

Identification of the reactive subunits of *Aspergillus umbrosus* involved in the antigenic response in farmer's lung

K. KAUKONEN, L. J. PELLINIEMI*, J. SAVOLAINEN and E. O. TERHO

Department of Clinical Allergology and *Laboratory for Electron Microscopy, University of Turku, Finland

Summary

Background Farmer's lung (FL) is the most common form of extrinsic allergic alveolitis and the fungus *Aspergillus umbrosus* has frequently been associated with FL among Finnish farmers. IgG and IgA class antibodies against *Aspergillus umbrosus* are found in the serum of FL patients and healthy exposed farmers. So far the immunologically reactive subunits of *Aspergillus umbrosus* are unknown.

Objectives The purpose of the study was to identify and localize the reactive subunits of *Aspergillus umbrosus* that are involved in the antigenic response in FL.

Methods The distribution of the primary antibody on the outer wall layers of 4- and 11-day-old fungi *Aspergillus umbrosus* was studied by pre-embedding immunolabelling with fluorescein isothiocyanate (FITC)- and gold-conjugated antibodies. The surface carbohydrates of the outer wall layers were studied using FITC-conjugated lectins.

Results FITC-conjugated antibodies from FL patient sera reacted with spores (conidia), phialides and hyphae, whereas the fruiting body, cleistothecia, remained unstainable. Immunofluorescence labelling was confirmed by immunolabelling electron microscopy, and gold-conjugated IgG antibodies were found on the outer cell walls of spinulose conidia, and in a less degree on the outer cell walls of hyphae and phialides. Only single gold particles were found scattered on the surfaces of cleistothecia. The surface carbohydrates of *Aspergillus umbrosus* conidia, vesicles, phialides and hyphae had a high affinity to GlcNAc β 1- and Man α 1-/Glc α 1- specific lectins and only GlcNAc β 1-specific lectins also reacted with the outer cell walls of cleistothecia. Blocking of the antigenic sites of *Aspergillus umbrosus* by preincubating antigen-coated enzyme linked immunosorbent assay (ELISA) plates with lectins caused some inhibition, indicating that sites with the highest affinity for lectins were not always antigenic sites.

Conclusion Gold- and FITC-conjugated antibodies were distributed mostly on the outer wall layers of conidia, hyphae and phialides of *Aspergillus umbrosus* and GlcNAc β 1-linked lectins had the strongest affinity to the outer wall layers.

Keywords: antigen localization, *Aspergillus umbrosus*, *Eurotium rubrum*, farmer's lung, fluorescein-conjugated lectins, gold labelling, immunocytochemistry

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Introduction

Farmer's lung (FL) is the most common form of extrinsic

allergic alveolitis, an entity of pulmonary hypersensitivity diseases, also known as hypersensitivity pneumonitis. Continuous exposure to high concentrations of inhaled antigens from mouldy hay or straw leads to sensitization and development of the disease. FL results in long-lasting impairment of lung function,

Correspondence: Dr K. Kaukonen, Department of Clinical Allergology, University of Turku, MediCity Research Laboratory, 20520 Turku, Finland.

even permanent damage of the lungs [1]. In Finland, exposure to the fungi of the genus *Aspergillus* and to thermophilic actinomycetes, especially *Thermoactinomyces vulgaris*, has been associated with FL [2–4]. The disease is characterized by alveolar lymphocytosis, and high titres of microbe-specific IgG antibodies are often found in the serum of FL patients [5].

It is assumed that inhaled whole fungal organisms, that is mycelial fragments and spores, act as antigens giving rise to the production of antibodies [6]. Polysaccharides located on the surface of cell walls react with antibodies and lectins [7]. The specificity of lectins for particular saccharides has been used successfully in the characterization of surface carbohydrates on the surface of cell walls [6,8,9]. Lectins have also been used as competitive inhibitors of antibody binding to surface carbohydrate antigens [10]. We have shown earlier that Man α 1-/Glc α 1- and GlcNAc β 1- linkage -specific lectins have affinity for antigen extracts of *A. umbrosus* [11]. In an attempt to identify reacting subunits of the fungal organism, we localized antigenic epitopes and polysaccharides on the cell surface of *A. umbrosus* by pre-embedding labelling with gold- and fluorescein isothiocyanate (FITC)-conjugated antibodies and FITC-conjugated lectins in this study.

