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Development of an Immunochromatographic Lateral-Flow Device for Rapid Serodiagnosis of Invasive Aspergillosis[▽]

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***Aspergillus fumigatus* is a cosmopolitan saprotrophic fungus that is second only to *Candida* species as a cause of invasive fungal infections in immunocompromised humans. Current immunodiagnostic tests for invasive aspergillosis (IA) are based on the detection of circulating galactomannan (GM) in a patient's serum by using a rat monoclonal antibody (MAb), EB-A2, that binds to tetra (1→5)-β-D-galactofuranoside, the immunodominant epitope in GM. The potential cross-reactivity of MAb EB-A2 with non-*Aspergillus* fungi, with contaminating GM in β-lactam antibiotics and foodstuffs, and with bacterial lipoteichoic acids has prompted efforts to discover non-GM antigens that can act as surrogate markers for the diagnosis of IA. This paper describes the development of a mouse MAb, JF5, that binds to a protein epitope present on an extracellular glycoprotein antigen secreted constitutively during the active growth of *A. fumigatus*. The MAb was used to develop an immunochromatographic lateral-flow device (LFD) for the rapid (15-min) detection of *Aspergillus* antigens in human serum. The test is highly specific, reacting with antigens from *Aspergillus* species but not with antigens from a large number of clinically important fungi, including *Candida* species, *Cryptococcus neoformans*, *Fusarium solani*, *Penicillium marneffei*, *Pseudallescheria boydii*, and *Rhizopus oryzae*. The LFD was able to detect circulating antigen in serum samples from patients suspected of having or shown to have IA on the basis of their clinical symptoms and results from tests for GM and fungal (1→3)-β-D-glucan. The ease of use of the LFD provides a diagnostic platform for the routine testing of vulnerable patients who have an elevated risk of IA.**

The dramatic increase in the numbers of opportunistic infections of humans caused by *Aspergillus* species over the last decade is associated with a rise in the numbers of solid-organ transplants and the use of aggressive cancer therapies and other immunomodulating treatments (4, 15). The rate of mortality due to invasive aspergillosis (IA) has increased by 357% over the last 25 years; and IA has become one of the leading causes of death in immunocompromised patients, with mortality rates ranging from 60 to 90% (21), even following the recent introduction of new broad-spectrum antifungal agents. The most common species of *Aspergillus* causing invasive disease include *Aspergillus fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans* (7, 25). Other less common species can also cause the disease, but *A. fumigatus* accounts for ~90% of all cases of IA (7).

In the absence of a single "gold standard" test for diagnosis of the disease, the definitive diagnosis of IA encompasses data from clinical, radiological, serological, molecular biological, mycological, and histopathological sources. It is imperative that a diagnosis be made without delay, since the prognosis worsens significantly in the absence of recognition and effective treatment. The rapid detection of IA by immunodiagnostic methods has centered around the detection of fungal galactomannan (GM) (16, 24, 25). Monoclonal antibodies (MAbs) have successfully been used to detect GM, and they form the basis of commercial laboratory-based tests, such as the Platelia *Aspergillus* enzyme-linked immunosorbent assay (ELISA) kit that incorporates a rat MAb (MAb EB-A2) directed against tetra (1→5)-β-D-galactofuranoside, the immunodominant epitope in the antigen (23, 31, 32). Immunoassays for GM detection are a significant asset for the management of patients at risk of IA because of detection of the antigen in the early stages of disease progression. Despite their widespread use, recent studies have revealed significant variation in performance. While the specificity of the GM assay is consistently >85%, the sensitivity of the assay can vary considerably from 29% to 100% and the rate of false-positive reactivity can vary from 5% in adults to 83% in newborn babies (39). False-positive results have been attributed to the cross-reaction of MAb EB-A2 with GM from non-*Aspergillus* fungi (8, 12, 25, 34, 39); with galactoxylomannan from *Cryptococcus neoformans* (5, 6); with lipoteichoic acid from intestinal bifidobacteria in the gastrointestinal microbiota of neonates (22); with the cancer prodrug cyclophosphamide (10); and with the GM in food, drink, and infant milk formulas (1). Contamination of β-lactam antibiotics with *Penicillium* GM may account for the serum reactivity of patients receiving piperacillin-tazobactam or amoxicillin-clavulanic acid (2, 20, 39, 40), although these reports have been disputed (46). There is therefore scope in IA immunodiagnostics for tests that employ MAbs directed at epitopes other than those present on GM. While a "panfungal" test that detects fungal (1→3)-β-D-glucan has been used for the diagnosis of invasive fungal infections (24, 25), its lack of specificity means that it is unable to discriminate between *Aspergillus* species

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and other opportunistic pathogens, which compromises the ability to select the most appropriate antifungal agent. In contrast, an ELISA used to detect the Afmp1p cell wall antigen of *A. fumigatus* in a patient's serum provides a high degree of specificity but does not allow the detection of IA caused by other *Aspergillus* species (45). Furthermore, combinations of antibody and antigen testing of serum samples are required to provide serodiagnostic sensitivities for *A. fumigatus* IA detection comparable to those of tests for GM.

The development of a noninvasive immunodiagnostic test that is rapid, reliable, and relatively inexpensive and that detects surrogate (non-GM and non-Afmp1p) markers for IA would allow the routine testing of vulnerable patients who have an elevated risk of infection, such as allogeneic hematopoietic stem cell transplant recipients, patients with hematological malignancies, and recipients of solid-organ transplants, especially of the lung. The aim of this paper is to report on the development of a mouse hybridoma cell line secreting an *Aspergillus* protein-specific MAb (MAb JF5) and its utilization in the development of a lateral-flow device (LFD) for the rapid serodiagnosis of IA. The assay exploits the lateral-flow technology that has been used to date in tests for the detection of viruses, bacteria, and toxins (11, 13, 28–30) and, most famously, for the home pregnancy tests first introduced by Unipath in 1988. While immunochromatographic assays have been developed for the identification of *Candida* species (19) and for the detection of fungi in soil (36, 37), this is the first time, to the best of the author's knowledge, that an LFD has been developed for the detection of *Aspergillus* antigens in human serum.

Current diagnostic tests for IA are confined to laboratories equipped to perform tests for the detection of GM or β-glucan or nucleic acid-based diagnostic tests. The simplicity of the LFD format allows it to be used with minimal training and provides an additional diagnostic platform for the management of IA in high-risk patient groups. The ability of the LFD to detect *Aspergillus* antigens in clinical samples is demonstrated with sera from IA patients.

