Sialic Acid-Dependent Recognition of Laminin and Fibrinogen by *Aspergillus fumigatus* Conidia

JEAN-PHILIPPE BOUCHARA,^{1*} MYRIAM SANCHEZ,¹ ALAIN CHEVAILLER,²
AGNES MAROT-LEBLOND,³ JEAN-CLAUDE LISSITZKY,⁴ GUY TRONCHIN,¹
AND DOMINIQUE CHABASSE¹

Laboratoire de Parasitologie-Mycologie¹ and Laboratoire d'Immunologie,² Centre Hospitalier Universitaire,
and Laboratoire de Parasitologie-Mycologie, Faculté de Pharmacie,³ Angers, and Laboratoire
d'Immunologie, INSERM U387, Hôpital Sainte-Marguerite, Marseille,⁴ France

Received 15 January 1997/Returned for modification 23 February 1997/Accepted 18 April 1997

In an attempt to define the molecular basis of the adherence of *Aspergillus fumigatus* conidia to the host tissues, a step which might be mediated by the recognition of basement membrane laminin or fibrinogen, we analyzed the binding of these glycoproteins by flow cytometry and a microtiter plate adherence assay. Flow cytometry revealed that the binding of fluorescein isothiocyanate-labeled laminin to conidia was saturable and specific. Moreover, the ability of conidia to bind laminin increased with their maturation. Competition experiments showed a cross-reactivity between laminin and fibrinogen binding and a lack of interactions with glycosaminoglycans. In addition, the binding of laminin was not inhibited by the different adhesive synthetic peptides tested. Furthermore, the microtiter plate assay of adherence to chymotrypsin degradation products of laminin or fibrinogen purified by gel filtration suggested a unique binding site common to sequential degradation fragments or the presence of multiple binding sites on the two ligands. Therefore, the role of carbohydrates in the recognition process was investigated. Among the carbohydrates tested, constitutive of the conidial wall or of the oligosaccharide side chains of laminin and fibrinogen, only *N*-acetylneuraminic acid and sialyllactose inhibited the binding of these glycoproteins to conidia. In conclusion, these results strengthen the idea that the laminin and fibrinogen receptors in *A. fumigatus* are identical and suggest an interaction mediated by a sialic acid-specific lectin of the conidial wall.

During the past two decades, the development of organ or bone marrow transplantations and the widespread use of immunosuppressive therapy have led to a marked increase in the prevalence of aspergillosis. Thus, fungi have been reported as major hospital pathogens and the aspergilli have been reported as a major cause of death in patients with hematological malignancies (4, 18). *Aspergillus fumigatus*, which is the main causative agent of these mycoses, is usually a saprophyte growing in a humid environment on decaying organic matter (5). Infestation of the host occurs by inhalation of the airborne conidia, which are rapidly eliminated in healthy patients by mucociliary clearance and the alveolar macrophages. Therefore, *A. fumigatus* is an opportunistic pathogen that is responsible for various respiratory infections such as sinusitis, allergic bronchopulmonary aspergillosis, and aspergilloma. The most severe clinical forms, however, are observed in severely immunocompromised patients, particularly in leukemic patients and bone marrow transplant recipients (20).

Elucidation of the mechanisms allowing the establishment of the fungus within the host and the initiation of disease has been the subject of much scientific investigation in the past few years. Thus, it has been suggested that the development of the fungus within the host requires the adhesion of conidia to the bronchopulmonary epithelium or to the subepithelial basement membrane and the production of various enzymes, including proteases and phospholipases, which mediate the subsequent invasion of the underlying tissues (10). Adherence of the fungus to the host tissues could be mediated by interactions

with fibrinogen or laminin (8). Fibrinogen, the major plasma glycoprotein, plays a key role in inflammatory reactions, and the recognition of the fibrin(ogen) deposits formed at the surface of wounded epithelia constitutes a mechanism of microbial attachment to mucosal surfaces. Numerous microorganisms such as *Staphylococcus aureus*, group A, C, and G streptococci, and some bacteria associated with periodontal diseases, *Porphyromonas gingivalis* and *Prevotella intermedia*, bind fibrinogen by saturable and specific receptors (for a review, see the work of Patti et al. [32]). Moreover, we have previously demonstrated the binding of fibrinogen to germ tubes of the opportunistic fungus *Candida albicans* (2). In addition, the role of laminin, the main structural component of basement membranes, in the adherence phenomena is now well documented (3). This large glycoprotein of 900 kDa is composed of three nonidentical polypeptide chains assembled by disulfide bonds in a cross-shaped structure. Interactions with laminin are crucial for a number of biological processes that require cell adhesion, including diapedesis, cellular cohesion inside tissues, metastasis of cancer cells, and infections. Indeed, laminin receptors have been reported on cells that normally interact with basement membranes, for example, epithelial or endothelial cells, muscle cells, and neuronal cells, but also on cells that extravasate such as macrophages, polymorphonuclear leukocytes, and human or animal tumor cells. Among the laminin receptors on mammalian cells already described, many of them belong to the integrin family. These transmembrane proteins recognize different ligands by their tripeptide sequence RGD, which is common to a large variety of proteins (44), including laminin and fibrinogen as well as fibronectin, whose binding to *A. fumigatus* conidia has been described elsewhere (33). However, some integrins link to laminin via its peptide sequence IKVAV, localized in the frag-

* Corresponding author. Mailing address: Groupe d'Etude des Interactions Hôte-Parasite, Laboratoire de Parasitologie-Mycologie, Centre Hospitalier Universitaire, 4 rue Larrey, 49033 Angers Cedex 01, France. Phone: 33 02 41 35 34 72. Fax: 33 02 41 35 36 16.

ment E_g. Nonintegrin receptors are also described, mainly a 67-kDa high-affinity laminin receptor which recognizes the pentapeptide YIGSR highly specific for laminin and localized in laminin P₁. Another 67-kDa protein interacts with both laminin and elastin from connective tissues by some hydrophobic peptide sequences, LGTIPG for laminin and VGVAPG for elastin (23).

