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Binding of Human Fibronectin to *Aspergillus fumigatus* Conidia

M. CARMEN PEÑALVER,[1] JOSE E. O'CONNOR,[2] JOSE P. MARTINEZ,[1] AND M. LUISA GIL[1]*

Departamento de Microbiología y Ecología, Facultad de Farmacia,[1] and Departamento de Bioquímica y Biología Molecular, Facultad de Medicina,[2] Universitat de Valencia, Valencia, Spain

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***Aspergillus fumigatus* conidia exhibited the ability to bind purified human fibronectin, whereas mycelial forms did not bind the ligand, as detected by an indirect immunofluorescence assay with an antifibronectin polyclonal antibody after incubation of the cells with fibronectin. Flow cytometry confirmed that binding of the ligand to conidia was dose dependent and saturable. Pretreatment of the cells with trypsin markedly reduced binding, which suggested a protein nature for the binding sites present at the surface of conidia. Intact conidia were also able to adhere to fibronectin immobilized on microtiter plates. When adhesion experiments were performed in the presence of soluble fibronectin or antifibronectin antibodies, a significant reduction (from 88 to 92%) in the binding of conidia was noticed, thus suggesting that adhesion to the immobilized ligand was specific. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting with fibronectin and antifibronectin antibody of whole conidial homogenates and 2-mercaptoethanol extracts from isolated conidial cell walls allowed identification, among the complex array of protein and glycoprotein species present in both cell-free preparations, of two polypeptides with apparent molecular masses of 23 and 30 kDa which specifically interact with fibronectin.**

Aspergillus fumigatus is a saprophytic fungus with a worldwide distribution and is sometimes recognized as an opportunistic pathogen. Inhalation of spores of the fungus causes allergic asthma and bronchopulmonary aspergillosis, respiratory diseases with limited fungal growth. The fungus is also capable of causing non-immunology-associated disorders such as aspergilloma, in which colonization of preexisting pulmonary cavities causes formation of a fungal ball, and invasive aspergillosis in immunosuppressed individuals, in which *A. fumigatus* invades the lung parenchyma and disseminates to other organs (4, 18). This last clinical form is often fatal, and its prevalence has increased markedly over the past 30 years (3). This has been the result of an increased use of immunosuppressive treatments for individuals with hematological malignancies and those undergoing organ transplantation (10), and more recently, invasive pulmonary aspergillosis has been also detected in patients with AIDS (12, 23).

Elucidation of the host defense mechanisms in aspergillosis has been the focus of many investigations in recent years. However, the mechanisms by which this fungus persists in the lung and causes diseases in certain individuals are still unclear. It has been suggested that adhesion of the conidia, the infectious propagules, to host cells and/or mucosal surfaces is a primary and crucial step in the establishment of infection (6). Previous studies have shown that in vitro, conidia of *A. fumigatus* are able to adhere to fibrinogen, laminin, and complement via proteins (receptors?) of the outer cell wall (1, 5, 11, 17, 18, 32, 35). The proteins laminin and fibrinogen are candidates for mediating adherence of conidia to the extracellular matrix, to basement membranes, and to the fibrin and fibrinogen deposits formed in response to the inflammatory reactions at the surfaces of wounded epithelia (11).

Fibronectin is a disulfide-linked dimeric glycoprotein present in a soluble form in blood plasma and other body fluids and in a fibrillar form in extracellular matrices. The major function of fibronectin is probably related to its ability to mediate substrate adhesion to mammalian cells, a process that involves the binding of specific cell surface receptors to discrete domains in the fibronectin molecule (27, 29). Fibronectin also interacts with several species of bacteria (28, 30, 33) and with the opportunistic pathogen *Pneumocystis carinii* (24). Binding of bacteria to the adhesion proteins represents a mechanism of attachment of bacteria to the tissues (2). Furthermore, fibronectin also binds to another fungal species, *Candida albicans*, and is a candidate for mediating the adherence of *C. albicans* to the extracellular matrix and basement membranes (15, 16, 25, 31).

Since fibronectin appears to be involved in the attachment of several pathogenic microorganisms to host tissues (2, 14), it seemed of interest to determine whether *A. fumigatus* cells were also able to interact with the ligand. In the present paper, we describe the characteristics and specificity of fibronectin binding to the surface of *A. fumigatus* conidia and report the characterization of cell wall-related conidial proteins that may represent receptors for fibronectin. The results presented may help in understanding how this fungal species interacts with the host.

MATERIALS AND METHODS

Microorganism and growth conditions. *A. fumigatus* (strain 2071) obtained from the Colección Española de Cultivos Tipo was used throughout this work. The organism was grown on plates of Vogel's N medium (36) for 30 days at 25°C. Mature conidia were harvested by washing the surfaces of the plates with a sterile 0.05% solution of Tween 20 in glass-distilled water. Conidia were pelleted from the suspension by centrifugation (1,500 × *g*, 10 min), washed with sterile glass-distilled water, and quantified by microscopic counting in a hemocytometer chamber. The absence of mycelial contamination was assessed by microscopic examination. Conidia were used directly in some experiments or, alternatively, were inoculated at a final concentration of 10^7 cells per ml into YPD (1% [wt/vol] Bacto Yeast extract, 2% [wt/vol] Bacto Peptone, 2% [wt/vol] glucose) liquid medium to obtain mycelial growth. Incubation was carried out until the spores germinated (usually after about 8 h at 37°C). Mycelial elements were harvested by centrifugation and washed twice in phosphate-buffered saline (PBS).

Assay of fibronectin binding to cells by indirect immunofluorescence. Conidia were resuspended in 100 μl of PBS containing 0.5 mg of human fibronectin

* Corresponding author. Mailing address: Departamento de Microbiología y Ecología, Facultad de Farmacia, Universitat de Valencia, Avda. Vicente Andrés Estellés, s/n, 46100-Burjasot, Valencia, Spain. Phone and fax: 34-6-3864770. Electronic mail address: Jose.Pedro.Martinez@uv.es.

(essentially free of other adhesive proteins and fibrinogen; see below) per ml at a final concentration of 10^9 conidia per ml in the assay. After incubation for 3 h at 37°C with gentle agitation in a gyratory incubator, the conidia were washed three times with PBS, resuspended in rabbit anti-human fibronectin antibody (1:10 dilution) in PBS plus 1% bovine serum albumin (BSA), and incubated for 1 h at 37°C. The cells were washed and incubated for 30 min at 37°C in fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin antibody (1:10 dilution) in PBS plus 1% BSA. Finally, the conidia were washed again with PBS and resuspended in 100 μl of PBS. Drops (10 to 15 μl) of these suspensions were placed on glass slides and examined (wet mountings) with a Zeiss photomicroscope III equipped for epifluorescence (UV filter no. 487702; excitation line, 365/366 nm). Control experiments were performed by omitting incubation of cells with fibronectin.

