growth conditions influence the production of metabolites such as allergens, a fact which indicates that the detection of molds or their products in the indoor environment does not automatically mean that allergens are present (10).

The aim of the investigations described here was the detection of common antigens of *Alternaria alternata*, antigens that occur under all described culture conditions, and that may serve as (semi-) quantitative marker antigens for exposure to *A. alternata* and its products, and as marker antigens for exposure to allergens of *A. alternata* under various growth conditions. The criterion for a marker antigen is twofold: the antigen has to be a common antigen and it has to occur before and/or during the occurrence of allergens.

The choice of *A. alternata* was based on a survey of the occurrence of molds in Dutch dwellings (11). One of the main detected genera was *Alternaria*, the species *A. alternata* being the most important representative of the genus. The growth conditions tested in the investigations described here were temperatures of 22°C and 30°C, and water activities (*A_w*) of 0.99 and 0.96. These temperatures closely resembled the average and peak temperatures in Dutch dwellings, and the *A_w* resembled the highest possible and the lowest possible occurrences necessary for germination and growth.

Material and methods

Culture

A. alternata (CBS 326.65) was cultured in a 7-l bioreactor with controller (Applikon, Schiedam, The Netherlands) in Czapek-Dox broth (Difco Laboratories) for 21 days. Culturing was performed at 22°C and 30°C, an average and a rather extreme indoor temperature, respectively, and at a high Aw of 0.99 and at such a low Aw (0.96) that germination and growth of the mold still took place. Low Aw conditions were achieved by adding 0.10 g ml⁻¹ calcium chloride (CaCl₂, Merck, Darmstadt, Germany) to the medium. The oxygen concentration in the medium was monitored and kept above 75%.

Production of extracts

During culture, 225-ml samples were taken at regular intervals. From each sample mycelium and culture filtrate (CF) were separated by passing through a Whatman GF/B filter. After addition of protease inhibitors (pepstatin [Merck, Darmstadt, Germany], 2 µg ml⁻¹; disodium-ethylenediaminetetraacetic acid [EDTA] [Acros Organics, Geel, Belgium], 2 mM; phenylmethanesulfonylfluoride [Merck, Darmstadt, Germany], 1 mM), the CF was concentrated by an Amicon Stirred Cell equipped with a 3-kDa cutoff filter, and dialyzed overnight against distilled water. The remaining fluid was called CF extract. One-tenth of the CF extract was kept apart for protein determination, and the remainder was lyophilized.

The mycelium was suspended in 0.05 M ammonium bicarbonate to create suspensions of 30% (v/v) with a minimal volume of 10 ml. The mycelium suspensions were sonified in melting ice four times over 6 min at an energy level of 20% with a Branson 450 sonicator. If necessary, sonification was prolonged with cycles of 6 min until a breakdown level of the septated hyphae was achieved of at least 95% (microscopically determined). After sonification, protease inhibitors were added and the suspensions were centrifuged (10 000 g, 30 min, 5°C) to separate soluble and insoluble components. Insoluble parts that could not be removed at the g force mentioned were separated from the supernatant by ultracentrifugation (100 000 g, 30 min, 5°C). The resulting supernatant was dialyzed against distilled water (twice overnight); after dialysis, the solution was called water-soluble extract (WS). One-tenth of the WS extract was kept apart for protein determination, and the remainder was lyophilized.

Protein determination and redissolving of CF and WS extracts

Protein concentrations in the one-tenth parts of CF and WS extracts were determined according to Lowry (12). All lyophilized extracts were redissolved in equal

amounts of distilled water to such an extent that the protein concentration in the extract with the highest amount of protein was 2.5 mg/ml.

Polyacrylamide electrophoresis

Redissolved extract was mixed with lysis buffer (sodium dodecyl sulfate, 0.27 M; TRIS [hydroxymethyl]-aminomethane, 40 mM; EDTA, 4 mM; bromophenol blue, 0.3 M; pyronin Y, 0.66 M; and glycerol, 40%) in the proportion of three to one. The mixed extracts were put on top of two 10% polyacrylamide gels together with blanks (made up from three parts distilled water with one part lysis buffer) and molecular weight (MW) markers (Amersham; myosin [220 kDa]; phosphorylase b [97.4 kDa]; bovine serum albumin [66 kDa]; ovalbumin [46 kDa]; carbonic anhydrase [30 kDa]; and trypsin inhibitor [21.5 kDa]). Electrophoresis was carried out in a Mini-PROTEAN II electrophoresis system (BioRad) equipped with a 1000/500 power supply (BioRad). After electrophoresis, the two gels were used for blotting procedures.