MATERIALS AND METHODS

Fungal culture. All fungi were cultured on Sabouraud agar (SA) under a 16-h fluorescent light regimen.

Development of MAb, preparation of immunogen, and immunization regimen. Mice were immunized with lyophilized mycelium (LM) of *A. fumigatus* AF293. Minimal medium [19 mM $(NH_4)_2PO_4$, 0.5% (wt/vol) yeast extract, 7 mM sodium citrate, 2 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $CaCl_2 \cdot 2H_2O$, and 50 mM glucose adjusted to pH 5.5 with 1 N HCl] was sterilized by autoclaving at 121°C for 15 min. Three-week-old SA petri dish cultures of the fungus were flooded with 20 ml distilled H_2O (dH_2O), and the conidia were suspended by gentle agitation with an inoculation loop. Spore suspensions were filtered through Miracloth to remove the mycelium, and the filtrate containing the conidia was transferred to 1.5-ml microcentrifuge tubes. The conidia were washed three times with dH_2O by repeated vortexing and centrifugation at 12,000 × *g* for 5 min and were finally suspended in dH_2O to give a concentration of 10^6 conidia/ml solution. Flasks containing 150 ml of medium were inoculated with 200 μl of the conidial suspension and incubated with shaking (150 rpm) for 24 h at 37°C. The mycelium was collected by filtering the contents of each flask through Miracloth, snap frozen in liquid N_2, and lyophilized.

One milligram of LM was suspended in 1 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.2]). Six-week-old BALB/c white female mice were given four intraperitoneal injections (300 μl per injection) of immunogen at 2-week intervals and a single booster injection 5 days before fusion.

Production and screening of hybridomas and determination of antibody specificity. Hybridoma cells were produced by the method described elsewhere (35), and the supernatants were screened by ELISA against soluble antigens extracted from LM in PBS and immobilized to the wells of Maxisorp microtiter plates (50 μl per well). For antibody specificity tests, fungi were grown on SA and surface washings prepared in PBS as described by Thornton (35). Protein concentrations, determined spectrophotometrically at 280 nm (Nanodrop; Agilent Technologies Limited, Berkshire, United Kingdom), were adjusted to 64 μg/ml buffer, and 50-μl volumes were used to coat the wells of microtiter plates. After coating of the plates overnight at 4°C, the wells were washed four times with PBS containing 0.05% (vol/vol) Tween 20 (PBST) and once each with PBS and dH_2O and air dried at 23°C in a laminar-flow hood. The plates were stored in sealed plastic bags at 4°C in preparation for screening of the hybridoma supernatants by ELISA, as described below.

ELISA. Wells containing immobilized antigens were successively incubated with hybridoma supernatant for 1 h, followed by goat anti-mouse polyvalent (immunoglobulin G [IgG], IgA, and IgM classes) peroxidase conjugate (Sigma Chemical Company, Poole, United Kingdom) diluted 1 in 1,000 in PBST for a further hour. Bound antibody was visualized by incubation of the wells with tetramethylbenzidine substrate solution for 30 min, and the reactions were stopped by the addition of 3 M H_2SO_4. Absorbance values were determined at 450 nm with an MRX automated microplate reader (Dynex Technologies, Billingshurst, United Kingdom). The wells were given four 5-min rinses with PBST between incubations. Working volumes were 50 μl per well, and control wells were incubated with tissue culture medium (TCM) containing 10% (vol/vol) fetal calf serum. All incubation steps were performed at 23°C in sealed plastic bags. The threshold for the detection of antigen by ELISA was determined from the control means (2× TCM absorbance values) (33). These values were consistently in a range from 0.050 to 0.100. Consequently, absorbance values >0.100 were considered positive for the detection of antigen.

Determination of Ig subclass and cloning procedure. The Ig class of the MAbs was determined with a commercial mouse MAb isotyping kit (ISO-1), according to the manufacturer's instructions (Sigma). Hybridoma cell lines were cloned by limiting dilution; and the cell lines were grown in bulk in a nonselective medium, preserved by slowly freezing them in fetal bovine serum-dimethyl sulfoxide (92:8 [vol/vol]), and stored in liquid nitrogen.

Epitope characterization by protease digestion. Microtiter wells containing immobilized antigens were incubated with pronase (0.25 U per well; Protease XIV; Sigma) or trypsin (Sigma) solution (1 mg/ml in PBS) at 37°C or 4°C for 5 h and washed three times with PBS. Wells incubated with trypsin were treated for 10 min with a 0.1-mg/ml solution of trypsin inhibitor (Sigma) and given three more washes with PBS. Controls received PBS without pronase or trypsin and inhibitor but were otherwise treated similarly. The wells were assayed by ELISA with MAb JF5 as described above. There were six replicates for each treatment.

Epitope characterization by periodate oxidation. The immobilized antigens were treated with sodium *meta*-periodate (20 mM $NaIO_4$ in 50 mM sodium acetate buffer [pH 4.5]), whereas the control wells received only buffer. After incubation for the appropriate time period in darkness at 4°C, the wells were washed three times with PBS and assayed by ELISA with MAb JF5 as described above. There were four replicates for each treatment.

Antigen purification, PAGE, and Western blotting. Antigen was purified from PBS extracts of LM by affinity chromatography with a Protein A IgG Plus Orientation kit (Pierce Biotechnology, Rockford, IL) containing immobilized MAb JF5. Ascitic fluid was prepared from JF5 hybridoma cells in female BALB/c mice (Eurogentec s.a., Belgium). The mice were injected with 10^6 hybridoma cells washed in PBS, and after 3 weeks, approximately 5 ml of ascitic fluid was recovered from each mouse and was stored at −20°C prior to use. For preparation of the affinity column, 15 μl of ascitic fluid was mixed with 2 ml of binding buffer and the solution was applied to the protein A-agarose matrix. Crude PBS antigen extract was then incubated with the immobilized antibody and bound antigen was eluted with 0.1 M glycine-HCl (pH 2.8) buffer. Polyacrylamide gel electrophoresis (PAGE) was carried out by using the system of Laemmli (14) with 4 to 20% (wt/vol) gradient polyacrylamide gels (Bio-Rad Laboratories Limited, Hemel Hempstead, United Kingdom) under denaturing conditions. Purified antigen was mixed with Laemmli buffer and denatured by heating at 95°C for 10 min in the presence of β-mercaptoethanol before it was loaded onto the gel. The proteins were separated for 1.5 h at 23°C (165 V). Prestained, broad-range markers (Bio-Rad) were used for molecular mass determinations. For Western blotting, the separated proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were washed three times with PBS and then blocked for 16 h at 4°C with PBS containing 1% (wt/vol) bovine serum albumin (BSA). The blocked membranes