Flow cytometry analysis. Binding of fibronectin to *A. fumigatus* conidia was analyzed by flow cytometry. Aliquots (containing about 10^7 cells) of the conidial suspensions were incubated with various amounts of fibronectin in PBS, and immunofluorescence assays were performed by the procedure described above. The cells were subsequently fixed in 1% paraformaldehyde solution in PBS. All flow cytometry analyses were performed on an EPICS Elite Cell Sorter (Coulter Electronics, Inc., Hialeah, Fla.), using an air-cooled argon-ion laser tuned at 488 nm and 15 mW. Cell debris and aggregates were excluded on the basis of the light scatter properties of the particles (13). The flow rate was kept at approximately 500 events (cells) per s. The filter settings for collecting fluorescein isothiocyanate-related green fluorescence were as follows: 488-nm blocking filter, 550-nm dichroic filter, and 525 (± 5)-nm band pass filter. Green fluorescence was amplified logarithmically. Twenty thousand events were collected as monoparametric histograms of log fluorescence as well as list mode data files. When necessary, histograms or list mode files were analyzed off-line with the Elite Stand-alone software. Equal numbers of cells were processed similarly but with omission of incubation with fibronectin as negative controls.

In some experiments, the conidial suspensions were incubated with increasing amounts (1 to 25 μg/ml) of trypsin for 30 min at 37°C. Proteolytic treatment was stopped by three washes with PBS containing 3% BSA. The trypsin-treated cells were washed with PBS and assayed for fibronectin binding by flow cytometry as described above, using in this case 0.5 mg of fibronectin per ml for the initial incubation.

Surface labelling of conidia with biotin. Conidia were washed with 100 mM phosphate buffer (pH 8) and resuspended at 5×10^{10} conidia per ml in the same buffer containing 10 mg of *n*-hydroxysuccinimido–biotin per ml (*n*-hydroxysuccinimido–biotin was previously dissolved in dimethyl sulfoxide). After incubation for 1 h at 28°C in a shaking bath, the cells were recovered and washed four times with 50 mM phosphate buffer (pH 6) and then once with 10 mM phosphate buffer (pH 7.4). Biotinylated conidia were incubated with Extravidin-peroxidase conjugate at a 1:100 dilution in 10 mM Tris-hydrochloride buffer (pH 7.4) containing 0.9% NaCl (TBS buffer) and 0.05% Tween 20 (TBST) plus 1% BSA (TBSTB buffer). After incubation for 1 h at room temperature with agitation, the biotinylated conidia were washed in PBS and used for adherence assays.

Adherence assays on microtiter plates. Wells of microtitration plates (Nunc-Immunoplate I [A/S Nunc]) were coated with different amounts of fibronectin dissolved in PBS (200 μl of the different fibronectin solutions per well) for 1 h at 37°C and then overnight at 4°C. Unoccupied sites at the plastic surface were subsequently blocked by adding to each well 200 μl of a 0.1% BSA solution in PBS (1 h at 37°C). The wells were washed three times with PBS, and then aliquots (100 μl) of a suspension (10^8 cells per ml) of biotinylated, Extravidin-peroxidase-labelled conidia were added to them. The plate was incubated for 60 min at 37°C, after which nonadherent cells were removed by washing with PBS containing 0.05% Tween 20. After addition of the substrate mixture containing *o*-phenylenediamine, the plate was incubated in the dark, and the color reaction was stopped by adding 25 μl of 3 M H_2SO_4 to each well. The color intensity was determined at 492 nm with an automated plate reader (Labsystems Multiskan MCC/340). Results, expressed as optical density at 492 nm, are the means for quadruplicate wells with standard deviations. Statistical analysis of data was performed by means of Dunnett's *t* test for multiple comparisons.

Preparation of conidial extracts. Conidia were mixed with lysis buffer (100 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA, 5 mM dithiothreitol, 5 μg of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride) to give a very dense suspension. An equivalent volume of Ballotini glass beads (0.5 mm in diameter) was then added to the suspension, and the conidia were broken by shaking in a Vortex mixer for 30-s periods with 1-min cooling intervals. Cell breakage was assessed by examination in a phase-contrast microscope. After removal of the glass beads, the cell walls were sedimented (1,200 × *g* for 10 min) from the cell-free homogenate; washed three times with chilled distilled water; resuspended in 10 mM phosphate buffer (pH 7.4) containing 1% (vol/vol) 2-mercaptoethanol (βME), 5 mg of aprotinin per ml, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride; and incubated for 30 min at 37°C in a rotary shaker. The cell walls were subsequently sedimented, and the supernatant fluid was recovered, dialyzed against distilled water at 4°C, and concentrated by freeze-drying (βME extract). βME-extracted walls were washed three times with chilled glass-distilled water and then boiled for 5 min with a 2% sodium dodecyl sulfate (SDS) solution in glass-distilled water. After treatment, the suspensions were centrifuged at 10,000 × *g* for 15 min. The proteins eventually present in the supernatant were precipitated with 75% (vol/vol) (final concentration) ethanol at 4°C for 16 h. The precipitates were recovered by centrifugation at 27,000 × *g* for 30 min and resuspended in water (SDS extract). The total protein contents in the different samples were determined by the method of Lowry et al. (22) with BSA as the standard.

PAGE and Western blotting techniques. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed basically as described by Laemmli (19) with minor modifications (8). Electrophoretic transfer of polypeptides from polyacrylamide gels to nitrocellulose paper (Western blotting) was carried out as described previously (8).

Blotted proteins were assayed for fibronectin binding as follows. The nitrocellulose membranes were blocked with 3% BSA in TBS buffer for 1 h at room temperature and then incubated for 6 h with agitation in PBS containing human fibronectin (80 μg/ml). After being washed four times (10 min per wash) with TBST buffer, the nitrocellulose sheets were incubated for 1 h with agitation with rabbit anti-human fibronectin antibody (1:500 dilution) in TBSTB buffer. The blots were washed with TBST and incubated with peroxidase-labelled goat anti-rabbit immunoglobulin at a 1:3,000 dilution in TBSTB. Finally, the blots were washed again, and reactive bands were developed with hydrogen peroxide and 4-chloro-1-naphthol as the chromogenic reagent.