Immunoblot

Blotting on polyvinylidene difluoride (PVDF, Millipore) membranes was performed with a TransBlot Cell (BioRad) equipped with a 250/2.5 power supply (BioRad) at 50 V for 1 h with blot buffer, according to Towbin (13). After the blot procedure, free binding places on the membranes were blocked with rinsing-buffer "high" (sodium chloride, 0.45 M; TRIS, 10 mM; Tween 20, 0.5%; pH 7.4) for 1 h.

For the detection of antigens, one membrane was incubated for 45 min in 1000-fold diluted rabbit serum directed against cell-wall antigens from *A. alternata* CBS 326.65 (produced in accordance with Wilson & Hearn [14]). The serum was diluted in rinsing-buffer "high". After serum-incubation, the membrane was washed twice for 15 min with rinsing-buffer "high" and rinsing-buffer "low" (sodium chloride, 0.15 M; TRIS, 10 mM; Tween 20, 0.5%; pH 7.4), respectively. After incubation of the membrane in conjugate solution (peroxidase conjugated goat antirabbit [Biosource Int., Camarillo, CA, USA] diluted 2500-fold in rinsing buffer "low") for 45 min, washing the membrane twice for 15 min with rinsing-buffer "low", and once for 15 min with distilled water, bands were visualized by incubating the membrane in substrate solution (5 µl 30% hydrogen peroxide and 100 µl stock solution [60 mg ml⁻¹ tetramethylbenzidine and 200 mg ml⁻¹ diethylsulphosuccinate in dimethylsulfoxide] in 10 ml buffer [citric acid, 5 mM; disodium hydrogen phosphate, 10 mM; ethanol, 25%]). Visualization of the bands was stopped by removing the membranes from the substrate solution and soaking them in distilled water as soon as the five largest marker bands could be clearly distinguished.

The protocol for the detection of allergens was largely

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Table 1. Molecular weights (MW) of antigens in CF extracts and concurrence under various culture conditions

1)*	2)	3)	4)	5)	6)	7)	8)	9)
Aw	High	High	Low	Low		Common antigens		
Temperature	30°C	22°C	30°C	22°C		22°C and 30°C	22°C	30°C
	MW (Da)	MW (Da)	MW (Da)	MW (Da)	Mean MW (kDa)	High and low Aw	High and low Aw	High and low Aw
	260 021			261 664	260–261			
		239 328			239			
	186 258	195 968		186 812	187–196		X	
				175 102	175			
	168 749	163 836			164–169			
		147 257			147			
				129 933	128–130			
	126 801	126 240			126–127			
		121 556			122			
		103 599	103 906		104			
		102 483		101 943	102		X	
	100 193			100 125	100			
		97 623		96 926	97–98		X	
		95 788	96 116		96			
	94 154	93 059		94 064	93–94		X	
		90 455	92 298	91 385	90–92		X	
		89 569			89.5			
		87 285	87 838	88 368	87–88		X	
	85 387		85 282		85			X
	83 443	84 469			83–84			
	82 315	82 620			82–82.5			
	81 189	80 961			81			
	78 031	78 425			78			
		76 898			77			
	73 979	74 962			74–75			
	73 525	72 849			73–73.5			
		71 709	72 393		72			
	68 316	68 142	68 934	68 331	68	X	X	X
		65 396	65 616		65–66			
		64 445		64 485	64		X	
	62 809		63 162		63			X
		61 398		61 452	61		X	
		60 856			60.5			
	59 441	58 316			58–59			
	57 767	57 141	57 852	57 521	57–57.5	X	X	X
	54 550	54 194	53 913	54 263	54–55	X	X	X
		53 665			53.5–54			
	51 477	51 342			51			
	50 448	50 786			50–50.5			
		49 826		49 834	49.8		X	
	48 165	48 339	48 458		48			X
	46 285	46 631			46–47			
	43 088	44 413		44 698	43–45		X	
	43 230	43 249	43 145		43			X
	43 049	42 087		42 176	42–43		X	
		41 401	40 937	41 527	41–42		X	
			40 225		40			
		39 757	39 781	39 502	39.5–40		X	
	39 398	39 276			39			
	38 622			38 492	38–39			
		37 998		37 440	37–38		X	
	35 795	35 900		36 233	36		X	
	34 987	35 447		35 422	35		X	
		33 283			33			
	32 706	32 836		32 297	32–33		X	
	31 554	31 354		31 780	31–32		X	
		30 499			30			
	28 434				28.4			
	28 257				28.2			
	26 419				26			
	24 591				25			
n=	35	49	16	26		3	21	7