TABLE 1. Details of organisms and results of ELISA specificity tests

Organism	Isolate no.	Source[a]	Absorbance (450 nm)[b]
Absidia corymbifera	101040	CBS	0.027
Absidia glauca	1	CRT	0.032
Absidia spinosa	3	CRT	0.000
Acremonium atrogriseum	306.85	CBS	0.083
Acremonium blochii	424.93	CBS	0.006
Alternaria alternata	42	CRT	0.000
Apophysomyces elegans	658.93	CBS	0.007
Subgenus *Aspergillus*			
Section *Aspergillus*			
Eurotium amstelodami	34	CRT	0.866
Section *Restricti*			
Aspergillus restrictus	116.50	CBS	0.938
Subgenus *Fumigati*			
Section *Fumigati*			
Aspergillus fumigatus	181	CRT	1.020
Aspergillus fumigatus	AFC	CRT	0.935
Aspergillus fumigatus	AF293	SK	1.213
Neosartorya fischeri var. *fischeri*	681.77	CBS	1.105
Section *Cervini*			
Aspergillus cervinus	537.65	CBS	0.667
Subgenus *Ornati*			
Section *Ornati*			
A. ornatus (*Hemicarpenteles ornatus*)	184	CRT	1.381
Subgenus *Clavati*			
Section *Clavati*			
Aspergillus clavatus	514.65	CBS	1.307
Subgenus *Nidulantes*			
Section *Nidulantes*			
Aspergillus nidulans (*Emericella nidulans* var. *nidulans*)	542.83	CBS	1.133
Aspergillus nidulans	A4	FGSC	1.237
Aspergillus nidulans	A26	FGSC	1.075
Emericella quadrilineata	591.65	CBS	1.045
Section Versicolores			
Aspergillus versicolor	599.65	CBS	1.120
Section *Usti*			
Aspergillus ustus	209.92	CBS	0.510
Section *Terrei*			
Aspergillus terreus var. *terreus*	601.65	CBS	1.186
Section *Flavipedes*			
Aspergillus niveus (*Fennelia nivea*)	261.73	CBS	1.085
Subgenus *Circumdati*			
Section *Wentii*			
Aspergillus wentii	229.67	CBS	0.000
Section *Flavi*			
Aspergillus flavus	91856iii	IMI	1.053
Aspergillus oryzae	29	CRT	0.963
Section *Nigri*			
Aspergillus niger	102.40	CBS	1.433
Aspergillus niger	121.49	CBS	1.155
Aspergillus niger	522.85	CBS	1.057

Continued on following page

TABLE 1—*Continued*

Organism	Isolate no.	Source[a]	Absorbance (450 nm)[b]
Aspergillus niger	553.65	CBS	1.066
Section *Circumdati*			
Aspergillus ochraceous	625.78	CBS	1.249
Section *Candidi*			
Aspergillus candidus	266.81	CBS	0.541
Aureobasidium pullulans	657.76	CBS	0.015
Botrytis cinerea	R2	CRT	0.077
Candida albicans	SC5314	SB	0.000
Candida dubliniensis	8500	CBS	0.015
Candida glabrata	4692	CBS	0.000
Chaetomium globosum	147.51	CBS	0.013
Cladosporium herbarum	159.59	CBS	0.067
Cryptococcus neoformans	5728	CBS	0.010
Cryptococcus neoformans	7779	CBS	0.009
Cunninghamella bertholletiae	182.84	CBS	0.012
Exophiala dermatitidis	153.94	CBS	0.024
Fusarium oxysporum f. sp. *melonis*	422.90	CBS	0.000
Fusarium oxysporum f. sp. *pisi*	260.50	CBS	0.005
Fusarium solani	224.34	CBS	0.034
Fusarium solani	80	CRT	0.056
Fusarium solani var. *petrophilum*	102256	CBS	0.006
Fusarium verticillioides	539.79	CBS	0.000
Geotrichum capitatum	327.86	CBS	0.014
Mucor fragilis	4	CRT	0.033
Mucor hiemalis var. *silvaticus*	50	CRT	0.002
Paecilomyces variotii	339.51	CBS	0.163
P. variotii	17.1	CRT	0.143
Penicillium brevicompactum	210.28	CBS	0.571
Penicillium cinnabarinum	39	CRT	0.885
Penicillium chrysogenum	105	CRT	1.248
Penicillium citrinum	139.45	CBS	0.556
Penicillium cyclopium	123.14	CBS	0.630
Penicillium dierckxii	250.66	CBS	0.629
Penicillium expansum	106	CRT	1.141
Penicillium jensenii	43	CRT	1.115
Penicillium islandicum	338.48	CBS	0.004
Penicillium marneffei	101038	CBS	0.093
Penicillium marneffei	669.95	CBS	0.057
Penicillium melinii	218.30	CBS	0.486
Penicillium purpurogenum	364.48	CBS	0.006
Penicillium roqueforti	221.30	CBS	0.347
Penicillium simplicissimum	220.30	CBS	0.500
Penicillium spinulosum	108	CRT	1.290
Penicillium variabile	385.48	CBS	0.037
Phialophora verrucosa	225.97	CBS	0.021
Pseudallescheria boydii	835.96	CBS	0.004
Rhizomucor miehei	360.92	CBS	0.005
Rhizopus microsporus var. *rhizopodiformis*	102277	CBS	0.020
Rhizopus oryzae	146.90	CBS	0.016
Rhizopus oryzae	395.54	CBS	0.010
Rhizopus sexualis var. *sexualis*	209090	IMI	0.000
Rhizopus stolonifer	G1	CRT	0.000
Saksenaea vasiformis	133.90	CBS	0.030
Scedosporium prolificans	742.96	CBS	0.010
Scedosporium prolificans	100391	CBS	0.025
Stachybotrys chartarum	485.48	CBS	0.017
Talaromyces flavus	437.62	CBS	0.051
Talaromyces stipitatus	266.91	CBS	0.046
Trichoderma longibrachiatum	446.95	CBS	0.000
Trichoderma pseudokoningii	500.94	CBS	0.000
Verticillium coccosporum	GD2/B8	CRT	0.000
Wallemia sebi	196.56	CBS	0.043

[a] CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; FGSC, Fungal Genetics Stock Centre, University of Missouri, Kansas City; CRT, C. R. Thornton; IMI, International Mycological Institute, Egham, England; SB, S. Bates, School of Biosciences, University of Exeter; SV, S. Krappman, Institute of Microbiology and Genetics, Department of Molecular Microbiology and Genetics, Georg-August-University, Gottingen, Germany.

