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β-1,2- and α-1,2-Linked Oligomannosides Mediate Adherence of *Candida albicans* Blastospores to Human Enterocytes In Vitro

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Candida albicans **is a commensal dimorphic yeast of the digestive tract that causes hematogenously disseminated infections in immunocompromised individuals. Endogenous invasive candidiasis develops from *C. albicans* adhering to the intestinal epithelium. Adherence is mediated by the cell wall surface, a domain composed essentially of mannopyranosyl residues bound to proteins, the N-linked moiety of which comprises sequences of α-1,2- and β-1,2-linked mannose residues. β-1,2-linked mannosides are also associated with a glycolipid, phospholipomannan, at the *C. albicans* surface. In order to determine the roles of β-1,2 and α-1,2 oligomannosides in the *C. albicans*-enterocyte interaction, we developed a model of adhesion of *C. albicans* VW32 blastospores to the apical regions of differentiated Caco-2 cells. Preincubation of yeasts with monoclonal antibodies (MAbs) specific for α-1,2 and β-1,2 mannan epitopes resulted in a dose-dependent decrease in adhesion (50% of the control with a 60-μg/ml MAb concentration). In competitive assays β-1,2 and α-1,2 tetramannosides were the most potent carbohydrate inhibitors, with 50% inhibitory concentrations of 2.58 and 6.99 mM, respectively. Immunolocalization on infected monolayers with MAbs specific for α-1,2 and β-1,2 oligomannosides showed that these epitopes were shed from the yeast to the enterocyte surface. Taken together, our data indicate that α-1,2 and β-1,2 oligomannosides are involved in the *C. albicans*-enterocyte interaction and participate in the adhesion of the yeasts to the mucosal surface.**

Candida species are part of the commensal flora of the mucosa and skin in humans and other vertebrates. In immunocompromised or intensive-care patients, increased mucosal proliferation secondary to use of broad-spectrum antibiotics, together with reduced host defenses and physical alteration of the mucosal barriers, may result in bloodstream invasion. Altogether, candidemia accounts for ~10% of nosocomial bloodstream infections, and *C. albicans* is the causative agent in 50 to 70% of disseminated candidiasis (13, 18, 20, 36, 48).

Molecular typing methods have shown an overall genetic similarity between *C. albicans* strains obtained from blood cultures and colonizing strains obtained from the gastrointestinal tracts of the same patients, confirming endogenous acquisition as the main source of invasive candidiasis (40, 47). On the basis of this model, adhesion of the yeasts to the epithelium of the digestive tract is a prerequisite for colonization and a critical step in the pathogenesis of invasive candidiasis. Characterization of the adhesins and ligands involved in the *C. albicans*-enterocyte interaction thus appears to be a necessary approach to developing strategies aimed at reducing mucosal colonization and preventing bloodstream invasion.

Interaction of *C. albicans* with host cell surfaces is mediated by the yeast cell wall, a complex and dynamic structure containing glucan, chitin and mannoproteins (reviewed in reference 6). The outermost layers of the *C. albicans* cell wall are made of phosphopeptidomannan (PPM), a polymer of mannose residues and proteins commonly referred to as mannan (3, 6). Mannan has been shown to play a role in adherence (27), immunomodulation (11), and antigenic variability (43). The PPM glycan moiety is composed of O-linked and N-linked oligomannosides. The N-linked part consists of a backbone of α-1,6-linked mannopyranose residues with branches composed of α-1,2- and α-1,3-linked mannopyranose units and terminal β-1,2 linkages in *C. albicans* serotype A (7). Short branches composed of β-1,2-linked mannopyranose residues are linked to PPM through phosphodiester bridges in *C. albicans* serotypes A and B. These side chains are referred to as the acid-labile fraction of PPM, since they are cleaved by mild acid treatment (45). β-1,2 oligomannosidic chains have also been identified on a 14- to 18-kDa glycolipid, referred to as phospholipomannan (PLM) (46), that is expressed at and shed from the *C. albicans* cell wall (25, 39).

β-1,2 mannosidic linkages are uncommon structures whose presence has been reported in only few bacterial and yeast species (30, 35). In *C. albicans*, their presence was first identified on PPM by Shibata et al. (41). These oligomannoside sequences are involved in the adhesion of *C. albicans* to the macrophage membrane, at least in part through binding to galectin 3, a member of a family of carbohydrate binding proteins implicated in a variety of biological functions (17, 25). β-1,2 oligomannosides also generate protective antibodies (22) and induce cytokine production (26). These unique carbohy-

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drate sequences thus appear to play a key role in the *C. albicans*-host balance.

Despite the importance of the digestive tract as the main source of invasive candidiasis, few groups have reported analyses of the *C. albicans*-enterocyte interaction at the cellular and molecular levels (44, 49, 50). In the present paper, we describe a model of adhesion of *C. albicans* blastospores to the human enterocyte cell line Caco-2 and its use to analyze the role of oligomannosides with distinct anomere-type linkages, either α or β, in the attachment of *C. albicans* blastospores to Caco-2 cells. Indeed, galectin-3, which binds β-1,2 oligomannosides on the macrophage (17), is also expressed in intestinal epithelial cells (1, 8). Moreover, recent studies with a mouse model of candidiasis showed that oral administration of synthetic β-1,2 oligomannosides could reduce colonization of the gut, presumably by competing with the natural flora for binding to enterocytes (12). We were thus interested in understanding the basis of this phenomenon at the cellular and molecular levels.