Laminin-binding proteins are also detected at the surface of bacteria, for example, *S. aureus*, *Streptococcus pyogenes*, *Escherichia coli*, and *Treponema denticola* (32), and of protozoa, e.g., *Toxoplasma gondii* (19), *Trichomonas vaginalis* (36), and *Leishmania donovani* (21). Moreover, it has been suggested that laminin mediates the adhesion of several pathogenic fungi such as *Candida albicans* (9, 25, 31), *Paecilomyces farinosus* (34), *Paracoccidioides brasiliensis* (42), *Histoplasma capsulatum* (30), *Rhizopus oryzae* (6), and some fish pathogenic yeasts, namely, *Rhodotorula rubra*, *Rhodotorula glutinis*, and *Candida zeylanoides* (41).

In this work, we attempted to define the molecular basis of the adherence in *A. fumigatus*. We present the flow cytometric analysis of the binding of laminin to *A. fumigatus* conidia. Moreover, microtiter plate adherence assays and inhibition experiments of the binding of laminin and fibrinogen allowed us to identify the binding sites on these glycoproteins.

MATERIALS AND METHODS

Microorganism and growth conditions. *A. fumigatus* CBS 113.26, initially isolated from soil and obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands), was used throughout. The organism was routinely maintained on YDA (yeast extract, peptone, dextrose, agar) plates supplemented with chloramphenicol (0.1%). Unless specified otherwise, conidia were obtained from cultures grown for 5 days at 37°C by flooding the agar plates with distilled water and scraping the aerial mycelium. Then, the hyphal fragments, the conidial heads, and the possible bits of agar were eliminated from the suspension by filtration through 25-μm-pore-size nylon Monyl filters (Scrynel NY 25 HC; Zürcher Beuteltuchfabrik A.G., Rüschlikon, Switzerland). The conidia were pelleted by centrifugation (1,200 × g, 5 min), washed in phosphate-buffered saline (0.15 M, pH 7.2; PBS), and quantified by hemocytometer counts.

For some experiments, suspensions of conidia were prepared from 24-h to 7-day-old cultures as described above.

Extracellular matrix components. Laminin was extracted from the mouse Englebreth-Holm-Swarm sarcoma tumor and purified by ion-exchange chromatography on a QA-Trisacryl M column (IBF Biotechnics, Villeneuve-La-Garenne, France) followed by gel filtration on a Fractogel TSK HW50 (Merck, Darmstadt, Germany) column (29). Commercially available human fibrinogen, grade L (Diagnostica Stago, Asnières, France), which often contains small amounts of other adhesive proteins such as fibronectin, was purified by two successive chromatographies on gelatin-Sepharose 4B (Pharmacia-Biotech Europe GmbH, Orsay, France) and heparin-Sepharose 4B (Pharmacia-Biotech) columns as described previously (2). The efficiency of the purification of laminin and fibrinogen was controlled by polyacrylamide gel electrophoresis (PAGE). Human plasma fibronectin and the glycosaminoglycans hyaluronic acid, heparan sulfate, keratan sulfate, and chondroitin sulfate A and B were from Sigma. All reagents were stored as aliquots at -80°C until used.

Fluorescent labeling of proteins. Laminin, purified human fibrinogen, fibronectin, and bovine serum albumin (BSA; IBF Biotechnics) were labeled with 5-fluorescein isothiocyanate (FITC; Sigma, St. Quentin Fallavier, France) by a procedure adapted from Clark and Shepard (15). The labeling was performed by mixing the protein solutions with a 10-fold-lower volume of 0.5 M carbonate buffer (pH 9.5) and then dialyzing the solutions overnight at 4°C under constant shaking against 0.05 M carbonate buffer containing 0.1 mg of FITC per ml. After incubation, the labeled protein was separated from the free FITC by gel filtration through a Sephadex G-25M column (model PD 10; Pharmacia-Biotech) previously equilibrated in PBS and saturated with 1 ml of a 0.1% BSA solution in PBS. One milliliter of the solution was loaded on the column, and elution was performed in PBS. The eluate was collected in fractions of 500 μl, and purification was monitored by measuring the absorbance at 280 nm (A_{280}) and the fluorescence intensity with a Fluoroskan II (Labsystems, Helsinki, Finland) with an excitation wavelength of 485 nm and an emission wavelength of 515 nm. Fractions containing the labeled protein were pooled, and the protein content was estimated by the method of Bradford (11). The labeled protein solutions were stored as aliquots at -80°C until used.

For fluorescent labeling of laminin and fibrinogen, 10 mM EDTA or 0.5% citrate sodium, respectively, was added to all reagents to prevent their polymerization.

Flow cytometric analysis of binding. Binding of the different host proteins to *A. fumigatus* conidia was analyzed by flow cytometry as described previously (16). Unless stated otherwise, 5×10^6 conidia were incubated for 30 min at 37°C under constant shaking with 250 μl of a 25-μg/ml FITC-conjugated protein solution. The cells were then washed, pelleted by centrifugation, and fixed with 1% formaldehyde in PBS.

Cell surface fluorescence was quantified on a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems Europe, Erembodegem, Belgium) equipped with an air-cooled 15-mW argon-ion laser operating at 488 nm. The standard filter configuration was used for acquisition of the data. Cell debris was excluded by gating the conidia on the basis of their rectilinear forward and side light scatter properties. Each sample was collected in a list mode file of 10,000 events, and data were analyzed with the LYSIS II software from Becton-Dickinson. The data presented correspond to fluorescence frequency distribution histograms (relative number of conidia versus relative fluorescence intensity expressed in arbitrary units on a logarithmic scale).