Methods

Human sera

Sera from three FL patients in an acute phase of the disease and sera from three healthy individuals were used in the study. The FL patients fulfilled internationally accepted diagnostic criteria [12]. Serum samples were obtained from the Department of Pulmonary Diseases, Kuopio University Hospital, Finland.

Preparation of *Aspergillus umbrosus*

Strain M 118 (isolated from mouldy hay in Finland by Dr M. Kotimaa, Kuopio Regional Institute of Occupational Health, Kuopio, Finland) was cultivated as described earlier [13]. Four- and 11-day-old fungal mass, washed with phosphate buffered saline (PBS) pH 7.4, was cut into 3 × 3 mm pieces and dried on glass slides for light microscopic examination and in polystyrene microtitre plates for electron microscopy at + 50 °C for 30 min. The specimens were stored at –20 °C. The crude antigen of *A. umbrosus* for the enzyme-linked immunosorbent assay (ELISA) was prepared as described earlier [13].

Taxonomy of the fungus

According to the presently valid code of nomenclature,

Aspergillus umbrosus is included within *Eurotium rubrum*. The name *Aspergillus* should be now reserved only for the conidial (asexual) form of the fungus, while *Eurotium* is the name for the sexual stage and the whole fungus. The name *A. umbrosus* is used throughout this study for both sexual and asexual stages of the fungus in order to be in accordance with our earlier studies.

Structure of *A. umbrosus*

Generic characteristics of *Eurotium* are seen in Fig. 1 [14]. The *Eurotium* colony consists of a thread-like mycelium composed of vegetative cells or hyphae (H) from which conidiophores comprising a stipe (stalk) with a swollen apical vesicle (V) rise which bears a single layer of elongated phialides on which the asexual conidia (spores) (C) are formed. The sexual stage, arising after nuclear fusion, comprises cleistothecia (Cl) within which enlarged cells known as asci (As) develop containing, at maturity, eight ascospores (A).

Immunofluorescence assay

Serum samples diluted 1:100 in PBS were spread on slides, blocked with 1% bovine serum albumin in PBS (BSA-PBS), incubated at + 4 °C for 24 h and washed with PBS. The specimens were made to react with FITC-conjugated second antibodies and incubated at + 4 °C for 1 h. The second antibodies were used at the following dilutions: rabbit α -human IgA 1:40 (Behring, Marburg, Germany), goat α -human IgG non-diluted (Immunoconcepts, Sacramento, CA, USA), and rabbit α -human

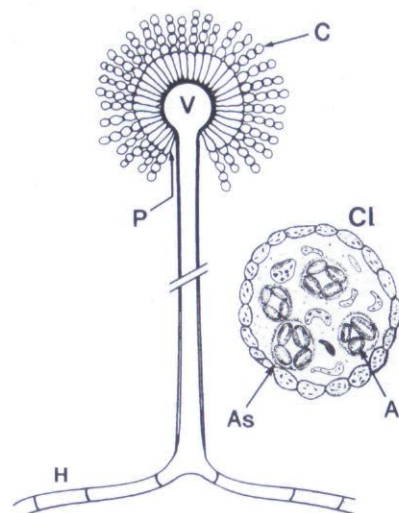


Fig. 1 Structural characteristics of *Aspergillus*. Conidiophore comprises of a vesicle (V), phialides (P) and conidia (C), and the stipe of the vesicle joints it to the vegetative mycelia, hyphae (H). The sac-formed cleistothecium (Cl) contains ascospores (A) within asci (As).

