

Monoclonal antibodies bind identically to both spores and hyphae of *Aspergillus fumigatus*

K. E. REIJULA, V. P. KURUP, A. KUMAR and J. N. FINK

Research Service, VA Medical Center, and the Department of Medicine, Allergy-Immunology Division, Medical College of Wisconsin, Milwaukee, WI, U.S.A.

Summary

Immunoelectron microscopy (IEM) was used to determine the binding of six monoclonal antibodies (MoAbs) produced against *Aspergillus fumigatus* antigens present on or within the conidia and hyphae of the fungus. Antigen-antibody complexes were demonstrated in EM using labelled colloidal gold particles (15 nm). Three out of 6 MoAbs (C9, F12 and H10) reacted only with the cytoplasmic components of *A. fumigatus* while the remaining three (B12, F6G5 and D6E6) showed reactivity to both cytoplasm and cell wall of the conidia and hyphae. The results indicate that IEM is of considerable value in determining and selecting monoclonal antibodies having specific reactivity with diverse antigenic components.

Clinical and Experimental Allergy, Vol. 22, pp. 547–553. Submitted 5 March 1991; revised 26 November 1991; accepted 28 November 1991.

Introduction

Aspergillus fumigatus is an ubiquitous fungus involved in a number of clinically important diseases in man [1–4]. Demonstration on *A. fumigatus*-specific circulating antibodies in the sera of patients is a significant characteristic in the diagnosis of *A. fumigatus*-induced diseases [1]. Pure and standardized antigens are necessary in order to develop dependable and reliable methods to be used in the diagnosis of these diseases [5,6]. Therefore, attempts have been made to produce monoclonal antibodies (MoAbs) against *A. fumigatus* in order to purify specific *A. fumigatus*-antigens [7,8]. Several MoAbs against specific components of *A. fumigatus* antigens have been produced in our laboratory some of which were then used to purify more relevant *A. fumigatus* antigens. Several of these antigens showed reactivity to both IgG and IgE antibodies from the sera of patients with allergic bronchopulmonary aspergillosis (ABPA) and aspergilloma [9].

Attempts have also been made to purify subcellular fractions of *A. fumigatus* in order to elucidate the allergens of the microbe which may play a significant role in the immunopathogenesis of *A. fumigatus*-induced diseases [10–13]. Several partially purified and characterized antigens have been isolated and evaluated for their usefulness in the antibody detection assays. However, it

has not been resolved if antigens from mycelium, culture filtrate, cell wall fractions or membranous components act as triggers in the pathogenesis of the disease.

In the present study we have developed an immunoelectron microscopic method (IEM) by which the binding and localization of antibody to antigen in the conidia and hyphae of *A. fumigatus* can be precisely determined. Six MoAbs to *A. fumigatus* and one non-specific control were investigated by IEM for their ability to bind either to the cell wall or to the intracellular components of the *A. fumigatus* conidia and hyphae. This technique demonstrated antigenic components in the cell wall and cytoplasm of both conidia and hyphae.

Materials and methods

Production of MoAbs

The production and characterization of the MoAbs have been described previously [7,8]. Briefly, BALB/c mice were immunized with culture filtrate antigens of *A. fumigatus* by subcutaneous inoculation using 500–1000 µg of partially purified antigens in 0.1 ml sterile physiological saline, mixed with an equal volume of complete Freund's adjuvant. Two subcutaneous injections at 3-day intervals were given to each mouse. Intraperitoneal injections were then given with antigens alone at weekly intervals for 4 more weeks. Two weeks after the last injection, the animals were bled from the tail, and the

Correspondence and present address: Dr K. Reijula, Oulu Regional Institute of Occupational Health, PO Box 451, SF-90101 Oulu, Finland.

serum was tested for anti-*Aspergillus* antibodies by biotin-avidin-linked immunosorbent assay (BALISA) [14]. Those animals demonstrating high levels of antibodies were boosted with antigen and an immune stimulant immunotone (American Biotechnology Company, Rockville, Maryland, U.S.A.). Immunotone injected mice (100–200 µg/animal) consistently gave more hybridomas than those mice injected with antigens alone.