[b] Each value represents the mean of replicate values. Threshold absorbance value for detection of antigen, ≥0.100.

were incubated with the MAb JF5 supernatant diluted 1 in 2 with PBS containing 0.5% (wt/vol) BSA (PBSA) for 2 h at 23°C. After the membranes were washed three times with PBS, they were incubated for 1 h with goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma) diluted 1 in 15,000 in PBSA. The membranes were washed twice with PBS and once with PBST, and the bound antibody was visualized by incubation in substrate solution. The reactions were stopped by immersion in dH_2O and air dried between sheets of Whatman filter paper. Modification of the JF5 antigen with peptide-*N*-glycosidase (PNGase) was carried out prior to electrophoresis and Western blotting, according to procedures described elsewhere (3).

Immunofluorescence and immunogold electron microscopy of *A. fumigatus* conidia and germlings. Immunogold labeling was performed with germlings of *A. fumigatus* AF293. Germlings were prepared by incubating washed conidia in normal human serum (Biosera, Ringmer, United Kingdom) or in sterile filtered (pore size, 0.2 μm) 1% (wt/vol) glucose solution for 16 h at 37°C with gentle mixing. The germlings were pelleted by centrifugation, and low-temperature embedding of the material was carried out as described elsewhere (38). Immunolabeling was carried out with MAb JF5 and goat anti-mouse 20-nm-diameter gold conjugate (British Biocell International, Cardiff, Wales) as the secondary reporter molecule. Control grids were incubated with TCM instead of the MAb supernatant but were otherwise treated the same. For the immunofluorescence studies, the washed conidia were suspended in glucose solution and transferred to the wells of multiwell slides. After incubation at 37°C for 16 h, the slides were air dried and fixed as described by Thornton (35). The wells were incubated for 1 h with 50 μl of the MAb JF5 supernatant or TCM only. The slides were washed three times with PBS with gentle agitation and incubated for a further 30 min with goat anti-mouse polyvalent fluorescein isothiocyanate conjugate (Sigma) diluted 1 in 40 in PBS. The slides were given three 5-min rinses with PBS, and the wells were overlaid with coverslips mounted in PBS-glycerol mounting medium (Sigma). The slides were examined with a Zeiss Axiophot microscope fitted with epifluorescence by using a UV excitation filter of 365 nm and an absorption filter of 420 nm. All incubation steps were performed at 23°C in a moist environment, and the slides were stored at 4°C in the dark in petri dishes containing moistened Whatman no. 1 filter paper.

Configuration of the LFD. The LFD consisted of a G&L Diecut 1734 backing card; Whatman 17chr and 1281 top and sample pads, respectively; and a Whatman Immunopore 5 μM nitocellulose membrane. MAb JF5 was conjugated to 40-nm-diameter gold particles, applied to the release pad at 100 units of conjugate/cm, and dried for 16 h at 37°C. The test line antibody consisted of MAb JF5 at 0.5 mg protein/ml of PBS containing 1% (wt/vol) BSA, while a commercial rabbit anti-mouse Ig acted as the control line.

Sensitivity and specificity of the LFD. Affinity-purified antigen (protein concentrations were determined as described above) was diluted into normal human serum or PBS, and 100-μl samples were applied to the LFD. Unspiked serum and PBS acted as the negative controls. After 15 min, the results were recorded as positive for the presence of *Aspergillus* antigen (two lines) or negative (a single control line only). The specificity of the LFD was determined by growing fungi in normal human serum. Replicate 1-ml serum samples contained in 1.5-ml Eppendorf tubes were inoculated with 10^4 washed conidia from filamentous fungi (*Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Fusarium solani*, *Pseudallescheria boydii*, and *Rhizopus oryzae*) or an equivalent number of washed yeast cells (*Candida albicans* and *Cryptococcus neoformans*). The tubes were incubated at 37°C with shaking (100 rpm) for 48 h, and fungal propagules were precipitated by centrifugation. One hundred-microliter samples of neat, cell-free supernatants were applied to LFD devices and the results were recorded as described above. The growth of the filamentous fungi and the yeast *Candida albicans* was determined by visual appraisal of hyphal development or by increases in the turbidity of serum samples (*C. neoformans*). Unspiked serum incubated under the same conditions acted as the negative control.

Further tests of the specificity of the LFD were conducted with serum containing the β-lactam antibiotics penicillin G (Melford Laboratories Limited, Ipswich, United Kingdom), amoxicillin (Fluka), and piperacillin (Sigma); the β-lactamase inhibitor tazobactam (Sigma); the cancer prodrug cyclophosphamide (Sigma); and lipoteichoic acids from the bacteria *Enterococcus faecalis* and *Staphylococcus aureus* (both from Sigma). Following reconstitution, 100-μl volumes of solutions containing 5 mg of solid/ml serum (lipoteichoic acids) or 50 mg solid/ml serum (antibiotics, tazobactam, and cyclophosphamide) were applied to the LFDs and the results were recorded as described above. Unspiked serum acted as the negative control, while serum samples containing purified antigen and test chemicals acted as positive controls. Three replicates were performed for each test.

LFD detection of antigen in sera from patients with IA. The ability of the LFD to detect circulating antigen in humans with IA was tested with sera collected from patients with known or suspected IA and from healthy controls. The samples were kindly provided during a blind assessment of assay sensitivity and specificity conducted in collaboration with Elizabeth Johnson (Bristol Health Protection Agency). The samples had previously been tested by using the Platelia GM enzyme immunoassay (EIA) and a panfungal β-glucan test (Fungitell). One hundred-microliter samples of undiluted serum or serum diluted 1 in 10 in normal human serum were applied to the LFDs, and the results were recorded as described above. Three replicates were performed for each sample.

TABLE 2. Absorbance values from ELISAs with protease-treated antigens by using MAb JF5 of IgG3

Temp (°C)	Absorbance (450 nm)[a]			
	Pronase	Pronase control	Trypsin	Trypsin control
4	0.559 ± 0.022	1.134 ± 0.048	1.097 ± 0.002	1.217 ± 0.046
37	0.399 ± 0.006	1.088 ± 0.025	0.701 ± 0.003	1.181 ± 0.050

[a] Each value represents the mean of replicated values ± standard error.