All experiments were performed with PBS (supplemented with 10 mM EDTA [PBS-EDTA] for the analysis of the binding of laminin or with 0.5% citrate sodium [PBSC] for the analysis of fibrinogen binding). They were repeated twice and included a negative control experiment performed by incubating conidia in the absence of any ligand to ascertain the absence of autofluorescence. Moreover, samples of conidia incubated with the fluorescent ligand alone or with a mix of the fluorescent ligand and a 10-fold molar excess of unlabeled ligand were also analyzed as controls in all inhibition experiments.

Analysis of the binding parameters. Influence of the concentration of the labeled protein solution was determined by incubating the conidia with solutions of the fluorescent ligand of increasing concentrations ranging from 0.1 to 100 μg/ml.

The specificity of the binding was assessed by incubating the cells with the fluorescent ligand (125 μl at 50 μg/ml) and an equal volume of a 10-fold excess of unlabeled ligand. The fluorescence intensity of cells incubated with FITC-labeled laminin was also compared to that of cells incubated with an equal amount of FITC-conjugated BSA.

Competition experiments of the binding of laminin were also performed in the presence of a 10-fold molar excess of unlabeled fibrinogen or BSA. Likewise, conidia were incubated simultaneously with FITC-laminin and different glycosaminoglycans (hyaluronic acid, heparan sulfate, keratan sulfate, and chondroitin sulfate A and B) at 100 or 500 μg/ml. The following peptides which mimic adhesive recognition sequences found in both laminin and fibrinogen were also used: RGD-containing peptides (RGD, RGDS, GRGDS, GRGES, GRGDSP, and GRADSPK) and GPRP. Similarly, peptides specific for laminin (IKVAV, YIGSR, YIGSR-NH₂, CDPGYIGSR, CDPGYIGSR-NH₂, and LGTIPG), fibrinogen (fibrinogen γ-chain 12-mer peptide), or elastin (VGVAPG) were tested. All synthetic peptides were from Neosystem (Strasbourg, France) except for the peptide VGVAPG, which was from Sigma. The peptide concentration in competition assays (250 μg/ml) corresponded to a 3,000- to 10,000-fold molar excess, depending on the peptides.

Finally, as described for the binding of fibrinogen to *A. fumigatus* conidia (16), the influence of the age of the conidia on their ability to bind laminin was also investigated. Towards this aim, conidia obtained simultaneously from 24-h to 7-day-old cultures were incubated with FITC-labeled laminin alone or in the presence of a 10-fold excess of unlabeled ligand. After incubation and subsequent washing and fixation, the fluorescence intensity of the cells was quantified by flow cytometry.

Influence of carbohydrates on the binding of laminin, fibrinogen, and fibronectin. The possible role of carbohydrates in the recognition of laminin, fibrinogen, and fibronectin by *A. fumigatus* conidia was studied by inhibition experiments of the binding of the fluorescent ligands. Different carbohydrates constitutive of the conidial cell wall or of oligosaccharide chains of the ligands and related sugars, i.e., glucose, galactose, mannose, glucosamine, galactosamine, N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-galactosamine (GalNAc), N-acetylneuraminic acid (NANA), lactose, sialyllactose, maltose, and melibiose, were tested at 20 or 200 mM. Bovine submaxillary mucin and its desialylated form, asialomucin, from Sigma were also used at 20 or 200 μg/ml to confirm the role of sialic acid residues.

Microtiter plate adherence assay. Adherence was quantified in 96-well flat-bottom microtitration plates (Immunoplate I; Nunc, Roskilde, Denmark) as described previously (16). The microtiter plates were coated by the addition of 100 μl per well of a 100-μg/ml protein solution in PBS (PBSC for fibrinogen). The plates were then incubated for 1 h at 37°C with gentle shaking; the protein solution was discarded, and the wells were washed three times in PBS (5 min each wash). Unoccupied sites at the plastic surface were blocked by the addition of a 0.1% BSA solution in PBS (200 μl per well).

After incubation for 1 h at 37°C and overnight at 4°C, followed by three washes in PBS, the conidial suspension (10⁷/ml) was loaded into the coated wells (200 μl per well) and the plates were incubated for 30 min at 37°C with gentle shaking. Nonadherent cells were removed by washing (three times, 5 min each wash) in PBS supplemented with 0.05% (wt/vol) Tween 20. Conidia adherent to the plastic surface were fixed with 2.5% glutaraldehyde in PBS for 15 min. After three additional washes in PBS, adhesion was quantified by phase-contrast microscopy at a ×600 magnification with an Olympus IMT-2 microscope connected to a Hitachi M-920 K monitor through a Hitachi HV-720 E video camera. Ten

FIG. 1. Influence of the concentration of the FITC-labeled laminin solution. Conidia of *A. fumigatus* were incubated for 30 min at 37°C with FITC-laminin solutions of increasing concentration. The fluorescence intensity at the cell surface was then measured by flow cytometry. *x* axis, fluorescence intensity; *y* axis, number of fluorescent cells. The solid black curve shows the fluorescence of conidia incubated in PBS-EDTA. The graphs shown are representative of two independent experiments.

randomly selected fields (9,000 μm^2 each) were counted for each well. Moreover, for each experiment, wells uncoated but simply saturated with BSA were also overlaid with the conidial suspension. Adhesion to these wells was considered as the background and subtracted from the values obtained for the coated wells.

Influence of enzyme treatment of laminin and fibrinogen on the adhesion of conidia. To specify the binding sites for *A. fumigatus* conidia, laminin (600 $\mu\text{g}/\text{ml}$ in PBS) and fibrinogen (1 mg/ml in PBSC) were incubated with chymotrypsin (Sigma) at 37°C for various times ranging from 5 min to 24 h at a 1:50 (wt/wt) enzyme/substrate ratio. The reaction was then stopped by the addition of 10 μl of a 100 mM phenylmethylsulfonyl fluoride (Fluka) stock solution in methanol. After control of the proteolysis by sodium dodecyl sulfate (SDS)-PAGE, wells of microtiter plates were coated by the addition of 100 μl per well of an appropriate dilution of the obtained solutions (100 μg of protein per ml). Adhesion of conidia to these wells was evaluated as described above. Likewise, the ability of the degradation products (at 250 $\mu\text{g}/\text{ml}$) to inhibit the binding of the fluorescent ligand to the conidia was evaluated by flow cytometry.