Miscellaneous. Human fibronectin and mouse laminin were obtained from Boehringer Mannheim; fibronectin was found to be free of other adhesive proteins by SDS-PAGE and Coomassie blue staining (fibronectin migrated as a single 220-kDa band) and to be free of fibrinogen by Western immunoblotting with an antifibrinogen antibody as the probe. Culture medium compounds were purchased from Difco. Rabbit anti-human fibronectin and fluorescein-conjugated goat anti-rabbit immunoglobulin antibodies were from Sigma Chemical Co. Peroxidase-labelled goat anti-rabbit immunoglobulin and gel electrophoresis and blotting reagents were from Bio-Rad. All other chemicals were from Sigma Chemical Co.

RESULTS

Fibronectin binding to *A. fumigatus* cells. The strong fluorescence observed in conidia of *A. fumigatus* indicated their interaction with fibronectin (Fig. 1B), yet cell-to-cell variations in fibronectin binding were detected. When the immunofluorescence assay was performed with germ tubes, no reactivity at the surfaces of mature (long) hyphal extensions was detected (Fig. 1D and F); only the mother cell surface from which mycelial filaments emanated was labelled, but a very weak and diffuse fluorescence on the surfaces of some young (short) mycelial forms was eventually noticed (Fig. 1D). In any case, the intensity of fluorescence in the mother conidia (Fig. 1D, arrows) was found to be lower than that in nongerminated cells (Fig. 1B), which suggests that germination caused qualitative and/or quantitative changes in the conidial surface that may affect binding of fibronectin to cells. Fluorescence was dependent on the previous interaction of the cells with fibronectin, since no fluorescent elements were observed when the cells were incubated with the indicator antibodies only.

Flow cytometry analysis of fibronectin binding. The characteristics of fibronectin binding to *A. fumigatus* conidia were further investigated by flow cytometry. Analysis of cells incubated in solutions of fibronectin at various concentrations ranging from 0.1 to 2 mg/ml showed that the ligand bound to the cells in a dose-dependent manner. Representative histograms are shown in Fig. 2A, whereas the mean channel of fluorescence intensity (linear scale) is represented in Fig. 2B. The intensity of the fluorescence detected at the surface of conidia increased with the concentration of the ligand in the solution, attesting to the saturability of binding, which reached a plateau at a fibronectin concentration of 0.5 mg/ml (Fig. 2). Incubation of conidial suspensions with saturating concentrations of fibronectin revealed the existence of at least two distinct conidial subpopulations; one of them represented 30% of the positive cells and showed an intensity of fluorescence higher than that of the rest of the cells present in the suspension (Fig. 2A).

Effect of proteolytic treatment on fibronectin binding. To characterize the biochemical nature of the binding sites, conidia pretreated with trypsin were analyzed for their ability to bind fibronectin by flow cytometry (Fig. 3). Proteolytic treat-

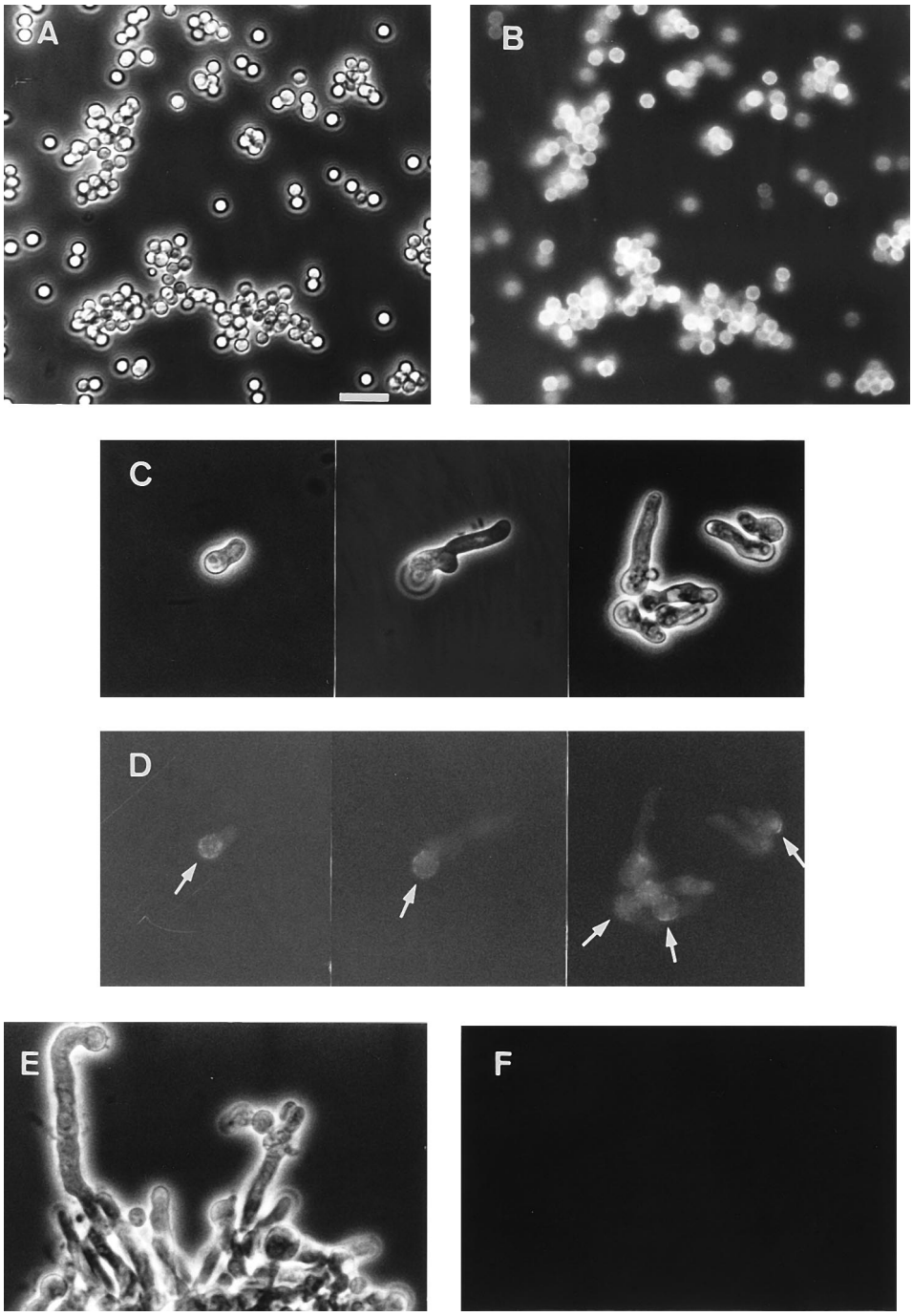


FIG. 1. Binding of fibronectin to *A. fumigatus* conidia (B) and germ tubes (D and F) detected by indirect immunofluorescence. Organisms were incubated with fibronectin, rabbit antifibronectin antibodies, and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G as described in Materials and Methods. (A, C, and E) Phase-contrast microscopy; (B, D, and F) UV illumination (fluorescence). Note the fluorescence at the conidial surface (B). The hyphal surface visualized by phase-contrast microscopy (C and E) was not labelled or exhibited a very weak fluorescence detectable only in young mycelial forms (D and F). Bar, 10 μm.

ment of the cells strongly reduced binding of the ligand to cells. The effect was dependent on the concentration of trypsin used. Thus, trypsin at concentrations of 1, 2.5, and 25 μg/ml inhibited binding of fibronectin to *A. fumigatus* conidia by 63, 86, and 93%, respectively (Fig. 3B). The conidial subpopulation showing a higher fluorescence intensity and representing 30% of the positive cells present in the suspension (Fig. 2A; Fig. 3A, non-trypsin-treated sample) was not detected following trypsin treatment of cells (Fig. 3A).