The spleen cells were separated and fused with mouse myeloma cells (Pe-NS-1/Ag4-1). The method followed was the same reported earlier [15], where the fused cells were cultured in hypoxanthine, aminopterin and thymidine medium. Positive wells were evaluated for the antibodies against *A. fumigatus* antigen by BALISA. *A. fumigatus*-specific antibody-secreting cells were cloned by using limiting dilution method. Hybridomas, thus repeatedly cloned and purified, were used to produce ascitic fluids in syngenic mice primed with pristaine. The ascitic fluids were collected and tested for anti-*A. fumigatus* antibodies by BALISA.

Several MoAbs against *A. fumigatus* were isolated from different fusions. Six antibodies (C9, F12, H10, F6G5, D6E6, B12) were selected for the present study. Ascitic fluid obtained from mice inoculated intraperitoneally with myeloma cells (NS-1) in pristaine-primed animals was used as control. No *A. fumigatus*-specific antibodies were detected in the fluids by ELISA or by Western blots. The immunoglobulin isotypes of the MoAbs were determined by using commercial typing reagents (Zymed, 458 Carltion Court, South San Francisco, California, U.S.A.) using BALISA. The binding of the MoAbs to different *A. fumigatus* antigen preparations were studied by BALISA, crossed immunoelectrophoresis, rocket electrophoresis and by immunoblotting.

SDS-PAGE and Western blotting

A. fumigatus antigen (a mixture of culture filtrate and mycelial extract containing 2.5 mg of protein/ml) was electrophoresed into a 0.5% sodium dodecyl sulphate-polyacrylamide vertical gel with a 7.5–20% linear gradient measuring 150 × 150 × 1 mm [16]. A current of 30 mA per gel was applied for 3 to 3.5 hr with continuous cooling at 10°C as described previously [8,9,13]. After electrophoresis the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immunobilon; Millipore Corporation, Bedford, Massachusetts, U.S.A.) by using a semi-dry electrotransfer system (ABN, American Bionetics, Inc., Hayward, California, U.S.A.) as determined by staining the gels for proteins with Coomassie Brilliant Blue R250 before blotting and silver stain after blotting. These results were compared with colloidal gold stained PVDF membranes containing the blotted

proteins. The membranes were blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) for 1 hr to saturate the free protein binding sites on the membrane. After air drying the membranes were cut into small strips and stored at –20°C until used. These strips were processed as previously described with minor differences [7]. The strips were incubated with appropriate dilutions of monoclonal antibodies made in 1% BSA PBS for 1 hr at 37°C. The strips were then washed with PBS containing 0.05% Tween-20 for three washes each of 5 min, followed by treatment with goat anti-mouse antibodies of the appropriate isotypes. Again, the strips were incubated as before and washed. Rabbit anti-goat IgG coupled to biotin was added, incubated and washed as above. This was followed by treating the strips with streptavidin peroxidase for 30 min. After incubation the strips were washed and developed with 4 chloro-1-naphthol in Tris buffered saline containing 0.15% H₂O₂. The reaction was stopped after 10 min and the bands were compared. All dilutions of the reagents were determined by studying positive and negative sera and comparing signal to noise ratio. Antigen profile was studied by staining the PVDF membrane with colloidal gold while Concanavalin A (Con-A) binding components were determined by treating the blots with Con-A biotin and developing with streptavidin peroxidase as described above.

Preparation of *A. fumigatus* for immunoelectron microscopy (IEM)

Conidia from a 5-day-old culture of *A. fumigatus* on Sabouraud's agar were harvested in 15 ml sterile physiological saline and the suspension was inoculated into a synthetic broth [5,6]. *A. fumigatus* was grown in the medium at 37°C for 4 and 72 hr. The conidia and hyphae were then washed several times and resuspended in 2 ml of a fixative solution (4% paraformaldehyde–1% glutaraldehyde) at 4°C under vacuum in a dessicator for 8 hr [17]. The fixed growth was then washed three times in PBS (pH 7.4) after which the pellet of cells was post-fixed in 1% osmium tetroxide for 15 min, dehydrated in alcohol and embedded in Spurr® (Polysciences Inc., Warrington, Pennsylvania, U.S.A.). Ultrathin sections were cut on the LKB Ultratome III and grids with thin sections were used in immunochemical stainings. No germ tubes or hyphae were detected at 4 hr, although the majority of the conidia were swollen. At 72 hr hyphae, swollen conidia and germ tubes were detected.