RESULTS

Production of hybridoma cell lines and isotyping of MAbs. A single fusion was performed. Cell lines were selected for further study on the basis of the strength of the MAb reaction in the ELISA. The JF5 cell line was selected and was subcloned three times. The MAb from the subcloned JF5 cell line belonged to the IgG3 class.

MAb specificity tests. MAb JF5 was tested for specificity against a wide range of related and unrelated fungi (Table 1). It reacted with antigens from *Aspergillus* species and related fungi from the teleomorphic genera *Emericella*, *Eurotium*, and *Neosartorya*. It cross-reacted with antigens from certain *Penicillium* species but not with *Penicillium* species in the subgenus *Biverticillium* or teleomorphic *Talaromyces* species whose *Penicillium* anamorphs belong to this subgenus. It cross-reacted weakly with antigens from the closely related fungus *Paecilomyces variotii* but did not react with antigens from a wide range of unrelated fungi, including the well-documented invasive pathogens *Candida albicans*; *Cryptococcus neoformans*; or the emerging pathogens *Fusarium solani*, *Pseudallescheria boydii*, and *Rhizopus oryzae* (9, 26, 41, 42).

Characterization of antigen and effects of protease and periodate. A reduction in MAb binding in the ELISA following treatment with pronase shows that its epitope consists of protein. Consequently, the reductions in MAb JF5 binding following the digestion of immobilized antigen with pronase showed that the antibody binds to a protein epitope (Table 2). More specifically, the sensitivity of the epitope to trypsin indicated that JF5 binds to a protein epitope containing positively charged lysine and arginine side chains. Reductions in antibody binding following chemical digestion of an antigen with periodate shows that its epitope is carbohydrate. Consequently, the lack of reduction of JF5 binding in the ELISA following periodate treatment of immobilized antigen (Table 3) showed that its epitope does not contain carbohydrate moieties.

PAGE and Western blotting. The affinity-purified antigen eluted from the column as a single peak containing 0.340 mg protein/ml of buffer. The diffuse binding pattern in Western blotting studies showed that the antigen bound by MAb JF5 is glycosylated and is a pattern consistent with the binding of

TABLE 3. Absorbance values from ELISAs with periodate-treated antigens by using MAb JF5 of IgG3

Time (h)	Absorbance (450 nm)[a]	
	Periodate	Control
20	1.212 ± 0.013	1.245 ± 0.013
5	1.180 ± 0.010	1.204 ± 0.010
4	1.178 ± 0.012	1.219 ± 0.010
3	1.180 ± 0.014	1.211 ± 0.014
2	1.205 ± 0.015	1.234 ± 0.009
1	1.223 ± 0.013	1.259 ± 0.008

[a] Each value represents the mean of replicated values ± standard errors.

MAbs to extracellular glycoproteins in *A. fumigatus* (32). Deglycosylation of the antigen with the enzyme PNGase showed that the protein moiety of the glycoprotein bound by MAb JF5 has an approximate molecular mass of 40 kDa and has an *N*-glycosylated component (Fig. 1, lane B).

Immunofluorescence and immunogold electron microscopy of conidia and germlings. Immunofluorescence studies showed that the antigen was absent from the surface of ungerminated spores but was present on the hyphal surface of germlings and was secreted from the hyphal tip (Fig. 2). Immunogold electron microscopy showed that the antigen was present in the hyphal cell wall, in septa, and in a capsule-like layer surrounding cells (Fig. 3).

Sensitivity and specificity of the LFD. There was strong detection of the affinity-purified antigen in the LFD tests (Fig. 4), with an assay sensitivity of 37 ng protein per ml of serum. In PBS only, the sensitivity of the assay was 1.25 ng protein per ml. After 48 h of growth of the fungi in human serum, there was strong detection of the antigen in serum spiked with 10^4 conidia of *A. fumigatus* AF293 (Fig. 4) and with other *Aspergillus* species (results not shown). No antigen was detected in serum inoculated with the other fungi tested (Fig. 4), despite prolific growth. No false-positive reactions were exhibited with the β-lactam antibiotics tested or with tazobactam, cyclophosphamide, or bacterial lipoteichoic acids. The chemicals did not inhibit the detection of the purified antigen (results not shown).

Detection of antigen in IA sera. The JF5 antigen was detected in sera from patients with known or probable IA infection (Table 4). No false-negative results were found with sera from healthy individuals. LFD test results were similar to those for GM detection by the Platelia EIA. However, three of the samples (samples 1655, 1665, and 1667) from patients diagnosed with IA on the basis of clinical symptoms gave positive reactions with the LFD but were negative by the GM test. One of these samples (sample 1655) and two others (samples 1537 and 1538) gave negative LFD reactions when they were used undiluted but gave positive reactions when they were diluted 10-fold in normal serum. This was likely due to a high-dose hook effect in which the high serum antigen concentrations impaired antigen-antibody binding. The results for all other samples were the same when they were used neat or diluted. Examples of negative and positive reactions with sera are shown in Fig. 4.

DISCUSSION

This paper describes the development of an LFD for the rapid serodiagnosis of IA. The LFD incorporates a murine MAb, MAb JF5, raised against a protein epitope on an N-linked glycoprotein antigen present in the hyphal cell wall and septa of *A. fumigatus* and that is secreted constitutively at the hyphal apex. Specificity tests showed that MAb JF5 reacted strongly with antigens from species of fungi in the genus *Aspergillus* and the closely related species *Eurotium amstelodami* (teleomorph of *Aspergillus amstelodami*), *Emericella nidulans* (teleomorph of *Aspergillus nidulans*), and *Neosartorya fischeri* (teleomorph of *Aspergillus fischeri*). It cross-reacted weakly with antigens from the closely related fungus *Paecilomyces variotii*. Cross-reaction with antigens from a number of *Penicillium* species was also exhibited, but not with antigens from *Penicillium islandicum*, *Penicillium purpurogenum*, or *Penicillium variabile* or with the endemic human pathogen *Penicillium marneffei*, a species of *Penicillium* belonging to the subgenus *Biverticillium* (17). This was confirmed by the absence of cross-reactivity with *Talaromyces* species (*Talaromyces flavus* and *Talaromyces stipitatus*) whose *Penicillium* anamorphs belong to this subgenus (17, 43). Recently, Schmechel et al. (27) showed that mouse MAbs raised against spores of *Aspergillus versicolor* also cross-reacted with *Penicillium* antigens but, similarly, did not cross-react with antigens from these three species. Analogous cross-reactivity of the antigalactomannan rat MAb EB-A2 with *Penicillium* and *Paecilomyces* species has also been shown (32, 34). However, unlike MAb EB-A2, the MAb reported on here, MAb JF5, does not cross-react with *Acremonium*, *Alternaria*, *Botrytis*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Wangiella*, or *Wallemia* species,