Attachment of conidia to immobilized fibronectin. In an attempt to assess whether binding of fibronectin may have a role in the process of adherence of conidia to host tissues during infection, an assay was developed to determine the ability of the cells to interact with the ligand immobilized on wells of microtiter plates, an experimental approach that mimics the in vivo conditions. As shown in Fig. 4A, *A. fumigatus* conidia adhered strongly to fibronectin, in a dose-dependent manner. Thus, coating of the wells with increasing concentra-

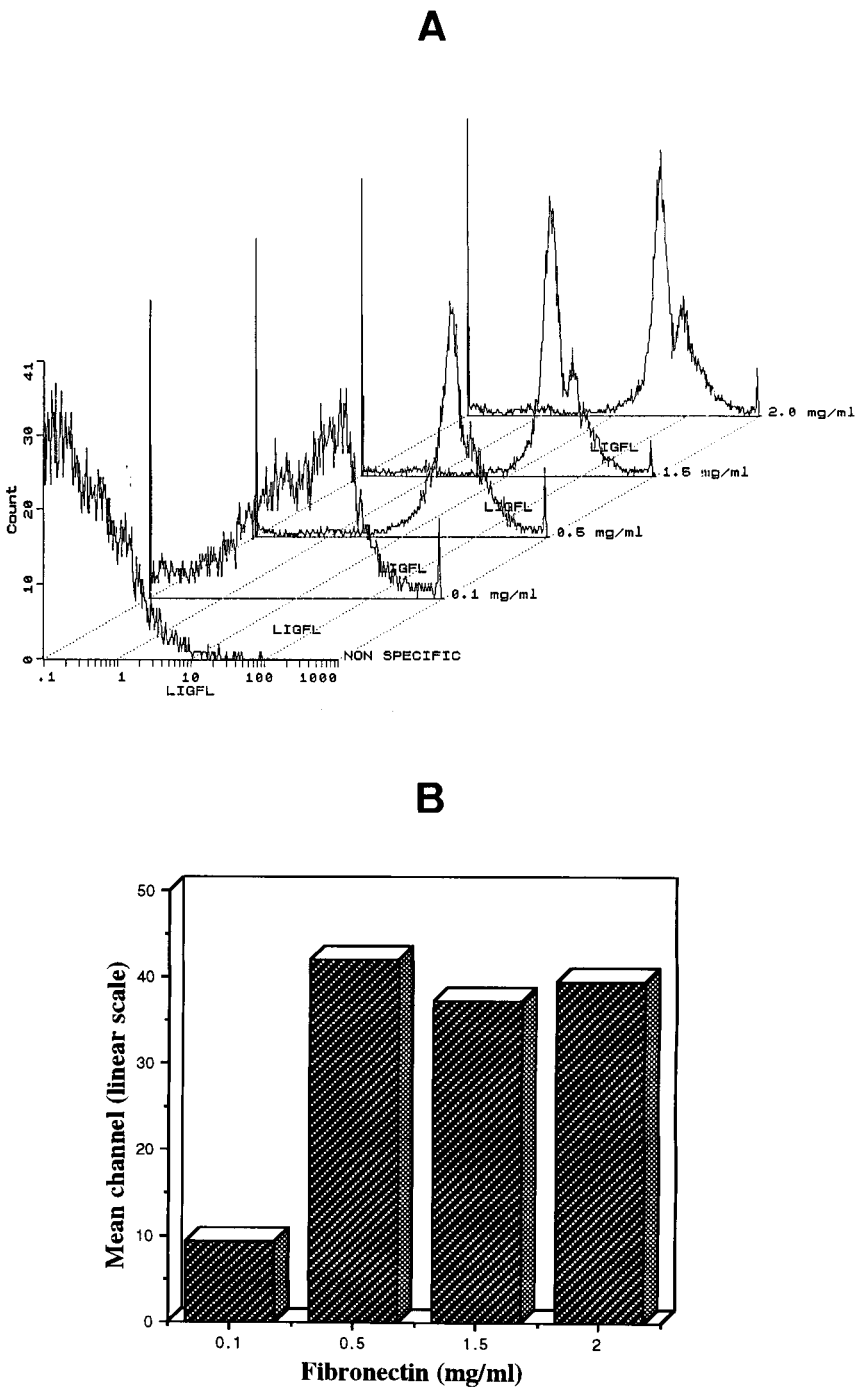


FIG. 2. Flow cytometric analysis of the binding of fibronectin to conidia. Mature conidia were incubated with PBS only (nonspecific) or in the presence of various amounts of fibronectin, and the immunofluorescence assay was performed as described in Materials and Methods. The intensity of fluorescence at the surface of conidia was measured by flow cytometry. (A) Representative histograms. *x* axis, log of fluorescence intensity (LIGFL); *y* axis, number of fluorescent cells. (B) Fluorescence mean channel (less background) represented on a linear scale.

tions of fibronectin (from 10 to 100 μg/ml) resulted in an increase in the number of adherent conidia (Fig. 4A). Maximal adhesion was found at a coating fibronectin concentration of 50 μg/ml (Fig. 4A). Hence, the concentration of 50 μg/ml was selected for all subsequent experiments on attachment to immobilized fibronectin. The attachment was also dependent on the concentration of conidia in the suspension added to the wells (data not shown). A concentration of 10^8 conidia per ml, which allowed the best distinction between coated and control wells, was retained. Background attachment of conidia to uncoated plastic (control wells) did not exceed 18% of the corresponding values for adhesion to fibronectin-coated wells. In order to assess whether surface labelling of conidia with the biotin-avidin-peroxidase complex may affect binding of the cells to the immobilized ligand, adhesion of labelled and unlabelled conidia to fibronectin-coated microtiter wells was de-