The chymotrypsin degradation products of laminin or fibrinogen were also separated by fast-performance liquid chromatography through HiLoad 16/60 Superdex 75 (prep grade; Pharmacia-Biotech). After the addition of phenylmethylsulfonyl fluoride, samples (2 ml) were applied to the column at a flow rate of 1 ml/min and elution was performed in PBS (PBSC for fibrinogen degradation products). Chromatography was monitored by measuring the $A_{280\text{nm}}$ and 2-ml fractions were collected. The efficiency of the material contained in these fractions to support the adhesion of conidia was then evaluated as described above. For each fraction, a single well was coated with 100 μl of the fraction. Moreover, 1 ml of the fractions which allowed adhesion was dialyzed overnight at 4°C against distilled water, freeze-dried, and then analyzed under reducing or non-reducing conditions by SDS-PAGE on 5 to 15% polyacrylamide linear gradient gels or 12.5% polyacrylamide resolving gels (28). After electrophoresis, proteins were visualized by staining with Coomassie brilliant blue R-250 and their molecular masses were calculated from the migration of standard proteins from Pharmacia-Biotech.

RESULTS

Saturability and specificity of the binding of laminin, fibrinogen, and fibronectin. Conidia of 5-day-old cultures were incubated with solutions of FITC-labeled laminin, fibrinogen, or fibronectin of increasing concentrations ranging from 0.1 to 100 $\mu\text{g}/\text{ml}$. Flow cytometric analysis of the fluorescence of 10,000 cells showed that the fluorescence intensity at the cell surface increased progressively with the concentration of the protein solution. As illustrated in Fig. 1 for laminin, the bind-

ing was dose dependent, reaching a plateau for a concentration of the fluorescent ligand of 25 $\mu\text{g}/\text{ml}$. This concentration was retained for subsequent experiments. Incubation of the cells without any ligand confirmed the absence of autofluorescence of the conidia.

Specificity of the binding was demonstrated by incubating the cells with an excess of unlabeled ligand. As shown for laminin binding in Fig. 2, this resulted in a marked decrease in the fluorescence intensity (mean channel fluorescence intensity of 423 but 1,110 in the absence of unlabeled laminin). The fluorescence of cells incubated with an equal amount of FITC-labeled BSA, a protein which is devoid of adhesive properties, is included in Fig. 2 as a control. FITC-BSA bound very weakly to the conidia, and the mean fluorescence intensity (mean channel fluorescence intensity, 26) was lower than that of cells incubated with FITC-labeled laminin and a 10-fold excess of unlabeled laminin.

Influence of the age of the conidia on their ability to bind laminin. Incubation of conidia from 24-h- to 7-day-old cultures with FITC-labeled laminin alone or in the presence of a 10-fold excess of unlabeled laminin showed that the expression of the laminin receptors at the surface of conidia was consistent with their maturation. The total binding (fluorescence intensity of cells incubated with FITC-laminin alone) progressively increased with the age of the cells (Table 1). Moreover, the specific binding which corresponded to the difference between the total binding and the nonspecific binding evaluated by incubating the cells with a mix of FITC-laminin and unlabeled laminin also increased with the age of the cells, reaching a plateau for conidia from 5-day-old cultures. This suggested an increasing density of the receptor sites at the cell surface.

Cross-reactivity between the binding of laminin and fibrinogen to *A. fumigatus* conidia. Competition experiments demonstrated the cross-reactivity between laminin and fibrinogen binding (Fig. 2). A marked reduction in the cell surface fluorescence was noted for conidia incubated with FITC-laminin

FIG. 2. Specificity of the binding of laminin to *A. fumigatus* conidia. Conidia of *A. fumigatus* were incubated with FITC-laminin alone or in the presence of unlabeled laminin, fibrinogen, or BSA. After 30 min at 37°C, the intensity of fluorescence at the cell surface was measured by flow cytometry. x axis, fluorescence intensity; y axis, number of fluorescent cells. The solid black curve shows the fluorescence of conidia incubated in PBS-EDTA. The fluorescence of conidia incubated with FITC-BSA is presented as a control. The graphs shown are representative of two independent experiments.

and a 10-fold molar excess of unlabeled fibrinogen (fluorescence mean channel, 294). Likewise, the binding of FITC-fibrinogen to *A. fumigatus* conidia was inhibited by laminin (data not shown). Conversely, no inhibition of the binding of FITC-laminin was observed in the presence of a 10-fold molar excess of BSA (fluorescence mean channel, 1,192).

To study the possible role of the glycosaminoglycans in the adherence of *A. fumigatus* conidia, competition experiments of the binding of FITC-laminin were also performed in the presence of these compounds at 100 or 500 µg/ml, followed by flow cytometric analysis of the cell surface fluorescence. The fluorescence intensity at the cell surface was not modified by the glycosaminoglycans tested, i.e., hyaluronic acid, heparan-sulfate, keratan-sulfate, or chondroitin sulfate A or B, regardless of the concentration used (data not shown).

To identify the binding sites on the laminin molecule, competition experiments of the binding of FITC-labeled laminin were performed with synthetic peptides corresponding to the adhesive recognition sequences common to laminin and fibrinogen, i.e., the RGD adhesive sequence and the sequence GPRP, which contributes to the binding of fibrinogen to human platelets (12, 35). None of them inhibited the binding of FITC-laminin (data not shown). Similarly, synthetic peptides which mimic adhesive peptide sequences specifically found in laminin, i.e., YIGSR, YIGSR-NH₂, CDPGYIGSR, CDPGYIGSR-NH₂, IKVAV, and LGTIPG, also failed to inhibit the binding of FITC-laminin. Likewise, no inhibition was observed in the presence of the fibrinogen γ-chain 12-mer peptide, which binds to human platelets and to *S. aureus* (17), and with the hydrophobic peptide VGVAPG, which corresponds to the elastin binding site for the 67-kDa elastin-laminin receptor (23).