MATERIALS AND METHODS

Growth and preparation of *C. albicans* strain for adherence assay. *C. albicans* strain VW32 (serotype A) (5) was used throughout this study. This strain, which was originally isolated from a patient with human renal candidiasis, has been employed in prior studies of *C. albicans*-macrophage interactions (16, 17, 25) and in the chemical characterization of *C. albicans* β-mannosidic epitopes (15, 46). Stock cultures were maintained at −20°C on Sabouraud dextrose agar. For adhesion experiments, stock cultures were plated on Sabouraud dextrose agar and incubated at 37°C. After 18 to 20 h of culture, the yeasts were recovered, washed with phosphate-buffered saline (PBS), and resuspended in PBS. The yeast concentration was determined by hemocytometer and growth on Sabouraud dextrose agar. For experiments using heat-killed yeasts, blastospores were treated at 100°C for 15 min, washed in PBS by centrifugation, and resuspended in PBS.

Growth and differentiation of Caco-2 cells. Caco-2 cells were obtained from the American Type Culture Collection (HTB 27) and were cultivated in the absence of antibiotics and antifungal agents. Cells (passages 4 to 15) were grown to confluence in 25-cm^2 flasks at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (3 volumes) with Ham F12 (1 volume) containing 10% fetal calf serum, glutamine, and 1 g of glucose/liter. For adhesion experiments, ~4 × 10^5 cells were seeded on each 11-mm-diameter glass coverslip in 24-well plastic dishes. The cultures were maintained at 37°C and 5% CO_2, and the medium of each culture well was replaced every other day until it was used.

Fluorescent probes and antibodies. Monoclonal antibody (MAb) EBCA1 is a rat immunoglobulin (IgM) that reacts with α-linked mannose (24). MAb 5B2 is a mouse-rat chimeric IgM that reacts with β-linked mannose (23). The control MAb A255 is a mouse IgM that recognizes a protein of the respiratory syncytial virus. It was provided by P. Pothier (Dijon, France). Uvitex 2B was purchased from LD Bio Diagnostics (Lyon, France). For immunofluorescence experiments, the secondary antibodies Alexa Fluor 488 goat anti-mouse IgM (μ chain) and Alexa Fluor 488 goat anti-rat IgM (μ chain) (1/200 dilutions in PBS; Molecular Probes, Leiden, The Netherlands) were employed for MAbs 5B2 and EBCA1, respectively. Detection of actin in confocal-microscopy experiments was performed with Alexa Fluor 568-phalloidin (1 U of phalloidin per well; Molecular Probes). For double-labeling experiments, the secondary antibody Alexa Fluor 594 goat anti-rat IgM (1/200 dilution in PBS; Molecular Probes) was employed for MAb EBCA1.

Carbohydrates. D-Glucose, D-galactose, galactosamine, *N*-acetylglucosamine, D-mannose, D-fucose, L-fucose, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, D-xylose, D-lactose, *N*-acetyllactosamine, D-mannosamine, mannan from *Saccharomyces cerevisiae*, hyaluronic acid, fetuin, asialofetuin, laminarin, heparin, fucoidan, and chondroitin sulfate A and C were purchased from Sigma-Aldrich Chimie (Saint Quentin Fallavier, France) and stored according to the recommendations of the manufacturer. D-Rhamnose and α-methylmannoside were purchased from Acros Organics (Noisy le Grand, France) and stored at 4°C. α-1,2 tetramannosides and β-1,2 tetramannosides were synthesized according to a previously described protocol (12) and stored at 4°C until they were used. All carbohydrates were diluted extemporaneously in PBS for attachment inhibition assays.

Adherence of *C. albicans* blastospores to Caco-2 cells. Monolayers for adhesion experiments were used 15 to 21 days after being seeded. The medium in each well was aspirated and replaced by 300 μl of PBS preincubated at 37°C and containing 10^3 blastospores. After 30 min at 37°C, the monolayers were washed three times to remove nonadhering blastospores, fixed in 2% glutaraldehyde for 10 min, and washed twice. For detection of adherent blastospores, Uvitex 2B (1/100 dilution in PBS) was added to each well for 5 min in the dark at room temperature. The coverslips were rinsed, counterstained with 0.2% Evans Blue in PBS for 30 s, rinsed three times, and mounted inverted on microscope slides. In all the experiments described, the Caco-2 monolayers were washed with PBS at 37°C. Attachment was quantified by immunofluorescence microscopy using a Zeiss Axioscop 2 microscope with an excitation filter at 360 nm and a suppression filter at 460 nm. The percentage of adhesion in each culture was determined as the ratio of the number of adherent yeasts on the entire surface of the coverslip to the inoculum evaluated by quantitative culture. Each condition was tested in triplicate, and three separate experiments were performed.

Attachment inhibition assays. The effects of MAbs 5B2 and EBCA1 on the attachment of *C. albicans* blastospores to Caco2 cells were studied by using the adherence assay described above. Yeasts were suspended in PBS and preincubated with purified MAbs at 60, 6, or 3 μg/ml for 1 h at 37°C under mild agitation. Agglutinated blastospores were pelleted by a 3-min centrifugation at 200 × *g*. Supernatants containing nonagglutinated blastospores were diluted with PBS, and 10^3 yeast cells were transferred to Caco-2 monolayers. The adhesion experiment was then performed as described above. In parallel, 20-μl fractions of each supernatant were recovered, placed on immunofluorescence slides, air dried, reacted with secondary antibodies, and examined with a Zeiss axioscope 2 microscope to assess the labeling of the blastospores with primary MAbs. All experiments included two positive control wells (cocultures in PBS alone without inhibiting antibody) and duplicate testing of attachment with each antibody concentration. Three separate experiments were performed.