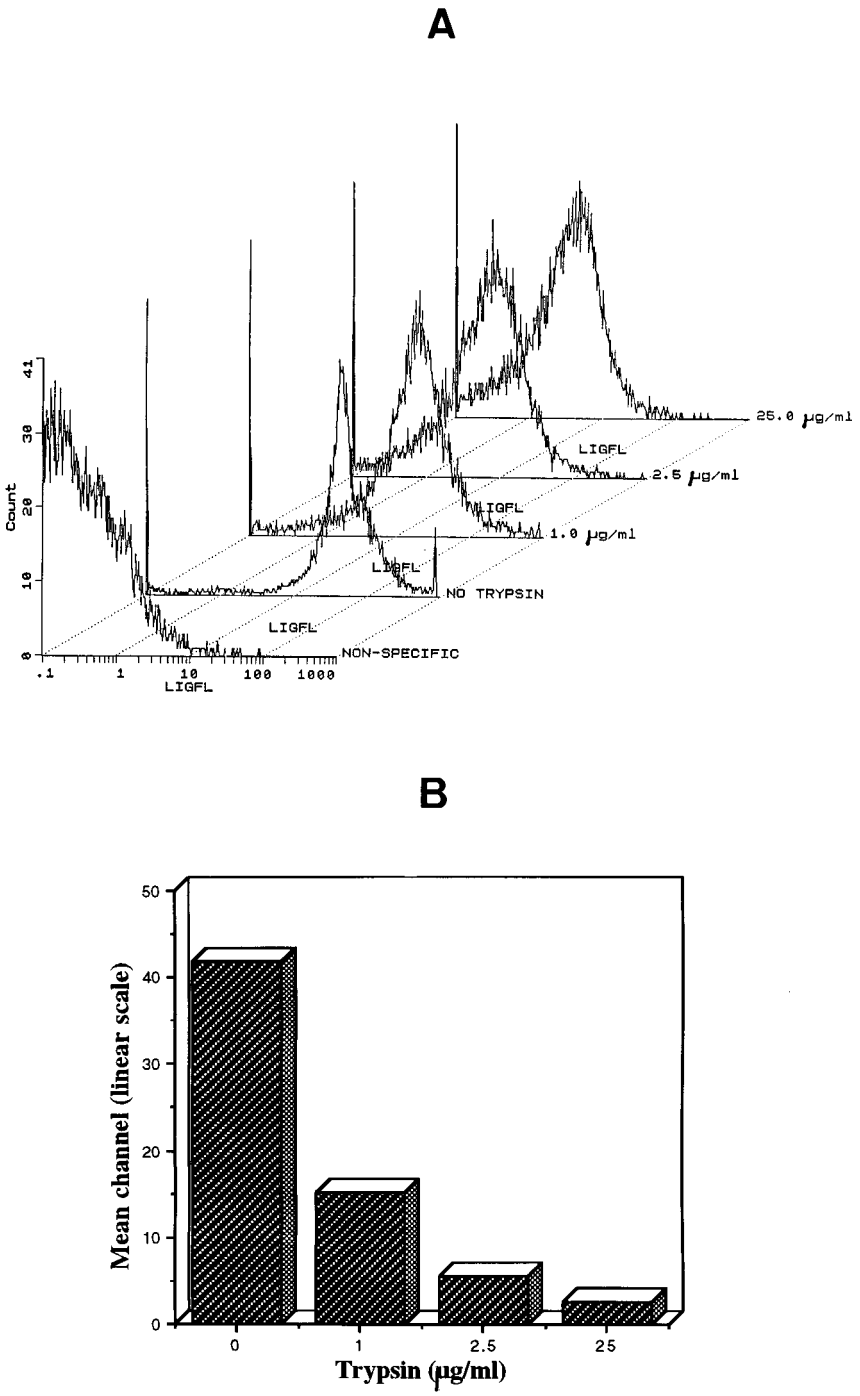
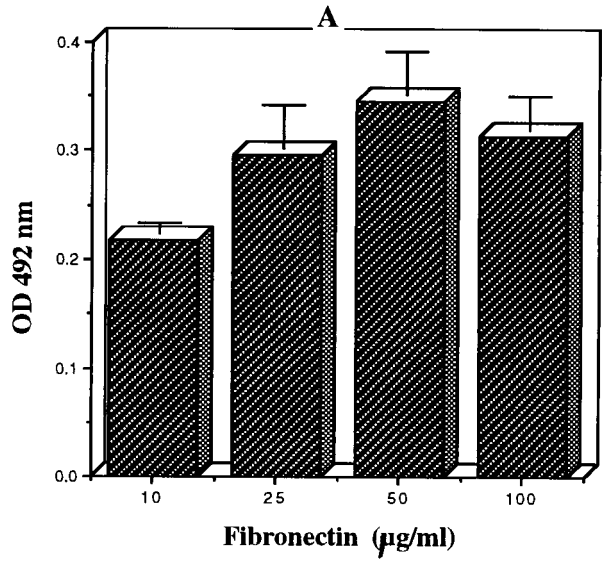


FIG. 3. Influence of trypsin pretreatment of conidia on binding of fibronectin. Conidia treated with different trypsin concentrations (0 to 25 μg/ml) were incubated with PBS (nonspecific) or in the presence of fibronectin (0.5 mg/ml) and assayed for immunofluorescence as described in Materials and Methods. The intensity of fluorescence at the surface of conidia was measured by flow cytometry. (A) Representative histograms. *x* axis, log of fluorescence intensity (LIGFL); *y* axis, number of fluorescent cells. (B) Fluorescence mean channel (less background) represented on a linear scale.

termined by microscopic counting. The adherent conidia were fixed with 2.5% glutaraldehyde in PBS for 15 min and counted in 10 randomly selected microscopic fields per well. No significant differences between the mean values of triplicate well countings for biotin-labelled and unlabelled conidia were found (standard deviations differed by less than 6%).

The adhesion of conidia to immobilized fibronectin was specific. As shown in Fig. 4B, the number of adherent conidia decreased when binding experiments were done in the presence of 500 μg of soluble fibronectin per ml (88% inhibition) or in the presence of different concentrations (1:50 and 1:100 dilutions) of specific antifibronectin antibodies (91 to 92% inhibition). Adhesion to immobilized fibronectin was also