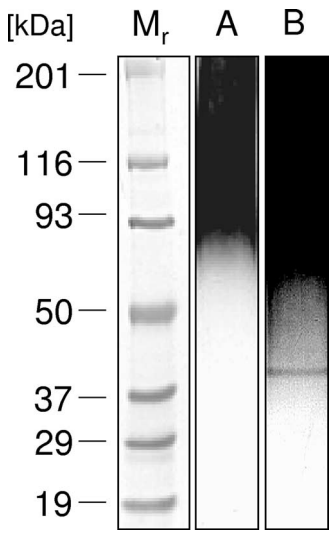

FIG. 1. Analysis of affinity-purified antigen by PAGE and Western blotting. Lane M_r, molecular mass marker; lane A, Western immunoblot with MAb JF5 after separation of purified antigen by sodium dodecyl sulfate-PAGE under reducing conditions; the well was loaded with 0.2 μg of protein; lane B, Western immunoblot with MAb JF5 after treatment of purified antigen with PNGase and separation by sodium dodecyl sulfate-PAGE under denaturing conditions; the well was loaded with 0.2 μg of protein.

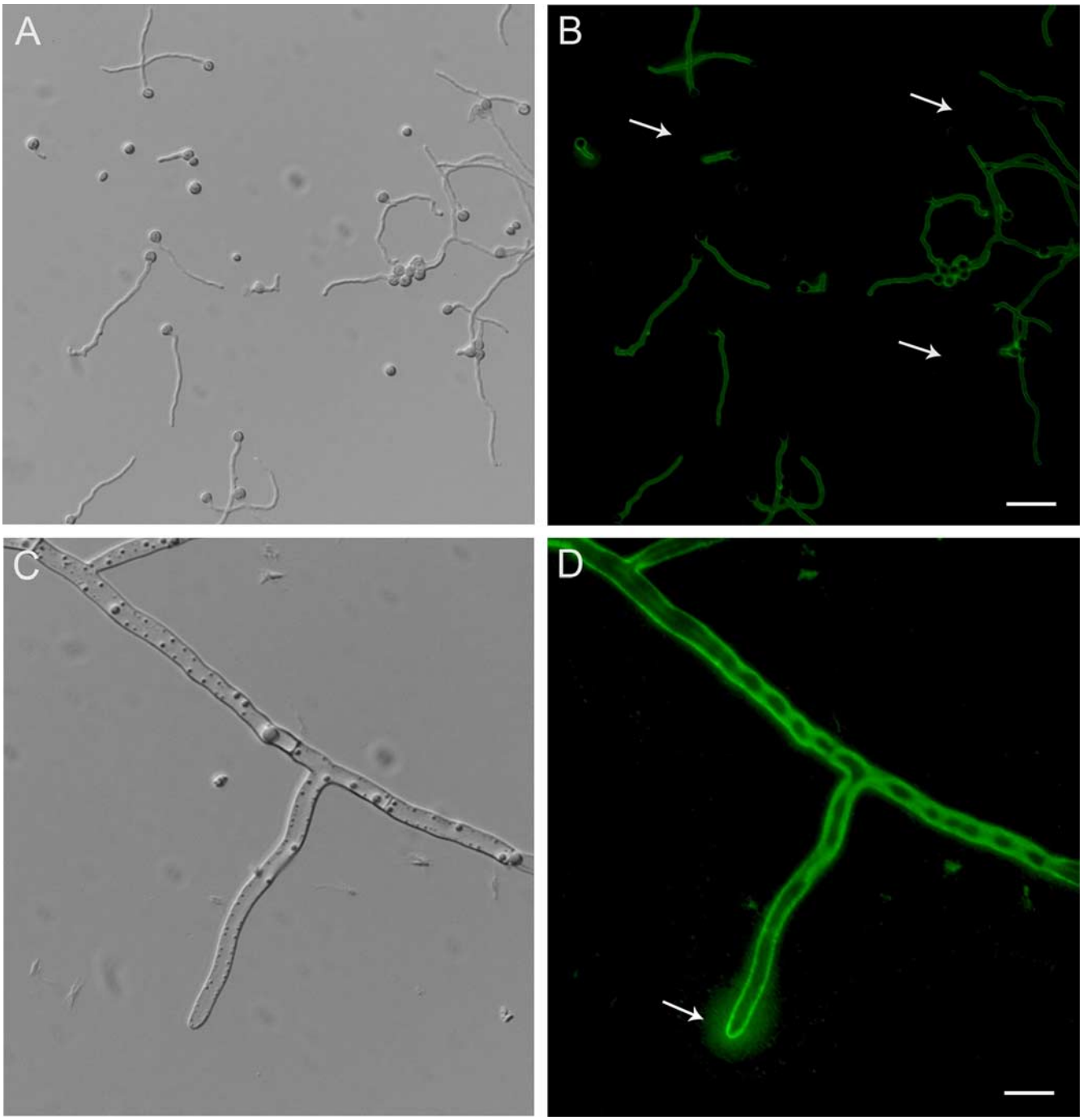

FIG. 2. Photomicrographs of *A. fumigatus* AF293 cells immunostained with MAb JF5 and anti-mouse polyvalent Ig fluorescein isothiocyanate. (A) Germlings examined under a bright-field microscope. (B) Same slide shown in panel A but examined under epifluorescence. Note the intense staining of the cell walls of the germ tubes but the lack of staining in ungerminated conidia (arrows). (C) Hypha examined under a bright-field microscope. (D) Same slide shown in panel C but examined under epifluorescence. Note the intense staining of the cell wall and secretion of the antigen at the growing tip (arrow). Bars, 6 μm.

fungi identified to be possible causes of false-positive responses in GM-based diagnostic tests (8, 12, 34). MAb JF5 therefore displays greater specificity than MAb EB-A2, and while cross-reactivity with *Penicillium* remains an issue, it is unlikely to represent a significant problem. With the exception of the endemic pathogen *P. marneffei*, there are very few reports of *Penicillium* species as etiologic agents of invasive diseases in humans (18, 41). Likewise, while invasive infections caused by *Paecilomyces* species have been reported, they are also extremely rare (9, 41, 42).