To determine the effect of synthetic β-1,2 and α-1,2 tetramannosides and control carbohydrates on adhesion, Caco-2 monolayers were incubated at 37°C in PBS containing the following concentrations of the carbohydrates tested. D-Glucose, D-galactose, galactosamine, *N*-acetylglucosamine, D-mannose, D-fucose, L-fucose, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, D-xylose, D-lactose, *N*-acetyllactosamine, D-mannosamine, D-rhamnose, and α-methylmannoside were tested at 10, 50, and 250 mM. Mannan from *S. cerevisiae*, hyaluronic acid, fetuin, asialofetuin, laminarin, heparin, fucoidan, and chondroitin sulfate A and C were tested at 0.2, 1, and 5 mg/ml. For β-1,2 mannotetraoses and α-1,2 mannotetrasoses, the concentrations tested were 0.3, 1.5, and 7 mM. After 30 min, 10^3 yeast cells were added to each well and coincubated with Caco-2 cells for 30 min at 37°C. The adhesion experiment was then performed as described above. All experiments included a series of positive control wells (cocultures in PBS alone without inhibiting carbohydrates) and triplicate testing of attachment with each carbohydrate concentration. Three separate experiments were performed for each carbohydrate tested.

In both series of inhibition experiments, the results of adhesion without inhibiting antibodies or competing carbohydrates were set to 100%, and adhesion in the presence of antibodies or carbohydrates was expressed as the ratio of the percentage of adhesive yeasts in the presence of a given concentration of antibody or carbohydrate to the percentage of adhesive yeasts in positive control wells.

Immunofluorescence confocal microscopy. Cultures for immunofluorescence confocal microscopy were performed in 24-well culture dishes containing removable porous inserts (0.1-μm pore diameter; BD Falcon). Approximately 0.5 × 10^5 cells were seeded in the upper compartment. The cultures were maintained at 37°C and 5% CO_2, and the medium in each well was replaced every other day. The monolayers were used 15 to 21 days postseeding. The adhesion assays were performed as described above, with an inoculum of 10^5 yeast cells per well for cocultures of 30 to 60 min. When longer interactions were analyzed, an inoculum of 10^3 yeast cells per well was used. Following incubation, the monolayers were washed three times to remove nonadhering blastospores. They were then fixed for 10 min at room temperature in a 3.7% formaldehyde solution in PBS. After two more washes, the monolayers were extracted with 0.1% Triton X-100 in PBS for 3 to 5 min. Each well was then washed twice, and the monolayers were reacted with Alexa Fluor 568-phalloidin for 20 min at room temperature for F-actin labeling in Caco-2 cells. After two washes, the monolayers were incubated with MAb 5B2 or EBCA1 (30 μg/ml; 1 h at 37°C). The monolayers were washed twice and reacted with Alexa Fluor-labeled secondary antibodies. The preparations were examined by confocal laser scanning microscopy on a Leica TCS 4000 microscope. The cell monolayers were optically sectioned in horizontal

(*x-y*) or vertical (*x-z*) planes every 0.8 μm. Five separate experiments were performed to verify the absence of internalization of reactive yeasts at 30 or 60 min postinfection, and 20 microscopic fields were examined for each culture.

Double-labeling experiments on infected monolayers. Caco-2 monolayers grown on glass coverslips were infected with 10^3 blastospores per well 15 to 21 days postseeding. At 12 h postinfection, the monolayers were washed to remove nonadherent blastospores. They were then fixed for 10 min at room temperature in a 3.7% formaldehyde solution in PBS. After two washes, the monolayers were reacted sequentially with MAb EBCA1, Alexa Fluor 594 goat anti-rat IgM, MAb 5B2, and Alexa Fluor 488 goat anti-mouse IgM at the concentrations given above. All incubations were performed for 1 h at 37°C and were followed by two washes. The monolayers were examined on a Nikon E600 Eclipse microscope.

Statistical analysis. The within-day and between-day repeatabilities were studied by one-way analysis of variance. In attachment inhibition assays, the differences among 5B2, A255, and EBCA1 antibodies, as well as those between α-1,2 and β-1,2 tetramannosides, were studied, along with the concentrations, by two-way analysis of variance. The concentration associated with a 50% inhibition (IC_{50}) was estimated with its standard error by modeling the inhibition curve according to the following equation: $y = a/(a + x)$, where y is the percentage of inhibition, x is the concentration, and a is the value of x associated with a y of 50%; the minimization algorithm used for this nonlinear regression was the Gauss-Newton type.

RESULTS

Characterization of a model of adhesion of *C. albicans* blastospores to Caco-2 cells. In order to identify the molecular partners involved at the initial stage of the *C. albicans*-enterocyte interaction, we developed a model of adhesion of *C. albicans* blastospores to Caco-2 cells. The binding of blastospores to monolayers increased from 10 to 60 min of incubation. After 60 min, clusters of blastospores indicating multiplication of the inoculum were observed, which precluded accurate evaluation of the initial attachment process. Moreover, germ tubes, hyphae, and pseudohyphae were detected, and these fungal elements express surface molecules, i.e., putative adhesins, distinct from blastospores (31). Our model was thus optimized to analyze *C. albicans* attachment after a 30-min interaction. Since the degree of maturation of Caco-2 cells increases until the cells are differentiated 14 to 21 days after being seeded (37), we evaluated the attachment of *C. albicans* to Caco-2 monolayers from 5 to 40 days postseeding. The binding of blastospores increased from 5 to 15 days and reached a plateau after 15 days. When the monolayers were infected with 10^3 blastospores 15 to 21 days postseeding, an average of 120 to 250 adherent yeast cells were detected after a 30-min interaction. Altogether, the mean within-day coefficient of variation of the model was 9%, whereas the between-day variance gave a coefficient of variation of 29%.