Immunogold staining (IGS)

Colloidal gold with a size of 15 nm were prepared according to the method of Frens [18]. Rabbit anti-goat

IgG (affinity purified heavy chain specific) was obtained from Sigma Chemicals (St Louis, Missouri, U.S.A.) and coupled with the prepared colloidal gold [19]. The stabilized antibody labelled colloidal gold solution could then be stored at 4°C for at least 6 months; the solution was discarded when clumping of gold particles was detected.

Wells of polyvinyl microtitre plates (Nunc-Immuno-plate, Denmark) were used for the staining procedure. The grids were gently transferred to the microtitre plate wells containing PBS, pH 7.4 with 5% non-fat dry milk (Carnation dry milk), 0.05% (v/v) Tween-20 and 0.1% sodium azide (PBS-Milk-Tween) with the specimens facing upwards and incubated for 30 min. After washing three times for 2 min each with Tris buffer (TBS, pH 8.2) containing 0.1% (w/v) BSA and 0.1% sodium azide (TBSA), 200 µl of serum dilutions were added to the microplate wells. All dilutions of test reagents were prepared in PBS-Milk-Tween. The detection of the binding sites of the MoAbs to *A. fumigatus* was carried out by using 1:50 dilutions of MoAbs. After incubating at 22°C for 1 hr, the grids were washed three times in TBSA

for 2 min each and incubated in 200 µl of goat anti-mouse IgM (1:200, Sigma, St Louis, Missouri, U.S.A.) for 1 hr at 22°C. The grids were then treated at 1:20 dilution of rabbit anti-goat IgG colloidal gold (15 nm) conjugate prepared as described above and incubated at 22°C for 1 hr. The grids were washed once in TBSA, followed by two washes each in PBS and double distilled water. After air drying the specimens were examined in RCA EMU4 electron microscope at 50 kV.

Results

All six MoAbs reacted with *A. fumigatus* antigens by immunoblot assay (Fig. 1). The number of antigenic components reacting with the MoAb varied from 1 to 4. All of the antigens which reacted with the MoAbs also demonstrated strong binding to Con-A (Fig. 1, Lane G).

Examination of *A. fumigatus* ultrathin sections incubated with the six MoAbs revealed the presence of gold particles both in the conidia and hyphae of the microbe. Incubation with the control ascitic fluid failed to show any attachment of the gold particles (Fig. 2). The six MoAbs which showed reactivity with the conidia and hyphae could be divided into two groups according to the localization of the gold particles in the thin sections (Table 1). Three out of six MoAbs (C9, F12 and H10)