* Column number.

the same as for the detection of antigens. After blotting and blocking, the membrane was incubated overnight in undiluted IgE-allergic positive human serum (International Enzymes, Inc., Fallbrook, CA, USA, cat. no. 2401). This serum contains IgE against various epidermals, foods, trees, grasses, molds, and weeds. With respect to *Alternaria*, the serum is of IgE antibody class 3. Incubation with conjugate was performed with 800-fold diluted peroxidase-conjugated murine monoclonal antihuman IgE (Centraal Laboratorium voor de Bloedtransfusiedienst, Amsterdam, The Netherlands). Washings and substrate incubation were similar to those described for the detection of antigens.

Evaluation of gel patterns

Antigen blots and allergen blots were scanned by a GS700 Densitometer (BioRad) with the Molecular Analyst program (BioRad). After optimization of the visual image of the patterns on gels and blots, the bands were converted into molecular weights (MWs) with the MW markers on both gels and blots as references. For further processing, the data were transferred to and tabled in Microsoft Excel.

Per series of extracts and per staining, MWs were compared, and corresponding MWs (visually identical bands with the same MW and Rf value) were put in the same rows of the table. From corresponding MWs, the average was determined, and the deviation in percentage for each MW on the same line was calculated. The

maximum deviation per series of extracts and per staining was calculated and used to determine a range for each average MW.

The mean MWs calculated from antigen blots from all series were compared to determine which MWs were found in all series.

The MWs from the allergen blots were not examined as extensively as the MWs on the antigen blots. The blots were merely used to investigate whether and at what stage of the culture allergens were formed.

No comparisons were made between results from WS extracts and CF extracts because it is not clear what happens to molecules when they cross the cell wall during excretion. Excretion might cause changes in MW, but whether this change is an increase or decrease is not clear.

Results

Detection of antigens

The MWs of all antigens, in kDa, found in the CF extracts of the four different culture conditions can be found in columns 2–5 of Table 1. The figures in this table all derive from antigen blots, as described under “Evaluation of gel patterns” in the Material and methods section. Fig. 1 shows the antigen blot of CF samples from day 0 to day 21 (as indicated) of the culture at 30°C and high Aw.

