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Experimental Evidence for the Role of Lipids in Adherence of *Candida* spp. to Human Buccal Epithelial Cells

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Lipids extracted from *Candida albicans* and *C. tropicalis*, but not from the weakly adherent *C. pseudotropicalis*, significantly blocked in vitro adherence of the respective yeast cells to buccal epithelial cells. The percentage of reduction from control values ranged between 16.4 and 42.1%, depending on the species, the strain, and the solvent used for lipid extraction. The constituent lipid classes of both the acetone and chloroform-methanol extracts of *C. albicans* ATCC 10231 were qualitatively and quantitatively analyzed. The individual classes were isolated by preparative thin-layer chromatography and then tested for their effects on the adherence of this strain to buccal epithelial cells. Individual phospholipids, sterols, and steryl esters blocked adherence significantly (between 15.5 and 55.7% reduction). Triacylglycerols and free fatty acids showed no effect whatsoever. The same results were obtained when standard lipid samples were investigated.

The adherence of *Candida albicans* to human epithelial cells as the initial stage in the development of fungal infection has been the subject of several studies (8, 9, 10, 20, 21, 24; R. D. King, A. L. Morris, R. L. Taylor, and E. E. M. Moody, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, F23, p. 122). Attempts to determine the surface component(s) of this fungus involved in its adherence to host cells have revealed so far that saccharides, particularly mannose, or mannose-containing compounds are effective (4, 11, 13, 20, 23). Although compounds such as lipids are also constituents of the cell envelope including the cell wall of *C. albicans* (1), there are no reports in the available literature on their relation to adherence.

The main objective of this study was to shed light on the possible role of certain lipid classes in the adherence of *Candida* cells to buccal epithelial cells (BEC) in vitro.

MATERIALS AND METHODS

Standard lipid samples. Cholesterol, cholesteryl palmitate, triolein, oleic acid, and palmitic acid were purchased from E. Merck AG (Darmstadt, Federal Republic of Germany). Standard phospholipid samples were prepared from freshly extracted (5) egg yolk lipids by repeated precipitation in acetone until the preparation proved pure by thin-layer chromatographic (TLC) analysis.

Organisms. Three species of *Candida* were used in the present study: *C. albicans*, *C. tropicalis*, and *C. pseudotropicalis*. Three strains of the first species were investigated. *C. albicans* ATCC 10231 was obtained as lyophilized samples (isolated from bronchomycosis) from the American Type Culture Collection, Rockville, Md. The other two strains, *C. albicans* KCCC 13878 and *C. albicans* KCCC 14172, as well as *C. tropicalis* KCCC 13605 and *C. pseudotropicalis* KCCC 13709, were isolated on Sabouraud agar medium (Difco Laboratories, Detroit, Mich.) from the oral cavities of patients undergoing head and neck radiation therapy at the Kuwait Cancer Control Centre (7) and lyophilized. The identities of these isolates were confirmed as described earlier (2).

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Preparation of yeast cells. Liquid yeast cultures were established by incubating lyophilized samples overnight at 37°C in yeast nitrogen base medium (Difco) supplemented with 2.5% (wt/vol) glucose and used for inoculation. Flasks, each containing 50 ml of a minimal medium (ammonium sulfate [1 g/liter], monopotassium sulfate [1 g/liter], glucose [10 g/liter]; pH 6.0) were each inoculated with 1 ml of the overnight culture, and the cells were grown for another 24 h at 37°C. The yeast cells were harvested by centrifugation at 1,200 × *g* for 10 min and washed twice with distilled water ready for the adherence assay.

Preparation of BEC. BEC were collected from healthy 20- to 30-year persons by gently rubbing the mucosal surface of the cheek with a sterile tongue depressor. The cells were washed twice with Hanks balanced salt solution (HBSS) (18) and harvested by centrifugation at 500 × *g* for 10 min. Only freshly prepared samples of BEC were used in the adherence assay.

Scanning electron microscopy. Glutaraldehyde in cacodylate buffer (0.5% [vol/vol]) was added to the yeast-BEC mixture at the end of the assay period (see below). This mixture was allowed to stand at room temperature for 5 min and then centrifuged. The pellet was suspended in 5% (vol/vol) buffered glutaraldehyde and left overnight at 4°C. The mixture was then filtered through a 20-μm filter, and the cells were washed twice with HBSS and processed for scanning electron microscopy as described elsewhere (6).