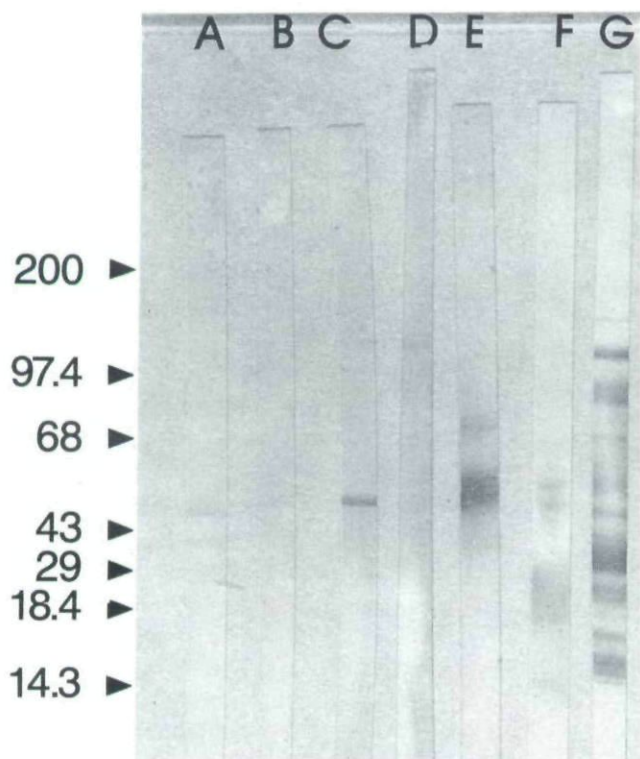


Fig. 1. Immunoblot of *A. fumigatus* antigens reacting with monoclonal antibodies. A, B₁₂ showing one band. B, H₁₀ with one weak band. C, C₉ showing one strong band. D, F₁₂ with one band. E, D6E6 showing 2 to 3 bands. F, F6G5 with 4 bands. G, antigen showing Concanavalin A binding activity.



Fig. 2. Thin section of an *A. fumigatus* conidia incubated with ascitic fluid of a control mouse (NS-1) and stained with anti-mouse IgM without visible gold particles in the specimen ($\times 13990$).

Table 1. Diversity of the reaction of *A. fumigatus* monoclonal antibodies (MoAb)

MoAb	Cytoplasm	Cell wall
C9	+	—
F12	+	—
H10	+	—
F6G5	+	+
D6E6	+	+
B12	+	+

**Fig. 3.** A thin section of an *A. fumigatus* conidia incubated with monoclonal antibody F12 and stained with immunogold procedure. Note the gold particles in the cytoplasm of the cell ($\times 13\,900$).

reacting with *A. fumigatus* conidia and hyphae consistently showed gold particles bound to the cytoplasm, and not on the cell walls (Figs 3 and 4). The remaining three (B12, F6G5 and D6E6) were reacting with both cell wall and cytoplasm of *A. fumigatus* conidia and hyphae as evidenced by the accumulation of colloidal gold particles on these structures (Fig. 5).

The number of gold particles adhering to the conidia or hyphae varied from one MoAb to the other. In the cytoplasm of *A. fumigatus* hyphae the attachment of the gold particles could be located in the electron dense areas or in areas closely connected with the endoplasmic

reticulum (Fig. 6). On the other hand, the conidia demonstrated gold particles at high magnification on the edge of the outer layer of the cell wall.

Discussion

All three antibodies reacting with cell walls also showed binding to cytoplasmic antigens. Two of the three antibodies showing binding to cell wall showed multiple immunoreactive bands in Western blots, while all three antibodies which reacted with cytoplasmic antigens showed only one band each. The significance of this finding is not known at present although non-specific crossreactivity may be a reason for that. However, other features such as antigenic characteristics and structure also may play a role in this phenomenon.

The present study demonstrates for the first time the presence of cell wall and cytoplasm specific MoAbs to *A. fumigatus* antigens. In previous studies we have used MoAbs to purify specific antigens from *A. fumigatus* [7]. These purified antigens showed binding activity against both IgE and IgG antibodies in the sera of patients with *Aspergillus*-induced diseases [7]. In our recent study, where these sera were used, no qualitative difference could be demonstrated in the antibody response to the conidial and mycelial antigens of *A. fumigatus* [20]. With the availability of cell wall and cytoplasm specific MoAbs the antibody response of patients can be further characterized. In addition, specific antigens involved in the diseases can be evaluated which may provide further understanding of the immunopathogenesis of the disease.

Elevated levels of specific IgG and IgE antibodies to *A. fumigatus* have been reported in several *Aspergillus*-induced diseases [21,22]. By using immunogold and immunogold-silver assay with light microscopy we have demonstrated *A. fumigatus* specific antibodies in the human sera [21, 22]. Gold particles bound to the cell walls of fixed slide cultures of *A. fumigatus* could be visualized directly under a light microscope or after enhancement with silver. Because of the relatively few epitopes reacting with the monoclonal antibodies on the sections of *A. fumigatus* spores and hyphae it is not feasible to demonstrate the attached gold particles by light microscopy. In accordance with the current findings, Kauffman *et al.* [24] have earlier shown that the immunological properties of the *A. fumigatus* spore plasma extracts were comparable with mycelial and/or culture filtrate extracts. In their study, however, components that were released spontaneously from spores demonstrated low biological activity.