Table 1. Summary of the used lectins

Lectin	Carbohydrate specificity	Inhibiting sugar
Concanavalin A (ConA)	Man α 1-, Glc α 1-	α -methylmannoside, α -methyl glucoside
Lycopersicon esculentum lectin (LEL)	GlcNAc β 1-	N-acetylglucosamine
Wheat germ agglutinin (WGA)	GlcNAc β 1-	N-acetylglucosamine
Soybean agglutinin (SBA)	GalNAc α 1-3Gal-	N-acetylgalactosamine
Peanut agglutinin (PNA)	Gal β 1-3GalNAc-	Galactose
<i>Ricinus communis</i> agglutinin (RCA ₁₂₀)	Gal β 1-3(4)GlcNAc-	Lactose
<i>Ulex europaeus</i> agglutinin (UEA I)	Fuc α 1-	Fucose

IgA-IgG-IgM 1:40 (Dako, Copenhagen, Denmark). Then, 3% formaldehyde was added for 1 h to stop the growth of the fungi. All incubations were carried out in a humid chamber. After washings, the slides were mounted with a coverslip and examined with an Olympus UV light microscope. The method was a modification of that by John *et al.* [15].

Immunolabelling electron microscopy

Fungal mass was incubated with serum samples, diluted 1:100 in PBS containing 1% bovine serum albumin, 0.05 mol/L Tris, pH 7.4, 0.15 mol/L NaCl and 0.05 mol/L 0.05% sodium azide (BSA-PBST), in a humid chamber at +4 °C for 24 h and washed with PBS 5 $\times$ 5 min. Thereafter, 5 nm colloidal gold particles coated with goat α -human IgG (Zymed, San Francisco, CA, USA) were diluted 1:10 in BSA-PBST and incubated in a humid chamber for 1 h at room temperature. After washing, the preparations were fixed with 5% glutaraldehyde in 0.16 mol/L s-Collidin buffer, postfixed in 1% OsO₄ and 1.5% potassium ferrocyanide in water, dehydrated in alcohol, embedded in epoxy resin (Glycidether 100, Merck, Darmstadt, Germany), and sectioned with ultratome (Reichert Ultracut E, Reichert-Jung, Vienna, Austria). The preparations were examined with a JEOL JEM 100 C electron microscope.

Affinofluorescence assay

Table 1 shows the names of FITC-conjugated lectins (Vector Laboratories, Burlingame, CA, USA) and their nominal specificities. Lectin specificity was tested by preincubating each lectin in a 0.2 mol/L solution of an appropriate inhibitory sugar. All lectins were diluted in PBS, supplemented with 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂, to contain 0.05–0.2 mg/mL of lectin conjugate. The lectins were incubated on slides in a humid chamber at +4 °C for 24 h and washed carefully. The slides were mounted with a coverslip and examined as above.

Enzyme-linked immunosorbent assay

A. umbrosus-specific IgG and IgA enzyme-linked immunosorbent assays (ELISA) were carried out largely as described earlier [12]. Polystyrene microtitre plates were coated with *A. umbrosus* crude antigen 0.3 mg/mL in PBS, and unoccupied binding sites were saturated with 1% normal sheep serum in PBS (NSS-PBS). The wells were incubated with serum samples diluted 1:250 in NSS-PBS for 2 h. Thereafter, alkaline phosphatase-conjugated swine α -human IgG and IgA (Orion Diagnostica, Helsinki, Finland), diluted 1:300 and 1:100 in NSS-PBS,