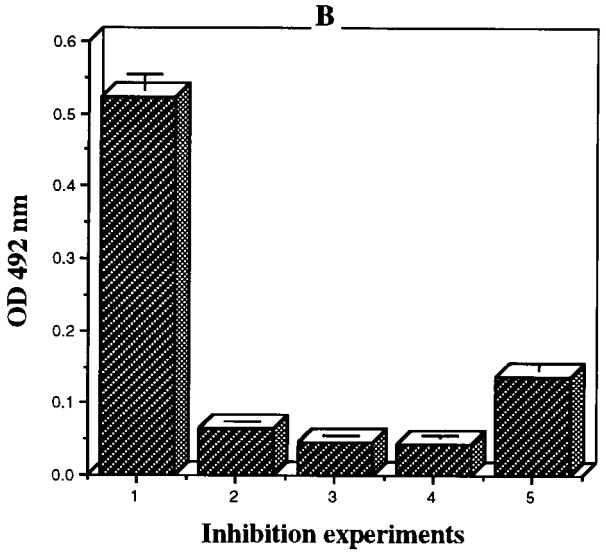


FIG. 4. Attachment of *A. fumigatus* conidia to immobilized fibronectin. (A) Biotin-Extravidin-peroxidase-labelled conidia were allowed to adhere to wells of microtiter plates coated with fibronectin at different concentrations (10 to 100 μg/ml). Nonadherent cells were removed by washing, and the amount of adherent conidia was estimated indirectly by measuring the optical density at 492 nm (OD_{492}) of the colored reaction product produced by peroxidase (see Materials and Methods). (B) Specificity of conidial attachment to immobilized fibronectin. Wells of microtiter plates were coated with a solution of fibronectin at 50 μg/ml. Conidia were allowed to adhere without any inhibitor (experiment 1) or in the presence of soluble fibronectin at 500 μg/ml (experiment 2), specific antibodies at a dilution of 1:100 (experiment 3) or 1:50 (experiment 4), or soluble laminin at 500 μg/ml (experiment 5). The amount of adherent conidia in the wells was estimated as described for panel A. The results shown in both panels are the means of quadruplicate determinations from three independent experiments with standard deviations.

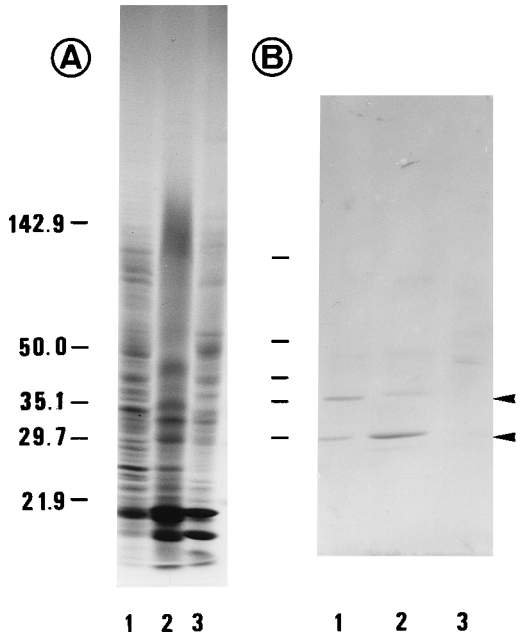


FIG. 5. Identification by ligand affinity blotting of polypeptides from *A. fumigatus* conidia with the ability to bind fibronectin. Whole conidial homogenates (lanes 1) and materials released by treatment of isolated conidial cell walls with βME (lanes 2) and with SDS after βME treatment (lanes 3) were analyzed by SDS-PAGE on 5 to 15% gradient gels followed by Coomassie blue staining (A) or, alternatively, were immunoblotted with fibronectin and antifibronectin antibody (B) (see Materials and Methods). The positions of standard proteins with known molecular masses (expressed in kilodaltons) run in parallel are shown at the left of each panel. Arrowheads indicate two polypeptides of 23 and 30 kDa that bound fibronectin.

markedly inhibited by the presence of 500 μg of soluble laminin per ml in the assay (74% inhibition).

Finally, the effects of several sugars (glucose, galactose, and mannose), which are found in the fibronectin molecule and which are also present in fungal cell wall moieties, on the ability of conidia to bind to the immobilized ligand were assayed. Preincubation of the cells in a 100 mM solution of each sugar did not modify binding of conidia to fibronectin (data not shown).

Identification of cell components that bind fibronectin. Ligand affinity binding experiments with individualized protein components present in different conidial extracts, following basically the protocol previously used by our group to identify receptors for fibrinogen and laminin in *C. albicans* (9, 21), were used to identify species that were eventually able to bind fibronectin. When analyzed by SDS-PAGE under reducing conditions on slab gradient gels (5 to 15% acrylamide) followed by staining with Coomassie blue, cell-free homogenates (Fig. 5A, lane 1) and cell wall βME (Fig. 5A, lane 2) and SDS (Fig. 5A, lane 3) extracts were seen to contain a complex array of polypeptide chains (16 to 35 species, depending on the extract analyzed) with molecular masses ranging from 17 to 150 kDa. Binding of fibronectin was observed with only two components, with molecular masses of approximately 23 and 30 kDa, which were present in the cell-free homogenate (Fig. 5B, lane 1) and βME extract (Fig. 5B, lane 2) preparations. This was a specific reaction, as indicated by the absence of bands following detection with the specific antifibronectin antibodies when previous incubation of the nitrocellulose blots in the fibronectin solution was omitted. As expected from the observed inhibitory effects of several substances on the ability of conidia to bind to immobilized fibronectin (Fig. 4), polyclonal antifibronectin antibodies added to the reaction mixture blocked binding of the ligand to the separated conidial polypeptides on the nitrocellulose blots, which additionally supported the contention of the specificity of the ligand affinity binding experiments mentioned above.

DISCUSSION

Attachment of *A. fumigatus* cells to the bronchioloalveolar epithelium is thought to be a crucial step for the establishment of aspergillosis. The ability to adhere to epithelial cells may represent a mechanism by which the fungus (particularly the conidia, which are the infecting forms) avoids entrapment within the mucus that covers the respiratory ducts and physical removal by the movement of the ciliary cells, thus playing a role in pathogenesis. However, little is known about how this process occurs. In this context, it has been reported that basement membrane and serum proteins such as laminin and fibrinogen may be involved in the interaction of *A. fumigatus* conidia with the host tissues (1, 5, 11, 35), but information on the potential ability of the fungus to interact with other animal ligands is not currently available.

In this communication, we report that *A. fumigatus* conidia were able to specifically bind to fibronectin, a glycoprotein component of extracellular matrices, whose major function is to mediate substrate adhesion to mammalian cells but which appears also to be a candidate for mediating the adherence of several prokaryotic and eukaryotic pathogenic microorganisms to host tissues (2, 14–16, 24, 25, 27–31, 33). Indirect immunofluorescence revealed that conidia but not hyphae had the ability to interact with fibronectin, as has been described for binding of some other ligands, such as fibrinogen, to conidial cells (1). The fact that the human fibronectin preparation used in this work was free of other adhesive contaminant proteins supports the contention of the specificity of the interaction described here.