Additionally, we have recently demonstrated by using colloidal gold binding that sera from patients with ABPA, invasive aspergillosis and aspergilloma reacted to *A.*

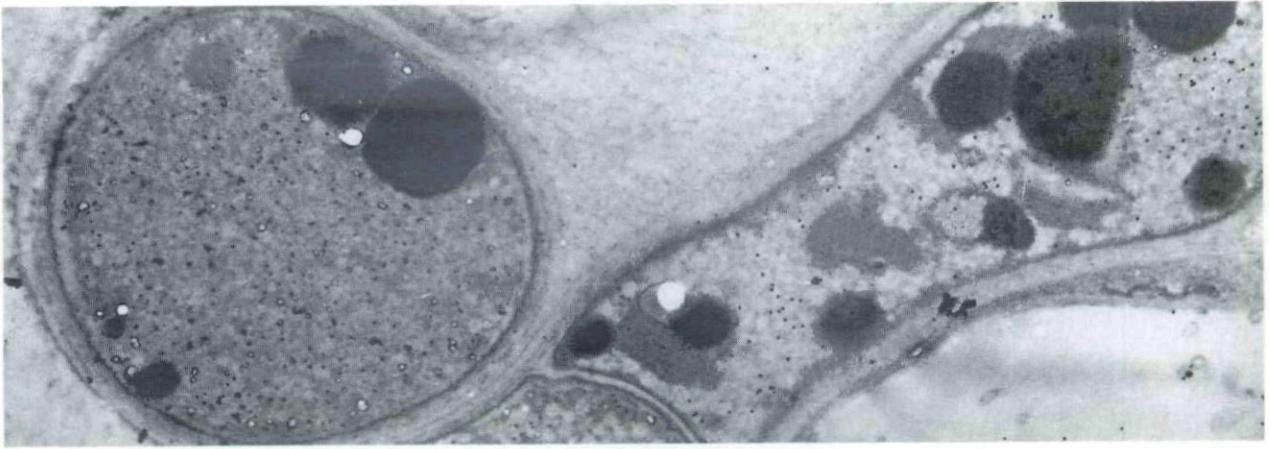


Fig. 4. *A. fumigatus* conidia and hypha with gold particles in the cytoplasm when MoAb C9 and immunogold staining were used ($\times 10\,250$).



Fig. 5. A fragment of *A. fumigatus* hypha after IEM-staining by using F6G5. Gold particles appear both in the cytoplasm and cell wall of the microbe ($\times 13\,990$).

fumigatus spores and hyphae [20]. When thin sections of *A. fumigatus* were incubated with the patients' sera followed by goat anti-human IgG and rabbit anti-goat IgG conjugated with colloidal gold, it was found that the gold particles had the tendency to bind to the cell wall of *A. fumigatus*. On the other hand, no colloidal gold could be demonstrated in the cytoplasm of the microbe. The fixation of the *A. fumigatus* spores and hyphae were similar in our previous experiments [20] and in the present study and therefore, the difference in the reactivity of different monoclonal antibodies is likely due to epitope specificity which is also evidenced by the Western blot results (Fig. 1).

Osmium tetroxide which was used in post-fixation of the microbial specimens in the present study may alter antigenic components in the cell and decrease antibody binding. In previous literature, however, post-osmium