Extraction, analysis, and fractionation of yeast lipids. Yeast cells, prepared as described above, were used for the extraction of lipids with acetone, as well as with chloroform-methanol (5).

Fresh cells were shaken with acetone for 10 min at room temperature. Acetone was evaporated under reduced pressure in an atmosphere of nitrogen, and the residue was weighed. It was then dissolved in acetone and stored at −20°C under nitrogen.

Total yeast lipids were also extracted from fresh cells with chloroform-methanol (2:1 [vol/vol]) three times and purified by established procedures (5). The pure lipids dissolved in chloroform-methanol were stored at −20°C under nitrogen. The lipid content of dry yeast cells was calculated.

The lipid extracts were analyzed by TLC on silicic acid plates. Apolar compounds were resolved by the solvent system hexane-diethyl ether-acetic acid (90:10:1 [vol/vol/vol]) (14). Polar classes were analyzed by two-dimensional chromatography with chloroform–methanol–7 N ammonium hydroxide (65:35:4 [vol/vol/vol]) in the first direction and chloroform-methanol-acetic acid-water (170:25:25:4 [vol/vol/vol/vol]) in the second direction (16). The spots were visualized with iodine vapor or by charring at 220°C after the plates were sprayed with 50% H_2SO_4. The individual compounds were identified by comparing their chromatographic behavior with that of authentic samples and by using specific spray reagents (3, 22, 25). The relative proportions of the different fractions charred on TLC plates were determined with a densitometer (Beckman Instruments, Inc., Fullerton, Calif.). Saccharides in the acetone extracts were determined spectrophotometrically (26).

Sterols were isolated by preparative TLC, eluted with chloroform-diethyl ether-ethanol (1:1:1 [vol/vol/vol]), and analyzed by gas-liquid chromatography (Pye Unicam model 204; glass column, 1.83 m by 2 mm [inside diameter] packed with 3% QV-17 on Chromosorb W-HP [100/120 mesh]). Saccharides extracted with acetone were resolved by paper chromatography (15).

Individual lipid classes were separated by preparative TLC in pure form and tested for their effects on adherence. Steryl esters, triacylglycerols, free fatty acids, and sterols were isolated by preparative unidimensional chromatography, whereas the individual phospholipids were isolated by preparative two-dimensional chromatography (up to five chromatograms were prepared for each analysis) with the solvents specified above.

Adherence assay. Yeast cells prepared as described above were suspended in HBSS to yield a concentration of 10^7 cells per ml. BEC were suspended in HBSS to yield a concentration of 2×10^5 cells per ml. A 2-ml portion of the yeast suspension was mixed with 2 ml of the BEC suspension in a sterile screw-cap bottle. The mixture was shaken at 37°C for 2 h and then filtered through a 20-μm (pore size) filter (Retsch, Idar-Oberstein, Federal Republic of Germany) to remove nonadhering yeast cells. The BEC on the filter were washed twice with 5-ml portions of HBSS and finally suspended in 5 ml of HBSS. A drop of this suspension was mounted on a glass slide, air dried, heat fixed, and stained with crystal violet for 1 min. Adherence was determined microscopically by counting the mean number of yeast cells adhering to every 100 BEC.

The adherence values thus obtained were those of the control experiments. To study the effect of the total lipid extracts, samples extracted from 5×10^9 cells were evaporated to dryness and directly emulsified or suspended in the assay mixture. These amounts gave final concentrations of 175 μg/ml for chloroform-methanol extracts and 80 μg/ml for acetone extracts. To test the effect of individual lipid classes, total lipid samples extracted from 5×10^9 cells were first fractionated by preparative TLC and the individual classes were eluted, dried, and directly shaken into the assay mixtures. Standard lipid samples (175 μg/ml each), saccharides, and inositol (10 mM each) were directly included in the assay mixtures. The mean numbers of yeast cells adhering to every 100 BEC were then determined in each case.

Cells of *C. albicans* ATCC 10231, as well as BEC suspended in HBSS, were pretreated separately with acetone and chloroform-methanol lipid extracts from yeast cells, 80 and 175 μg/ml, respectively. The mixtures were incubated for 30 min at 37°C on an electric shaker. The cells were harvested, washed twice with HBSS, resuspended in this buffer, and counted, and their adherence to each other was assayed.