Binding of fibronectin to *A. fumigatus* conidia was further investigated by flow cytometry analysis, which not only confirmed observations made by immunofluorescence microscopy but also revealed that binding of fibronectin to cells was saturable. We found that 10^7 conidia were labelled at saturation with 100 μl of a 0.5-mg/ml fibronectin solution. When a saturating fibronectin concentration was used in the flow cytometry experiments, about 30% of conidia showed an intensity of fluorescence higher than that of the rest of the cells. This observation suggests the existence of a subpopulation of conidia that (i) overexpress cell surface fibronectin-binding components or (ii) possess additional fibronectin binding sites with greater affinity for the ligand. A greater sensitivity of such additional binding sites to proteolytic attack would explain why this subpopulation was not detected in the flow cytometry analysis of cell suspensions treated with trypsin (Fig. 3A).

The strong reduction of the ability of cells to bind fibronectin caused by trypsin treatment, as evidenced by flow cytometry assays, suggested a protein nature for the binding sites eventually present at the conidial cell surface. Furthermore, no evidence for a lectin-type interaction with the ligand was found, since several sugars (glucose, galactose, and mannose) did not inhibit binding of fibronectin. In any case, the possibility that digestion with the protease releases protein moieties containing fibronectin-binding motifs defined by sugars different from those tested cannot be completely ruled out.

It has been reported that fibronectin was inefficient in induction of the attachment of *A. fumigatus* conidia (11). However, the use of microtiter plates coated with fibronectin allowed us to establish that this extracellular matrix component was able to promote adhesion of conidial cells. Although it is possible that the discrepancy between our results and those of Coulot et al. (11) may be due to the different strain and experimental conditions used here, and although further investigations are required to establish whether a distinct domain(s) of the fibronectin molecule is able to promote differential interaction with *A. fumigatus* conidia, the results presented in this communication are not unique; other authors have recently reported that spores from another *A. fumigatus* strain bind to several extracellular matrix proteins, such as fibrinogen, laminin, type I and IV collagens, and fibronectin, yet in those experiments conidia showed the least binding with fibronectin (7).

Adhesion to immobilized fibronectin was found to be dose dependent and significant at low coating concentrations. The specificity of adhesion between conidia and fibronectin was demonstrated by the inhibition caused by both soluble fibronectin and antifibronectin antibodies. Interestingly, addition of exogenous laminin reduced the attachment to fibronectin-coated wells; likewise, soluble fibronectin inhibited binding of conidia to immobilized laminin (data not shown). Other authors have demonstrated that soluble laminin also inhibited attachment of conidia to immobilized fibrinogen (11) and that soluble fibrinogen reduced their adhesion to immobilized laminin (35). The results presented here in combination with data from other authors suggest that a common receptor for several animal serum and extracellular matrix proteins (fibrinogen, laminin, and fibronectin) may be present at the surface of *A. fumigatus* conidia, although the existence of distinct receptors for each ligand cannot be completely ruled out. In this context, distinct receptors present in close proximity may lead to a steric hindrance phenomenon in which one receptor site may be obscured by another ligand bound to its own receptor at the cell surface.

Two polypeptides of 23 and 30 kDa that bind fibronectin were identified in whole conidial homogenates and βME extracts from isolated conidial cell walls by ligand affinity blotting, yet the reducing sulfhydryl agent was apparently unable to solubilize detectable amounts of either fibronectin-binding polypeptide from the surface of intact conidia. However, one may expect that these two species (or at least one of them) are exposed at the surface of conidial cells (immunofluorescence assays indicated that fibronectin binding occurs at the cell surface level), a location which is consistent with a putative role as receptors for the ligand. The extreme hydrophobicity and resistance to chemical degradation of the outermost cell wall layer of *Aspergillus* conidia, which is characterized by the presence of interwoven fascicles of clustered proteinaceous microfibrils (rodlets [26, 34]), along with the low solubilizing effect exerted by βME (this reducing agent extracts no more than 15% of the total protein content present in the wall of intact *C. albicans* cells [20]), may account for the absence of detectable polypeptides (including the 23- and 30-kDa fibronectin-binding species) in the supernatants obtained following treatment of intact conidia with the reducing agent. In this context, breakage and relaxation of the wall structure as a consequence of the ballistic cell disruption procedure used to obtain the conidial homogenates may result in an increase of the solubilizing efficiency of βME. The bands detected here may represent different stages of modification of the same fibronectin-binding protein or different binding proteins that may recognize the same or different sites on the fibronectin molecule, although further work is required to identify the fibronectin domains that promote its interaction with *A. fumigatus* conidia and particularly with the two conidial polypeptides detected here.

The results described in this paper expand current knowledge on the number of the extracellular matrix components with which *A. fumigatus* is able to interact. The presence of receptors for fibronectin on the surface of conidia, the infectious airborne form of the fungus, may play an important role

in the pathogenesis of aspergillosis, promoting their adhesion to host tissues and initiation of infection. Since fibronectin has the ability to interact with fibrinogen, whose synthesis is stimulated during inflammation caused by mechanical injuries and/or by fungal, bacterial, or parasitic infections of tissues, this may represent an additional way to increase the efficiency of pathogen adhesion.