Influence of chymotrypsin digestion of laminin and fibrinogen on their ability to bind to *A. fumigatus* conidia. Adhesion of conidia was evaluated after incubation of laminin or fibrinogen with chymotrypsin for various times and coating the mi-

croter plates with the obtained degradation products. Despite significant proteolysis attested by SDS-PAGE, adhesion of the conidia remained almost unchanged during the first 4 h of digestion by chymotrypsin (data not shown). Thereafter, it markedly decreased. Likewise, the degradation products drastically inhibited the binding of the fluorescent ligand to the conidia until a 4-h incubation of the protein with chymotrypsin; thereafter, their ability to block the binding decreased. Thus, an incubation time of 4 h was retained for subsequent experiments, which consisted of the separation of the digestion products by chromatography and the evaluation of their ability to support the adhesion. Numerous chromatographic peaks which corresponded to polypeptides of different molecular masses, ranging from 250 to 20 kDa for laminin (Fig. 3B) and from 90 to 25 kDa for fibrinogen (Fig. 4B), supported the adhesion of conidia (Fig. 3A and 4A).

TABLE 1. Influence of the age of conidia on their ability to bind laminin^a

Age of conidia	Fluorescence mean channel		
	PBS-EDTA	FITC-laminin	FITC-laminin + unlabeled laminin
24 h	5	24	8
2 days	3	108	29
3 days	3	147	45
4 days	3	206	73
5 days	4	251	83
7 days	4	262	99

^a Conidia of *A. fumigatus* from 24-h- to 7-day-old cultures were incubated for 30 min at 37°C in PBS-EDTA or with FITC-laminin with or without a 10-fold excess of unlabeled laminin. The intensity of fluorescence at the cell surface was then measured by flow cytometry. Results, which are representative of two independent experiments, are expressed as the fluorescence mean channel.

FIG. 3. Influence of digestion of laminin by chymotrypsin on the adherence of conidia. (A) Adhesion of conidia to the degradation products of laminin. After incubation with chymotrypsin for 4 h at 37°C, the cleavage products were separated by gel filtration. The dotted line represents the A_{280} . Wells of microtiter plates were then coated with a fixed volume (100 μ l) of the different fractions of chromatography, and the adherence of conidia was quantified by use of the standard assay. The continuous line represents the number of adherent conidia for 10 randomly selected fields. (B) SDS-PAGE analysis of the different fractions of chromatography on 5 to 15% polyacrylamide gradient gels under reducing conditions. The lanes are designated by the numbers of the corresponding fractions. The molecular masses (MM) of the protein standards (in kilodaltons) are indicated on the left.

Role of sialic acid residues in the binding of laminin and fibrinogen. The experiments described above suggested a unique binding site shared by different fragments resulting from sequential proteolysis or the presence of multiple binding sites on the two ligands. Therefore, we investigated the influence of carbohydrates constitutive of the oligosaccharide chains of laminin and fibrinogen, or related to these sugars, on the binding of these glycoproteins. Among the carbohydrates, only NANA and, to a lesser extent, sialyllactose inhibited the binding of laminin (Fig. 5). A dose-dependent inhibition of the binding was found with NANA since 40 and 77% inhibitions were obtained with this sugar at 20 and 200 mM, respectively. Similar results were obtained for fibrinogen binding, which was

inhibited by 80% in the presence of 200 mM NANA and by 37% with sialyllactose (Table 2). In contrast, the binding of FITC-fibronectin was only slightly affected by carbohydrates, with 32% inhibition with 200 mM NANA and 23% inhibition with 100 mM sialyllactose. However, due to the acid pH of the NANA solutions used (2.5 at 200 mM), part of the inhibition of the binding of FITC-laminin or fibrinogen may have been caused by a pH shift.

To confirm the role of sialic acids in the recognition process, the ability of the bovine submaxillary mucin to inhibit the binding of FITC-labeled laminin, fibrinogen, or fibronectin was evaluated and compared to that of asialomucin (Table 2). The binding of the different ligands was inhibited by mucin in a

FIG. 4. Influence of digestion of fibrinogen by chymotrypsin on the adherence of conidia. (A) Adhesion of conidia to the degradation products of fibrinogen. After incubation with chymotrypsin for 4 h at 37°C, the cleavage products were separated by gel filtration. The dotted line represents the A_{280} . Wells of microtiter plates were then coated with a fixed volume (100 μ l) of the different fractions of chromatography, and the adherence of conidia was quantified by use of the standard assay. The continuous line represents the number of adherent conidia for 10 randomly selected fields. (B) SDS-PAGE analysis of the different fractions of chromatography on 12.5% polyacrylamide gels under nonreducing conditions. The lanes are designated by the numbers of the corresponding fractions. The molecular masses (MM) of the protein standards (in kilodaltons) are indicated on the left.

FIG. 5. Effect of carbohydrates on laminin binding. Conidia of *A. fumigatus* were incubated with FITC-laminin without any potential inhibitor or in the presence of different carbohydrates at a final concentration of 200 mM, except for sialyllactose, which was at 100 mM. After 30 min at 37°C, the intensity of fluorescence at the cell surface was measured by flow cytometry. x axis, fluorescence intensity; y axis, number of fluorescent cells. The solid black curve shows the fluorescence of conidia incubated in PBS-EDTA. The graphs shown are representative of two independent experiments.

dose-dependent manner. For example, a 60% inhibition was noted for laminin binding in the presence of 20 μ g of mucin per ml, and the binding was completely abolished with 200 μ g of mucin per ml. Conversely, the binding of the three ligands was only slightly inhibited by asialomucin regardless of the concentration used.