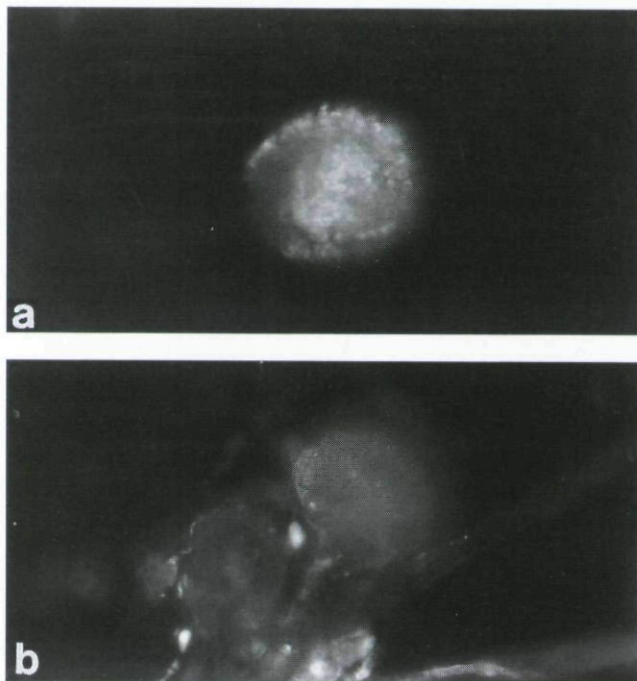


Fig. 2 Light-microscopic detection of antigenic sites of 4-day-old *Aspergillus umbrosus* by FITC-conjugated IgG antibodies. (a) Binding on the walls of conidia and phialides detected with serum from FL patient 1. (b) No binding detected with serum from a healthy control subject 1. Magnification: $\times$ 670.

respectively, were incubated on the plates for 2 h, and p-nitrophenylphosphate was added. The enzyme reaction was stopped with NaOH, and absorbances were measured at a wavelength of 405 nm.

Lectin inhibition ELISA

Biotinylated lectins ConA, LEL, SBA, PNA, RCA₁₂₀ and UEA I (Vector Laboratories, Burlingame, CA, USA) were used as competitive inhibitors of IgG antibody binding to *A. umbrosus* crude antigen in the ELISA. The ELISA was carried out as described above, except that the wells

were preincubated with lectins at six dilutions (1–0.01 mg/mL) before incubation with serum samples.

Results

Immunofluorescence labelling

FL patients had *A. umbrosus*-specific IgG and IgA antibodies that recognized the walls of *A. umbrosus* conidia, phialides, stipes of conidiophores and hyphae, whereas the fruiting bodies, cleistothecia, remained unstained (Fig. 2a). Non-specific fluorescence was detected with