A key problem in setting up the model was to verify that yeasts counted after washings were adherent yeasts, i.e., yeasts that were attached to the surfaces of the cells as opposed to blastospores that could have been internalized by enterocytes. The Uvitex 2B dye met this requirement. This marker is excluded from live phagocytes (29) and has been employed to identify nonphagocytized yeasts in a model of *Candida*–endothelial-cell interaction (19). Moreover, fluorescence confocal microscopy analysis of permeabilized monolayers at 30 and 60 min of coculture confirmed that *C. albicans* blastospores were present at the apical surfaces of the cells only (Fig. 1). No internalized yeasts were detected in these experiments.

Inhibition of *C. albicans* adhesion to Caco-2 cells by MAbs specific for α-linked or β-1,2-linked oligomannosides. To establish the role of α- and β-mannosidic sequences in the *C. albicans*–Caco-2 interactions, we first performed neutralizing experiments with 5B2 and EBCA1, two MAbs that bind β-1,2 and α-1,2 mannoside epitopes, respectively (23, 24). An isotype-matched irrelevant MAb was used as a control. Since 5B2 and EBCA1 agglutinated *C. albicans* blastospores, yeasts treated with the MAbs were centrifuged and adhesion experiments were performed with the nonagglutinated blastospores present in the supernatants. Fractions of these nonagglutinated yeasts were assayed in parallel by immunofluorescence to verify that they bound the MAbs (Table 1). In experiments performed with 60 μg of MAbs/ml, the percentages of adhesion of *C. albicans* to Caco-2 cells were 91.4 ± 7.17, 53 ± 6.21, and 49 ± 5.56% for A255 (control), 5B2 (anti-β-1,2 mannoside), and EBCA1 (anti-α-1,2 mannoside) antibodies, respectively (Fig. 2), and statistical analysis showed that both anti-mannoside antibodies differed significantly from the A255 control antibody ($P < 0.05$). The increased adherence of blastospores treated with MAb 5B2 at 6 μg/ml (114 ± 18.2%) was significant ($P < 0.05$) compared to that of A255 (95.0 ± 6.08%) (Fig. 2). These results reflect a significant interaction between antibody and concentration, as the difference between antibodies is much higher at 60 than at 6 μg/ml. Other attachment percentages did not differ significantly.

Competitive inhibition of *C. albicans* adhesion to Caco-2 cells by glycans. We next performed competitive assays using

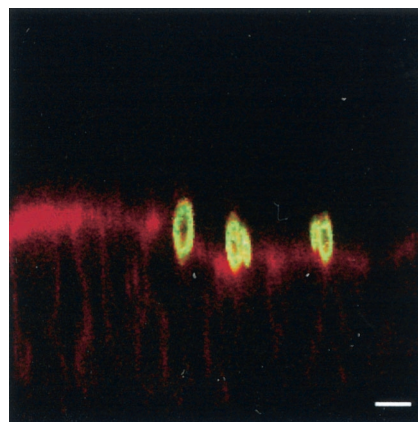

FIG. 1. Confocal laser scanning microscopy of Caco-2 monolayers at 30 min postinfection with *C. albicans* blastospores. Monolayers sectioned in vertical planes (*x-z*) were processed for double labeling using Alexa fluor 568-phalloidin (red) and MAb 5B2 (green), and a merged image is presented. The apical region of the cell is identified by strong reactivity of phalloidin with the actin network of the brush border. Yeast cells are present at the surfaces of the cells. Bar, 5 μm.

TABLE 1. Immunofluorescence reactivities of fractions of yeast suspensions exposed to MAbs 5B2, EBCA1, and A255 in attachment inhibition assays[a]

MAb	Reactivity[b]		
	3 μg/ml	6 μg/ml	60 μg/ml
5B2	−	±	++
EBCA1	−	±	++
A255	−	−	−

[a] See Fig. 2.
[b] −, no reactivity; ±, weak reactivity; ++, strong reactivity.

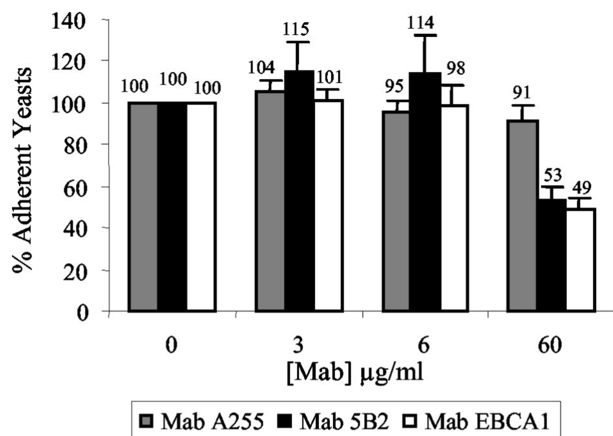

FIG. 2. Effects of MAbs 5B2 (anti-β-1,2 mannose), EBCA1 (anti-α-1,2 mannose), and A255 (control antibody) on attachment of *C. albicans* blastospores to Caco-2 monolayers. Yeast cells were incubated with 3, 6, and 60 μg of antibodies/ml or with PBS alone prior to and during adhesion. Attachment is expressed as a percentage of that of the controls to which no antibody was added. The values are the means plus standard deviations of three independent experiments, including testing in duplicate wells.