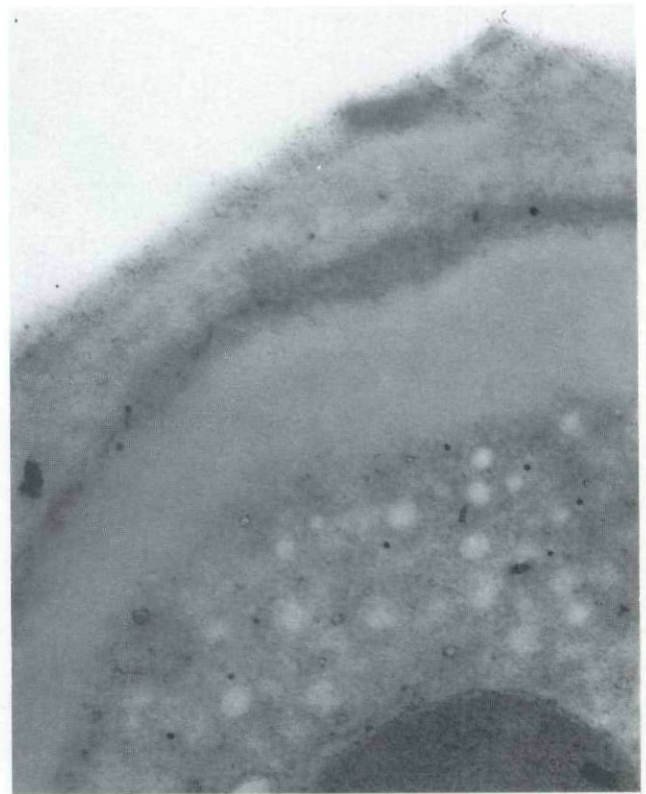


Fig. 6. IEM of *A. fumigatus* conidia incubated with D6E6 showing the attachment of gold particles to the electron dense areas in the cytoplasm ($\times 25\,920$).

reactivation treatment with sodium metaperiodate has been introduced to improve the antigen-antibody binding in osmium treated specimens [25].

IEM has recently been used to detect antigenic determinants of *Streptococcus pneumoniae* [26]. According to

Skov Sørensen *et al.* [26], pneumococcal capsular and cell wall antigens were demonstrable by using specific antibodies, colloidal gold particles and immunostaining carried out before the embedding of the specimens. In concordance with their method, in the present study thin sections were prepared first followed by the immunostaining. By this procedure, close proximity of the intracellular antigenic determinants to the MoAbs and gold particles could be maintained in contrast to immunostaining which has been performed before embedding. Cochrane *et al.* [27] obtained good results when thin sections and post-embedding immunostaining were used to detect antigens of *Plasmodium brasilianum* inside the cytoplasm of infected erythrocytes.

It can be concluded from the present study that the specific reactivity of epitope specific MoAbs can be demonstrated in thin sections of both *A. fumigatus* spores and hyphae using colloidal gold binding in IEM.

Acknowledgments

The authors wish to thank the technical assistance of Ivory Parks, Laura Castillo, Abe Resnick and Nancy Elms. This investigation was partly supported by a grant from the National Institute of Health (AI-23071), by the Veterans Affairs, and by The Finnish Work Environment Fund, The Foundation for the Study of Allergy, The Finnish Anti-Tuberculosis Association and Farmer's Social Insurance Institution, Finland (KER).