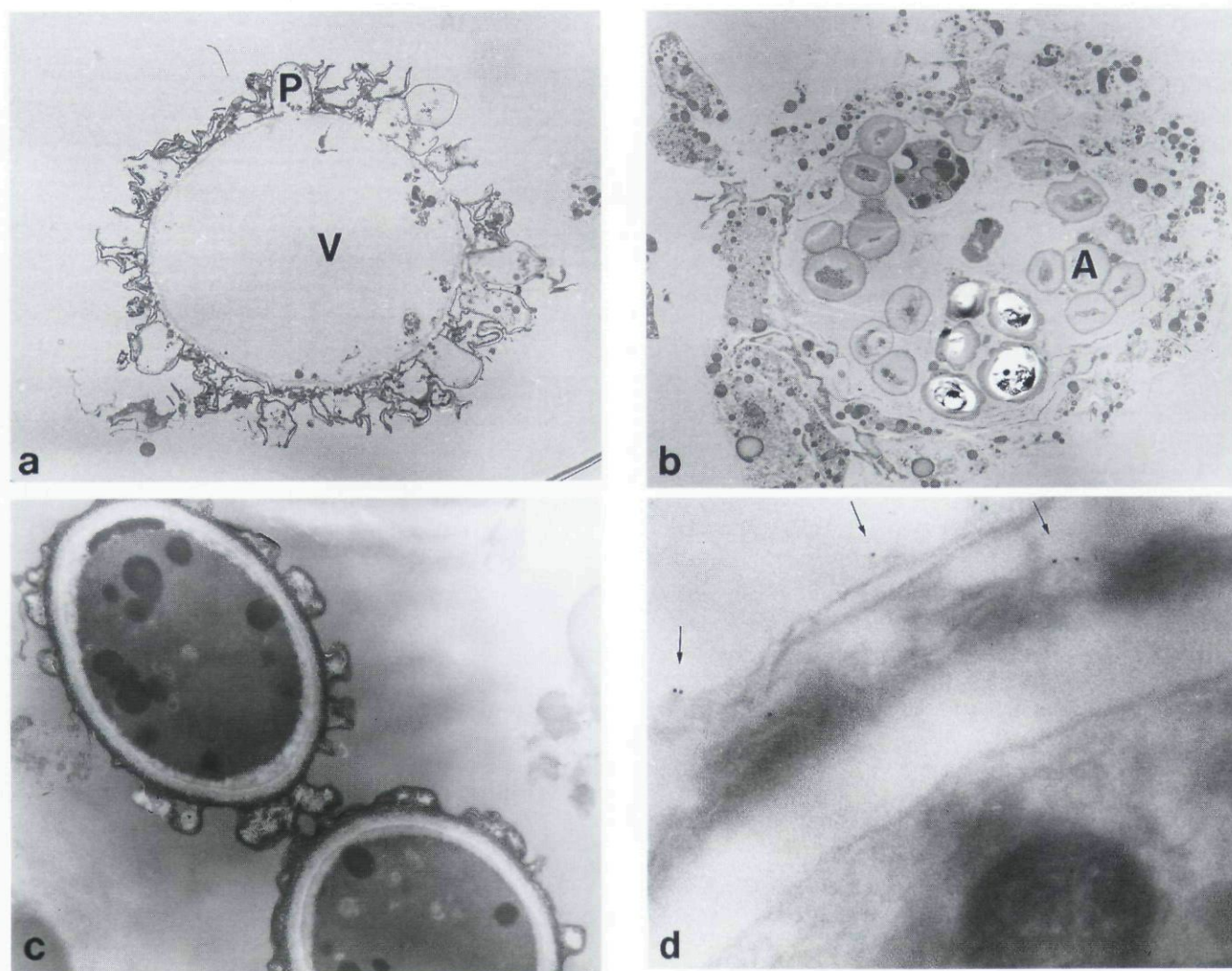


Fig. 3 The electron microscopic structure of 4-day-old *Aspergillus umbrosus*. (a) Vesicle (V) covered by phialides (P). (b) Maturing ascospores (A) within a cleistothecium. (c) Two spinulose conidia. (d) Immunolabelling electron microscopical detection of the antigenic outer cell wall of conidium by pre-embedding labelling with 5 nm-gold particles (arrows) coated with IgG antibodies. While the degree of the immunogold labelling is classified as negative (–; no gold label found), weakly positive (+), moderately positive (++) and strongly positive (+++), this particular labelling is classified as strongly positive. Magnifications a,b: $\times 1800$; c: $\times 14\,800$; d: $\times 108\,800$.