DISCUSSION

Adhesion of microorganisms to the host tissues is now considered an essential event in the development of infections. The recognition of the host cells by the pathogen requires the presence at their surface of complementary molecules. Various host proteins such as laminin, collagen, fibronectin, fibrinogen, and the C3 component of complement have been proposed as cellular ligands of microorganisms (32). These glycoproteins are normally unexposed at the surface of epithelia and endothelia. However, any type of trauma that damages the host tissue may lead to the exposure of basement membranes and therefore to the accessibility of their components, which are laminin, collagen IV, entactin/nidogen, and heparan-sulfate proteoglycans. Moreover, the subsequent inflammatory reaction results in the deposition of fibrinogen and of some components of the complement system. For example, besides inducing neutropenia, the chemotherapy used in preparation for bone marrow transplantation, one of the main predisposing factors for invasive aspergillosis (20), kills not only cancer cells but also epithelial or endothelial cells and therefore could facilitate the adherence of the fungus. Likewise, an increase in the prevalence of aspergillosis in AIDS patients due to previous bacterial, viral, or parasitic bronchopulmonary infections, which led to epithelial tissue damage allowing the colonization of the respiratory tract by the fungus, has recently been reported (24).

Previous works have shown the presence in *A. fumigatus* of laminin and fibrinogen receptors that are localized at the surface of resting or swollen conidia and on mother cells of germ

tubes (1, 7, 38). Moreover, laminin and fibrinogen support the adhesion of conidia (8, 16, 38). Here, we show that the binding of laminin to *A. fumigatus* conidia is saturable and specific. Binding was inhibited in the presence of a 10-fold excess of unlabeled laminin. Conversely, no inhibition was observed in the presence of an equimolar concentration of BSA.

The glycosaminoglycans, which constitute side chains of proteoglycans located preferentially at the surface of the host cells, seem to be involved in the adherence of microorganisms such as *Plasmodium falciparum*, *Trypanosoma cruzi*, and the gonococci (14, 32). Immobilized onto microtiter plates, these components were unable to induce the adhesion of *A. fumigatus* conidia (8). However, their immobilization on an inert surface could modify their spatial conformation and lead to a loss of their binding capability. For example, *C. albicans* does not adhere to immobilized glycosaminoglycans (25). Conversely, these components in a soluble form inhibit the adhesion of the yeast to immobilized laminin (26) as a consequence of an interaction of the highly negative charged glycosaminoglycans with laminin or of a steric hindrance of the laminin receptors by the direct binding of glycosaminoglycans to the cell surface. In our experiments, no inhibition of the binding of FITC-laminin was noted with the glycosaminoglycans, allowing the exclusion of an interaction with *A. fumigatus* conidia.

Furthermore, our results reinforce previous experiments suggesting the presence in *A. fumigatus* conidia of receptors with a broad specificity which interact with both laminin and fibrinogen. In addition to the similar binding pattern that we have previously mentioned for these two host proteins in resting or swollen conidia and germ tubes (1, 38), we found by flow cytometry that the ability of conidia to bind laminin correlated with their maturation as it occurs for fibrinogen binding (16). The fluorescence intensity and therefore the density of receptor sites at the conidial surface increased with the age of the cells, reaching a plateau for conidia 3 to 5 days old. Likewise, fibrinogen inhibited the fixation of FITC-laminin. This result agrees with that of Tronchin et al. (38) showing an inhibition of

TABLE 2. Effect of carbohydrates on the binding of laminin, fibrinogen, and fibronectin to *A. fumigatus* conidia

Carbohydrate	Concn	% Binding relative to the control		
		FITC-laminin	FITC-fibrinogen	FITC-fibronectin
None		100	100	100
Glucose	20 mM	93	99	104
	200 mM	93	97	114
Galactose	20 mM	98	109	93
	200 mM	92	97	98
Mannose	20 mM	98	110	108
	200 mM	101	104	97
Glucosamine	20 mM	103	108	ND ^a
	200 mM	103	71	ND
Galactosamine	20 mM	98	106	ND
	200 mM	89	66	ND
GlcNAc	20 mM	98	105	106
	200 mM	97	89	105
GalNAc	20 mM	97	104	103
	200 mM	100	92	90
NANA	20 mM	60	44	116
	200 mM	23	20	68
Lactose	20 mM	97	109	96
	200 mM	92	93	97
Maltose	20 mM	92	85	91
	200 mM	73	86	94
Sialyllactose	20 mM	98	91	96
	100 mM	41	63	77
Mucin	20 µg/ml	40	24	39
	200 µg/ml	1	3	5
Asialomucin	20 µg/ml	96	78	101
	200 µg/ml	88	75	71

^a ND, not determined.

the adhesion of conidia to immobilized laminin in the presence of fibrinogen, and with that of Coulot et al. (16) which showed an inhibition by laminin of the fixation of FITC-fibrinogen and of the adhesion to immobilized fibrinogen.

Precise identification of the binding sites on the laminin and fibrinogen molecules was achieved by competition experiments of the binding of the fluorescent ligands and microtiter plate adherence assays. Of the potential inhibitors we examined, our attention has been focused primarily on the well known adhesive peptide motifs of laminin, fibrinogen, and elastin. However, the synthetic peptides tested failed to inhibit the binding of FITC-laminin, confirming previous experiments performed with FITC-labeled fibrinogen (16). The situation in *A. fumigatus* differs from that reported for *C. albicans* and *Histoplasma capsulatum*, which recognize laminin through their peptide sequences CDPGYIGSR (31) and IKVAV (30), respectively.