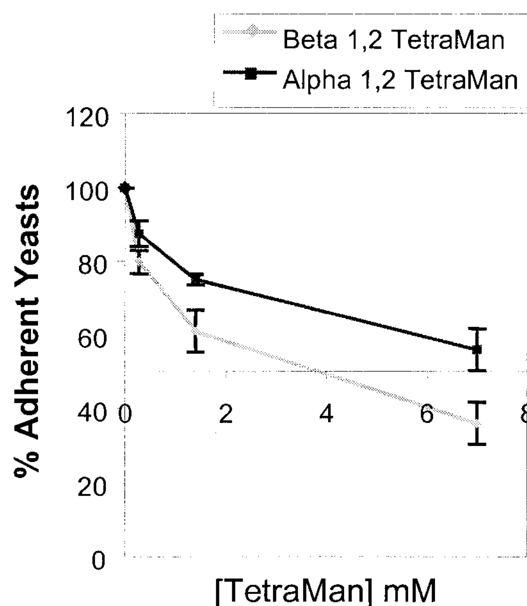

FIG. 3. Effects of synthetic mannotetraoses (TetraMan) on attachment of *C. albicans* blastospores to Caco-2 monolayers. The monolayers were incubated with β-1,2 mannotetraoses and α-1,2 mannotetraoses at 0.3, 1.5, and 7 mM prior to and during adhesion. Attachment is expressed as a percentage of that of the control cultures to which no competing carbohydrate was added. The values are the means ± standard deviations of three independent experiments, including testing in triplicate wells.

synthetic β-1,2 and α-1,2 tetramannosides with antigenic profiles that mimic the antigenicities of their natural counterparts in the *C. albicans* cell wall (12). A panel of mono- and disaccharides and complex glycans was also evaluated for the ability to inhibit *C. albicans* binding to Caco-2 cells. In these experiments, the concentration associated with a 50% inhibition was correctly estimated, since its coefficient of variation (the standard error divided by the estimate) ranged from 9 to 19% between products. The concentrations at which synthetic β-1,2 and α-1,2 tetramannosides inhibited 50% of *C. albicans* attachment to Caco-2 monolayers (IC_{50}s) were 2.58 (standard error, 0.481) and 6.99 (standard error, 1.32) mM, respectively (Fig. 3), and these two glycans were the most potent inhibitors of *C. albicans* adhesion to Caco-2 cells (Fig. 4). Moreover, when the analysis took into account the IC_{50} range (IC_{50} ± 2 standard errors), the results for β-1,2 oligomannosides ranged from 1.62 to 3.54 mM and differed significantly from those for all other glycans tested, including α-1,2 oligomannosides (IC_{50} range, 4.36 to 9.64 mM). None of the complex glycans reduced adherence at 1 mg/ml or below.

Shedding of antigens containing α-1,2- and β-1,2-linked mannose residues during coculture of *C. albicans* with Caco-2 cells. As antigens containing β-1,2 mannose residues are shed during the interaction of *C. albicans* with macrophages (25), we were next interested in determining whether shedding of α-1,2 or β-1,2 mannosides occurred during the *C. albicans*-Caco-2 interaction. To this end, cocultures of *C. albicans* with Caco-2 cells were subjected to indirect immunofluorescence detection of β-1,2 and α-1,2 mannosides with MAbs 5B2 and EBCA1, respectively. At 30 min postinfection, examination of the monolayers showed that MAb 5B2 detected islands of reactive material close to the yeasts (Fig. 5A). A more diffuse localization of the labeling was observed after 12 h of coculture (data not shown). Upon examination in the *x-z* orientation, the 5B2-reactive material was localized at the surfaces of the monolayers (Fig. 5B). When heat-killed blastospores were used, the percentage of adhesion was low (~2.5% of the inoculum), and no reactive material was detected at the surfaces of the monolayers with MAb 5B2 (data not shown). Similar experiments were performed with MAb EBCA1, and clusters of reactive material were detected at the surfaces of the monolayers in the vicinity of the yeasts (data not shown). Similarly, no reactivity of MAb EBCA1 was detected upon incubation of the monolayers with heat-killed blastospores (data not shown). Double labeling of infected monolayers with 5B2 and EBCA1 MAbs (Fig. 6) showed that both antibodies essentially bound to the same domains of the monolayers (Fig. 6D). However, within a reactive domain, not all spots of 5B2-reactive material colocalized with spots of EBCA1-reactive material (Fig. 6D).