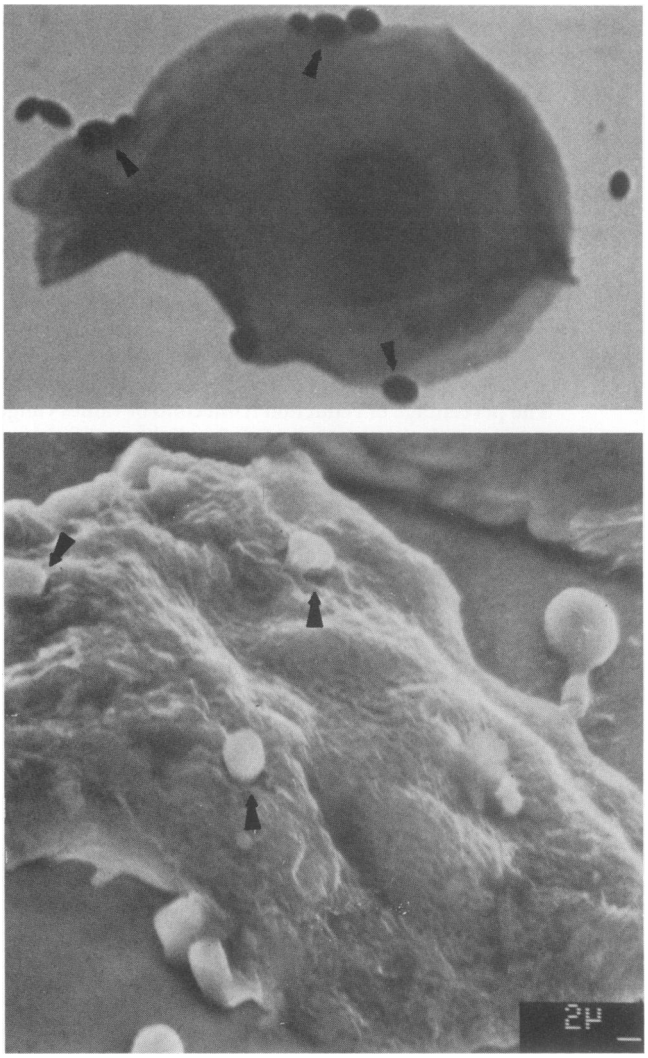

FIG. 1. A light micrograph (top) and a scanning electron micrograph (bottom) of *C. albicans* cells adhering to BEC. It should be noted that the yeast cells (arrows) are embedded in shallow depressions of the BEC.

Each assay was carried out in duplicate, and the Student *t* test was used to evaluate the differences in adherence values. A P value of <0.05 was considered significant.

RESULTS

Microscopic observations. Figure 1 shows typical yeast cells adhering to BEC. In most cases, each yeast cell was found embedded in a shallow depression of the BEC surface as shown on the photographs.

Effects of the total lipid extracts on adherence. Table 1 presents data on the adherence of various *Candida* species and strains to BEC in the absence or presence of total lipid extracts from the corresponding yeast cells.

The three strains of *C. albicans* showed similar adherence results and exhibited higher affinities for BEC than did *C. tropicalis* and *C. pseudotropicalis*. Of the three species

TABLE 1. Effect of lipid extracts from *Candida* cells on their adherence to BEC

Organism	Mean adherence (±SE) in[a]:		
	Control	Acetone extract (% reduction, *P*)	Chloroform-methanol extract (% reduction, *P*)
C. albicans ATCC 10231	523 ± 25	373 ± 25 (28.7, <0.001)	353 ± 31 (32.5, <0.001)
C. albicans KCCC 13878	570 ± 25	330 ± 27 (42.1, <0.001)	350 ± 27 (38.6, <0.001)
C. albicans KCCC 14172	565 ± 26	364 ± 25 (35.6, <0.001)	381 ± 25 (32.6, <0.001)
C. tropicalis KCCC 13605	421 ± 25	352 ± 22 (16.4, <0.04)	340 ± 24 (19.2, <0.005)
C. pseudotropicalis KCCC 13709	277 ± 23	284 ± 21 (NS)	276 ± 20 (NS)