The cleavage of laminin and fibrinogen by chymotrypsin also led to numerous fragments able to support the adhesion of conidia, suggesting a unique binding site common to sequential degradation fragments or the presence of multiple binding

sites on these glycoproteins. Strikingly, laminin and fibrinogen are two heavily glycosylated proteins with closely related N-linked bi-, tri-, or tetra-antennary carbohydrate chains composed of N-acetyl lactosamine or poly lactosamine side chains with a terminal sialic acid residue branched by mannose residues on the glycosidic core (27, 37). Thus, we investigated the possible role of carbohydrates in the recognition process. Among the carbohydrates tested, only NANA and sialyllactose inhibited the binding of both laminin and fibrinogen. Likewise, bovine submaxillary mucin, another glycoprotein rich in terminal sialic acid residues, also inhibited the binding of the fluorescent ligands, and its desialylated form, asialomucin, was much less efficient in this inhibition. Together, these results suggest the presence at the conidial surface of a lectin which binds to the terminal sialic acid residues of the carbohydrate chains of these glycoproteins. Interestingly, such a mode of recognition of extracellular matrix components is also reported for some malignant cells (13) and for various gram-negative bacteria. For example, S fimbriae of *E. coli* (43) and *Helicobacter pylori* (40) recognize laminin through its terminal sialyl- α (2,3) galactoside residues.

The pathogenic scheme of many organisms is comprised of a multifactorial scenario, and for instance, it seems likely that several distinct ligand-receptor systems contribute to the adherence in *A. fumigatus*. A sialic acid-specific lectin of the conidial wall seems to be involved in the binding of laminin and fibrinogen. Conversely, the binding of fibronectin was only slightly inhibited by NANA and sialyllactose, suggesting that it is mediated by another receptor system. This is consistent with the previous report of Peñalver et al. (33) which showed that fibronectin binding is mediated by two polypeptides of 23 and 30 kDa of the conidial wall, whereas our ligand-blotting experiments revealed that laminin binding involves a 72-kDa component (39). Likewise, during the course of this work, Gil et al. (22) showed that the peptide RGD inhibits the adhesion of conidia to immobilized fibronectin and the binding of soluble fibronectin but not the binding of laminin. Further studies are needed to elucidate the structure of this lectin which is used as a laminin and fibrinogen receptor in *A. fumigatus* and which may represent an attractive target for the development of new antimicrobial agents such as vaccines or adhesin blockers.

ACKNOWLEDGMENTS

This work was supported by grants from the Association Française de Lutte contre la Mucoviscidose and from the Ligue de Recherche contre le Cancer.

REFERENCES

1. Annaix, V., J. P. Bouchara, G. Larcher, D. Chabasse, and G. Tronchin. 1992. Specific binding of human fibrinogen fragment D to *Aspergillus fumigatus* conidia. *Infect. Immun.* **60**:1747-1755.
2. Annaix, V., J. P. Bouchara, G. Tronchin, J. M. Senet, and R. Robert. 1990. Structures involved in the binding of human fibrinogen to *Candida albicans* germ tubes. *FEMS Microbiol. Immunol.* **64**:147-154.
3. Beck, K., I. Hunter, and J. Engel. 1990. Structure and function of laminin: anatomy of a multidomain glycoprotein. *FASEB J.* **4**:148-160.
4. Bodey, G. P. 1988. The emergence of fungi as major hospital pathogens. *J. Hosp. Infect.* **11**:411-426.
5. Bodey, G. P., and S. Vartivarian. 1989. Aspergillosis. *Eur. J. Clin. Microbiol. Infect. Dis.* **8**:413-437.
6. Bouchara, J. P., N. Ait Oumeziane, J. C. Lissitzky, G. Larcher, G. Tronchin, and D. Chabasse. 1996. Attachment of spores of the human pathogenic fungus *Rhizopus oryzae* to extracellular matrix components. *Eur. J. Cell Biol.* **70**:76-83.
7. 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.
8. Bouchara, J. P., G. Renier, P. Coulot, P. Penn, G. Tronchin, and D. Chabasse. 1994. Recognition of fibrinogen and basement membrane components as mediators of the adherence of *Aspergillus fumigatus* conidia. *Colloids Surf. B* **2**:299-304.