DISCUSSION

Most studies of the adhesion of *C. albicans* to human cells were performed with buccal epithelial cells (4, 10, 33), esophageal epithelial cells (14), endothelial cells (19), fibroblasts (32), keratinocytes (34), HeLa cells (21), or macrophages (16), and although intestinal colonization is accepted as the main source of bloodstream dissemination (38, 40, 47), little is known about the molecular basis of attachment of *C. albicans* to enterocytes. Three recent studies have addressed the *C. albicans*-enterocyte interaction. Weide and Ernst showed that transcellular migration of *C. albicans* occurred during a 12- to 24-h interaction (49). Wiesner et al. demonstrated that the absence of a functional INT1 gene, a gene involved in filamentous growth, attachment to HeLa cells, and virulence, was associated with decreased adherence of *C. albicans* blasto-

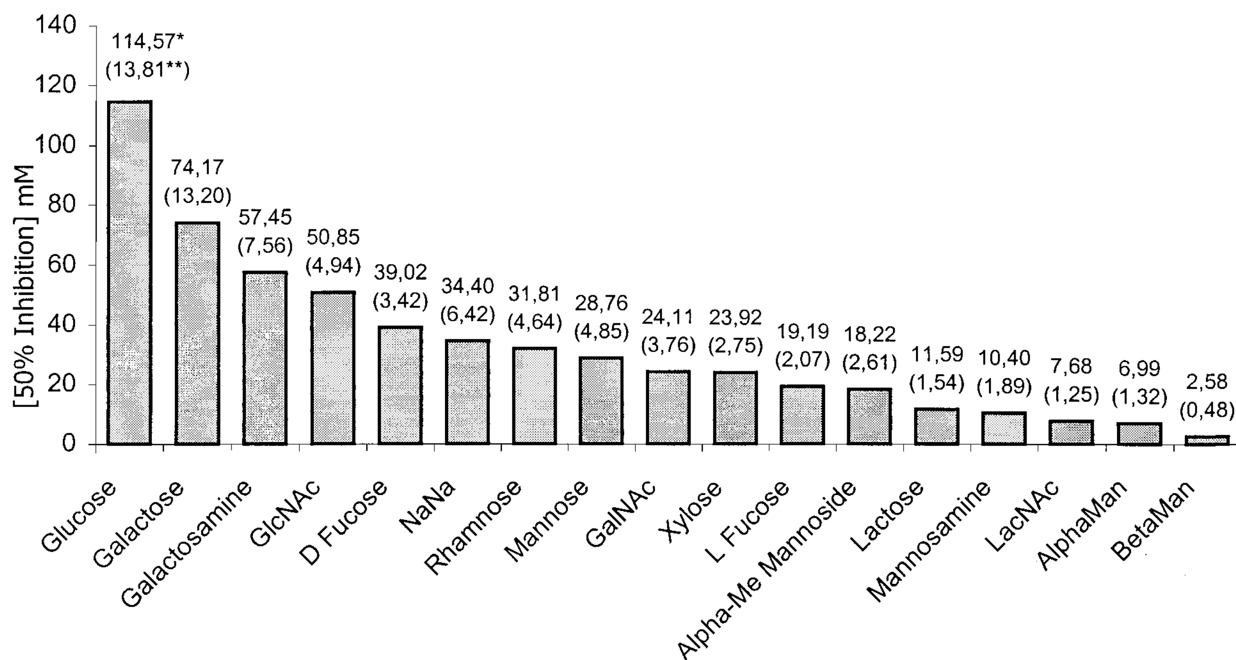

FIG. 4. Inhibition of adherence of *C. albicans* blastospores to Caco-2 monolayers by carbohydrates. GlcNac, *N*-acetylglucosamine; NaNa, *N*-acetylneuraminic acid; GalNac, *N*-acetylgalactosamine; Alpha-Me, α-methylmannoside; LacNac, *N*-acetyllactosamine; AlphaMan, α-1,2 tetramannoside; BetaMan, β-1,2 tetramannoside. The concentration of carbohydrate at which adhesion is 50% of adhesion in the absence of carbohydrate (IC_{50}) (*) was obtained by modeling the inhibition curves (as shown in Fig. 3) following the method described in Materials and Methods. **, standard error of the IC_{50}.

spores to enterocytes (50). In a model of undifferentiated Caco-2 cells, Timpel et al. showed that a *C. albicans* strain defective in PMT1, a gene encoding a mannosyltransferase involved in the O-glycosylation pathway, showed decreased adherence (44). We chose to study the attachment of *C. albicans* to Caco-2 cells. This cell line derives from a human colorectal adenocarcinoma, expresses characteristics of enterocytic differentiation upon reaching confluence (37), and has been used to study interactions of human intestinal cells with bacteria (51), virus (2), protozoa (28), and *C. albicans* (44, 49, 50). The limitation of this model is that Caco-2 cells do not produce mucins. Therefore, our system is not suitable to investigate to what extent mucins may modulate the *C. albicans*–Caco-2 interaction (10).