Current immunodiagnostic tests for invasive aspergillosis are based on the detection of circulating galactofuranose antigens

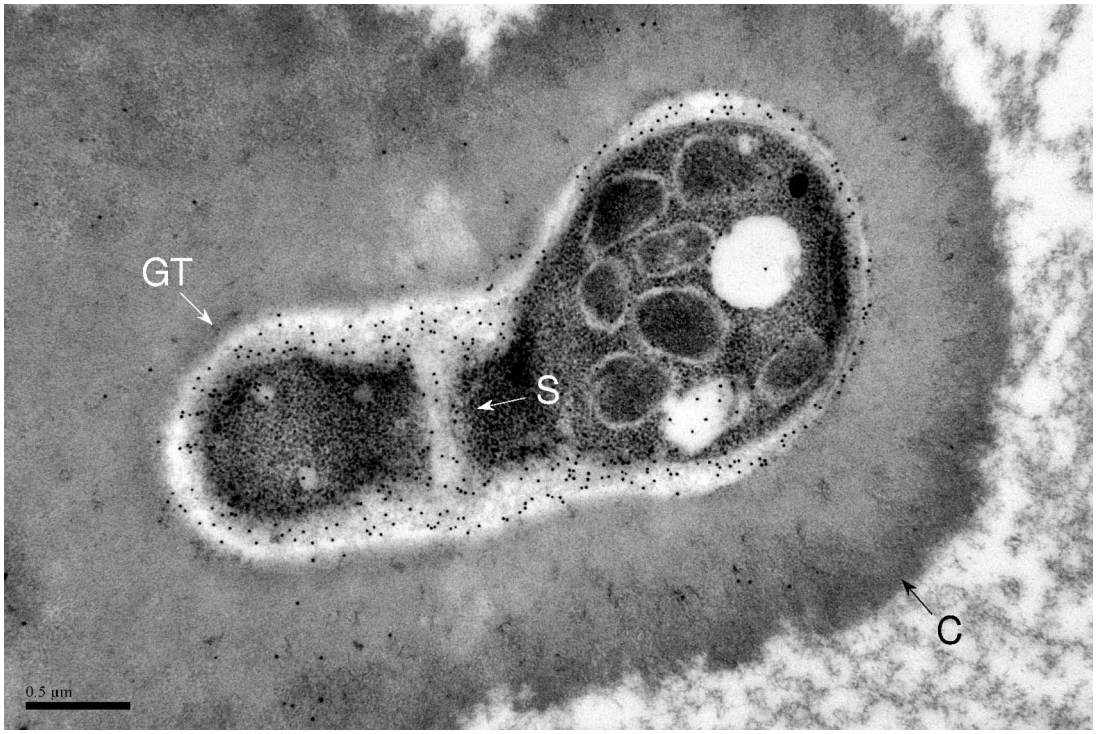

FIG. 3. Immunogold localization of the JF5 antigen in cells of *A. fumigatus* AF293. A longitudinal section of a germling grown in human serum shows the localization of the antigen in the cell walls of the germ tube (GT) and swollen conidium, in the septum (S), and in a surrounding capsular-like layer (C). Bar, 0.5 μm.

in human serum. The Platelia *Aspergillus* sandwich ELISA, which incorporates rat MAb EB-A2, is now commonly used to monitor patients at high risk for IA and provides a valuable tool for the early diagnosis of the disease. However, a number of issues hamper the use of the assay. False-negative results have been attributed to the heat pretreatment of serum samples that denatures protein but that may also eliminate protein-bound galactofuranose antigens, thereby leading to underestimation of serum reactivity (39). False-positive responses have also been reported, and the reasons for these have already been discussed. Consequently, surrogate markers of IA are desirable. Diagnostic tests that employ DNA detection by PCR have been developed (44), but such technology is restricted to laboratories equipped to perform such tests.

The recent observations by Morelle et al. (23) that circulating *Aspergillus* antigen may consist not only of fungal polysaccharide (GM) but also of glycoproteins raised the possibility that the antigen bound by MAb JF5 might act as a surrogate marker for the diagnosis of IA. Immunogold labeling studies showed that in the presence of human serum, the antigen was secreted into an extracellular capsule-like layer surrounding developing propagules of the fungus, reminiscent of the capsule induced in *C. neoformans* upon exposure to serum. LFD tests of human sera, in which the fungus and other angioinvasive species had been allowed to proliferate, showed that the antigen was also detectable in solution and that the test was specific for *Aspergillus* species. A useful property of the LFD is its potential to discriminate between active invasive growth of the fungus and quiescent spore production. Immunofluorescence studies showed that antigen production occurs at the growing tip of hyphae and is absent from ungerminated conidia. The absence of false-positive results with antibiotics and with bacterial lipoteichoic acids and the ability to use non-heat-treated serum samples provide additional benefits compared to tests based on GM detection.

The analytical sensitivity of the LFD was determined in the presence and the absence of serum proteins. The limit of detection of the LFD in saline buffer was 1.25 ng protein per ml, a level of sensitivity comparable to that of the Platelia GM EIA (1 ng per ml). However, in the presence of serum proteins, the sensitivity was reduced to 35 ng protein ml. Comparisons of sensitivities between the LFD, the Platelia GM EIA, and other assays such as the Afmp1p ELISA (45) are problematic since each assay detects a different *Aspergillus* antigen and each assay comprises different species of antibody (a mouse MAb, a rat MAb, and rabbit and guinea pig polyclonal antisera, respectively). Furthermore, the JF5 MAb binds to a protein epitope, whereas the rat MAb EB-A2 used in the Platelia EIA binds to a carbohydrate moiety, further complicating issues of assay sensitivity and its clinical significance. In the absence of a source of purified GM, a comparison of assay sensitivities cannot be made here. Consequently, the results of GM and LFD tests with clinical serum samples provide the most appropriate measure of accuracy of the LFD. A blind assessment of the sensitivities and specificities was conducted with serum samples from patients with known or suspected IA and healthy controls. The four control samples from healthy individuals gave negative results for IA in the GM and LFD tests. Of