9. Bouchara, J. P., G. Tronchin, V. Annaix, R. Robert, and J. M. Senet. 1990. Laminin receptors on *Candida albicans* germ tubes. *Infect. Immun.* **58**:48–54.
10. Bouchara, J. P., G. Tronchin, G. Larcher, and D. Chabasse. 1995. The search for virulence determinants in *Aspergillus fumigatus*. *Trends Microbiol.* **3**:327–330.
11. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
12. Burke, C., K. H. Mayo, A. P. N. Skubitz, and L. T. Furcht. 1991. ¹H-NMR and CD secondary structure analysis of cell adhesion promoting peptide F-9 from laminin. *J. Biol. Chem.* **266**:19407–19412.
13. Chammas, R., M. G. Jasiulionis, F. Jin, D. M. S. Villa-Verde, and V. N. Reinhold. 1994. Carbohydrate binding proteins in cell-matrix interaction. *Br. J. Med. Biol. Res.* **27**:2169–2179.
14. Chen, T., R. J. Belland, J. Wilson, and J. Swanson. 1995. Adherence of pilus⁺ *Opa*⁺ gonococci to epithelial cells in vitro involves heparan sulfate. *J. Exp. Med.* **182**:511–517.
15. Clark, H. F., and C. C. Shepard. 1963. A dialysis technique for preparing fluorescent antibody. *Virology* **20**:642–644.
16. 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.
17. Doolittle, R. F. 1984. Fibrinogen and fibrin. *Annu. Rev. Biochem.* **53**:195–229.
18. Fraser, D. W., J. L. Ward, L. Ajello, and B. D. Plikattis. 1979. Aspergillosis and other systemic mycoses. The growing problem. *JAMA* **242**:1631–1635.
19. Furtado, G. C., N. Slowik, H. K. Kleinman, and K. A. Joiner. 1992. Laminin enhances binding of *Toxoplasma gondii* tachyzoites to J774 murine macrophage cells. *Infect. Immun.* **60**:2337–2342.
20. Gerson, S. L., G. H. Talbot, S. Hurwitz, B. L. Strom, E. J. Lusk, and P. A. Cassileth. 1984. Prolonged granulocytopenia: the major risk factor for invasive pulmonary aspergillosis in patients with acute leukemia. *Ann. Intern. Med.* **100**:345–351.
21. Ghosh, A., L. Kole, K. Bandyopadhyay, K. Sarkar, and P. K. Das. 1996. Evidence of a laminin binding protein on the surface of *Leishmania donovani*. *Biochem. Biophys. Res. Commun.* **226**:101–106.
22. Gil, M. L., M. C. Peñalver, J. L. Lopez-Ribot, J. E. O'Connor, and J. P. Martinez. 1996. Binding of extracellular matrix proteins to *Aspergillus fumigatus* conidia. *Infect. Immun.* **64**:5239–5247.
23. Hinek, A. 1994. Nature and the multiple functions of the 67 kd elastin/laminin binding protein. *Cell Adhesion Commun.* **2**:185–193.
24. Klaphotz, A., N. Salomon, D. C. Perlman, and W. Talvera. 1991. Aspergillosis in the immunodeficiency syndrome. *Chest* **100**:1614–1618.
25. Klotz, S. A. 1990. Adherence of *Candida albicans* to components of the subendothelial extracellular matrix. *FEMS Microbiol. Lett.* **68**:249–254.
26. Klotz, S. A., and R. L. Smith. 1992. Glycosaminoglycans inhibit *Candida albicans* adherence to extracellular matrix proteins. *FEMS Microbiol. Lett.* **99**:205–208.
27. Knibbs, R. N., F. Perini, and I. J. Goldstein. 1989. Structure of the major concanavalin A reactive oligosaccharides of the extracellular matrix component laminin. *Biochemistry* **28**:6379–6392.
28. Laemmli, U. K. 1970. Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
29. Lissitzky, J. C., M. Bouzon, E. Loret, M. F. Poupon, and P. M. Martin. 1987. Laminin-mediated adhesion in metastatic rat rhabdomyosarcoma cell lines involves prominent interactions with the laminin E8 fragment. *Clin. Exp. Metastasis* **7**:469–480.
30. McMahon, J. P., J. Wheat, M. E. Sobel, R. Pasula, J. F. Downing, and W. J. Martin. 1995. Murine laminin binds to *Histoplasma capsulatum*: a possible mechanism of dissemination. *J. Clin. Invest.* **96**:1010–1017.
31. Ollert, M. W., R. Söhnchen, H. C. Korting, U. Ollert, S. Bräutigam, and W. Bräutigam. 1993. Mechanisms of adherence of *Candida albicans* to cultured human epidermal keratinocytes. *Infect. Immun.* **61**:4560–4568.
32. Patti, J. M., B. L. Allen, M. J. McGavin, and M. Höök. 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu. Rev. Microbiol.* **48**:585–617.
33. Peñalver, M. C., J. E. O'Connor, J. P. Martinez, and M. L. Gil. 1996. Binding of human fibronectin to *Aspergillus fumigatus* conidia. *Infect. Immun.* **64**:1146–1153.
34. Pendland, J. C., and D. G. Boucias. 1993. Variation in the ability of galactose and mannose-specific lectin to bind to cell wall surfaces during growth of the insect pathogenic fungus *Paecilomyces farinosus*. *Eur. J. Cell Biol.* **60**:322–330.
35. Plow, E. F., and G. Marguerie. 1982. Inhibition of fibrinogen binding to human platelets by the tetrapeptide glycyl-L-prolyl-L-arginyl-L-proline. *Proc. Natl. Acad. Sci. USA* **79**:3711–3715.
36. Silva-Filho, E., W. De Souza, and J. D. Lopes. 1988. Presence of laminin binding proteins in trichomonads and their role in adhesion. *Proc. Natl. Acad. Sci. USA* **85**:8042.
37. Townsend, R. R., E. Hilliker, Y. T. Li, R. A. Laine, W. R. Bell, and Y. C. Lee. 1982. Carbohydrate structure of human fibrinogen. Use of 300-MHz ¹H-NMR to characterize glucosidase-treated glycopeptides. *J. Biol. Chem.* **257**:9704–9710.
38. Tronchin, G., J. P. Bouchara, G. Larcher, J. C. Lissitzky, and D. Chabasse. 1993. Interaction between *Aspergillus fumigatus* and basement membrane laminin: binding and substrate degradation. *Biol. Cell* **77**:201–208.
39. Tronchin, G., K. Esnault, G. Renier, R. Filmon, D. Chabasse, and J. P. Bouchara. 1997. *Aspergillus fumigatus* conidia: expression and identification of a laminin-binding protein. *Infect. Immun.* **65**:9–15.
40. Valkonen, K. H., M. Ringner, A. Ljungh, and T. Wadström. 1993. High-affinity binding of laminin by *Helicobacter pylori*: evidence for a lectin-like interaction. *FEMS Immunol. Med. Microbiol.* **7**:29–38.
41. Vazquez-Juarez, R., F. Ascencio, T. Andlid, L. Gustafsson, and T. Wadström. 1993. The expression of potential colonization factors of yeast isolated from fish during different growth conditions. *Can. J. Microbiol.* **39**:1135–1141.
42. Vicentini, A. P., J. L. Gesztesi, M. F. Franco, W. De Souza, J. Z. De Moraes, L. R. Travassos, and J. D. Lopes. 1994. Binding of *Paracoccidioides brasiliensis* to laminin through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis. *Infect. Immun.* **62**:1465–1469.
43. Virkola, R., J. Parkkinen, J. Hacker, and T. K. Korhonen. 1993. Sialyloligosaccharide chains of laminin as an extracellular matrix target for S fimbriae of *Escherichia coli*. *Infect. Immun.* **61**:4480–4484.
44. Yamada, K. M. 1991. Adhesive recognition sequences. *J. Biol. Chem.* **266**:12809–12812.