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REFERENCES

1. **Annaix, V., J. P. Bouchara, G. Larcher, D. Chabasse, and G. Tronchin.** 1992. Specific binding of fibrinogen fragment D to *Aspergillus fumigatus* conidia. Infect. Immun. **60:**1747–1755.
2. **Beachey, E. H., C. S. Giampapa, and S. M. Abraham.** 1988. Adhesin-receptor mediated attachment of pathogenic bacteria to mucosal surfaces. Am. Rev. Respir. Dis. **138:**S45–S48.
3. **Bodey, G. P.** 1988. The emergence of fungi as major hospital pathogens. J. Hosp. Infect. **11:**411–426.
4. **Bodey, G. P., and S. Vartivarian.** 1989. Aspergillosis. Eur. J. Clin. Microbiol. Infect. Dis. **8:**413–437.
5. **Bouchara, J. P., A. Bouali, G. Tronchin, R. Robert, D. Chabasse, and J. M. Senet.** 1988. Binding of fibrinogen to the pathogenic *Aspergillus* species. J. Med. Vet. Mycol. **26:**327–334.
6. **Bouchara, J. P., G. Tronchin, and D. Chabasse.** 1994. Mechanisms and implications of the adherence phenomenons in *Aspergillus fumigatus*. Pathol. Biol. **42:**640–646.
7. **Bromley, I. M. J., and K. Donaldson.** 1995. Adherence of spores of *Aspergillus fumigatus* to pulmonary epithelial cells and to extracellular matrix proteins, abstr. P25, p. 68. *In* Abstracts of the 132nd Ordinary Meeting of the Society for General Microbiology.
8. **Casanova, M., M. L. Gil, L. Cardeñoso, J. P. Martínez, and R. Sentandreu.** 1989. Identification of wall-specific antigens synthesized during germ tube formation by *Candida albicans*. Infect. Immun. **57:**262–271.
9. **Casanova, M., J. L. López-Ribot, C. Monteagudo, A. Llombart-Bosch, R. Sentandreu, and J. P. Martínez.** 1992. Identification of a 58-kilodalton cell surface fibrinogen binding mannoprotein from *Candida albicans*. Infect. Immun. **60:**4221–4229.
10. **Cohen, J.** 1991. Clinical manifestations and management of aspergillosis in the compromised patient, p. 117–152. *In* D. W. Warnock and M. D. Richardson (ed.), Fungal infection in the compromised patient, 2nd ed. John Wiley & Sons, Chichester, United Kingdom.
11. **Coulot, P., J. P. Bouchara, G. Renier, V. Annaix, C. Planchenault, G. Tronchin, and D. Chabasse.** 1994. Specific interaction of *Aspergillus fumigatus* with fibrinogen and its role in cell adhesion. Infect. Immun. **62:**2169–2177.
12. **Denning, D. W., S. E. Follansbee, M. Scolaro, S. Norri, H. Edelstein, and D. A. Stevens.** 1991. Pulmonary aspergillosis in the acquired immunodeficiency syndrome. N. Engl. J. Med. **324:**654–662.
13. **Guasch, R. M., C. Guerri, and J. E. O'Connor.** 1995. Study of surface carbohydrates on isolated Golgi subfractions by fluorescent-lectin binding and flow cytometry. Cytometry **19:**112–118.
14. **Hook, M., L. M. Switalski, T. Wadstrom, and M. Lindberg.** 1989. Interactions of pathogenic microorganisms with fibronectin, p. 295–307. *In* D. F. Mosher (ed.), Fibronectin. Academic Press, Inc., New York.
15. **Kalo, A., E. Segal, E. Sahav, and S. Dyan.** 1988. Interaction of *Candida albicans* with genital mucosal surfaces: involvement of fibronectin in adherence. J. Infect. Dis. **157:**1253–1256.
16. **Klotz, S. A., and R. L. Smith.** 1991. A fibronectin receptor on *Candida albicans* mediates adherence of the fungus to extracellular matrix. J. Infect. Dis. **163:**604–610.
17. **Kozel, T. R., M. A. Wilson, T. P. Farrell, and S. M. Levitz.** 1989. Activation of C3 and binding to *Aspergillus fumigatus* conidia and hyphae. Infect. Immun. **57:**3412–3417.
18. **Kwon-Chung, K. J., and J. E. Bennett.** 1991. Aspergillosis, p. 201–247. *In* Medical mycology. Lea and Febiger, Philadelphia.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
20. **López-Ribot, J. L., M. Casanova, J. P. Martínez, and R. Sentandreu.** 1991. Characterization of cell wall proteins of yeast and hydrophobic mycelial cells of *Candida albicans*. Infect. Immun. **59:**2324–2332.
21. **López-Ribot, J. L., M. Casanova, C. Monteagudo, P. Sepúlveda, and J. P. Martínez.** 1994. Evidence for the presence of a high-affinity laminin receptor-like molecule on the surface of *Candida albicans* yeast cells. Infect. Immun. **62:**742–746.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
23. **Minamoto, G. Y., T. F. Barlam, and N. J. Van der Els.** 1992. Invasive aspergillosis in patients with AIDS. Clin. Infect. Dis. **14:**66–74.
24. **Narasimhan, S., M. Y. K. Armstrong, K. Rhee, J. C. Edman, F. F. Richards, and E. Spicer.** 1994. Gene for an extracellular matrix receptor protein from *Pneumocystis carinii*. Proc. Natl. Acad. Sci. USA **91:**7440–7444.
25. **Nègre, E., T. Vogel, A. Levanon, R. Guy, T. J. Walsh, and D. D. Roberts.** 1994. The collagen binding domain of fibronectin contains a high affinity binding site for *Candida albicans*. J. Biol. Chem. **269:**22039–22045.
26. **Parta, M., Y. Chang, S. Rulong, P. Pinto-DaSilva, and K. J. Kwong-Chung.** 1994. *HYP1*, a hydrophobin gene from *Aspergillus fumigatus*, complements the *rodletless* phenotype in *Aspergillus nidulans*. Infect. Immun. **62:**4389–4395.
27. **Pearlstein, E., L. I. Gold, and A. Garcia-Pardo.** 1980. Fibronectin: a review of its structure and biological activity. Mol. Cell. Biochem. **29:**103–137.
28. **Peterson, K. M., J. B. Baseman, and J. F. Alderete.** 1983. *Treponema pallidum* receptor binding proteins interact with fibronectin. J. Exp. Med. **157:**1958–1970.
29. **Ruoslahti, E.** 1988. Fibronectin and its receptors. Annu. Rev. Biochem. **57:**375–413.
30. **Signas, C., G. Raucci, K. Jonsson, P. E. Lindgren, G. M. Anantharamaiah, M. Hook, and M. Lindberg.** 1989. Nucleotide sequence of the gene for fibronectin-binding protein from *Staphylococcus aureus*: use of this peptide sequence in the synthesis of biologically active peptides. Proc. Natl. Acad. Sci. USA **86:**699–703.
31. **Skerl, K. G., R. A. Calderone, E. Segal, T. Sreevalsan, and W. M. Scheld.** 1983. In vitro binding of *Candida albicans* yeast cells to human fibronectin. Can. J. Microbiol. **30:**221–227.
32. **Sturtevant, J. E., and J. P. Latgé.** 1992. Interactions between conidia of *Aspergillus fumigatus* and human complement C3. Infect. Immun. **60:**1913–1918.
33. **Talay, S. R., P. Valentin-Weigand, P. G. Jerlstrom, K. N. Timmis, and G. S. Chatwal.** 1992. Fibronectin-binding protein of *Streptococcus pyogenes*: sequence of the binding domain involved in adherence of streptococci to epithelial cells. Infect. Immun. **60:**3837–3844.
34. **Thau, N., M. Monod, B. Crestani, C. Rolland, G. Tronchin, J. P. Latgé, and S. Paris.** 1994. *rodletless* mutants of *Aspergillus fumigatus*. Infect. Immun. **62:**4380–4388.
35. **Tronchin, G., J. P. Bouchara, G. Larcher, J. C. Lissitsky, and D. Chabasse.** 1993. Interaction between *Aspergillus fumigatus* and basement membrane laminin: binding and substrate degradation. Biol. Cell **77:**201–208.
36. **Vogel, H. J.** 1964. Distribution of lysine pathways in fungi: evolutionary implications. Am. Nat. **98:**435–466.

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