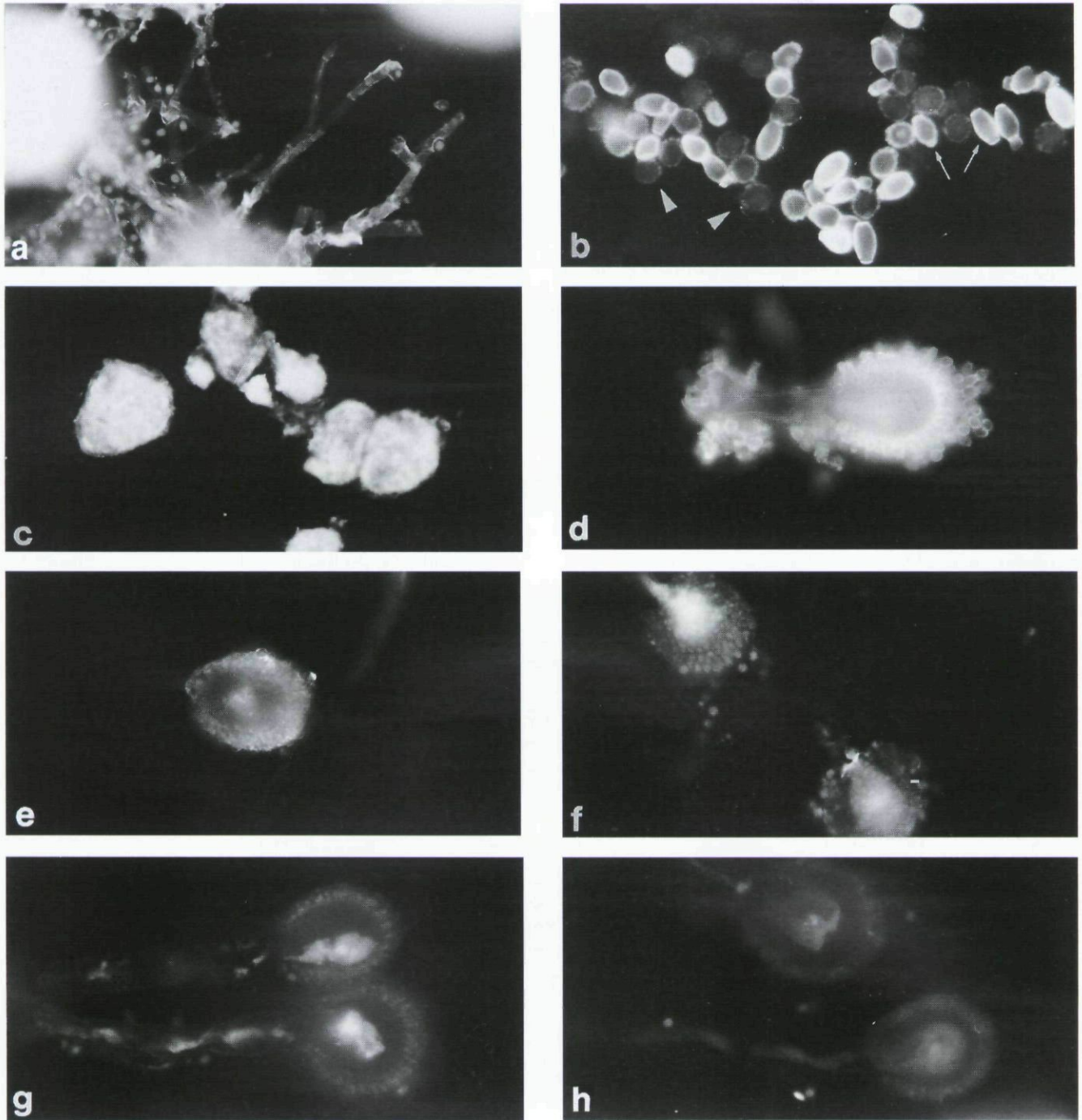


Fig. 4 Fluorescence microscopy of surface carbohydrates of 4-day-old *Aspergillus umbrosus* using fluorescein-conjugated lectins. Strong binding in (a) hyphae (b) young conidia (arrows), and (c) cleistothecia labelled with WGA, while fluorescence in older conidia was moderate (b: arrowheads). Binding seen in conidia, phialides and stipes of conidiophores (d) with LEL (e) with ConA, and (f) with PNA. No binding with (g) RCA or (h) with SBA, but instead, autofluorescence can be seen inside the vesicle. Magnifications a: $\times 570$; b: $\times 1,360$; c: $\times 130$; d, e, f, g, h: $\times 670$.

FITC-conjugated rabbit α -human IgA-IgG-IgM antibodies, and it was not completely abolished by preincubation of slides with 1% BSA, 1% NSS or 1% milk powder. By contrast, fluorescence binding was detected only with patient sera when rabbit α -human IgA and goat α -human IgG antibodies were used (Fig. 2b).