Corresponding antigens, based on MW, are placed on

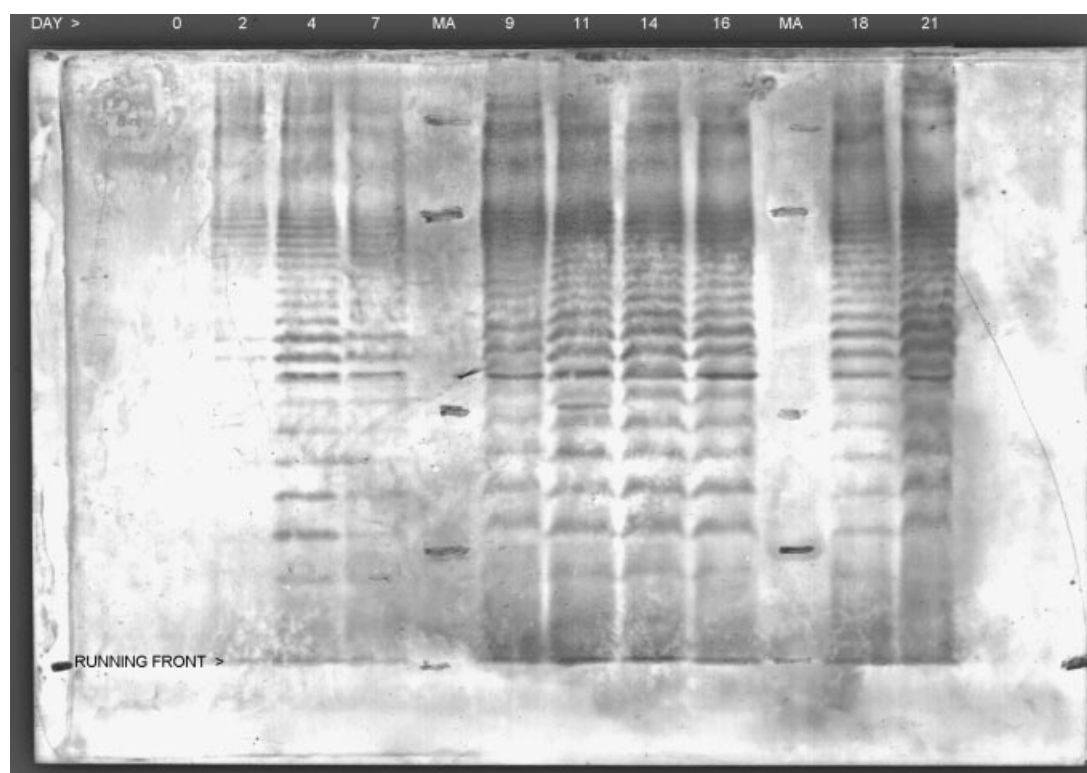


Figure 1. Antigen blot of culture filtrate samples from day 0 to day 21 of culture at 30°C and high water activity (MA: molecular weight marker).

Table 2. Molecular weights (MW) of antigens occurring in WS extracts plus concurrence under various culture conditions

1)*	2)	3)	4)	5)	6)	7)	8)	9)
Aw	High	High	Low	Low		Common antigens		
Temperature	30°C	22°C	30°C	22°C		22°C and 30°C	22°C	30°C
	MW (Da)	MW (Da)	MW (Da)	MW (Da)	Mean MW (kDa)	High and low Aw	High and low Aw	High and low Aw
	216 746		240 023		217–240			X
			193 448		193			
			173 685		174			
		166 748			167			
		164 905			165			
	149 926	153 323			150–153			
		147 143	146 071		146–147			
	137 346				137			
		134 173			134			
		132 523	133 228		133			
		130 026	128 663		129–130			
	125 737	122 085			122–126			
		115 854			116			
		113 466			113			
		109 042			109			
	105 742		106 742		106–107			X
	99 773		103 627		100–104			X
			97 811		98			
		96 587	96 937		97			
	95 111	95 128	95 229		95			X
		93 370			93			
		92 806	92 876		92.8			
	91 197		91 101		91			X
	89 915	90 236			90			
	88 810	88 069	88 335		88–89			X
		85 371			85			
		84 782	84 732		84.7			
	84 489	84 358			84			
		82 686			83			
		81 392	80 886		81			
		79 080		80 495	79–80		X	
		78 838			78.8			
		76 592	76 824		77			
		74 036			74			
		72 537	72 711		73			
		71 146		77 458	71		X	
		69 154	69 450		69			
		67 273			67			
			65 661		66			
	64 153	63 945	64 295	64 466	64	X	X	X
			62 721		63			
		60 846	60 624		61			
	59 743	59 806			60			
	56 378	56 552	57 106	57 151	56–57	X	X	X
	53 524	54 196	53 809		54			X
		52 950			53			
	51 336	52 656	51 127	51 635	51–52	X	X	X
	50 120			50 325	50			
		48 471			48.4			
		48 248			48.2			
		47 505	47 067		47–48			
		44 574	44 112		44–45			
			43 101	42 758	43			
		40 718	41 131		41			
		40 500	40 074		40			
		36 671	38 235		37–38			
		36 182	36 588		36–37			
			35 637		36			
		33 134			33			
		32 583	32 606		32.5–33			
		30 732	31 959		31–32			

Table 2. Continuation

1)*	2)	3)	4)	5)	6)	7)	8)	9)
Aw	High	High	Low	Low		Common antigens		
Temperature	30°C	22°C	30°C	22°C		22°C and 30°C	22°C	30°C
	MW (Da)	MW (Da)	MW (Da)	MW (Da)	Mean MW (kDa)	High and low Aw	High and low Aw	High and low Aw
		28 376			28			
		26 142			26.1			
		25 708			25.7			
		24 265			24.2			
		24 114			24.1			
n=	16	54	36	7		3	5	10

* Column number.

one line. The data show all MWs regardless of the moment found during culture. Column 6 shows the average MWs from the antigens per line.