C. albicans blastospores that bound anti-α-1,2 or anti β-1,2 mannoside MAbs showed a 50% decrease in adhesion to Caco-2 monolayers when exposed to higher antibody concentrations (Fig. 2). Despite the moderate (but statistically significant) increase in adhesion observed with MAb 5B2 at 6 μg/ml (Fig. 2), for which we have no explanation, the major attachment inhibition occurring upon exposure of blastospores to both anti-mannoside antibodies at 60 μg/ml suggests that α-1,2- and β-1,2-linked oligomannoside sequences present at the yeast cell surface are involved in attachment to Caco-2 cells. One limitation of these experiments is the use of nonagglutinated blastospores present in supernatant fractions after mild centrifugation of the yeast preparations. Indeed, despite a dose-dependent reactivity with anti-mannoside antibodies by immunofluorescence assay (Table 1), we cannot rule out the possibility that this blastospore population has adhesive properties distinct from their agglutinated counterparts. Moreover, immunoglobulins bound to the yeast surface could mask adhesive epitopes other than α-1,2 and β-1,2 oligomannosides. We therefore performed competitive experiments with synthetic α-1,2 and β-1,2 tetramannosides. Previous studies of macrophages showed that similar inhibition was achieved by incubating cells with native or synthetic β-1,2 mannotetraoses (16), demonstrating that synthetic molecules with a degree of polymerization of four mannose residues showed normal biological activity. Furthermore, α-1,2 and β-1,2 tetramannosides reacted normally with antibodies specific for α- and β-mannosidic epitopes in an enzyme immunoassay, indicating that the synthetic molecules retained the antigenicities of their native counterparts (12). The competing effect obtained with α-1,2 and β-1,2 tetramannosides is a striking result of the present investigation. The inhibitions obtained with both molecular species are dose dependent (Fig. 3). The specificity of this effect was established by comparing a panel of carbohydrates. None of the complex glycans tested reduced attachment at 1 mg/ml, a finding similar to that reported in a model of the adhesion of *Candida glabrata* to Hep2 cells (9). Other mono- and disaccharides inhibited *C. albicans* attachment to Caco-2 cells, with IC_{50}s ranging from 7.68 mM for *N*-acetyllactosamine to 114.57 mM for glucose (Fig. 4). Thus, among all carbohydrates tested, β-1,2 tetramannosides exhibited the strongest inhibitory effects on the adhesion of *C. albicans* to Caco-2 cells. This finding is consistent with the dramatic reduction in gastrointestinal colonization reported in the infant mouse model after oral administration of synthetic β-1,2 tetramannosides (12). Conversely, the inhibitory effect observed in our study

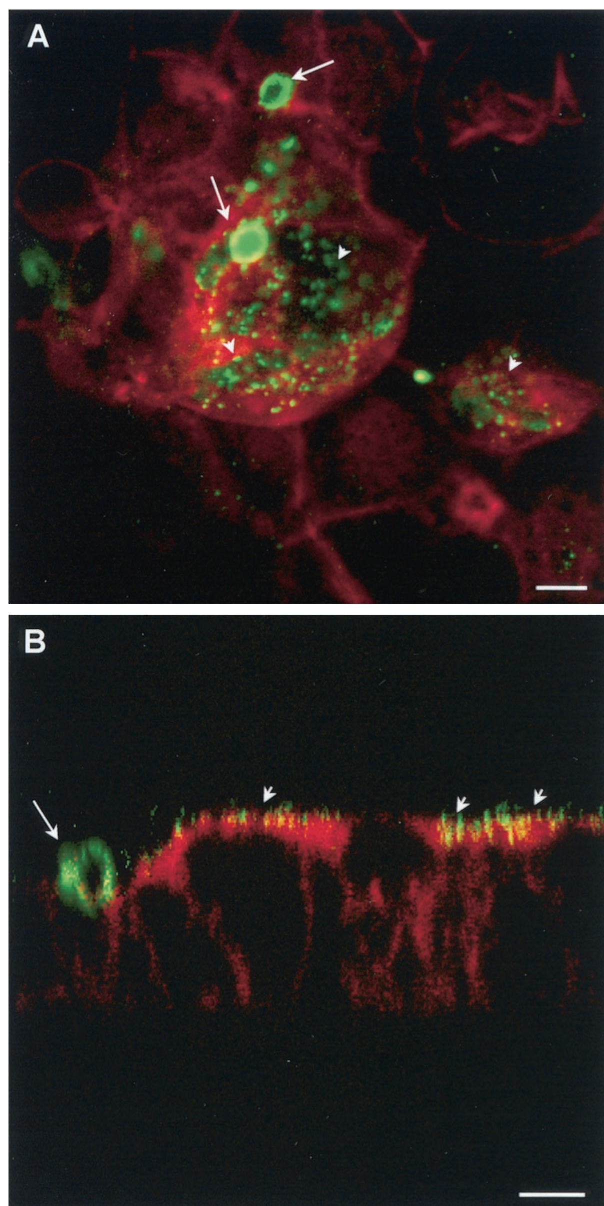

FIG. 5. Immunofluorescence confocal microscopy analysis of β-1,2 oligomannosidic epitopes after a 30-min (A) or 12-h (B) culture of Caco-2 cells with *C. albicans*. The monolayers were processed for double labeling using Alexa Fluor 568-phalloidin (red) and MAb 5B2 (green), and merged images are presented. (A) Islands of material reactive with MAb 5B2 (arrowheads) in the vicinity of *C. albicans* (arrows). (B) Analysis in the vertical plane shows that β-1,2 oligomannosidic epitopes shed by *C. albicans* (long arrow) are present at the apical surfaces of the cells (short arrows). Bars, 5 μm.

with synthetic α-1,2 tetramannosides contrasts with their lack of activity on gastrointestinal colonization in the mouse model (12). The reason why in vivo and in vitro data obtained with β-1,2 tetramannosides are correlated whereas those obtained with α-1,2 oligomannosides are not is unclear. In macrophages, oligomannosides with distinct anomere types of linkage have distinct receptors, since β-1,2 mannosides bind galectin 3 (17) and α-1,2 mannosides react with the macrophage mannose receptor (42). If α-1,2 and β-1,2 mannosides also bind distinct receptors at the enterocyte surface, one possible explanation is that Caco-2 cells express both receptors, whereas infant mouse enterocytes express receptors for β-1,2 mannosides only. An alternative hypothesis could be that enterocyte receptors for α-1,2 mannosides were saturated in the infant mouse model by ubiquitous α-mannosidic sequences expressed by the bacterial flora of the gastrointestinal tract in vivo.