Location of antigenic sites at the electron microscopic level

The electron microscopic appearance of the different structures present in *A. umbrosus* colonies are shown in Figs 3a-3c. Figure 3a shows the single layer of phialides on a vesicle, Fig. 3b shows a cleistothecium and asci and Fig. 3c shows spinulose conidia. The number and size of cleistothecia was considerably higher in 11-day-old than in 4-day-old fungal culture, and more maturing ascospores were seen in the asci of 11-day-old fungal culture. Pre-embedding immunolabelling showed gold-conjugated IgG antibody attachment to the surfaces of spinulose conidia (Fig. 3d). Gold particles were seen on the surfaces of immature conidia still forming on phialides, and also on the surfaces of phialides and hyphae. The antibody attachment was clearly strongest

to conidia. Gold particles were seen on the surfaces of hyphae and phialides, but in a less degree than on conidia, while more gold particles were seen on hyphae than on phialides. Morphologically distinct and distant structures, conidia and hyphae, were chosen for Table 2, that shows the gold- and fluorescence-conjugated antibody attachment and ELISA results for each patient separately (Table 2). Only single gold particles were detected on the cell surfaces of 4- and 11-day-old cleistothecia. In general, more gold particles were detected in 4-day-old fungal culture than in 11-day-old fungal culture. No reaction was noted with sera from healthy control subjects.

Characterization of surface carbohydrates

Binding of lectins to *A. umbrosus* was visualized with FITC-conjugated lectins. Table 3 and Fig. 4 show the results. The walls of conidia, vesicles, phialides and hyphae of *A. umbrosus* were strongly labelled with GlcNAc β 1-linkage-specific lectins WGA and LEL and with Man α 1-/Glc α 1- linkage-specific lectin ConA, whereas the reaction with the stipe wall of the conidiophores was weak.

Table 2. Summary of immunoassay results

Subject	Immunofluorescence*		Immunogold*		ELISA†	
	Conidia	Hyphae	Conidia	Hyphae	IgG	IgA
FL patient 1	++	++	+++	+	2.2	0.8
FL patient 2	++	+	++	+	2.1	0.8
FL patient 3	+	+	+	+	1.6	1.2
Control 1	—	—	—	—	0.1	0.1
Control 2	—	—	—	—	0.1	0.0
Control 3	—	—	—	—	0.0	0.0

* Binding was classified as negative (—), weakly positive (+), moderately positive (++) or strongly positive (+++).

† Absorbances from ELISA at a wavelength of 405 nm.

Table 3. Summary of affinolabelling* with lectins

Structure	ConA	LEL	WGA	SBA	PNA	RCA	UEA I
Conidia	++	+++	+++	—	+	—	+
Vesicle	++	+++	+++	—	+	—	+
Phialide	++	+++	+++	—	+	—	+
Hyphae	++	+++	+++	—	+	—	+
Cleistothecia	—	++	+++	—	—	—	—

* Binding was classified as negative (—), weakly positive (+), moderately positive (++) or strongly positive (+++).

The reaction with WGA was strongest, and the cleistothecial surface bound only with WGA and LEL. Gal β 1-3GalNAc- linkage-specific lectin PNA and fucose α 1-specific lectin UEA I reacted weakly with the walls of conidia, phialides and hyphae. SBA and RCA₁₂₀ showed no affinity for *A. umbrosus*. Similar binding activities were seen with both 4- and 11-day-old cultures. The different labelling pattern of the conidia with fluorescein-conjugated WGA (Fig. 4b) was suggested to depend on the age of the conidia, i.e. the even-edged younger conidia did label more strongly compared with the spinulose older conidia.

The blocking of antigenic sites was tested by making sera react with crude *A. umbrosus* antigen-coated ELISA wells pretreated with lectins studied. ConA and LEL caused 20% and 15% inhibition of antibody binding, respectively, at a concentration of 1.0 mg/mL.

Discussion

A. umbrosus is one of the *Aspergillus* species reproducing both sexually and asexually [14]. The anamorphic asexual genus is characterized by pale green spinulose conidia, emerging through phialides located on the vesicle [16], whereas the sexual ascospores mature in the cleistothecium. Recently, the ultrastructure of *A. umbrosus* has been defined by electron microscopy [17,18].