The antigens that were found in CF extracts under all described circumstances (marker antigens) are indicated (X) in column 7; namely, antigens with MWs of 54–55, 57–57.5, and 68 kDa. In columns 8 and 9, antigens have been marked (X) that occur in the CF of cultures at 22°C or in the CF of cultures at 30°C, respectively.

The MWs of the antigens that were found in WS extracts under the described circumstances are listed in Table 2. For the comparison of MWs among the different culture circumstances, the same columns are used as for the CF extracts in Table 1. Also in column 7, the antigens found under all circumstances (marker antigens) have been marked (X); namely, antigens with MWs of 51–52, 56–57, and 64 kDa. As in Table 1, in columns 8 and 9, antigens have been marked (X) that occur in the WS of cultures at 22°C or in the WS of cultures at 30°C, respectively.

Detection of allergens

The numbers of allergens detected in CF extracts were as

follows: 14 allergens in culture at 22°C and high Aw, 13 allergens in culture at 30°C and high Aw, seven allergens in culture at 22°C and low Aw, and 15 allergens in culture at 30°C and low Aw (data not shown in tabular form).

In the WS extracts of the culture at 22°C and high Aw, 26 allergens were found, six allergens at 30°C and high Aw, 20 allergens at 22°C and low Aw, and 24 allergens at 30°C and low Aw (data not shown in tabular form).

Occurrence of common antigens and allergens in CF extracts

Table 3 lists the common antigens in CF extracts, antigens occurring under all conditions applied in this research, correlated to the occurrence of allergens. Three antigens (54–55, 57–57.5, and 68 kDa) were found under all culture conditions. In the 22°C, low-Aw culture, all three antigens were detectable only at day 4. In the culture at 30°C and low-Aw allergens, the 68-kDa antigen was detected at day 18, the 57–57.5-kDa antigen at days 4 and 21, and the 54–55 kDa antigen was detected only at day 4. In the culture at 22°C and high Aw, the 68-kDa antigen was detected from day 0 until day 9, the 57–57.5-kDa antigen was found at day 14, and

Table 3. Correlation between occurrence of common antigens and allergens in CF extracts from cultures of all conditions

Day	0	2	4	7	9	11	14	16	18	21
68-kDa antigen at 22°C low Aw								+		
57–57.5-kDa antigen at 22°C low Aw								+		
54–55-kDa antigen at 22°C low Aw								+		
Occurrence of allergens at 22°C low Aw							X	X		
68-kDa antigen in 30°C low Aw									+	
57–57.5-kDa antigen in 30°C low Aw			+							+
54–55-kDa antigen in 30°C low Aw			+							
Occurrence of allergens at 30°C low Aw					X	X	X	X	X	X
68-kDa antigen at 22°C high Aw	+	+	+	+	+					
57–57.5-kDa antigen at 22°C high Aw							+			
54–55-kDa antigen at 22°C high Aw	+		+	+						
Occurrence of allergens at 22°C high Aw					X	X	X	X	X	X
68-kDa antigen at 30°C high Aw			+	+	+	+	+	+	+	+
57–57.5-kDa antigen at 30°C high Aw		+	+	+	+	+			+	
54–55-kDa antigen at 30°C high Aw		+	+	+	+	+	+	+	+	+
Occurrence of allergens at 30°C high Aw		X	X	X	X	X	X	X	X	X

Table 4. Correlation between occurrence of common antigens and allergens in WS extracts from cultures of all conditions

Day	0	2	4	7	9	11	14	16	18	21
64-kDa antigen at 22°C low Aw						+	+	+	+	+
56–57-kDa antigen at 22°C low Aw		+			+	+	+		+	+
51–52-kDa antigen at 22°C low Aw										+
Occurrence of allergens at 22°C low Aw		X			X	X	X	X	X	X
64-kDa antigen at 30°C low Aw					+					
56–57-kDa antigen at 30°C low Aw		+	+	+	+	+	+	+	+	+
51–52-kDa antigen at 30°C low Aw		+	+		+		+		+	
Occurrence of allergens at 30°C low Aw		X	X	X	X	X	X	X	X	X
64-kDa antigen at 22°C high Aw				+	+	+	+	+	+	+
56–57-kDa antigen at 22°C high Aw				+	+	+	+	+	+	+
51–52-kDa antigen at 22°C high Aw				+						
Occurrence of allergens at 22°C high Aw					X	X	X	X	X	X
64-kDa antigen at 30°C high Aw				+						
56–57-kDa antigen at 30°C high Aw				+	+	+	+	+		
51–52-kDa antigen at 30°C high Aw				+	+	+	+	+		
Occurrence of allergens at 30°C high Aw					X	X	X	X	X	X

the 54–55-kDa antigen was detected from day 0 until day 7 at varying times. In the culture at 30°C and high Aw, the 68-kDa antigen was found from day 4 until day 21, the 57–57.5-kDa and 54–55-kDa antigens were detected from day 2 until day 21, and the 57–57.5-kDa antigen was detected at varying intervals. Allergens in CF extracts appeared at the same temperature/Aw combinations from days 14, 9, 9, and 2 on, respectively.