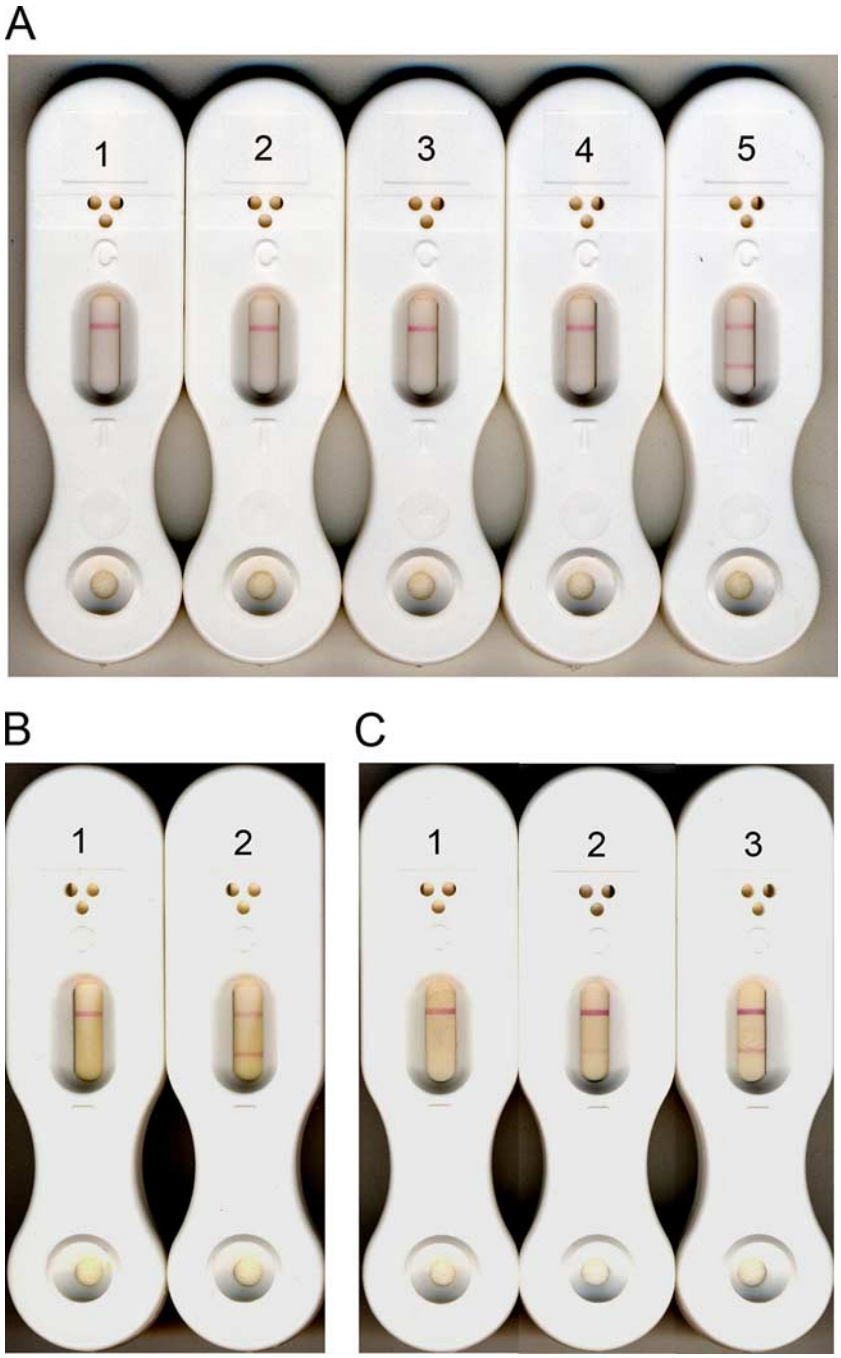

FIG. 4. Serum LFD tests. (A) LFD tests with normal human serum following inoculation with fungi and incubation for 48 h at 37°C. Negative reactions (single control line only) exhibited by *Candida albicans* (LFD 1), *Pseudallescheria boydii* (LFD 2), *Rhizopus oryzae* (LFD 3), and *Fusarium solani* (LFD 4) and a positive reaction (two lines) exhibited by *Aspergillus fumigatus* (LFD 5) are shown. (B) LFD tests of normal human serum (LFD 1) and serum spiked with affinity-purified antigen (LFD 2) at a concentration of 2.5 μg protein/ml. (C) Examples of results from LFD tests with serum samples from healthy individuals or patients with confirmed IA. Negative, weak, and strong reactions exhibited by specimens 9OHD (LFD 1), 1657 (LFD 2), and 1131 (LFD 3), respectively, are shown. Specimen numbers relate to those shown in Table 4.

the 12 probable or proven cases of IA according to EORTC criteria, 5 were determined to be IA positive according to the GM test results, while 8 were determined to be IA positive with the LFD. These results therefore suggest that the LFD has a greater clinical sensitivity for the diagnosis of disease, while it retains the specificity of the GM test. The strongest parity between the two immunoassays was found with the four samples deemed probable IA according to EORTC criteria. In these cases, strong positive results were recorded with both the GM and the LFD tests. Further comparative testing of the assays with samples from a larger cohort of patients is planned, but this work has shown the

TABLE 4. Results of LFD tests of serum samples from healthy individuals or from patients with known or suspected IA

Specimen no.	IA[a]	Platelia GM EIA index value	Platelia GM EIA result	Fungitell test β-glucan concn (pg/ml)	Fungitell test result	LFD result[b]
6OHD	No			45.90	Negative	−
7OHD	No			42.40	Negative	−
8OHD	No			44.30	Negative	−
9OHD	No			44.09	Negative	−
813	Yes	0.12	Negative	128.35	Positive	−
815	Yes	0.36	Negative	360.49	Positive	−
1263	Yes	0.16	Negative	111.72	Positive	−
1652	Yes	0.32	Negative	111.94	Positive	−
1655	Yes	0.35	Negative	104.13	Positive	+[c]
1657	Yes	0.71	Positive	122.23	Positive	±
1665	Yes	0.16	Negative	108.28	Positive	±
1667	Yes	0.30	Negative	142.19	Positive	±
1130	Probable	2.04	Positive	85.51	Equivocal	+
1131	Probable	1.52	Positive	219.61	Positive	+
1537	Probable	4.64	Positive	782.95	Positive	+[c]
1538	Probable	4.64	Positive	>500	Positive	+[c]

[a] Proven or probable cases of disease formally classified according to EORTC criteria.
[b] Reactions in LFD tests: −, no antigen detected; ±, weak reaction; +, strong reaction. The results for specimens 9OHD, 1657, and 1131 are shown in Fig. 4.
[c] Samples with a strong reaction at a 1 in 10 dilution in normal serum but negative undiluted.

potential of an LFD that detects a surrogate marker for IA to be a user-friendly diagnostic platform for the rapid and specific detection of the disease.

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