The small size of both asexual spinulose conidia and sexual ascospores of *Aspergillus*, 5–10 μ m in axial length, allows them to penetrate alveoli. Both hypha- and spore-like particles have been detected with rabbit antiserum to *Aspergillus* (*A.*) *fumigatus* in alveolar granulomas from lung biopsy specimens of patients with FL [19,20]. The results of our immunofluorescence studies on *A. umbrosus* are in agreement with earlier immunofluorescence studies, which have shown that both spores and hyphae of *Micropolyspora faeni*, *Thermoactinomyces candidus* and *Thermoactinomyces vulgaris* are responsible for antibody production in FL [21]. A similar reaction is caused by spores and hyphae of *A. fumigatus*, involved in allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis [22]. Diagnostic use of fluorescein-labelled antibody assays in hypersensitivity lung diseases has also been proposed, especially for evaluation of the disease process and treatment responses, since the results of such assays are available on the same day [21,23,24]. Today, more sensitive immunoassays and better characterized antigens have replaced immuno-fluorescein assays. FITC-conjugated antibodies were used in this study in an attempt to detect the antigenic sites of *A. umbrosus*.

Although IgG and IgE antibody binding to spores and hyphae of *A. fumigatus* in hypersensitivity lung diseases

has been demonstrated at the electron microscopic level [22], immunolabelling electron microscopy of *Aspergillus* epitopes related to FL has to our knowledge not been reported earlier. The finding of gold-conjugated IgG antibodies mainly on wall surfaces of the conidia and in a less degree on wall surfaces of hyphae and phialides of *A. umbrosus* confirmed the results of our immunofluorescence studies. In addition to the outer surface of conidia and mycelia, the cytoplasm and intracellular membranes of *A. fumigatus* also react with mouse monoclonal antibodies and polyclonal human antibodies to *A. fumigatus* [22,25]. The present method of immunolabelling whole fungus rather than ultrathin sections makes only the surface layers available for immunodetection. In fact, the inner cell walls and intracellular membranes of *A. umbrosus* conidia and hyphae may also be strongly antigenic, since it has been reported that intracellular membranes and cytoplasm induce extensive alveolitis in mice [26] and serodiagnosis of FL is more successful with ruptured mycelial than culture fluid antigen [27]. Immunolabelling before embedding may also affect staining. Therefore, we would like to establish a method for postembedding labelling of thin sections of *A. umbrosus* with FL patient sera.

The staining patterns of lectins depend on terminal sugars rather than the gross composition of the cell wall. All lectins studied are bound to residues at the non-reducing terminal sugars, whereas only ConA can also interact with internal mannosyl or glucosyl groups. The cell wall constituents of the hyphae and conidia of *Aspergillus* species included mannosyl, galactosyl, and glucosyl units [28,29]. The strongest reaction in this study was seen with WGA, which provides strong evidence for the concept that the conidia, hyphae and cleistothecia of *A. umbrosus* consist of mannans and glucans with terminal GlcNAc β 1- linkages. Our finding that the outer surface of *A. umbrosus* was labelled with ConA is partly consistent with the finding of Stoddard and Herbertson [9] who detected binding of FITC-conjugated ConA and SBA on the hyphal walls of *A. fumigatus*. The binding pattern of lectins, the antibody-binding inhibition with lectins and our earlier antigen analysis with biotinylated lectins suggest that the antigenic sites of *A. umbrosus* partly consist of GlcNAc β 1- and Man α 1-/Glc α 1- linkages [11].

The finding of hyphae- and spore-like particles in the alveolar granulomata [19,20], suggesting *in vivo* autolysis and break-down of fungal mass of *Aspergillus*, supports the concept of antigenic intracellular constituents. The present study supports the hypothesis that both fungal conidia and hyphae are responsible for antibody production in FL. This was the first time that the location of FL-related antigenic epitopes on the surface of an *Aspergillus*

fungus was shown at the electron microscopic level. The surface layers of *A. umbrosus* hyphae and conidia were visualized mainly using GlcNAc β 1- and Man α 1-/Glc α 1-linkage-specific FITC-conjugated lectins.

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