[a] Data are expressed as mean numbers of yeast cells adhering to 100 BEC. NS, Not significant.

studied, *C. pseudotropicalis* showed the least tendency to adhere. With the exception of *C. pseudotropicalis*, adherence of yeast cells to BEC was significantly blocked in the presence of the various lipid extracts (Table 1). Similar results were obtained when adherence was measured for each *Candida* species in the presence of lipid extracts from any of the two other species. Even the total lipid extracts from *C. pseudotropicalis* were as efficient as those from the other two species in blocking the adherence of the latter to epithelial cells. On the other hand, neither the lipid extracts from *C. albicans* nor those from *C. tropicalis* affected the adherence affinity of *C. pseudotropicalis*.

Adherence of cells pretreated with yeast lipids. Table 2 presents data on adherence of pretreated yeast cells to nontreated BEC and of nontreated yeast cells to pretreated BEC. It is apparent that adherence was significantly blocked in both cases compared with the control. Similar results were obtained when both yeast cells and BEC were pretreated with yeast lipids.

Composition of lipid classes in the lipid extracts. Because of the relatively large amount of work required for lipid analysis, this study was done on lipid extracts from *C. albicans* ATCC 10231 only.

Acetone-soluble lipids made up 0.3% of the cell dry weight, whereas chloroform-methanol-soluble lipids made up 0.7%. The acetone extract contained mainly apolar lipid classes, whereas the chloroform-methanol extract contained roughly equal proportions of apolar and polar compounds. Table 3 lists the relative proportions of the individual classes in the total lipids.

In both extracts, the apolar classes consisted of sterols and steryl esters in addition to smaller proportions of triacylglycerols and fatty acids (Fig. 2). The polar classes in the chloroform-methanol extract were mainly phospholipids whose identities are specified in Table 3 and in Fig. 3.

Gas-liquid chromatographic analysis showed that the free sterols isolated from the chloroform-methanol extract by preparative TLC consisted of 17.0% zymosterol, 24.6% ergosterol, 42.7% 24,28-dehydroergosterol, 11.7% 3-β-hydroxy-24-methylcholesta-diene, and 9.5% 4,4'-dimethylzymosterol.

The acetone extract, but not the chloroform-methanol extract, contained 2.3% saccharides. Paper chromatographic analysis showed that the major saccharide was mannose. Trace amounts of *N*-acetylglucosamine and glucose were also detected.

Effects of the constituent lipid classes of total lipids on adherence. Table 4 presents data on the effects of individual lipid classes, isolated by preparative TLC from the total lipids of *C. albicans* ATCC 10231, on the adherence of this organism to BEC.

These data indicate that all of the individual phospholipids, as well as the sterols and steryl esters, significantly blocked adherence. The nonphospholipid components in the polar fraction of the acetone extract (unidentified glycolipids that reacted positively to the α-naphthol spray reagent [22]) also blocked adherence. On the other hand, free fatty acids and triacylglycerols were not at all effective.

Effects of standard lipid classes and saccharides on adherence. We studied the effects of standard lipid samples, saccharides, and inositol on the adherence of *C. albicans* ATCC 10231 to BEC. Inositol was included in this test because it is a saccharidelike cyclic alcohol occurring as a constituent moiety in one of the effective phospholipids (diacylglycerophosphoinositols). The results of this experiment confirmed those of the previous one. Only egg phospholipids, cholesterol, and cholesteryl palmitate blocked adherence by 50.7, 38.9, and 21.7%, respectively, whereas triolein, oleic acid, and palmitic acid showed no significant effects.

TABLE 2. Effect of pretreatment of cells with total lipids extracted from yeast cells on their adherence to BEC

Pretreatment of:		Mean adherence (±SE) in[a]:	
Yeast cells	BEC	Acetone extract (% reduction, *P*)	Chloroform-methanol extract (% reduction, *P*)
−[b]	−[b]	516 ± 25	516 ± 25
+	−	283 ± 26 (45.1, <0.001)	350 ± 30 (32.2, <0.01)
−	+	273 ± 24 (47.0, <0.001)	351 ± 31 (32.0, <0.01)

[a] Data are expressed as mean numbers of yeast cells adhering to 100 BEC.
[b] Control.