Occurrence of common antigens and allergens in WS extracts

As shown in Table 2, three common antigens (64, 56–57, and 51–52 kDa) were found in WS extracts produced from cultures grown under all four conditions. Table 4 lists the occurrence of both the common antigens and the allergens under the respective conditions. In the culture at 22°C and low Aw, the 64-kDa antigen was detected from day 11 until day 21, the 56–57-kDa antigen from day 2 until day 21 at varying intervals, and the 51–52 antigen only at day 21. At 30°C and low Aw,

the 64-kDa antigen appeared only at day 9, the 56–57-kDa antigen from day 2 until 21, and the 51–52-kDa antigen at varying intervals between days 2 and 18. At 22°C and high Aw, the 64-kDa and the 56–57-kDa antigens appeared from day 7 until the end of the incubation period, while the 51–52-kDa antigen occurred only at day 7. At 30°C and high Aw, the 64-kDa antigen was formed only at day 7, and the 56–57- and 51–52-kDa antigens were detected at days 7–16. Allergens were detectable at the same temperature/Aw combinations from days 2, 2, 9, and 9 on, respectively.

Discussion

Exposure to molds and their products in the indoor environment may lead to the emergence of allergies (1). Methods to assess exposure to molds are available (2–7), but these methods are cannot indicate exposure to mold allergens. The detection of mold antigens is performed

Table 5. Correlation between occurrence of index antigens and allergens in CF extracts from cultures of all conditions

Day	0	2	4	7	9	11	14	16	18	21
54–55-kDa antigen at 22°C low Aw								+		
35-kDa antigen at 22°C low Aw							+			
32–33-kDa antigen at 22°C low Aw								+		
Occurrence of allergens at 22°C low Aw							X	X		
54–55-kDa antigen at 30°C low Aw			+							
35-kDa antigen at 30°C low Aw										
32–33-kDa antigen at 30°C low Aw										
Occurrence of allergens at 30°C low Aw					X	X	X	X	X	X
54–55-kDa antigen at 22°C high Aw	+		+	+						
35-kDa antigen at 22°C high Aw					+	+	+		+	
32–33-kDa antigen at 22°C high Aw						+				+
Occurrence of allergens at 22°C high Aw					X	X	X	X	X	X
54–55-kDa antigen at 30°C high Aw		+	+	+	+	+	+	+	+	+
35-kDa antigen at 30°C high Aw										
32–33-kDa antigen at 30°C high Aw										
Occurrence of allergens at 30°C high Aw		X	X	X	X	X	X	X	X	X

for different purposes, such as the study of antigenemia in systemic aspergillosis. Antigen detection does not depend on the viability of molds, a feature which makes the technique useful for the determination of exposure to molds and their products.

The investigations described here were carried out within the framework of a project to determine to what extent molds occurring in the indoor environment contribute to the emergence of asthma and other respiratory disorders. It is imaginable that, through the consumption of contaminated food or swallowing of inhaled material, mold allergens may be ingested and sensitization via the intestinal tract may take place. No proof of this hypothesis, however, could be found in the literature or through personal communication (15). Therefore, these investigations are limited to the contribution of inhalable allergens.

A. alternata is known to produce several allergens, most of which are so-called major allergens (16–18). IgE antibodies against those allergens are found most frequently in patient material. The objective of these investigations, however, was not to measure the prevalence of IgE against mold allergens in individuals, nor to detect major allergens. The aim was to establish the presence of allergens in extracts in order to achieve the true objective of our investigations; namely, to present a method capable of indicating the possible risk of exposure to mold allergens. To establish the presence of allergens in CF and WS extracts, we used a single allergenic serum, from a donor with IgE against a great number of allergens, including mold allergens. With only one detector serum, there was a chance of missing allergens in some extracts. Unfortunately, there are few commercial allergenic sera available in the quantities needed for these investigations. However, the principle of using marker antigens for the risk of exposure to mold allergens has been proven by the results presented here.