We next examined by immunofluorescence the fate of *C. albicans* α-1,2 and β-1,2 mannosidic epitopes during coculture with Caco-2 cells. The reactivity of MAbs 5B2 and EBCA1 with antigenic material localized at the surface of the cells (Fig. 5 and 6) suggests that α-1,2 and β-1,2 oligomannosides are released by *C. albicans* during interaction with Caco-2 monolayers and bind to the surfaces of the cells. No such labeling was observed upon incubation of monolayers with heat-killed blastospores. The distribution of α-1,2 and β-1,2 mannoside labeling on Caco-2 monolayers was heterogeneous and was localized close to *C. albicans* at 30 min postinfection (Fig. 5A). Low levels of antigen shedding relative to enterocyte surfaces may account for this observation, a hypothesis supported by the increased reactivity of monolayers upon prolonged incubation with *C. albicans*. Double-labeling experiments with EBCA1 and 5B2 antibodies identified domains of the monolayers that reacted with both antibodies. In such domains, merged images showed that clusters of α-1,2 and β-1,2 mannoside reactivity did not necessarily colocalize (Fig. 6D), an observation consistent with the existence of a cell-specific or nonsynchronous expression of receptors for α-1,2 and β-1,2 oligomannosides. The shedding of β-1,2 mannoside epitopes in association with *C. albicans* PLM and adhesion to the host cell membrane were previously reported during cocultures with macrophages (25). At present, we cannot decide whether β-1,2 mannosidic epitopes shed by *C. albicans* during coculture with enterocytes correspond to PLM or to PPM. However, the fact that some of the shed material reacted with 5B2 but not with EBCA1 suggests that PLM is involved, since the carbohydrate moiety of PLM is composed of β-1,2 oligomannosides only. Altogether, this body of informations highlights the need to characterize Caco-2 receptors for α-1,2 and β-1,2 oligomannosides of *C. albicans* and to determine the biological significance of this shedding process in the *Candida*–Caco-2 interaction.

Finally, Timpel et al. demonstrated recently that a homozygous *C. albicans* mutant defective in *PMT1*, a gene encoding a mannosyltransferase involved in the O-glycosylation pathway, showed reduced adherence to Caco-2 cells. This observation pointed to the role of O-linked carbohydrates in the adherence of *C. albicans* to enterocytes (44). Interestingly, the present paper indicates that β-1,2- and α-1,2-linked oligomannosides, two components of the *N*-glycan moiety expressed at the surface of *C. albicans*, participate in the adhesion of blastospores to Caco-2 cells. Therefore, and despite the fact that the monolayers employed in the two studies were presumably not in the same state of differentiation, both O-linked and N-linked carbohydrates seem to represent putative adhesins in the host-fungus interplay that takes place at the intestinal mucosal surface. The characterization of the glycosylation pathways of *C. albicans*, including their regulation and response to the yeast environment, will thus be pivotal to understanding the commensal-pathogen transition and the physiopathological events occurring in the early stages of invasive candidiasis.

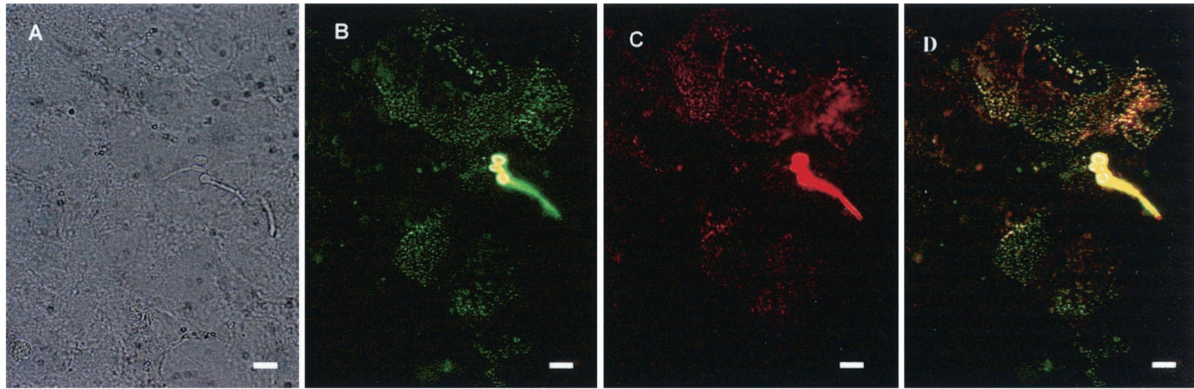

FIG. 6. Double-labeling experiment with MAbs 5B2 and EBCA1 on Caco-2 monolayers incubated for 12 h with *C. albicans*. (A to C) The same microscopic field was examined under phase-contrast microscopy (A), immunofluorescence microscopy for reactivity of MAb 5B2 with β-1,2 oligomannoside (green) (B), and immunofluorescence microscopy for reactivity of MAb EBCA1 with α-1,2 oligomannoside (red) (C). Both MAbs react with *C. albicans* blastospore and germ tube and with clusters of antigenic material at the surface of the cells. (D) Merged image of panels B and C. The secondary antibodies were Alexa Fluor 488 goat anti-mouse IgM (B) and Alexa Fluor 594 goat anti-rat IgM (C). Bars, 5 μm.

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