TABLE 3. Composition of lipid classes in lipid extracts from *C. albicans*

Lipid class	% of total lipids in[a]:	
	Acetone extract	Chloroform-methanol extract
Hydrocarbons	Tr	Tr
Steryl esters	50.5	9.5
Triacylglycerols	12.8	7.2
Fatty acids	13.0	11.4
Sterols	14.4	10.3
Diacylglycerophosphoethanolamines	Tr	11.4
Diacylglycerophosphoglycerols	Tr	3.1
Diacylglycerophosphocholines	Tr	17.0
Diacylglycerophosphoinositols	Tr	13.9
Diacylglycerophosphoserines	Tr	6.7
Phosphatidic acids	Tr	3.3
Unidentified nonphospholipid compounds	9.3	6.2

[a] Tr, Traces.

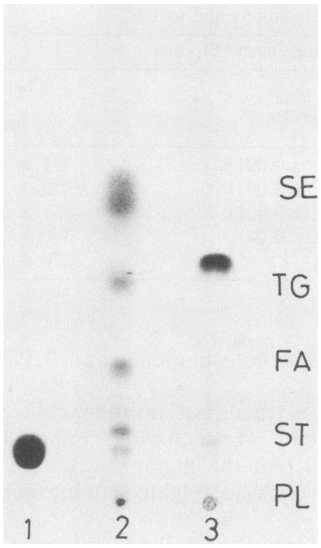

FIG. 2. A typical unidimensional chromatogram showing the composition of lipid classes in the acetone extract from *C. albicans*. Similar fractions were also identified in the chloroform-methanol extract. The sorbent and solvent were Silica Gel G and hexane-diethyl ether-acetic acid (90:10:1 [vol/vol/vol]), respectively, with visualization by charring. Lanes: 1, standard cholesterol; 2, sample (acetone extract); 3, triolein. Identities of the spots: SE, steryl esters; TG, triacylglycerols; FA, fatty acids; ST, sterols; PL, polar lipids.

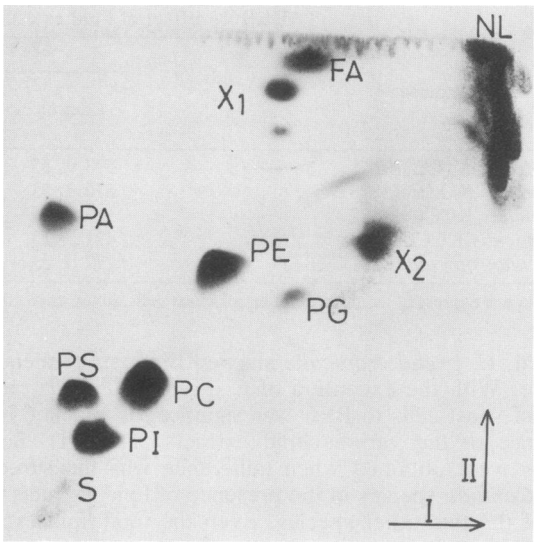

FIG. 3. A typical two-dimensional chromatogram showing the composition of lipid classes in the chloroform-methanol extract from *C. albicans*. The sorbent was Silica Gel G, and the solvents were (direction I) chloroform–methanol–7 N ammonium hydroxide (65:35:4 [vol/vol/vol]) and (direction II) chloroform-methanol-acetic acid-water (170:25:25:4 [vol/vol/vol/vol]). Visualization was by charring. Identities of the spots: NL, neutral lipids (steryl esters, triacylglycerols, sterols); FA, fatty acids; X_1 and X_2, unidentified (X_2 is a glycolipid); PA, phosphatidic acids; PE, diacylglycerophosphoethanolamines; PG, diacylglycerophosphoglycerols; PC, diacylglycerophosphocholines; PS, diacylglycerophosphoserines; PI, diacylglycerophosphoinositols; S, start.

Of the saccharides tested, only mannose (39.9% reduction) and, to a lesser extent, *N*-acetylglucosamine (36.2% reduction) were effective, whereas glucose was ineffective. Inositol, with adherence reduction of 37.0%, was as efficient as mannose in blocking adherence of yeast cells to BEC.