Some, or all, of these major allergens may have been present in our extracts, but, for our investigations, all, or at least as many as possible, allergens that can be formed are of interest. Yet, there is no information available on the allergens that are produced by *A. alternata* or what circumstances are necessary for production of these allergens. The investigations described here were carried out to assess the production of antigens and allergens under various culture circumstances and to determine whether certain antigens formed under all conditions (common antigens) and before and/or concurrent with the production of allergens can be found. Such antigens might be useful as markers for the risk of exposure to *A. alternata* allergens.

The interpretation of the results described here was carried out separately for the CF extracts and the WS extracts. With the methods used, it is not possible to determine whether identical MWs in CF and WS stand for identical molecules. However, this uncertainty of whether all allergens found in the CF are indeed CF

allergens, or result from decay of mycelium, is not specific to these *in vitro* experiments. As these experiments closely resemble the *in vivo* processes of sporulation, growth, and decay of *A. alternata*, the same questions will also arise *in vivo*. Therefore, the uncertainty of whether all allergens found in the CF at 30°C are indeed CF allergens or also result from decay of the mycelium resembles the situation that can occur *in vivo*.

Spores of *A. alternata* are known to be reservoirs of allergens (19, 20). Although the utmost care was taken to work with a submerged culture only, it was impossible to avoid the production of spores and thus to avoid the influence of spore allergens. Therefore, the presence in the CF or WS extracts of some allergenic material deriving from spores could not be excluded, but as no investigations were carried out, no indication of the extent of allergenic material in the extracts could be given.

There are indeed antigens that are formed under all culture conditions, in the CF extracts as well as in the WS extracts. Yet, only one of these common antigens, namely, the 56–57-kDa antigen in the WS extracts, may serve as a marker antigen, as it is found before, or concurrent with, the appearance of allergens (Tables 3 and 4). The other common antigens in CF and WS extracts are useless as marker antigens because of their infrequent, early, or too late appearance compared to the production of allergens.

When we held to the strict definition of marker antigen, no marker antigen could be detected in the CF extracts. However, a combination of CF antigens might serve as marker too. The occurrence of the combination of the 54–55-kDa antigen (occurring in both the 22°C and 30°C cultures; Table 1, columns 7, 8, and 9), the 35-kDa antigen, and the 32–33-kDa antigens in the 22°C cultures (Table 1, column 8) coincided with the occurrence of allergens (Table 5). As these antigens do not comply individually with the criteria for marker antigens but do have, in combination, the same properties, they might be referred to as index antigens. In the culture at 30°C at low Aw, the 54–55-kDa index antigen was detected only at day 4, and not at any other day. The experiments were carried out in a closed system, causing accumulation in the CF of antigens. Possibly, the total load of antigen from day 7 on was too high to detect the 54–55-kDa index antigen.

In the CF extracts, one allergen was found under all culture conditions. Although it could be used as such, this allergen is not indicated as a marker molecule because of the insensitivity of the allergen test. In the immunoblot test for the detection of antigens, the detector serum was used diluted 1000-fold, while for the detection of allergens the detector serum was used undiluted. The chance of missing this “marker” allergen was too high to provide a reliable test.

The number of allergens found in the cultures (for CF

extracts: 13 in 30°C high Aw, 14 in 22°C high Aw, 15 in 30°C low Aw, and seven in 22°C low Aw; for WS extracts: six in 30°C high Aw, 26 in 22°C high Aw, 24 in 30°C low Aw, and 20 in 22°C low Aw) was remarkably high, as compared to the number of allergens produced by *Aspergillus fumigatus* under the same culture conditions (10). That finding may explain the higher incidence of IgE titers against *A. alternata* than against *As. fumigatus* in Dutch children (21).

Except for the 30°C high-Aw culture, the number of allergens found in the WS extracts exceeded the number

found in the CF extracts. This may indicate that the decay of *A. alternata* is an even more important source of allergens than its excretion products.

The investigations described here are the result of tests under laboratory conditions. The usefulness of the marker antigens for establishing exposure to allergens, as reported here, still has to be established for natural conditions; for example, when using house-dust or air samples. Simulated indoor conditions and controlled mold growth on various materials might prove to be useful in such studies.

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