References

- Rosenberg M, Patterson R, Murtzer R, Cooper BJ, Roberts M, Harris KE. Clinical and immunological criteria for diagnosis of allergic bronchopulmonary aspergillosis. *Ann Intern Med* 1977; 80:405-14.
- Bardana EJ Jr. The clinical spectrum of aspergillosis 2. Classification and description of saprophytic, allergic and invasive variants of human diseases. *CRC Crit Rev Clin Lab Sci* 1980; 13:85-159.
- Greene R. The pulmonary aspergillosis: three distinct entities or spectrum of disease. *Radiology* 1981; 140:527-30.
- Fink JN. Hypersensitivity pneumonitis. *J Allergy Clin Immunol* 1984; 74:1-4.
- Kurup VP, John KV, Resnick A, Fink JN. A partially purified glycoprotein antigen from *Aspergillus fumigatus*. *Int Arch Allergy Appl Immunol* 1986; 79:263-9.
- Kurup VP, Resnick A, Scribner GH, Gunasekaran M, Fink JN. Enzyme profile and immunochemical characterization of *Aspergillus fumigatus* antigens. *J Allergy Clin Immunol* 1986; 78:1166-73.
- Kurup VP. Production and characterization of a murine monoclonal antibody to *Aspergillus fumigatus* antigen having IgG- and IgE-binding activity. *Int Arch Allergy Appl Immunol* 1988; 86:400-6.
- Kurup VP. Murine monoclonal antibodies binding to the specific antigens of *Aspergillus fumigatus* associated with allergic bronchopulmonary aspergillosis. *J Clin Lab Anal* 1989; 3:116-21.
- Kurup VP, Ramaswamy M, Greenberger PA, Fink JN. Isolation and characterization of a relevant *Aspergillus fumigatus* antigen with IgG- and IgE-binding capacity. *Int Arch Allergy Appl Immunol* 1988; 86:176-82.
- Kim J, Chaparas S, Buckley H. Characterization of antigens from *Aspergillus fumigatus*. IV. Evaluation of commercial and experimental preparations and fractions in the detection of antibody in aspergillosis. *Am Rev Respir Dis* 1979; 120:1305-11.
- Mishra S, Staib F, Rajendran C, Falkem U. Serodiagnostic value of culture filtrate antigens from aspergilli with septate phialides. *Sabouraudia* 1982; 20:63-74.
- Hearn V, MacKenzie D. Analysis of cell wall antigens of *Aspergillus* by two dimensional immunoelectrophoresis. *J Med Microbiol* 1981; 14:119.
- Piechura JE, Riefel RS, Daft LJ. Biochemical and immunochemical analyses of detergent solubilized antigens from membrane vesicles of *Aspergillus fumigatus*. *Can J Microbiol* 1987; 33:955-62.
- Kurup VP. Enzyme-linked immunosorbent assay in the detection of specific antibodies against *Aspergillus fumigatus* in patients sera. *Zbl Bakteriell Hyg (A)* 1986; 261: 509-16.
- Kurup VP, Elms N, Fink JN. Characterization of a monoclonal antibody against Concanavalin A binding antigen of *Aspergillus fumigatus*. *Hybridoma* 1991; 10:382-93.
- Kurup VP, Greenberger PA, Fink JN. Antibody response to low molecular weight antigens of *Aspergillus fumigatus* in allergic bronchopulmonary aspergillosis. *J Clin Microbiol* 1989; 27:1312-6.
- Reijula KE. Two common fungi associated with Farmer's lung: fine structure of *Aspergillus fumigatus* and *Aspergillus umbrosus*. *Mycopathologia* 1991; 113:143-9.
- Frens G. Controlled nucleation for the regulation of the particle size in monodisperse gold suspension. *Nature (Phys Sci)* 1983; 241:20-4.
- Geoghegan WD, Ackerman GA. Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin at colloidal gold for the indirect detection on Concanavalin A, wheat germ agglutinin and goat antihuman immunoglobulin G on cell surfaces at the electron microscopic level: a new method, theory and application. *J Histochem* 1977; 25:1187-200.
- Reijula KE, Kurup VP, Fink JN. Ultrastructural demonstration of specific IgG and IgE antibodies binding to *Aspergillus fumigatus* from patients with aspergillosis. *J Allergy Clin Immunol* 1991; 87:683-8.
- Brummund W, Resnick A, Fink JN, Kurup VP. *Aspergillus fumigatus* specific antibodies in allergic bronchopulmonary aspergillosis and aspergilloma. Evidence for a polyclonal response. *J Clin Microbiol* 1987; 25:5-9.
- Reijula KE, Kurup VP, Fink JN. The detection of *Aspergil-*

- lus fumigatus* antibodies in sera by immunogold-silver staining. J Microb Methods 1992, in press.
- 23 Gugnani HC, Reijula KE, Kurup VP, Fink JN. Detection of IgG and IgE antibodies to *Aspergillus fumigatus* in human sera by immunogold assay. Mycopathologia 1990; 109:33-40.
- 24 Kauffman HF, van der Heide S, Beaumont F, de Monchy JGR, de Vries K. The allergenic and antigenic properties of spore extracts of *Aspergillus fumigatus*: a comparative study of spore extracts with mycelium and culture filtrate extracts. J Allergy Clin Immunol 1984; 73:567-73.
- 25 Bendayan M, Zollinger M. Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. J Histochem Cytochem 1983; 31:101-9.
- 26 Skov Sørensen UB, Blom J, Birch-Andersen A, Henrichsen J. Ultrastructural localization of capsules, cell wall polysaccharide, cell wall proteins, and F antigen pneumococci. Infection and Immunity 1988; 1890-96.
- 27 Cochrane AH, Matsumoto Y, Kumboij KK, Mara M, Nussenzweig RS, Aikawa M. Membrane-associated antigens of blood stages of *Plasmodium brasilianum* parasite. Infection and Immunity 1988; 2080-8.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.