DISCUSSION

An essential prerequisite of a successful infection is adherence of the pathogen to the host. The molecular and biochemical bases of *C. albicans* adherence to BEC are still far from clear. What has been established so far is that a few chemical substances known to be constituents of the yeast cell wall are involved in adherence. Even in this respect the information is really meager; emphasis has been placed on saccharides, mainly mannose (13, 19), amino sugars (21), and glycoproteins (11). Our results confirm earlier findings regarding the blocking of yeast cell adherence to epithelial cells by mannose and *N*-acetylglucosamine (21) and show in addition that the saccharidelike cyclic alcohol inositol is as efficient as mannose in this respect.

Since the role of saccharides and saccharide derivatives in adherence has already been established, we first considered glycolipids; hence, the application of acetone in extraction. However, since this solvent does not dissolve phospholipids, which are major constituents of the membranes, chloroform-methanol was also used for extraction of total lipids.

It is interesting that of the lipid classes we tested only phospholipids, sterols, and steryl esters—the typical plasma membrane lipid classes—were found to be effective in the adherence assay (the unidentified glycolipids in the acetone extract are now under investigation). This result was true for

TABLE 4. Effects of constituent lipid classes of total lipids from *C. albicans* on its adherence to BEC

Lipid class	Mean adherence (±SE) in[a]:		
	Control	Acetone extract (% reduction, *P*)	Chloroform-methanol extract (% reduction, *P*)
Steryl esters	515 ± 28	423 ± 27 (17.9, <0.02)	435 ± 25 (15.5, <0.05)
Triacylglycerols	515 ± 28	510 ± 28 (NS)	522 ± 30 (NS)
Fatty acids	515 ± 28	503 ± 25 (NS)	498 ± 22 (NS)
Sterols	515 ± 28	416 ± 28 (19.2, <0.005)	413 ± 25 (19.8, <0.01)
Diacylglycerophosphoethanolamines	515 ± 28		277 ± 24 (46.2, <0.001)
Diacylglycerophosphoglycerols	515 ± 28		315 ± 26 (38.8, <0.001)
Diacylglycerophosphocholines	515 ± 28		309 ± 25 (40.0, <0.001)
Diacylglycerophosphoinositols	515 ± 28		228 ± 25 (55.7, <0.001)
Diacylglycerophosphoserines	515 ± 28		292 ± 24 (43.3, <0.001)
Other nonphospholipid compounds	515 ± 28	298 ± 25 (42.1, <0.001)	

[a] Data are expressed as mean numbers of yeast cells adhering to 100 BEC. NS, Not significant.

the lipid classes extracted from *C. albicans*, as well as for the standard lipid samples tested. Owing to the practical difficulty of obtaining masses of BEC which could yield amounts of lipids adequate for the tests, the role of these lipids in adherence was not studied. However, the plasma membranes of the BEC to which yeast cells directly adhere do contain phospholipids and sterols.

It is interesting that the sterol mixture isolated from the yeast lipids and cholesterol, certainly present in BEC membranes, blocked adherence. In this context it may be mentioned that estradiol was reported to block the adherence of *Escherichia coli* to epithelial cells (17). Our results also show that steryl esters could block adherence. It has been reported that the outer layers of the plasma membranes from animal cells also contain, in addition to phospholipids and sterols, large proportions of steryl esters (12). The activity of phospholipids in blocking adherence does not lie in their acyl moieties; our results indicated that neither palmitic acid nor oleic acid showed any effect on adherence.

The possible role of certain lipid classes in adherence should not be oversimplified. We found that lipids effective in the adherence of *C. albicans* and *C. tropicalis* could not affect the adherence of *C. pseudotropicalis*. In addition, lipids extracted from the weakly adherent *C. pseudotropicalis* significantly affected the adherence of *C. albicans* and *C. tropicalis* to BEC. The order of adherability of the three *Candida* species tested reflects the degree to which they are involved in the disease process (10).

Our results on blocking adherence by pretreating yeast cells, BEC, or both by lipids generally confirm similar findings on mannose (20). Reportedly, during pretreatment, mannose may block adherence sites on the cell surface resulting in blocking of subsequent adherence. This interpretation may also apply to lipid pretreatment.

It is concluded that not only saccharides but also certain lipid classes, viz., phospholipids, sterols, and steryl esters, are involved in adherence, but in association with other unknown factors. In this context, it is to be noted that both the individual saccharides and the individual lipid classes were almost equally effective in blocking adherence of *Candida* cells to BEC.

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